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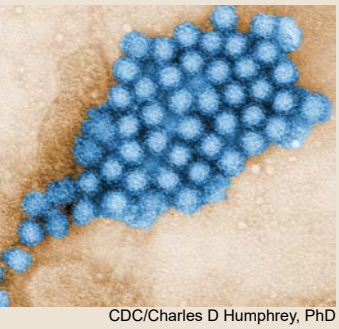
# MSMR

## MEDICAL SURVEILLANCE MONTHLY REPORT

CDC/Melissa Dankel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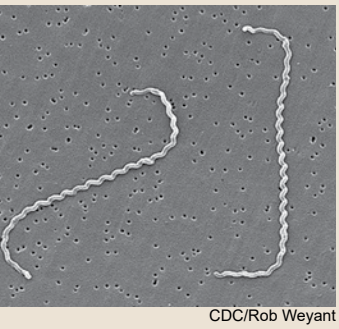
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## *Global Emerging Infections Surveillance (GEIS) Issue*

# Contributions from the Global Emerging Infections Surveillance (GEIS) Network

*Franca R. Jones, PhD (CDR, USN)*

As Chief of the Global Emerging Infections Surveillance (GEIS) section of the Armed Forces Health Surveillance Branch (AFHSB), I am pleased to introduce this month's issue of the *Medical Surveillance Monthly Report* (*MSMR*), which contains several reports of surveillance data from GEIS partners. Although GEIS partners regularly publish in other peer-reviewed journals,[1] with more than 100 publications between 2016 and 2017 alone,[2] the *MSMR* provides a unique platform for communicating results to a targeted audience of military public health and medical professionals. This collection of *MSMR* articles is part of a larger effort to increase the timeliness and availability of GEIS surveillance data to Department of Defense (DoD) personnel responsible for readiness and force health protection (FHP) decision making, particularly the Combatant Command (CCMD) Surgeons and their staffs. Consistent with that effort, GEIS will be actively engaging with GEIS partners to encourage additional manuscript submissions to the *MSMR*. This introduction provides an opportunity to summarize the variety of surveillance activities being carried out across the GEIS network.

Last year marked the 20th anniversary of the GEIS program. The DoD established GEIS in 1997 to address a Presidential Decision Directive, National Science and Technology Council–7, which tasked the DoD to improve infectious disease surveillance, prevention, and response.[3] GEIS achieves these goals through support to an interconnected global network of U.S. Army, Navy, and Air Force laboratory partners located in the U.S., South America, Africa, Europe, and Asia with extensive field, clinical, and laboratory biosurveillance capabilities **(Figure)**. Moreover, GEIS partners have developed strong and long-standing relationships with other U.S. government agencies, international organizations, and partner nations in strategically relevant locations to expand the capabilities and reach of the network in providing regional disease surveillance support. A

**FIGURE.** Map of core GEIS partner U.S. Army, Navy, and Air Force laboratories, by Geographic Combatant Command. U.S. Army laboratories include the Walter Reed Army Institute of Research (WRAIR), U.S. Army Medical Research Directorate–Georgia (USAMRD-G), U.S. Army Medical Research Directorate–Kenya (USAMRD-K), and Armed Forces Research Institute of Medical Sciences (AFRIMS). U.S. Navy laboratories include the Naval Medical Research Center (NMRC), Naval Health Research Center (NHRC), and U.S. Naval Medical Research Unit No. 2 (NAMRU-2), No. 3 (NAMRU-3), and No. 6 (NAMRU-6). Other core GEIS partners include U.S. Air Force School of Aerospace Medicine (USAFSAM) and Uniformed Services University of the Health Sciences (USU).

network approach ensures that GEIS partners are more than the sum of their parts by providing, for example, comparable data on circulating influenza virus strains for seasonal vaccine selection,[4-6] etiologic agents of traveler's diarrhea, and antimicrobial resistance patterns[7] around the world.

In 2015, GEIS, as a section of AFHSB, was integrated into the Defense Health Agency's (DHA) Public Health Division. Incorporation into the DHA elevated the GEIS section's role in Combat Support and highlighted the need to provide more timely surveillance data to the CCMDs. In addition to peer-reviewed publications, GEIS partners now provide timely updates on outbreaks and high-impact surveillance findings, which are communicated immediately to the relevant CCMDs, and generate monthly data reports for all ongoing surveillance projects with routine findings. These data are compiled into summary reports for each CCMD on a monthly basis, a process that provides timely and actionable surveillance information for FHP decision making to supplement more in-depth, but less timely, peer-reviewed publications.

The articles in the August 2018 issue of the *MSMR* provide only a small sample of the overall GEIS network's results, with a focus on disease surveillance among U.S. military populations. As comprehensive risk assessments require knowledge of emerging pathogens circulating more broadly, the GEIS network conducts disease surveillance activities in partner nation militaries, partner nation civilians, animal reservoirs, and arthropod vectors, in addition to U.S. military personnel. Such work in partnership with other nations broadens the scope of information for FHP decision making while improving the partner nations' understanding of their endemic infectious disease burdens and risks. These activities cover all six geographic CCMDs and address a wide diversity of militarily relevant infectious disease threats that fall under four GEIS Focus Areas:

**Antimicrobial-resistant infection** surveillance projects identify circulating antimicrobial-resistant pathogens that may have an impact on our global fighting force. Antimicrobial-resistant pathogens linked to healthcare-associated infections, wound infections, and sexually transmitted infections, as well as emerging resistance patterns are analyzed to provide information for military health initiatives such as improved antibiotic stewardship and medical countermeasures development. An increasing number of projects at the DoD overseas laboratories have focused on advanced molecular methods, which provide a more in-depth evaluation of genetic mechanisms of antimicrobial resistance on a near real-time basis. Over recent years, GEIS partner laboratories documented the emergence of carbapenem-resistant bacteria at Landstuhl Regional Medical Center in Germany,[8] found colistin-resistant *Klebsiella pneumoniae* in Thailand,[9] and characterized *Staphylococcus aureus* carriage among U.S. Navy submariners (see article by Millar et al. on page 5).

**Enteric infection** surveillance projects concentrate on the etiologic agents causing acute gastroenteritis or travelers' diarrhea, pathogens that often degrade military operational readiness. Surveillance activities cover acute gastroenteritis in U.S. military personnel (including recruit, shipboard, and forward-deployed populations) and in foreign military and civilian populations, study of travelers' diarrhea in immune-naive travelers,[10] and advanced characterization and antimicrobial susceptibility testing of enteric pathogens. Recent studies have described the causes of acute gastroenteritis among trainees at four recruit training centers (see article by Brooks et al. on page 8), the incidence of *Campylobacter concisus* and *C. ureolyticus* in pathogen-negative stool samples from travelers' diarrhea cases and asymptomatic controls,[11] resulting in the first report of these pathogens detected among travelers to Nepal and Thailand, and the epidemiology and patterns of illness among individuals infected with fluoroquinolone-resistant *Campylobacter* species and other diarrheal pathogens during a 2- to 3-week military exercise in Thailand.[12]

**Febrile and vector-borne infection** surveillance projects examine vector-borne and zoonotic pathogens associated with acute febrile illness in humans in three general areas: human infections and disease (see article by Chen et al. on page 13), vector distribution and pathogen presence in vectors and reservoirs,[13,14] and environmental drivers of exposure and infection.[15] Recently published studies have documented the transmission of scrub typhus outside of Asia,[16,17] the distribution of malaria resistance to major antimalarial drugs in Southeast Asia,[18] and the identification of rickettsial pathogens in mosquitoes in the Republic of Korea.[19]

**Respiratory infection** surveillance projects address rapid detection and response to respiratory pathogens, especially those with pandemic potential in humans. Surveillance includes U.S. military personnel, including recruit, shipboard, and deployed populations,[20] and foreign military and civilian populations. Other specific studies address the human–animal interface (designed to develop knowledge regarding zoonotic transmission of emerging respiratory pathogens), advanced characterization of viruses (designed to monitor viral drift and potential shift), and vaccine effectiveness and response studies.[21,22] Recent studies examined clinical and laboratory predictors of influenza virus infection among individuals with influenza-like illness,[23] biomarkers of antibody-dependent cellular cytotoxicity as a broader metric than neutralizing antibodies for assessing immune response to influenza vaccination,[24] and sampling strategies for detecting genetic diversity of influenza viruses (see article by Fries et al. on page 16).

* * *

In the 20 years since its establishment, GEIS has been a global leader in efforts to address militarily relevant infectious disease threats, informing readiness and FHP decision making. Pathogens continue to emerge at an astounding rate, and GEIS partners have been on the front line in detecting and responding to these threats: from the 2009 influenza A(H1N1) virus pandemic,[25] the emergence of MERS-CoV in Central Command,[26] the Ebola epidemic in Africa Command,[27,28] chikungunya[29,30] and Zika virus emergence[31,32] in Southern Command and Indo-Pacific Command, and the first identification of colistin-resistant bacteria in Northern Command.[33] As Rear Admiral Colin Chinn pointed out at the recent GEIS State of the Science meeting

in November 2017, "Those working in the laboratories in the U.S. and overseas are at the tip of the spear so that the Combatant Commands have the healthy forces necessary for their missions."[34] As mentioned, the articles in this issue represent only a small sample of what I anticipate will be many submissions to the *MSMR* from the GEIS network, in line with the *MSMR*'s goal to provide "public health information that is directly relevant to the health, safety, and well-being of Military Health System beneficiaries or the operational fitness of military members."[35,36]

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*Brief Report*

# Pre- and Post-Deployment Prevalence of *Staphylococcus aureus* Colonization Among U.S. Navy Submariners

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*Staphylococcus aureus* is a major cause of skin and soft tissue infection (SSTI). Military personnel in congregate settings (e.g., training, deployment) are at increased risk for *S. aureus* colonization and SSTI.[1-4] In these settings, a preponderance of SSTI risk factors (e.g., crowding, infrequent handwashing/bathing, skin microabrasions) favors the person-to-person transmission of *S. aureus* and leads to outbreaks of SSTI which, in turn, impose on the military a significant operational and healthcare burden. A significant proportion of SSTIs are caused by methicillin-resistant *S. aureus* (MRSA), a strain known for its enhanced virulence.[5]

Shipboard deployments have been associated with SSTI outbreaks and increased prevalence of *S. aureus*/MRSA colonization,[3,6] likely explained by the prolonged confinement of personnel in enclosed, crowded spaces.[7,8] Fifty percent of crew members on a U.S. Navy vessel were colonized with *S. aureus* (3.5% with MRSA),[7] whereas colonization rates of 32% (1% with MRSA) have been reported in the general U.S. population.[9] Among British submariners, pre- and post-deployment rates of *S. aureus* colonization were 36.9% and 43.5%, respectively.[8] Submarine deployments may represent periods of increased colonization and SSTI risk. This report describes a study of *S. aureus* nasal colonization and SSTI among two different crews of U.S. Navy submariners who deployed on a ballistic missile submarine (SSBN) in 2016. The results are reported herein.

## METHODS

For a 7-month period in 2016, an observational cohort study of *S. aureus* colonization and SSTI among U.S. Navy submariners was conducted. Submariners who were embarking on ballistic missile submarine deployments lasting 3–4 months were targeted for enrollment. Personnel from two consecutive crews who deployed on the same submarine participated in the study. All active duty military members on board (150 in each of the two crews) were eligible for participation. Enrollments occurred on the submarine approximately 1 week before deployment. Because of operational security, the study team was assigned a specific area in the galley of the submarine and did not have access to other areas of the submarine for recruitment. Independent duty corpsmen, assigned to the submarine, briefed the crew about the study before the enrollment visit, and flyers were used to advertise the study. All active duty personnel were invited to participate by the independent duty corpsmen using the overhead announcement system on the submarine.

Personnel who were interested in the study presented to the galley during specific hours for enrollment and were enrolled by the study team. The post-deployment visit was conducted at a clinic a few miles from the port. The independent duty corpsmen attempted to contact subjects who did not show up for their follow-up visit and those who missed the follow-up visit were considered lost to follow-up. Each crew was deployed on the submarine for a period of 3 months. Post-deployment nasal swabs were collected within 1 week of returning from deployment.

At the pre- and post-deployment visits, participants had *S. aureus* culture swabs taken from the anterior nares using dual BBL™ CultureSwab™ Collection and Transport System. Swabs were immediately placed on dry ice and stored at -70°C prior to shipping to the microbiology laboratory at the Naval Medical Center in Portsmouth, VA. Culture swabs were plated onto mannitol salt agar plates. Mannitol fermenting colonies were isolated and plated onto trypticase soy agar with 5% sheep's blood and incubated overnight. *S. aureus* isolates were identified based on colony morphology, Gram stain, latex agglutination testing (Staphaurex™, Remel, Lenexa, KS), and slide catalase testing. All *S. aureus* isolates underwent identification and susceptibility testing using MicroScan™ WalkAway-96 (Dade Behring Inc., Deerfield, IL), according to Clinical Laboratory Standards Institute methods.

Participant demographic characteristics and information on potential SSTI risk factors were collected at the time of enrollment using a self-administered questionnaire. A post-deployment questionnaire elicited information on incident SSTIs, treatment and outcome during deployment. Statistical analysis was performed using SAS/STAT© software version 9.3 (2012, SAS Institute, Cary, NC). This study was approved by the Uniformed Services University Infectious Disease Institutional Review Board (IDCRP-088).

## RESULTS

Study participants represent convenience samples of personnel from two crews deployed on a single SSBN. Of the 300 submariners (150 in each deployment) eligible for participation, 119 (39.7%) submariners were enrolled (59 from group 1, 60 from group 2) and had pre-deployment swabs collected. Of these, 97 (44 from group 1, 53 from group 2) returned for the follow-up visit and had post-deployment swabs collected.

The pre-deployment prevalence of *S. aureus* colonization was 38.9% in group

**TABLE.** Characteristics of U.S. Navy submariners enrolled in a study of *Staphylococcus aureus* colonization and skin and soft tissue infection

| | Group 1 | | Group 2 | | Total | |
|---|---|---|---|---|---|---|
| Participant characteristics | n=59 | | n=60 | | n=119 | |
| Median age, years (range) | 27.0 (19–46) | | 27.5 (19–43) | | 27.0 (19–46) | |
| Race/ethnicity | No. | % | No. | % | No. | % |
| White | 50 | 84.7 | 48 | 80.0 | 98 | 82.4 |
| Black | 4 | 6.8 | 6 | 10.0 | 10 | 8.4 |
| Mixed race/ethnicity | 5 | 8.5 | 5 | 8.3 | 10 | 8.4 |
| Asian | 0 | 0 | 1 | 1.7 | 1 | 0.8 |
| Sex | | | | | | |
| Male | 56 | 94.9 | 60 | 100 | 116 | 97.5 |
| Female | 3 | 5.1 | 0 | 0.0 | 3 | 2.5 |
| Rank | | | | | | |
| Officer | 5 | 8.5 | 2 | 3.3 | 7 | 5.9 |
| Jr. Enlisted (E1–E3) | 6 | 10.2 | 7 | 11.7 | 13 | 10.9 |
| Mid-level Enlisted (E4–E6) | 41 | 69.5 | 39 | 65.0 | 80 | 67.2 |
| Sr. Enlisted (E7–E9) | 7 | 11.9 | 12 | 20.0 | 19 | 16 |
| Pre-deployment survey | | | | | | |
| Had a skin infection in the past 12 months | 0 | 0.0 | 2 | 3.3 | 2 | 1.7 |
| Used antibiotics in the past 3 months | 4 | 6.8 | 3 | 5.0 | 7 | 5.9 |
| Nasally colonized at the pre-deployment visit | | | | | | |
| *S. aureus* | 23 | 38.9 | 7 | 11.7 | 30 | 25.2 |
| MRSA | 1 | 1.7 | 2 | 3.3 | 3 | 2.5 |
| Post-deployment survey | n=44 | | n=53 | | n=97 | |
| Shared/rotated bunk with ≥1 crew members | 2 | 4.5 | 18 | 33.9 | 20 | 20.6 |
| Washed hands or used hand sanitizer ≥4 times per day | 24 | 54.5 | 26 | 49.1 | 50 | 51.5 |
| Shared razor, towels, or bedding with other crew members | 0 | 0.0 | 0 | 0.0 | 0 | 0 |
| Changed uniform several times per week | 26 | 59.1 | 23 | 43.4 | 49 | 50.5 |
| Nasally colonized at the post-deployment visit | | | | | | |
| *S. aureus* | 10 | 22.7 | 3 | 5.7 | 13 | 13.4 |
| MRSA | 1 | 2.3 | 0 | 0.0 | 1 | 1 |

MRSA, methicillin-resistant *Staphylococcus aureus*

1 and 11.7% in group 2. The post-deployment prevalence of *S. aureus* colonization was 22.7% in group 1 and 5.7% in group 2 **(Table)**. The pre-deployment prevalence of MRSA colonization was 1.7% in group 1 and 3.3% in group 2. The post-deployment prevalence of MRSA colonization was 2.3% in group 1 and 0% in group 2. No cases of SSTI during either deployment were identified on the post-deployment surveys.

## EDITORIAL COMMENT

This study of *S. aureus* nasal colonization, conducted among two U.S. Navy submariner samples on 3-month training exercises, demonstrated moderate rates of overall *S. aureus* colonization, low rates of MRSA colonization, and a decrease in colonization prevalence from the pre- to the post-deployment period. No cases of SSTI were reported during either deployment.

Previous studies have shown that the shipboard setting is associated with SSTIs among military personnel,[3] and that *S. aureus* colonization, an important risk factor for SSTI, is common.[7,8] One cross-sectional study on a U.S. Navy vessel demonstrated a *S. aureus* colonization prevalence of 49% in the first 2 weeks of deployment.[7] Environmental samples were not collected in that shipboard study, but contamination of common-touch surfaces on the ship may have served as an important reservoir for *S. aureus*, as has been suggested in a military recruit setting.[10]

Lower than anticipated rates of colonization and a decrease in prevalence from the pre- to post-deployment visit were observed in the study samples. The reasons for these findings are not known, but the observations may be explained in part by limitations in the study design and sampling strategy. Samples were limited to two crews that were deployed on a single submarine. Moreover, the data are based on a convenience sample, as only 40% of eligible

submarine personnel agreed to participate in the study. It was not feasible to assess the representativeness of those who did participate (i.e., compare the characteristics of participants and non-participants in the study), because study personnel did not interact with non-participants. Lastly, it is possible that two swabs over a 3-month period are insufficient to capture the dynamics of *S. aureus* acquisition and colonization in this setting. This study was conducted among personnel deployed on a ballistic missile submarine and it is not known whether the risk of *S. aureus* colonization and/or SSTI differs for personnel deployed on other classes of submarines (e.g., fast attack submarines), or for personnel at other submarine bases in the U.S.

Previous studies have shown that the congregation of military personnel in shipboard settings increases the risk of outbreaks of acute respiratory infection, gastrointestinal infection, and SSTI.[3,11,12] Military personnel on submarines likely experience a number of SSTI risk factors (e.g., crowding, shared equipment) that may increase their risk of *S. aureus* acquisition, persistent colonization, and infection. The findings from this study did not demonstrate an increased risk of *S. aureus* colonization or SSTI. However, these results may have been due to significant limitations in the study design and sampling strategy. Nonetheless, SSTI prevention strategies, including personal hygiene optimization, proper wound care, and environmental cleaning measures among congregate military populations are warranted, as is ongoing surveillance for SSTI cases during deployment.

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# Surveillance for Norovirus and Enteric Bacterial Pathogens as Etiologies of Acute Gastroenteritis at U.S. Military Recruit Training Centers, 2011–2016

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An estimated 179 million cases of acute gastroenteritis (AGE) occur each year in the U.S. and AGE is commonly reported within both training and deployed U.S. military populations. Beginning in 2011, the Operational Infectious Diseases (OID) laboratory at the Naval Health Research Center (NHRC) has undertaken routine surveillance of four U.S. military training facilities to systematically track the prevalence of AGE and to establish its etiologies among U.S. military recruits. Employing both molecular and standard microbiological techniques, NHRC OID routinely assays for pathogens of direct military relevance, including norovirus genogroups I and II, *Salmonella*, *Shigella*, and *Campylobacter*. During its initial surveillance efforts (2011–2016), NHRC OID identified norovirus as the primary etiology of both sporadic cases and outbreaks of AGE among trainees.

Acute gastroenteritis (AGE) is defined as the rapid onset of diarrheal disease, with or without accompanying symptoms, such as nausea, vomiting, fever, or abdominal pain. Annually, AGE causes 1.8 million deaths worldwide in children younger than 5 years old.[1,2] In the U.S. alone, despite public health advances to improve food, water, and sanitation, AGE remains a major cause of morbidity and hospitalization, accounting for more than 1.5 million outpatient visits, 200,000 hospitalizations, and 300 deaths annually.[3-5] Furthermore, an estimated 179 million cases occur annually in the U.S. among people who do not seek medical attention.[5] AGE is one of the most common ailments affecting travelers and military populations. Polymerase chain reaction (PCR), along with other diagnostic techniques, have been utilized to identify both bacterial and viral causations, with norovirus (NoV) being identified as the primary etiologic agent.[6,7]

Among U.S. Department of Defense (DoD) military populations and other international military units, NoV has been implicated or suspected as the causative agent in several AGE outbreaks.[8,9] Results of an anonymous cross-sectional survey of U.S. military personnel deployed to Southwest Asia from 2003 through 2004 found that 76.8% of personnel stationed in Iraq and 54.4% in Afghanistan had experienced at least one episode of diarrhea.[6] It was found that diarrhea was most often associated with time spent off the regional base and with eating local food.[6] Additional studies have shown that among deployed U.S. military personnel, the bacteria *Salmonella*, *Shigella*, and *Campylobacter* were responsible for 5%–17%, 3%–7%, and 6%–10% of reported cases of gastroenteritis, respectively.[7] NoV incidence rates have been estimated to account for approximately 3% of all diarrhea within the same population.[10] As a result of crowding and subsequent ease of transmission, AGE due to NoV has been estimated to spike to over 70% during shipboard outbreaks.[9] These studies clearly showed that bacterial and viral etiologies, with an emphasis on *Salmonella*, *Shigella*, *Campylobacter*, and NoV have a significant impact on the morbidity, medical resources, and operational effectiveness of deployed troops.[11,12] Given the unpredictability and disruptive effect of infectious gastroenteritis on U.S. military training, readiness, and operational performance, AGE surveillance efforts are crucial to understanding the etiology and epidemiology of AGE within these "at-risk" populations.

The impact of these pathogens is also recognized in DoD recruit training populations. The enteric disease surveillance study implemented by the Naval Health Research Center Operational Infectious Diseases (NHRC OID) offers a unique opportunity to focus on this specific, high-risk AGE population. Recruit populations offer several advantages for studying highly communicable infectious diseases. First, recruit trainees live within a "crowded" environment (paramount for sustained transmission dynamics for infectious diseases). Second, trainees have excellent healthcare facilities at their disposal and are required to seek care when ill. Finally, military populations have demonstrated excellent research study participation and compliance (within the framework of approved institutional review board [IRB] protocols), allowing the collection of enrollment and follow-up data to be almost fully complete. For etiologic identification, NHRC OID has developed and implemented a sophisticated enteric diseases surveillance program with the capability to identify NoV and several significant bacterial pathogens in human clinical samples.

## METHODS

Study participation was strictly voluntary for all recruits who agreed to enroll from 12 May 2011 through 30 September 2016. Only presumptive infectious AGE patients were sought and enrolled as cases; those trainees who were deemed to have non-infectious AGE (e.g., dehydration, heat- or exercise-related AGE), were excluded from enrollment after initial evaluation.

The case definition of AGE was a trainee presenting for medical care after having experienced three or more episodes of diarrhea or vomiting in the preceding 24 hours, or a combination of episodes of diarrhea (two or more) or vomiting (two or more) accompanied by additional gastrointestinal-related symptoms (two or more), such as nausea or abdominal cramps. Since 2011, the standardized surveillance network has included Marine Corps Recruit Depot, San Diego, CA (initiated May 2011); Marine Corps Recruit Depot, Parris Island, SC (June 2011); Recruit Training Command, Great Lakes, IL (October 2011); and U.S. Army Training Center, Fort Leonard Wood, MO (April 2012).

This surveillance research was approved by the NHRC IRB in 2011. Following consent and enrollment, all enrolled trainees with AGE were asked to provide a stool sample. Stool samples were preserved in Cary-Blair transport medium and Campy-thioglycollate medium. Recruits unable to provide a stool specimen were asked to self-administer two rectal swabs, which were preserved in universal transport medium (UTM) and Cary-Blair medium. All inoculated media and remaining unpreserved stool were stored at 4°C. Collected specimens were shipped to the NHRC OID laboratory under refrigerated conditions on a weekly basis.

Each training site had a dedicated NHRC OID research assistant to conduct surveillance. Research assistants normally collected up to 10 specimens per week but could have collected up to three specimens per day during an AGE outbreak. An AGE outbreak was defined as an "unusual" (relative to weekly averages) number of recruits with AGE symptoms clustered by both time and place. More practically, an outbreak for the purposes of surveillance was defined as two or more associated cases of diarrhea and/or vomiting within a 24-hour period.

The NHRC OID laboratory is accredited by the College of American Pathologists and the DoD Clinical Laboratory Improvement Program. Additionally, NHRC OID has been certified to participate in the CaliciNet National Norovirus Outbreak Network. The Centers for Disease Control and Prevention (CDC) launched CaliciNet in 2009 to collect information on NoV strains associated with gastroenteritis outbreaks in the U.S. Participation is limited to federal, state, and local public health laboratories in the U.S. The NHRC OID laboratory is currently the only DoD laboratory participating in the program and has been certified to do so since 2011.[13]

### Data Management

The NHRC OID laboratory maintains a relational database containing demographic and epidemiologic information collected from case report forms as well as laboratory results. Summary statistics are frequently generated on all relevant variables, including demographics, symptoms, disposition, and functional outcomes. Enrollment and specimen collection may occur on a daily basis at each field site; laboratory testing occurs at least once per week.

### Laboratory Processing

*Detection of Salmonella, Shigella, and Campylobacter*

Stool specimen swabs received in Cary-Blair transport media were streaked onto tryptic soy agar with 5% sheep blood, MacConkey media, and xylose lysine deoxycholate agar plates. Inoculated plates were incubated for a total of 48 hours at 37°C, with primary analysis at 24 hours. Suspected *Salmonella* and *Shigella* colonies were isolated and identified using the BD Phoenix Automated Microbiology System (Becton Dickinson).

Swabs received in Campy-thioglycollate media were streaked onto Campy-CVA agar plates and incubated under microaerophilic conditions for 72 hours at 42°C. Suspected *Campylobacter* colonies were isolated and identified at the genus level by the following characteristics: cellular morphology, reaction on Gram staining, oxidase, and hippurate analysis. Single-swab samples received in Cary-Blair media were used to streak all of the aforementioned plates. Incubation times, temperatures, and subsequent analyses were identical to the procedure outlined above.

*Detection and characterization of NoV genogroups GI/II*

RNA was extracted from 20% saline suspensions of stool or directly from UTM using the QIAamp® Viral RNA Mini Kit (Qiagen Inc., Valencia, CA), according to the manufacturer's instructions. Bacteriophage MS2 virus (ZeptoMetrix) was added to the fecal suspension prior to extraction. Eluted RNA extractions were assayed for the presence of norovirus using a multiplex real-time RT-PCR assay developed by CDC for simultaneous detection of human NoV GI/GII, as well as an MS2 virus internal amplification control.[13] Strain identification was performed in accordance with CDC protocols and uploaded to the CaliciNet surveillance database.[13]

## RESULTS

During the 5-year surveillance period, approximately 3% of all recruits across the four training sites were diagnosed with AGE by a clinician. Although 1,940 subjects provided informed consent and were enrolled, four enrollees failed to provide samples, leaving 1,936 samples collected and submitted to the NHRC OID laboratory for analysis **(Table)**. Among the population experiencing AGE, rates of consent to participate in surveillance and contribution of research samples varied between sites. The highest average rate of participation among those approached to enroll was seen at Marine Corps Recruit Depot San Diego (47%), the lowest at Fort Leonard Wood (22%), though variation was seen across years at all sites. A minor seasonal trend was observed across all sites, with the highest participation occurring during winter months, suggestive of a positive association with AGE activity. Etiologic agents were

**TABLE.** Cumulative acute gastroenteritis (AGE) surveillance data, by recruit training site, May 2011–September 2016

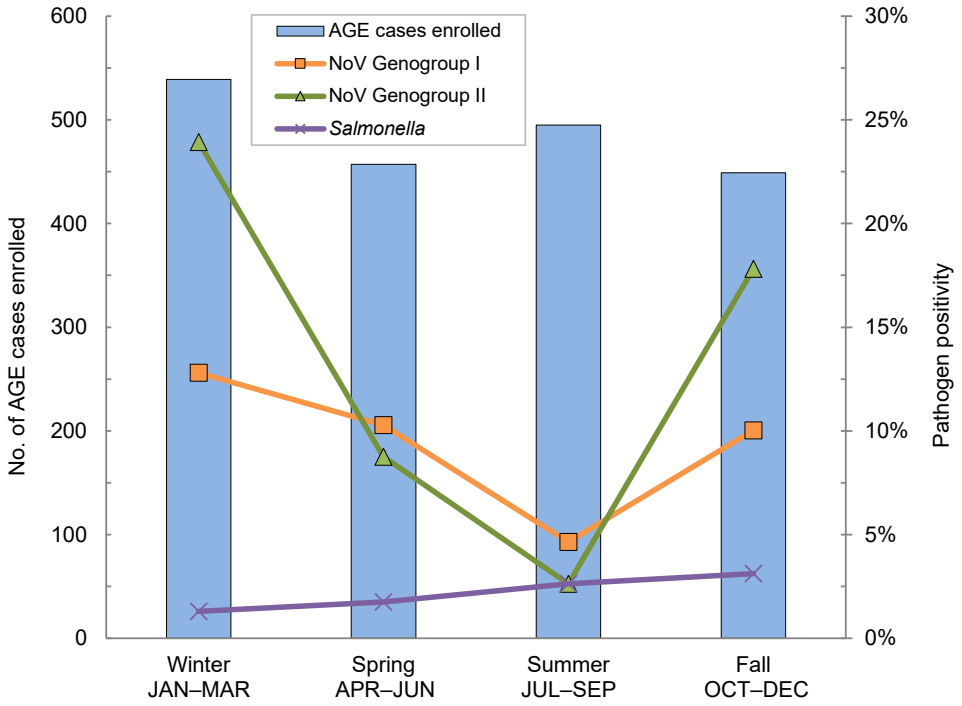
| Recruit training site | Total cases of clinician-diagnosed AGE | Total no. of recruits on site | No. of cases approached to enroll | No. of samples collected | Participation rate (%) | No. of norovirus positive | No. of *Salmonella* positive | No. of *Shigella* positive | No. of *Campylobacter* positive |
|---|---|---|---|---|---|---|---|---|---|
| Recruit Training Command, Great Lakes, IL | 6,940 | 198,279 | 2,283 | 458 | 20.1 | 75 | 1 | 0 | 2 |
| Marine Corps Recruit Depot, San Diego, CA | 2,365 | 84,796 | 1,061 | 502 | 47.3 | 102 | 25 | 0 | 0 |
| Marine Corps Recruit Depot, Parris Island, SC | 2,605 | 95,703 | 1,411 | 427 | 30.3 | 121 | 13 | 0 | 0 |
| U.S. Army Training Center, Fort Leonard Wood, MO | 2,604 | 97,331 | 2,442 | 549 | 22.5 | 148 | 3 | 1 | 2 |
| Totals | 14,514 | 476,109 | 7,197 | 1,936 | 26.9 | 446 | 42 | 1 | 4 |

identified in 491 (25%) samples. NoV GI/II accounted for 90% of all positive testing results. Bacterial agents (*Salmonella*, *Shigella*, and *Campylobacter*) accounted for the remaining 10% **(Table)**.

Self-administerd rectal swabs accounts for approximately 40% of all specimens collected during the surveillance period. Of those, only 15.6% of all swabs collected yielded a pathogen-positive result, compared to 32.5% of all stool samples tested **(data not shown)**. All four *Campylobacter* were isolated from stool, while 10 times as many *Salmonellae* were isolated from stool as from rectal swabs, and three times as many NoV were detected from stool as from rectal swabs **(data not shown)**. Although comparable numbers of samples (449–539) were cumulatively collected from trainees during all seasons of the year, sporadic NoV cases and outbreaks were most prevalent during the fall (28%) and winter (37%) versus the spring (19%) and summer (8%) months **(Figure 1)**. *Salmonella* was identified at a lower, consistent rate across total enrollments, regardless of the season **(Figure 1)**. NoV GI was most commonly identified in specimens collected during 2011, while NoV GII was the predominant genotype in circulation among all recruit training sites in 2012–2015 **(Figure 2)**.

As of 30 September 2016, a total of 18 NoV GI- and 26 NoV GII-associated outbreaks had been identified across all sites since surveillance began in 2011 **(Figure 3)**.

**FIGURE 1.** Cumulative numbers of acute gastroenteritis (AGE) cases enrolled[a] and percentage distribution of pathogens identified among U.S. military recruits, all sites, by season, May 2011–September 2016




[a]Four enrollees failed to provide samples.

## EDITORIAL COMMENT

The enteric disease surveillance program was established to describe the epidemiology of AGE and NoV among U.S. DoD military recruits. Historically, most AGE outbreaks have been attributed to bacterial pathogens. However, surveillance data spanning from 2005 to 2012 have identified viral agents as the most common etiology in AGE outbreaks in military operational settings, with NoV the most commonly identified.[10] Surveillance data collected by

**FIGURE 2.** Numbers of acute gastroenteritis (AGE) cases enrolled and percentage distribution of pathogens identified among U.S. military recruits, all sites, by year, May 2011–September 2016

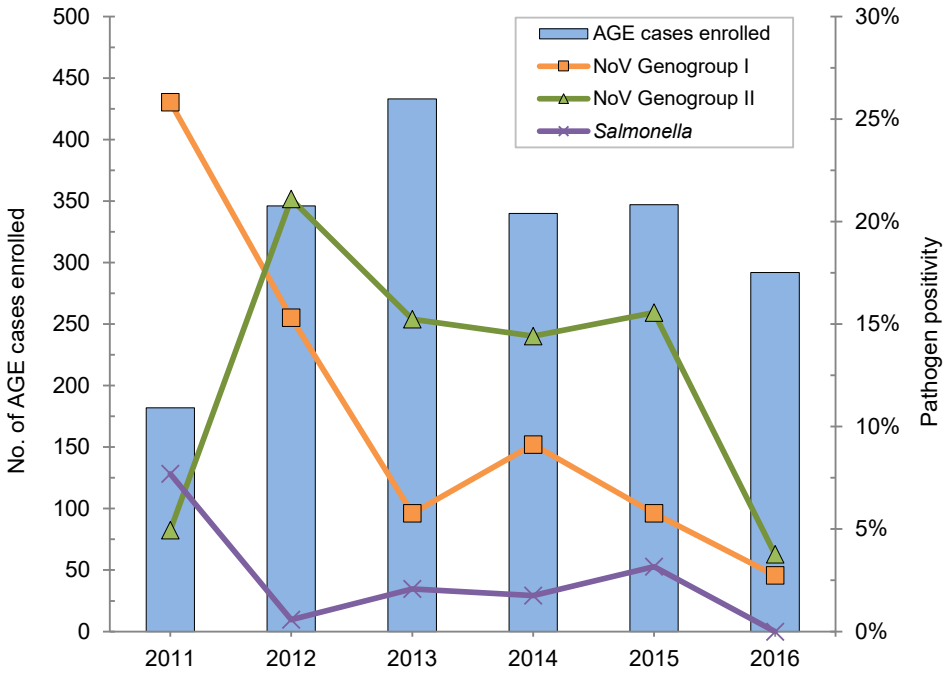
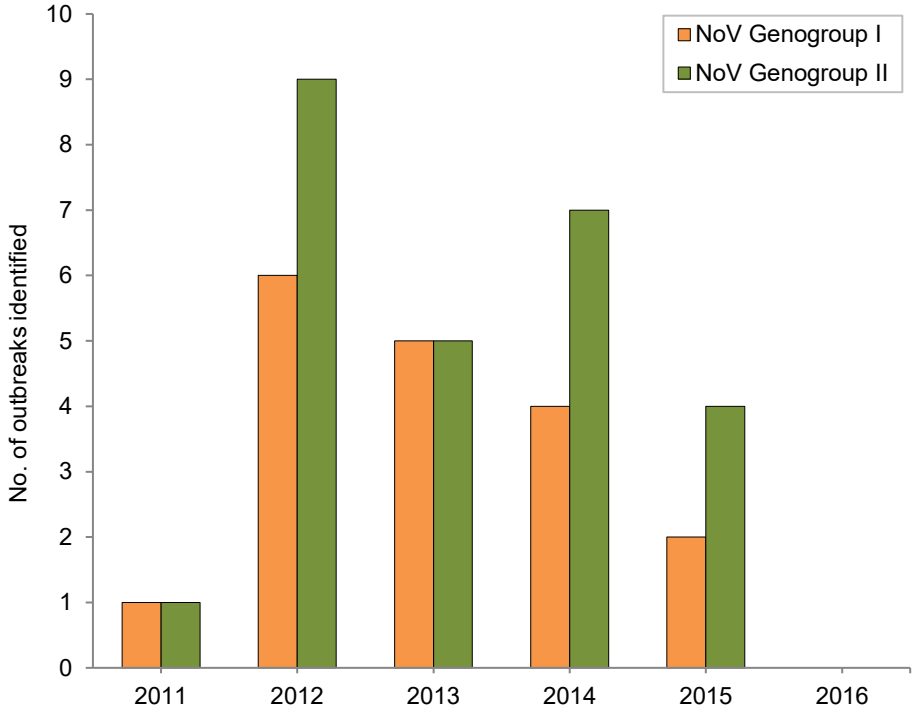



**FIGURE 3.** Numbers of norovirus outbreaks[a] identified by Naval Health Research Center Operational Infectious Diseases among U.S. military recruits, all sites, May 2011–September 2016




[a]Norovirus genogroup and genotype were reported to the CaliciNet national surveillance database per Centers for Disease Control and Prevention protocol.

NHRC OID support these findings. In this study, the observed trends relating to NoV seasonality, outbreak trends, and prevalence of GII circulation correspond to what has been previously described in the literature.[14,15] The reason for the dramatic drop in NoV burden in 2016 is unknown.

Among the enrollees with AGE studied in this report, etiologic agents were not identified by routine surveillance testing in approximately 75% of the total collected specimens. This pathogen recovery rate is low, compared with previous studies in which causative organisms were identified in approximately 45%–50% of all symptomatic cases of AGE.[16,17] The dissimilarity in NHRC OID's findings during the 2011–2016 surveillance period could be attributed to a variety of factors, including the study population, specimen type, testing methodologies, and pathogenic targets. The cited studies focus on long-term traveler and/or deployed military populations. Recruit populations are generally restricted to their respective facilities for the duration of their training and are not exposed to as many sanitation- and poverty-related AGE risk factors as the populations monitored in cited studies. Additionally, all trainees enrolled in the study met the required AGE case definition. The fact that such a high percentage were found to be pathogen-negative by the current methodologies could be indicative of the need to minimize the collection of self-administered rectal swabs and focus primarily on collecting and testing stool samples, the need for improved recovery methods, and/or the need for more sensitive detection techniques. Finally, low pathogen recovery rates could be due to the choice of bacterial and viral agents targeted in these initial efforts. More extensive testing of these samples would be required to determine whether other bacterial, viral, or parasitic pathogens are more prevalent than the currently targeted organisms.

Since its inception in 2011, NHRC OID's enteric disease surveillance program's initial surveillance efforts have succeeded in establishing a standardized method for tracking the incidence of, and determining common etiologies of, AGE among U.S. military recruits. Ultimately, data derived from this program have the potential to facilitate the development of

more targeted and effective AGE prevention and/or intervention policies and programs. Such policies and programs could help decrease the impact and burden of infectious gastrointestinal disease not only on military recruits and operational forces but on the general U.S. population as well.

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*Brief Report*

# Leptospirosis Seroconversion Surveillance Among U.S. Army Infantry Forces Assigned to South Korea, 2011–2014

*Hua-Wei Chen, PhD; Zhiwen Zhang, MS; Tatyana Belinskaya, MS; Wei-Mei Ching, PhD*

Leptospirosis is caused by spirochetes of the genus *Leptospira*. It is considered to be the most widespread zoonotic disease in the world.[1] A wide range of wild and domestic animals—notably cattle, pigs, dogs, rodents, and other small mammals—are natural reservoirs.[2] Infected animals excrete leptospires in their urine intermittently or regularly for months, years, or the rest of their lifetime.[3] In turn, human infection results from direct or indirect exposure to the urine of carrier animals.

In the Republic of Korea (ROK), human leptospirosis was first identified in 1984 as the cause of "epidemic pulmonary hemorrhagic fever."[4,5] There have been several more outbreaks reported between 1984 and 1999 in the ROK civilian population.[6] A leptospiral vaccination program was initiated in 1988 targeting high-risk groups in rural areas of the ROK and may have been responsible for the reduction of cases in the succeeding years. The program was discontinued in late 1997.[7] According to data from the Korea Centers for Disease Control and Prevention's National Notifiable Disease Surveillance System, the incidence of leptospirosis decreased between 1990 and 1999 (average annual incidence, 0.02 per 100,000 population) but has increased since then. From 2000 through 2013, there were 1,386 reported cases, and the average annual incidence was 0.22 per 100,000 population.[8]

The presence of leptospirosis in the ROK poses a potential threat to more than 40,000 U.S. Armed Forces personnel and their family members who reside in the ROK. Data or information relevant to the risk of leptospirosis to U.S. military personnel assigned to the ROK and their families is generally limited to case reports. The aim of the current study is to estimate the leptospirosis risk by measuring the frequency of acquisition of leptospiral antibodies among Army infantry soldiers assigned to the ROK.

## METHODS

The Defense Medical Surveillance System (DMSS), maintained by the Armed Force Health Surveillance Branch, was used to identify a cohort of U.S. Army infantry members who were assigned to the ROK for the first time for a minimum of 180 days from 1 January 2011 through 31 December 2014. Infantry soldiers were selected for study because they were more likely to work and train in field conditions that placed them at risk for leptospirosis infection. The Department of Defense Serum Repository was queried to identify serum specimens from infantry soldiers who had served in the ROK at any time during the years of interest. Only individuals with serum specimens collected within the 365 days before the start date of their ROK assignment (pre-tour) and additional serum specimens collected during the period 180 days after the start date to 180 days after the end date of ROK assignment (post-tour) were selected. If multiple qualifying pre-tour or post-tour specimens were available for an individual, then specimens collected closest to the ROK assignment start and end dates were selected. Service members born outside of the U.S. were excluded from the study. From the population of service members who met the inclusion criteria, 250 infantry soldiers were randomly selected for each year during the study period 2011–2014 to achieve the target sample size of 1,000. Because there were only 230 and 217 eligible subjects during 2012 and 2014, additional subjects were randomly selected from years 2011 and 2013 to achieve the target sample size. Overall, a total of 280 and 273 subjects were included for years 2011 and 2013. Information on sex, age, year stationed in the ROK, and number of prior overseas operational deployments was available for each specimen.

All 1,000 post-tour serum samples were screened at a dilution of 1:100 for immunoglobulin G against *Leptospira* with enzyme-linked immunosorbent assay (ELISA) using leptospiral recombinant antigens.[9] The post-tour serum samples with ELISA absorbances >0.32 at 405 nm were considered as positive for leptospiral-specific antibodies. Pre-tour samples were tested only if the corresponding service members' post-tour samples were positive. Seroconversion was defined as a change from a negative result to a positive result at a titer of at least 100 or a 4-fold rise in titers from the pre- to post- tour samples.

## RESULTS

The study population consisted of 1,000 males; of these, the majority were younger than 25 years old (69%) and most had had no prior operational deployments (70.6%) **(Table)**. The post-tour seropositivity among the infantry soldiers for antibodies against *Leptospira* at a titer >100 was 4.5% (45 of 1,000). Among them, 36 were also seropositive in their pre-tour specimens and only nine (0.9%) seroconverted during their assignment to ROK. Higher percentages of seropositive were found among those aged 30–34 years (8.2%) and 35–39 years (10.0%). Because only nine soldiers seroconverted in association with their assignment to ROK, there was no apparent correlation between seroconversion and

**TABLE.** Demographic and other characteristics of study sample

| | Total | | Seropositive case | | Seroconversion case | |
|---|---|---|---|---|---|---|
| Characteristic | N | % | N | % | N | % |
| Sex | | | | | | |
| Male | 1,000 | 100.0 | 45.0 | 4.5 | 9 | 0.9 |
| Age group (years) | | | | | | |
| 18–19 | 192 | 19.2 | 6 | 3.1 | 1 | 0.5 |
| 20–24 | 498 | 49.8 | 21 | 4.2 | 5 | 1.0 |
| 25–29 | 175 | 17.5 | 7 | 4.0 | 1 | 0.6 |
| 30–34 | 85 | 8.5 | 7 | 8.2 | 1 | 1.2 |
| 35–39 | 30 | 3.0 | 3 | 10.0 | 1 | 3.3 |
| 40+ | 20 | 2.0 | 1 | 5.0 | 0 | 0.0 |
| Stationed year | | | | | | |
| 2011 | 280 | 28.0 | 9 | 3.2 | 1 | 0.4 |
| 2012 | 230 | 23.0 | 14 | 6.1 | 2 | 0.9 |
| 2013 | 273 | 27.3 | 15 | 5.5 | 5 | 1.8 |
| 2014 | 217 | 21.7 | 7 | 3.2 | 1 | 0.5 |
| No. of prior operational deployments | | | | | | |
| 0 | 706 | 70.6 | 30 | 4.2 | 7 | 1.0 |
| 1 | 140 | 14.0 | 5 | 3.6 | 1 | 0.7 |
| 2 | 84 | 8.4 | 6 | 7.1 | 0 | 0.0 |
| 3 | 57 | 5.7 | 3 | 5.3 | 1 | 1.8 |
| 4+ | 13 | 1.3 | 1 | 7.7 | 0 | 0.0 |

age or number of prior operational deployments. The fact that five of the nine seroconverters were assigned to ROK during 2013 suggests an increased level of exposure for that year, but the small numbers overall preclude any firm conclusion about a temporal risk **(Table)**.

## EDITORIAL COMMENT

Leptospirosis is likely underreported among U.S. military personnel. In November 2014, there was a leptospirosis outbreak involving as many as 90 U.S. Marines who had been training at the Jungle Warfare Training Center, Camp Gonsalves, Okinawa, Japan.[10] One challenge to correctly diagnosing and promptly treating leptospirosis is that the symptoms of leptospirosis are easily confused with other febrile illnesses, such as dengue and malaria. Many cases of acute febrile illness are clinically diagnosed as malaria or dengue without using laboratory methods that can differentiate infectious pathogens including *Leptospira*. The lack of ready availability of laboratory support to diagnose leptospirosis undoubtedly contributes to underreporting. In addition, in endemic regions the majority of infections may not provoke serious symptoms and the requisite laboratory testing may never be performed.

This is the first published study for risk assessment of leptospirosis among U.S. Army soldiers assigned to the ROK. The distribution of the demographic and assignment characteristics of interest in the **Table** is comparable to the overall infantry population and other studies of Army infantry forces in ROK.[11,12] The results suggest a 0.9% seroconversion to leptospires, which is higher than the 0.2% seroconversion to scrub typhus and Japanese encephalitis among U.S. infantry forces in ROK.[11,12]

The standard microscopic agglutination test (MAT) is technically complex and time consuming and the culturing of live *Leptospira* to obtain the whole cell antigen is particularly labor intensive and requires special precautions to prevent infecting laboratory staff. In this study, ELISA was used instead of MAT because ELISA is much easier to perform and can test a large number of samples at the same time. Because the ELISA assay was about 90% sensitive as compared to MAT, it is likely there were some false negatives among the tested specimens. The 0.9% rate of seroconversion suggests a low but not zero level of risk for leptospirosis for infantry soldiers in the endemic region of the ROK. These results also indicate that some U.S. military personnel were exposed to leptospires before their assignment to ROK, as evidenced by the number of soldiers whose pre-tour serologic tests were positive.

Currently, there is no human vaccine available for leptospirosis in the U.S. The early diagnosis of the disease is important as antibiotic therapy with doxycycline or penicillin provides the greatest benefit when initiated early in the course of illness. The delay of antibiotic treatment of leptospirosis leads to higher morbidity and mortality. Soldiers who might be at an increased risk for infection should be educated on exposure risks and advised to consider preventive measures such as chemoprophylaxis; wearing protective clothing, especially footwear; and covering cuts and abrasions with occlusive dressings. Limited studies have shown that a weekly regimen of doxycycline before and continuing through the period of exposure can be used to prevent leptospira infections in endemic areas.[13-15] Military doctors should have heightened awareness of the possibility of leptospirosis in field settings worldwide and also when freshwater exposure occurs during training in the U.S.

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# Sampling Considerations for Detecting Genetic Diversity of Influenza Viruses in the DoD Global Respiratory Pathogen Surveillance Program

*Anthony C. Fries, PhD; William Gruner, MS; James Hanson, MS*

The Department of Defense (DoD) Global Respiratory Pathogen Surveillance Program annually monitors the genetic diversity of influenza viruses circulating in DoD beneficiary populations. This program relies on a global network of partners across the DoD to submit respiratory specimens throughout the influenza season. In previous seasons, representative specimens for sequencing were chosen because of cost and time restrictions associated with reliance on Sanger-based sequencing technology. The effect of this specimen prioritization for sequencing has not been previously examined in the respiratory surveillance program. Here, specimen prioritization was simulated by iteratively subsetting sequencing data sets from 1 October 2013 through 15 March 2017 to determine how prioritizing affects common metrics of genetic diversity. Prioritization of specimens did not meaningfully affect calculations of average influenza genetic diversity within seasons or subtypes. Because of the high genetic diversity of influenza, prioritizing resulted in fewer unique viruses and less accurate measures of geographic relationships although it still provided relevant estimates. Given the advent of cost-effective next-generation sequencing approaches, all programs should carefully consider how best to prioritize influenza sequencing to recover meaningful information on the evolutionary dynamics of the virus.

Genetically diverse influenza viruses represent an ever-evolving target requiring global efforts to track changes and monitor emerging strains.[1] The Department of Defense (DoD) Global Respiratory Pathogen Surveillance Program is an effort that annually monitors the circulation of influenza viruses in DoD healthcare beneficiaries by utilizing a network of collaborative partners across the military services.[2] This global network provides essential data, such as vaccine efficacy and genetic sequencing data, to collaborators at World Health Organization Collaborating Centers (WHO-CC) such as the Centers for Disease Control and Prevention (CDC) to assist in their selection of representative influenza strains for vaccine composition in subsequent influenza seasons.

Within the DoD program, respiratory specimens are collected at military treatment facilities (MTFs) and are shipped to and cultured at the U.S. Air Force School of Aerospace Medicine (USAFSAM) Epidemiology Laboratory where influenza-positive isolates are sequenced. Sequenced viruses are analyzed to examine essential aspects of virus evolution such as characterizing amino acid substitutions and genetic divergence measures. Phylogenetic trees are then constructed using statistical models that summarize the genetic diversity of the viruses to identify trends in viral relationships and global movement of strains in relation to current vaccine strains. Each of these analyses relies on the genetic variation that is contained within the captured data set; therefore, large data sets from broad geographic localities typically provide the most accurate representation of evolutionary dynamics of influenza viruses.[3]

In previous seasons, USAFSAM has utilized Sanger-based sequencing technology, which has limited the ability to sequence every influenza isolate that was cultured from submitted specimens, especially during the peak period of flu season. Specimens were instead prioritized for sequencing by selectively choosing specimens based on criteria that maximized the geographic and temporal distribution of the data as well as capturing severe or vaccine breakthrough specimens to efficiently and cost-effectively capture representative influenza genetic diversity. Other considerations included selection based on patient hospitalization and history of interaction with domestic animals. However, the effect of selecting representative specimens for sequencing on measures of genetic diversity has not been previously assessed in the surveillance program. In this study, sequence data were examined over four surveillance seasons to determine how specimen prioritization may affect surveillance efforts. Additionally, the effect of a shift to more efficient and higher-throughput next-generation sequencing methods and its potential impact on future surveillance efforts are discussed.

## METHODS

The surveillance period examined was 1 October 2013 through 15 March 2017 and represents two A(H1N1) pdm09 (2013–2014, 2015–2016) and two A(H3N2) (2014–2015, 2016–2017) predominant influenza seasons. All specimens discussed in these analyses were collected by the extensive network of partners within

the DoD Global Respiratory Pathogen Surveillance Program. The surveillance population for this program consists of active duty members from all services as well as other DoD beneficiaries, including dependents and retirees. Respiratory specimens were collected at MTFs from patients who presented with influenza-like illness (ILI), which was defined as the presence of fever (>100.5°F) and either cough or sore throat within 72 hours of symptom onset. The USAFSAM Epidemiology Laboratory tested all respiratory specimens using a real-time polymerase chain reaction (PCR) panel from the Centers for Disease Control and Prevention (CDC) (CDC Flu rRT-PCR Dx Panel) or, prior to the 2014–2015 season, an in-house laboratory developed test. In addition, specimens were cultured in primary monkey kidney cells for immunofluorescence-based testing and isolation of influenza viruses. In certain cases, sequencing was attempted on the original specimen when priority specimens tested positive by PCR but were negative for culture. Influenza viruses were sequenced for the hemagglutinin gene using Sanger-based sequencing chemistry on ABI 3130xL genetic analyzers per protocols developed in collaboration with the CDC. Sequences were assembled and analyzed using DNASTAR Lasergene,[4] BioEdit,[5] and MEGA software.[6]

For this study, hemagglutinin sequence data sets were compiled by influenza season (October–May): 2013–2014, 2014–2015, 2015–2016, and 2016–2017. Sequences were limited to locations inside the continental U.S. (CONUS) due to sample size considerations. To replicate the impact of specimen prioritization on measures of genetic diversity, an iterative approach was used whereby random subsets of sequence data sets were taken for a given season and subtype. Within a season, each subtype (e.g., A(H3N2)) was randomly sampled 50 times by varying percentages (25%, 50%, 75%) to capture a representative distribution of each molecular diversity measure for each percentage level. These values were then compared to the observed values in the complete data set for that subtype and season. For example, there were 695 A(H3N2) sequences in 2014–2015, so 50 data sets were replicated with 174 samples each (i.e., 25% percentage level) chosen at random from the 695 in the complete data set. Genetic metrics (discussed below) for each data set were compared to the 695 in the complete data set. Here, resampling without replacement was chosen so as to not bias estimates of the nucleotide diversity of influenza viruses.

The diversity at both a nucleotide (nt) and amino acid (aa) level were characterized using two categories of metrics that represent common measures of genetic diversity,[7] including metrics that represent average, summary characteristics of the entire sequence data set and metrics that represent raw counts of differences in the sequence data set. Average or summary metrics included the probability that two randomly chosen haplotypes (i.e., unique sequences) are different ($Hd_{nt}$ and $Hd_{aa}$—"nucleotide or amino acid haplotype diversity, respectively") and the average number of nucleotide or amino acid differences per site for two randomly chosen sequences ($\pi_{nt}$ and $\pi_{aa}$—"nucleotide or amino acid diversity, respectively"). Raw/count values included the number of nucleotide and amino acid haplotypes ($H_{nt}$ and $H_{aa}$) and the number of polymorphic/variable nucleotide or amino acid sites ($loci_{nt}$ and $loci_{aa}$). Of note, for a variable site to be considered real in these analyses and not an artifact of potential sequencing error, the substitution needed to occur in more than 0.5% of the sequences for that given data set.

Lastly, an analysis of molecular variance (AMOVA) test was used to determine whether the interpretation of the geographic distribution of influenza diversity would change due to prioritization. The AMOVA test uses a matrix of pairwise genetic distances among haplotypes to measure the amount of the total variation that may be attributable to some geographic grouping ($\varphi_{st}$).[8] For this test, the sequences were divided into Health and Human Services (HHS) regions as this was more statistically appropriate than either state or MTF-level groupings due to the greater variation and often low sample number represented at either the state or MTF level. The significance of each $\varphi_{st}$ value was assessed using a permutation framework where HHS regions were randomly assigned to influenza sequences, and the AMOVA was then rerun. The number of times that the observed $\varphi_{st}$ was greater than the permuted value (n=100) represented the p value. All analyses were conducted using R version 3.3.2[9] with packages poppr,[10] pegas,[11] and adegenet[12] (code available upon request).

## RESULTS

During the 4-year study period, USAFSAM sequenced an average of approximately 43% ± 5% of influenza-positive specimens received each season in the surveillance program. The data set analyzed here consisted of 2,497 sequences. The number of sequenced isolates (isolates per subtype per season) ranged from one A(H1N1)pdm09 influenza virus in 2014–2015 to 695 A(H3N2) influenza viruses in 2014–2015. For subsequent analyses, the A(H1N1)pdm09 data for seasons 2014–2015 and 2016–2017 were removed due to small sample sizes from CONUS sentinel sites (less than 20 per season), which affected calculations on the 25% data sets. The 100% category (i.e., non-subset) discussed below represents the observed value of the diversity metrics for the entire sequenced data set for a given season.

Genetic metrics varied among subtypes with the most diverse lineage being influenza A(H3N2) **(Table)**. Within subtypes, the summary values of π and Hd did not vary across the subsetting percentages **(Table, Figure 1)**. These summary metrics did not meaningfully change across seasons within a subtype suggesting a consistent amount of genetic diversity for a subtype over the surveillance period. The raw count metrics of H and loci did increase as the size of the data sets increased **(Table, Figure 2)**. Of particular note, these summary and count metrics should be interpreted together. For example, $Hd_{aa}$ can be interpreted as having a 97% probability of observing a new influenza A(H3N2) amino acid haplotype for the next surveillance specimen in the 2016–2017 season. Additionally, the number of loci did not always increase when the number of specimens for a given subtype was high (e.g., A(H3N2) 2014–2015) **(Table)**. This is an artifact of specifying a minor substitution frequency

**TABLE.** Common measures of genetic diversity[a] for influenza strains occurring in influenza seasons between 2013 and 2017 calculated on various data sets representing subset percentages of hemagglutinin sequences from each year

| | No. of specimens | $\pi_{nt}$ | $Hd_{nt}$ | $H_{nt}$ | $loci_{nt}$ | $\pi_{aa}$ | $Hd_{aa}$ | $H_{aa}$ | $loci_{aa}$ | $\varphi_{st}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| A(H1N1)pdm09 | | | | | | | | | | |
| 25% | | | | | | | | | | |
| 2013–2014 | 74 | 0.009 | 0.996 | 66 | 156 | 0.252 | 0.845 | 31 | 36 | 0.136 |
| 2015–2016 | 84 | 0.009 | 0.993 | 72 | 187 | 0.26 | 0.829 | 37 | 49 | 0.162 |
| 50% | | | | | | | | | | |
| 2013–2014 | 148 | 0.009 | 0.996 | 121 | 231 | 0.255 | 0.850 | 58 | 61 | 0.140 |
| 2015–2016 | 168 | 0.009 | 0.993 | 133 | 279 | 0.262 | 0.829 | 68 | 75 | 0.161 |
| 75% | | | | | | | | | | |
| 2013–2014 | 222 | 0.009 | 0.996 | 169 | 128 | 0.25 | 0.852 | 82 | 80 | 0.143 |
| 2015–2016 | 251 | 0.009 | 0.994 | 191 | 169 | 0.253 | 0.829 | 94 | 96 | 0.177 |
| 100%[b] | | | | | | | | | | |
| 2013–2014 | 296 | 0.009 | 0.996 | 216 | 152 | 0.25 | 0.852 | 105 | 95 | 0.144 |
| 2015–2016 | 335 | 0.009 | 0.994 | 245 | 210 | 0.25 | 0.830 | 117 | 112 | 0.172 |
| A(H3N2) | | | | | | | | | | |
| 25% | | | | | | | | | | |
| 2013–2014 | 7 | 0.012 | 1.000 | 7 | 56 | 0.728 | 0.972 | 6 | 23 | 0.372 |
| 2014–2015 | 174 | 0.013 | 0.997 | 149 | 318 | 0.8 | 0.971 | 99 | 91 | 0.053 |
| 2015–2016 | 20 | 0.014 | 0.995 | 19 | 106 | 0.824 | 0.980 | 17 | 36 | 0.154 |
| 2016–2017 | 126 | 0.014 | 0.996 | 108 | 268 | 0.775 | 0.969 | 75 | 77 | 0.072 |
| 50% | | | | | | | | | | |
| 2013–2014 | 14 | 0.012 | 1.000 | 14 | 83 | 0.774 | 0.973 | 12 | 33 | 0.379 |
| 2014–2015 | 348 | 0.013 | 0.997 | 273 | 247 | 0.804 | 0.970 | 165 | 121 | 0.062 |
| 2015–2016 | 40 | 0.014 | 0.996 | 37 | 154 | 0.831 | 0.981 | 31 | 47 | 0.163 |
| 2016–2017 | 252 | 0.014 | 0.996 | 201 | 197 | 0.783 | 0.970 | 129 | 104 | 0.083 |
| 75% | | | | | | | | | | |
| 2013–2014 | 21 | 0.012 | 1.000 | 21 | 101 | 0.774 | 0.971 | 17 | 40 | 0.403 |
| 2014–2015 | 521 | 0.013 | 0.997 | 386 | 227 | 0.801 | 0.970 | 223 | 141 | 0.063 |
| 2015–2016 | 60 | 0.014 | 0.996 | 54 | 188 | 0.842 | 0.982 | 44 | 55 | 0.168 |
| 2016–2017 | 379 | 0.014 | 0.996 | 287 | 243 | 0.781 | 0.970 | 177 | 123 | 0.082 |
| 100% | | | | | | | | | | |
| 2013–2014 | 28 | 0.012 | 1.000 | 28 | 112 | 0.75 | 0.971 | 21 | 45 | 0.396 |
| 2014–2015 | 695 | 0.013 | 0.997 | 488 | 219 | 0.8 | 0.969 | 276 | 157 | 0.063 |
| 2015–2016 | 80 | 0.014 | 0.996 | 69 | 215 | 0.85 | 0.981 | 55 | 61 | 0.173 |
| 2016–2017 | 505 | 0.014 | 0.996 | 369 | 207 | 0.8 | 0.970 | 222 | 137 | 0.083 |
| B/Victoria | | | | | | | | | | |
| 25% | | | | | | | | | | |
| 2013–2014 | 5 | 0.007 | 0.968 | 5 | 24 | 0.245 | 0.918 | 4 | 6 | 0.264 |
| 2014–2015 | 9 | 0.006 | 0.983 | 8 | 35 | 0.249 | 0.934 | 7 | 9 | 0.133 |
| 2015–2016 | 24 | 0.005 | 0.983 | 21 | 67 | 0.250 | 0.793 | 12 | 18 | 0.123 |
| 2016–2017 | 8 | 0.006 | 0.991 | 8 | 34 | 0.285 | 0.906 | 6 | 9 | 0.194 |
| 50% | | | | | | | | | | |
| 2013–2014 | 10 | 0.006 | 0.963 | 8 | 33 | 0.236 | 0.912 | 7 | 8 | 0.268 |
| 2014–2015 | 18 | 0.006 | 0.976 | 15 | 52 | 0.249 | 0.926 | 11 | 12 | 0.165 |
| 2015–2016 | 48 | 0.005 | 0.981 | 38 | 104 | 0.235 | 0.784 | 20 | 25 | 0.118 |
| 2016–2017 | 16 | 0.006 | 0.989 | 15 | 53 | 0.264 | 0.894 | 10 | 14 | 0.190 |
| 75% | | | | | | | | | | |
| 2013–2014 | 16 | 0.007 | 0.967 | 13 | 43 | 0.243 | 0.922 | 10 | 10 | 0.188 |
| 2014–2015 | 27 | 0.006 | 0.978 | 21 | 68 | 0.250 | 0.929 | 15 | 15 | 0.164 |
| 2015–2016 | 73 | 0.005 | 0.982 | 54 | 136 | 0.242 | 0.789 | 27 | 32 | 0.122 |
| 2016–2017 | 23 | 0.006 | 0.989 | 21 | 66 | 0.275 | 0.893 | 14 | 18 | 0.226 |
| 100% | | | | | | | | | | |
| 2013–2014 | 21 | 0.007 | 0.967 | 15 | 49 | 0.250 | 0.919 | 12 | 12 | 0.179 |
| 2014–2015 | 36 | 0.006 | 0.978 | 26 | 80 | 0.250 | 0.932 | 18 | 18 | 0.165 |
| 2015–2016 | 97 | 0.005 | 0.982 | 68 | 160 | 0.250 | 0.789 | 33 | 37 | 0.121 |
| 2016–2017 | 31 | 0.006 | 0.989 | 27 | 81 | 0.250 | 0.890 | 18 | 22 | 0.211 |
| B/Yamagata | | | | | | | | | | |
| 25% | | | | | | | | | | |
| 2013–2014 | 12 | 0.007 | 0.967 | 10 | 48 | 0.165 | 0.819 | 6 | 7 | 0.292 |
| 2014–2015 | 18 | 0.006 | 0.977 | 16 | 66 | 0.137 | 0.783 | 8 | 10 | 0.053 |
| 2015–2016 | 40 | 0.011 | 0.992 | 35 | 134 | 0.250 | 0.781 | 14 | 24 | 0.289 |
| 2016–2017 | 22 | 0.007 | 0.981 | 19 | 75 | 0.129 | 0.764 | 9 | 10 | 0.073 |
| 50% | | | | | | | | | | |
| 2013–2014 | 24 | 0.007 | 0.968 | 19 | 75 | 0.167 | 0.835 | 11 | 12 | 0.306 |
| 2014–2015 | 36 | 0.006 | 0.979 | 30 | 109 | 0.144 | 0.779 | 12 | 17 | 0.076 |
| 2015–2016 | 80 | 0.011 | 0.991 | 64 | 176 | 0.243 | 0.779 | 21 | 31 | 0.310 |
| 2016–2017 | 45 | 0.007 | 0.981 | 35 | 110 | 0.137 | 0.763 | 15 | 16 | 0.074 |
| 75% | | | | | | | | | | |
| 2013–2014 | 37 | 0.007 | 0.967 | 28 | 98 | 0.16 | 0.829 | 14 | 16 | 0.314 |
| 2014–2015 | 54 | 0.007 | 0.978 | 42 | 143 | 0.141 | 0.781 | 16 | 25 | 0.070 |
| 2015–2016 | 119 | 0.011 | 0.992 | 91 | 210 | 0.25 | 0.782 | 27 | 36 | 0.313 |
| 2016–2017 | 68 | 0.007 | 0.981 | 50 | 132 | 0.137 | 0.768 | 20 | 20 | 0.074 |
| 100% | | | | | | | | | | |
| 2013–2014 | 49 | 0.007 | 0.969 | 37 | 118 | 0.15 | 0.830 | 18 | 20 | 0.319 |
| 2014–2015 | 72 | 0.006 | 0.978 | 54 | 170 | 0.15 | 0.782 | 20 | 31 | 0.064 |
| 2015–2016 | 159 | 0.011 | 0.991 | 116 | 236 | 0.25 | 0.782 | 32 | 41 | 0.319 |
| 2016–2017 | 90 | 0.007 | 0.980 | 62 | 150 | 0.15 | 0.769 | 24 | 23 | 0.075 |

[a]The average number of nucleotide/amino acid differences per site for two randomly chosen sequences ($\pi_{nt}/\pi_{aa}$); the probability that two randomly chosen nucleotide/amino acid haplotypes are different ($Hd_{nt}/Hd_{aa}$); the number of nucleotide/amino acid haplotypes ($H_{nt}/H_{aa}$); the number of nucleotide/amino acid polymorphic sites ($loci_{nt}/loci_{aa}$); $\varphi_{st}$ is a measure examining the amount of genetic variation attributable to HHS region.

[b]100% represents the entire sequenced data set for a given season and subtype.

**FIGURE 1.** Average haplotype diversity measurements for amino acid sequences ($Hd_{aa}$) for each subtype by season across each of the representative percentage levels

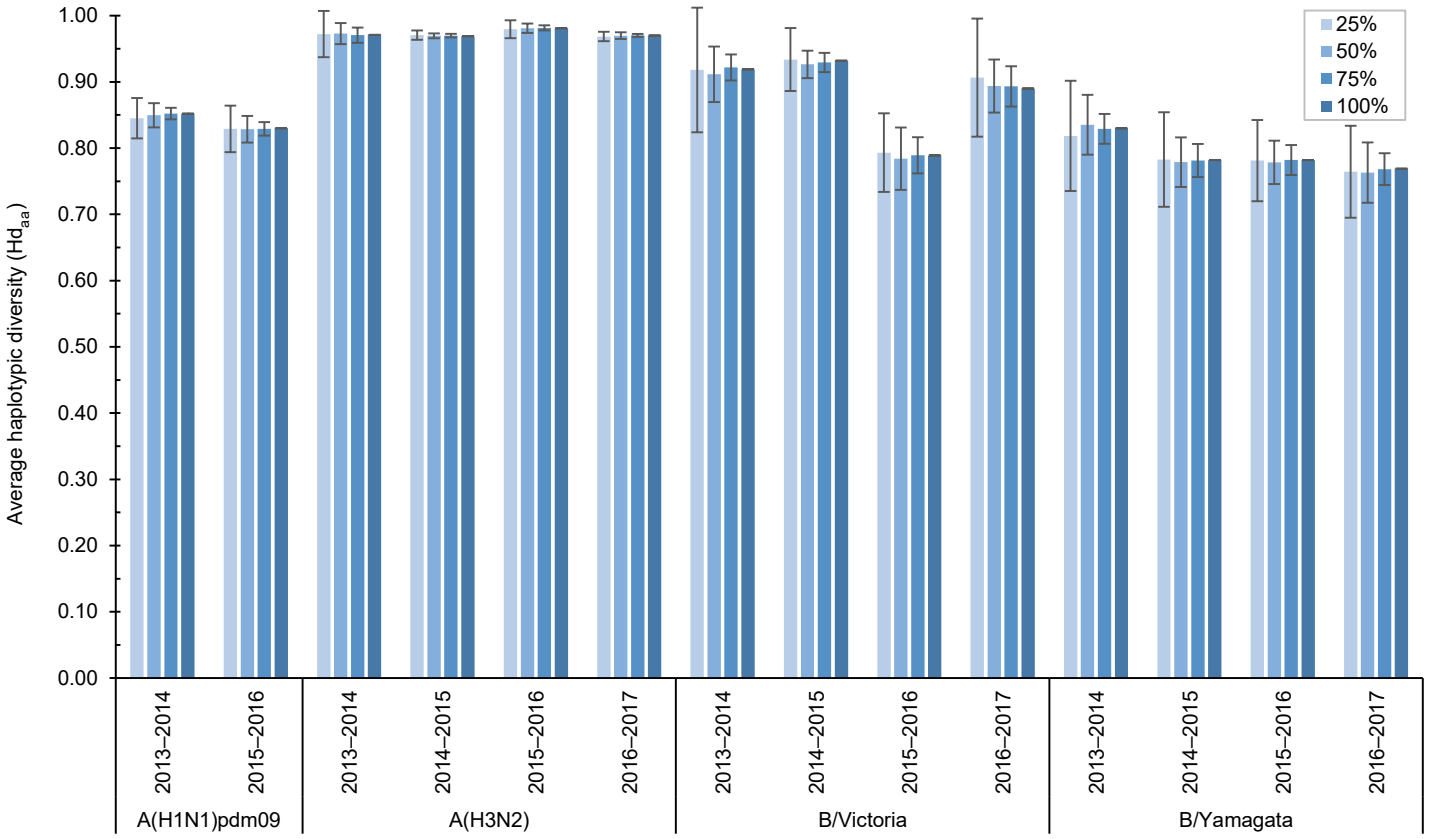



**FIGURE 2.** Average number of amino acid haplotypes ($H_{aa}$) for each subtype by season across each of the representative percentage levels

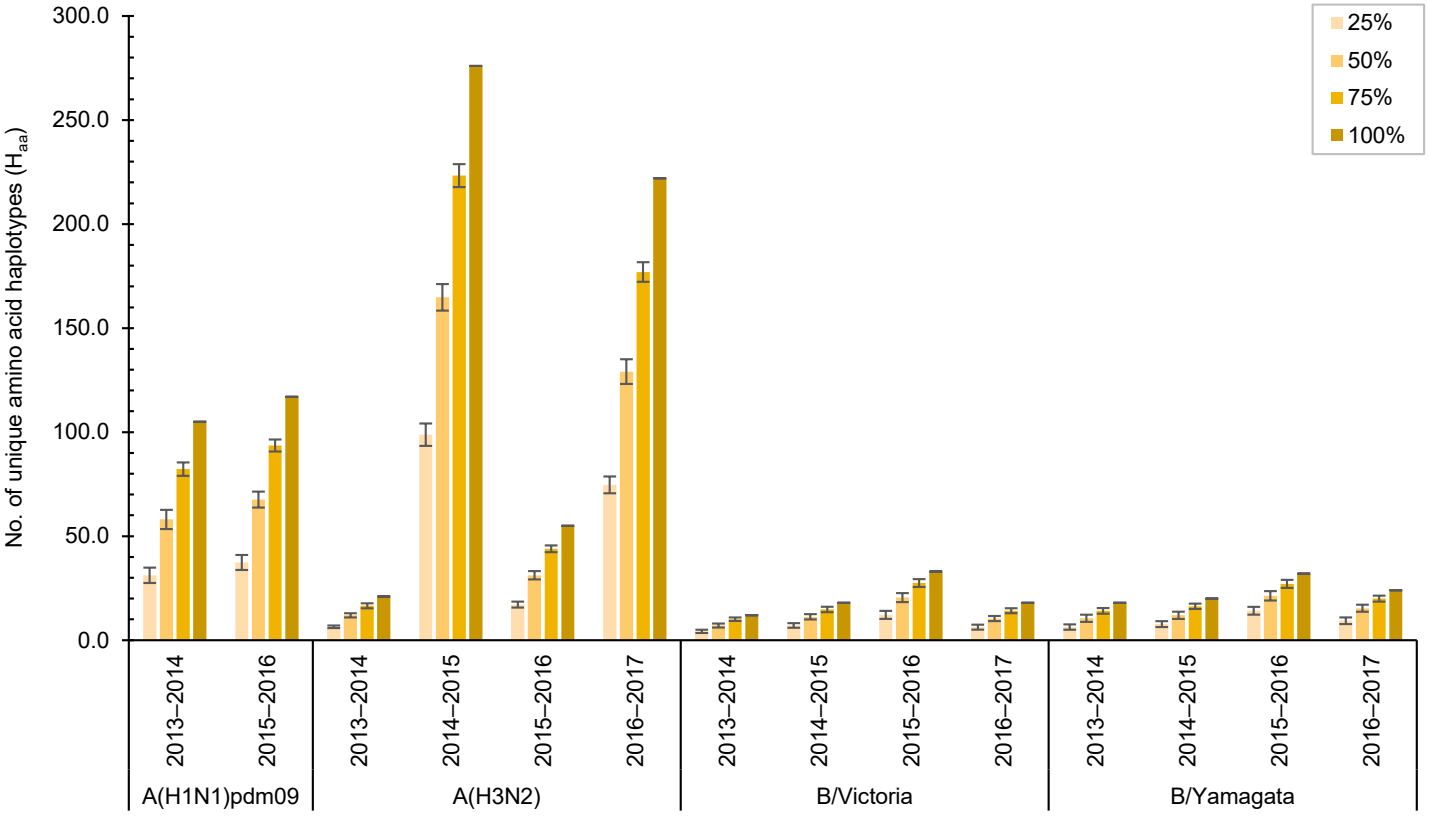
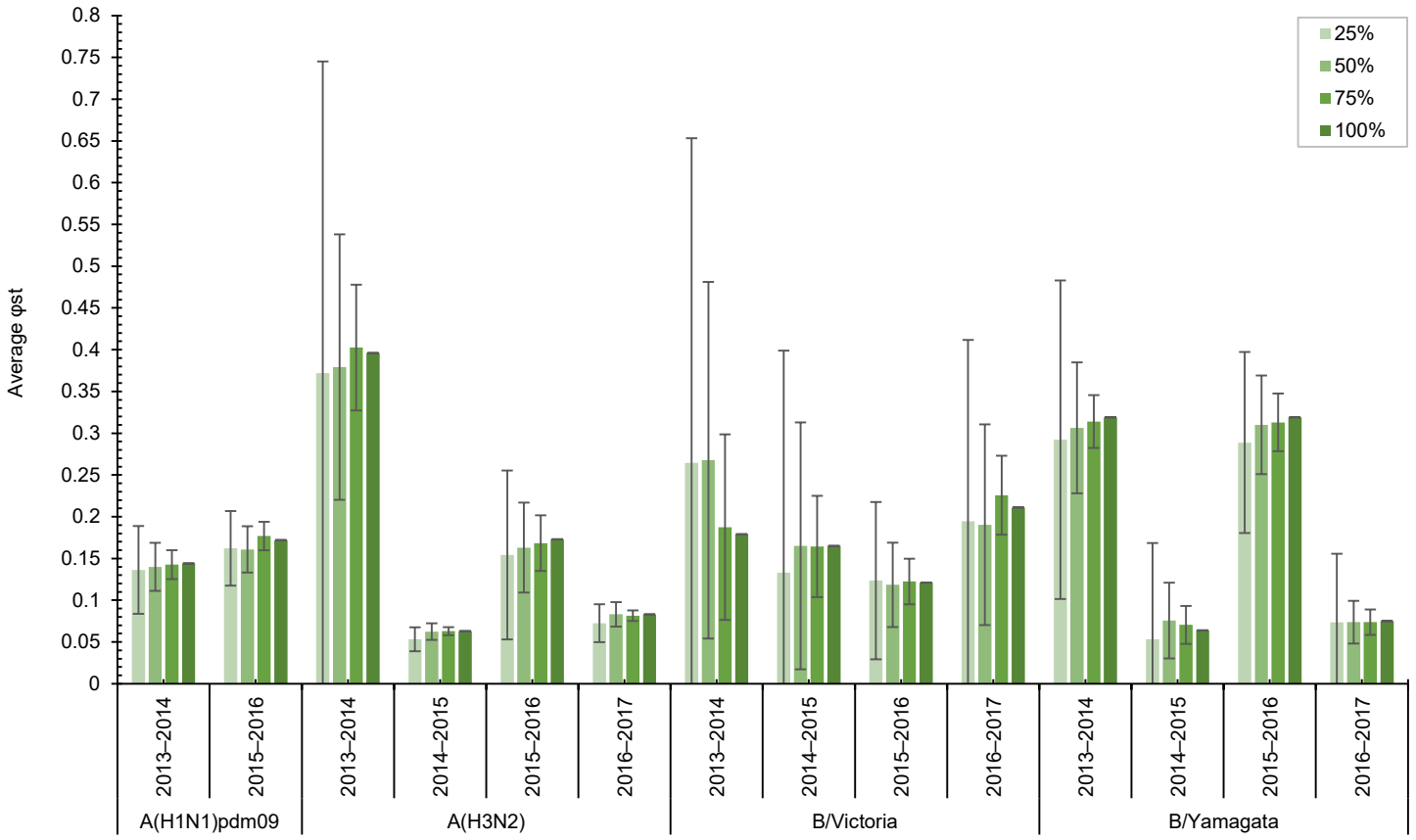


**FIGURE 3.** Average percentage of genetic variation explained by Health and Human Services regions ($\varphi_{st}$) for each subtype by season across each of the representative percentage levels. Significance for each 100% data set was significant at the 0.05 level.




of 0.5% to identify a true substitution. Furthermore, because the representative data set is subsetted to smaller numbers, the total number of sequences needed to classify a substitution at 0.5% decreases.

Using the AMOVA test, the amount of pairwise genetic diversity described in each subtype and season by region ($\varphi_{st}$) indicated that the distribution of sequences collected from HHS regions explained a portion of genetic variation observed in the 100% data sets **(Table, Figure 3)**, although it varied across subtypes. This result suggests that prioritizing for geographically diverse locations to capture more genetically divergent sequences is worthwhile especially if one has small numbers of specimens in any given year. However, the $\varphi_{st}$ values in subset data sets were highly variable indicating inaccurate measures at smaller subsets and an increased likelihood of misinterpreting the geographic relationships of viruses when prioritizing **(Figure 3)**. All $\varphi_{st}$ values were significant at $p \leq 0.01$.

## EDITORIAL COMMENT

This analysis showed that metrics that quantify the average diversity of a representative data set (Hd, $\pi$) are well estimated when prioritizing specimens. This is unsurprising, as diversity should not change as a function of the number of specimens collected because the mutation rate of the influenza virus is typically consistent in its host environment.[13,14] To this point, even in the 75% subset category, a consistently high probability of observing new nucleotide or amino acid haplotypes was identified, regardless of subtype, with every additional specimen sequenced.

Considering influenza's propensity to quickly sweep across the globe, surveillance systems must be vigilant for new and emerging strains and mutations.[15] Each new mutation represents an opportunity for a strain to gain a selective advantage. Admittedly, many unique haplotypes result from one-off mutations (i.e., a single nucleotide or amino acid change) that are potentially deleterious or never observed again[16] but still may be informative. First, one or two amino acid mutations can influence significant phenotypic advantages.[17] Second, models that predict how influenza will evolve in future seasons or what clades may dominate in subsequent years depend on accurate estimates of the frequencies of all mutations.[18] Therefore, sequencing suitable numbers of specimens to accurately represent mutational frequencies across the globe is essential for effective public health of both military and general populations.

The need to sequence greater numbers of specimens is often offset by the effort to minimize costs. The recent advent of next-generation sequencing (NGS) methods offers a cost-effective mechanism in which to genotype ever-increasing numbers of specimens.[19] With NGS onboarding in many key public health laboratories, it is now possible to quickly screen genotypes allowing public health agencies to identify specimens that are deemed worthy of further antigenic or phenotypic characterization. Additionally, NGS methods typically encompass full genome sequencing efforts that provide added information such as identifying reassortment events among strains[20] and/or characterizing resistance markers albeit with greater bioinformatics demands.[21] Nevertheless, while the cost of sequencing will continue to drop, prioritizing influenza viruses for sequencing based on useful geographical, temporal, or clinical characteristics appears to be an effective means to capture influenza dynamics.

Several limitations exist in this study. First, this work is not directly comparable to other WHO-CC surveillance efforts in that the USAFSAM surveillance population is comprised of highly-vaccinated, active duty service members that may be interconnected and not representative of the broader general public. Second, given the sample sizes among subtypes and regions, the analysis was limited to CONUS, and the results and interpretation may change if the data were to include an OCONUS (outside the continental U.S.) perspective, which would likely increase the effect of geography on genetic diversity. Third, the interpretation discussed in this study is related only to the hemagglutinin segment and may change considerably if compared to nonsurface segments such as the matrix protein, which represents lower diversity but would still contribute additional haplotypes with increased sampling.[22] Lastly, this analysis did not include any interpretation of the effect of subsetting sequencing efforts in relation to phylogenetic tree building and instead only alluded to the diversity that goes into phylogenies. Future studies would be well served in considering the impact of subsetting data sets on resulting phylogenies.

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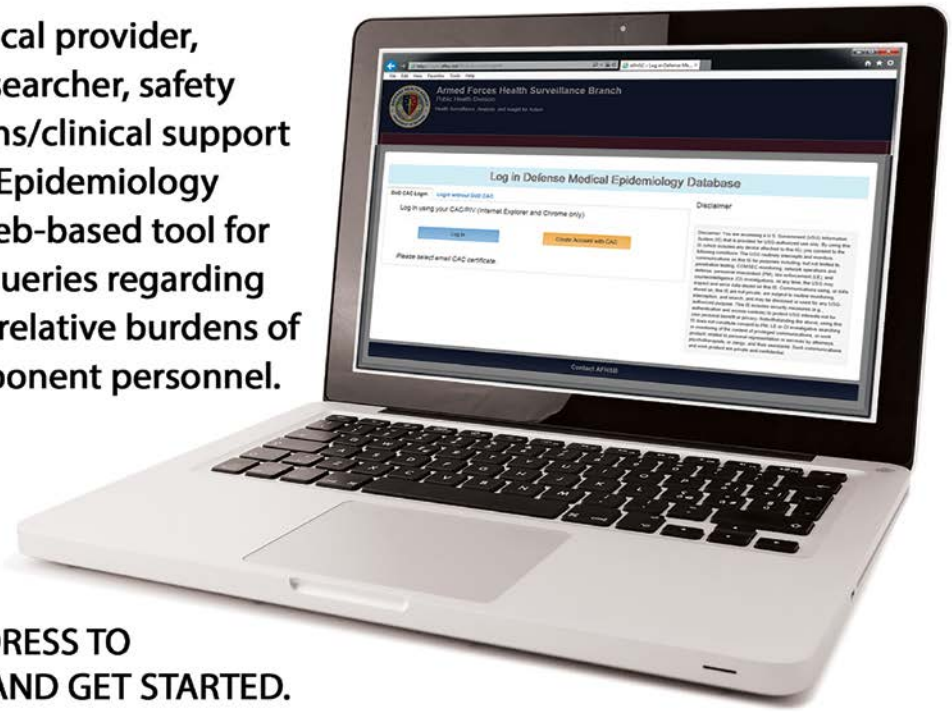


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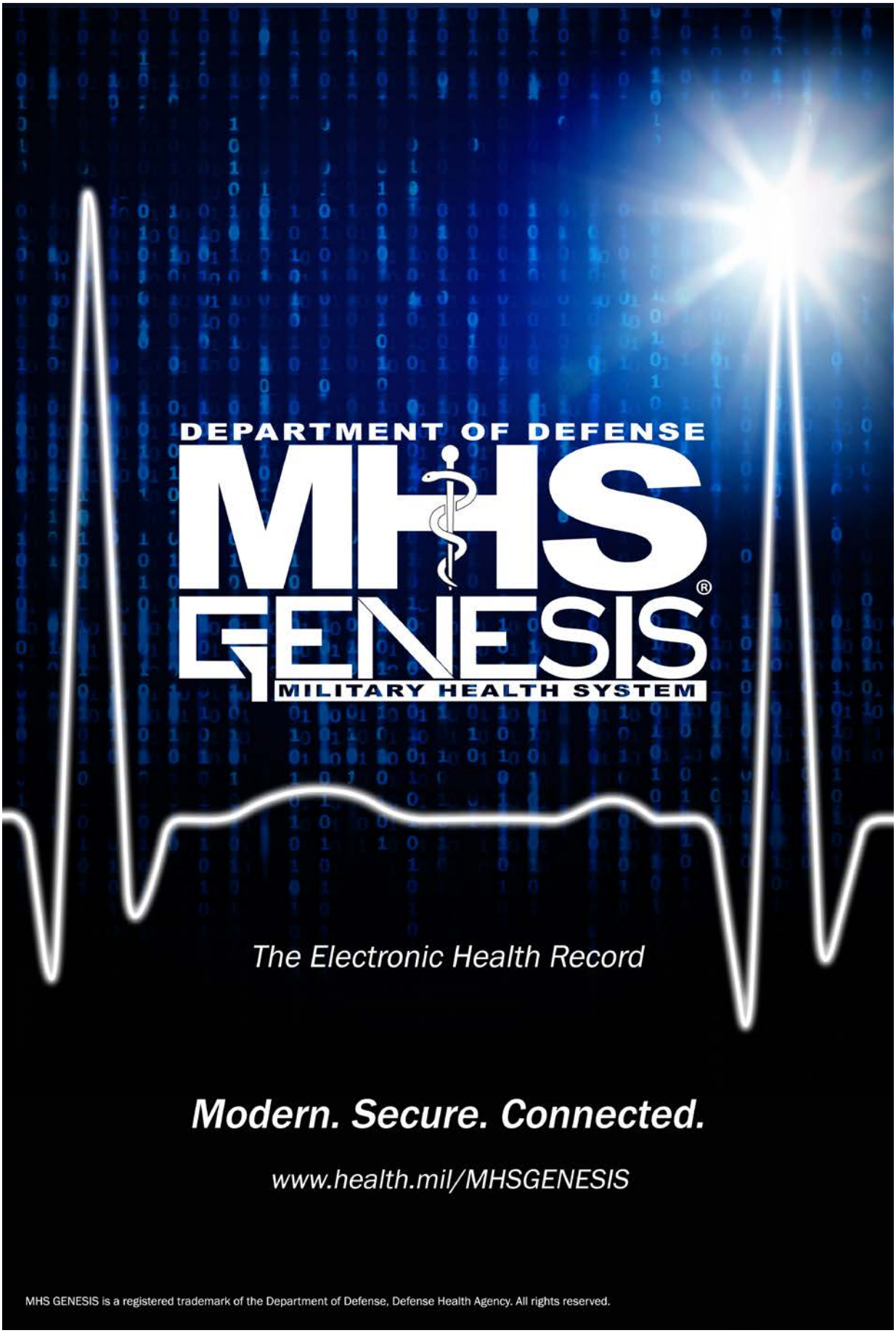
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