Applications of PCR (Real-Time and MassTag) and Enzyme-Linked Immunosorbent Assay in Diagnosis of Respiratory Infections and Diarrheal Illness Among Deployed U.S. Military Personnel During Exercise Balikatan 2009, Philippines

John Mark S. Velasco, MD, MPH; COL In-Kyu Yoon, MC USA*; COL Carl J. Mason, MC USA†; MAJ Richard G. Jarman, MS USA*; Ladaporn Bodhidatta, MD†; Chonticha Klungthong, PhD*; Sasikorn Silapong, BSc†; Maria Theresa G. Valderama, RMT*; Tippa Wongstitwilairoong, RMT, MS‡; LT Arturo G. Torres, MC USN§; LT Daniel P. De Cecchis, MC USN§; COL Julie A. Pavlin, MC USA‡*

ABSTRACT Laboratory-based surveillance for diarrheal and respiratory illness was conducted at the 2009 Republic of the Philippines–United States Balikatan exercise to determine the presence of specific pathogens endemic in the locations where the military exercises were conducted. Ten stool and 6 respiratory specimens were obtained from individuals meeting case definitions for diarrhea or respiratory illness. Stool specimens were frozen in dry ice and remotely tested using enzyme-linked immunosorbent assay for *Rotavirus*, *Astrovirus*, *Adenovirus*, *Entamoeba histolytica*, *Giardia,* and *Cryptosporidium* and polymerase chain reaction for enterotoxigenic *Escherichia coli*, *Campylobacter*, *Shigella*, *Vibrio*, *Salmonella,* and *Norovirus*. Eight (4 for *Campylobacter jejuni*, 2 for *Campylobacter coli*, 1 for *Norovirus* genogroup II, and 1 for both *Campylobacter coli* and enterotoxigenic *Escherichia coli*) of 10 samples were positive for at least 1 enteric pathogen. MassTag polymerase chain reaction for influenza A and B, respiratory syncytial virus groups A and B, human coronavirus-229E and human coronavirus-OC43, human metapneumovirus, enterovirus, human parainfluenza viruses 2,3, and 4a, human adenovirus, *Haemophilus influenzae*, *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Legionella pneumonia*, and *Mycoplasma pneumonia* was done on respiratory specimens. Out of 6 samples, 3 tested positive for *H. influenzae*; 1 tested positive for both *H. influenzae* and human parainfluenza virus 3; and 2 tested negative. Laboratory-based surveillance can be useful in determining etiologies of diarrheal and respiratory illness of deployed military personnel.

INTRODUCTION

Diarrheal and acute respiratory illnesses are considered important health concerns among military personnel during deployments.[1–3] Infectious diarrhea is one of the more common medical problems associated with military operations,[4] with the disease documented to cause significant loss in person-days and increase in health care utilization.[4–6] Acute respiratory infections and influenza can also have significant operational implications especially in the setting of local outbreaks.

The U.S. military has made contributions to the study of diarrheal[7] and respiratory diseases,[8] and it also regularly conducts military exercises with foreign militaries to promote increased readiness and interoperability and to provide humanitarian relief needs. The Republic of the Philippines–United States (RP–US) Exercise Balikatan, which translates from Tagalog into "shoulder-to-shoulder," is an annual military exercise, which brings together the U.S. and Philippine armed forces for bilateral exercises in the Philippines covering approximately 10 days. Conditions existing during field operations such as crowded living conditions may have untoward effects on operational readiness and efficiency of military forces and could make them more susceptible to disease outbreaks (e.g., respiratory and diarrheal).[9] Laboratory-based disease surveillance can be useful during military operations to confirm the presence of specific pathogens and lead to more accurate assessments of the local infectious disease threat. This information may also be useful in formulating effective prevention and treatment strategies for these acute illnesses in deployed military personnel. Because of the frequent lack of on-site diagnostic capabilities, specimens may need to be frozen on-site and tested remotely using more advanced diagnostic capabilities such as polymerase chain reaction (PCR).

*Department of Virology, Armed Forces Research Institute of Medical Sciences, 315/6 Rajvithi Road, Bangkok, Thailand.

†Department of Enteric Diseases, Armed Forces Research Institute of Medical Sciences, 315/6 Rajvithi Road, Bangkok, Thailand.

‡Global Emerging Infections Surveillance and Response System, Armed Forces Research Institute of Medical Sciences, 315/6 Rajvithi Road, Bangkok, Thailand.

§31st Marine Expeditionary Unit, Camp Hansen, Marine Corps Base Camp Smedley D. Butler, Unit 35621, FPO AP 96606-5621, Okinawa, Japan.

The views expressed in this article are those of the authors and do not reflect the official policy or position of the Department of the Army, Department of Defense, or U.S. Government.

Here, we describe the use of this laboratory-based surveillance strategy to characterize diarrheal and respiratory disease burden among Marines and Navy personnel deployed during the RP–U.S. Exercise Balikatan 2009.

METHODS

Study Patients

Specimens were collected from U.S. active duty personnel of the 31st Marine Expeditionary Unit (MEU) deployed from April 16 to 26, 2009 for the military exercise in Luzon, Philippines, at Crow Valley and an aviation combat element composed mostly of Marines and some Navy support personnel who were deployed at Clark Air Base and presenting for medical care with a chief complaint of acute diarrhea or acute respiratory symptoms. The exact number of U.S. military personnel who would normally seek care at these aid stations was not available. However, the medical units on site were responsible for the medical care of about 3,000 U.S. military personnel during the exercise.

Study Sites

Two Battalion Aid Stations (BASs) located in Crow Valley (one at main camp and one at forward camp), Capas, Tarlac, and one BAS at Clark Air Base in Angeles, Pampanga, all located in Northern Luzon, were selected as surveillance sites (Fig. 1). Crow Valley is a flood-prone valley with average temperatures ranging from 70°F to 93°F annually. During the summer months when Balikatan is conducted, the average rainfall is usually less than two inches, but during the rainy months, the small stream running across the valley becomes a deep river. Makeshift shanties operated by local civilian vendors offering various viands such as cooked meats, vegetables, rice, etc., which served as "cafeterias," were set up near the U.S. and Philippine camps. These "cafeterias" sold local food and drinks and were frequented by both U.S. and Philippine forces. The forward camp where the actual military exercises took place was located ~8 km from the Crow Valley main camp. Food for U.S. personnel in the forward camp was mostly sourced from Meals Ready to Eat (MRE), whereas those situated in the Crow Valley main camp had an option to eat at the mess area of the camp, consume their MREs, or eat at the local "cafeterias."

The other surveillance site was the BAS located within Clark Air Base. The U.S. personnel based in Clark were housed in accommodations within the Clark Freeport Zone area. The site is urbanized with restaurants, cafeterias, and malls. There were other BAS facilities serving troops in various other Balikatan sites in the Philippines, but because of logistical constraints, only the locations mentioned above were covered by the surveillance.

Initial coordination was done together with the U.S. 31st MEU and the U.S. Army Medical Component–Armed Forces Research Institute of Medical Sciences (USAMC–AFRIMS). Supplies including viral transport media (VTM; Remel, Lenexa, Kansas) and sterile Dacron swabs for respiratory specimen collection, cups for stool specimen collection, and dry ice were provided to the 31st MEU BAS medical staff by USAMC–AFRIMS. BAS medical staff was briefed about appropriate specimen collection and handling before start of actual collection. U.S. active duty personnel who presented for medical care with a chief complaint of acute diarrhea (3 or more unformed stools during a 24-hour period within 72 hours of presentation) or acute respiratory symptoms (history of fever [≥100.4°F] with cough or sore throat) were considered eligible for inclusion in the surveillance program. If the patient gave his verbal agreement, one stool specimen (for acute diarrheal symptoms) or two nasal swabs (for acute respiratory symptoms) were collected and frozen on-site. Stool specimens were collected at the BAS by providing stool cups to patients to collect samples. Approximately 4–10 g of stool were collected, and 3 aliquots of stool (approximately 1 g each) were prepared. Stool specimens were placed immediately on dry ice after aliquoting. For respiratory specimens, 2 nasal swabs were collected from each patient. A Dacron-tipped swab was inserted 1–2 cm into each nostril, rotated for approximately 5 seconds, and placed into a tube containing VTM. Nasal specimens were placed immediately on dry ice. In addition, a questionnaire about clinical symptoms, demographics, and exposures was completed by the BAS staff. All specimen containers and corresponding clinical questionnaires were anonymized by labeling only with a unique identification number and the date of collection at the field site with no link between the identification number and the patient's name. The specimens were transported on dry ice from the field site to Manila (2-hour drive away) every 3–4 days and placed in a –70°C freezer for

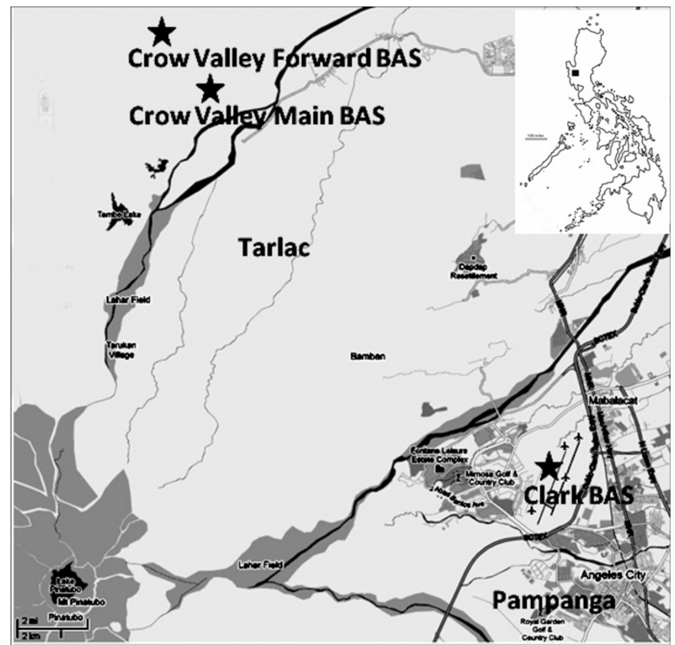

FIGURE 1. Map showing approximate locations of BASs. (Source: Google maps)

more extended storage. After the conclusion of the exercise, the specimens were shipped on dry ice to the AFRIMS laboratory in Bangkok, Thailand, for advanced testing.

Laboratory Testing

At the AFRIMS laboratory, enzyme-linked immunosorbent assay for *Rotavirus*, *Astrovirus,* and *Adenovirus* (Ridascreen; R-Biopharm, Darmstadt, Germany) and *Entamoeba histolytica*, *Giardia,* and *Cryptosporidium* (ProSpecT; Thermo Fisher Scientific Remel Products, Lenexa, Kansas) was performed on frozen stool specimens following the manufacturers' instructions. Real-time reverse transcriptase PCR (rRT-PCR) assays were performed on stool specimen extracts to detect enterotoxigenic *Escherichia coli* (ETEC; producing heat-labile toxin, heat-stable toxin Ia, and heat-stable toxin Ib), *Campylobacter jejuni, C. coli, Shigella spp., Vibrio cholera, Salmonella typhi,* and nontyphoidal *Salmonella*. Nucleic acid extracts were prepared using a NucliSens Magnetic Nucleic Acid Isolation Kit (cat. no.1055260; BioMérieux, Durham, North Carolina) on a 10% stool suspension following the manufacturer's instructions. Purified nucleic acid extracts were tested immediately by real-time PCR or stored at –70°C until ready for testing. Target genes of specific pathogens tested in this study were as follows: heat-labile toxin (LT), heat-stable toxin Ib (STIb), and heat-stable toxin Ia (STIa) for ETEC;[10,11] transport protein (ceuE) for *C.jejuni* and *C. coli*;[12] invasion plasmid antigen H (ipaH) for *Shigella spp.*;[13] hemolysin A (hlyA) and cholera toxin (ctx) for *V. cholera;*[14,15] flagellin gene (fla) for *Salmonella spp.*;[16] open reading frame (ORF) 1–ORF 2 junction for *Norovirus* GI and GII;[17] and Vi antigen (viaB) for *S. typhi*.[18] Reaction mixtures were incubated in the ABI7900 Sequence Detection System (Applied Biosystems, Foster City, California) or Rotor-Gene 6000 System (Corbett Research, Sydney, Australia). The assays were considered positive when the number of cycles to detect the amplified product for a pathogen was less than 40 units.

Viral RNA Extraction and Detection of Influenza Viruses by rRT-PCR (Respiratory Samples)

QIAamp Viral RNA Mini kit (QIAGEN, Valencia, California) was used to extract viral RNA from 140 µL of liquid mixed with the swab sample inside each of the VTM tubes. RNA was used as a template in the one-step rRT-PCR using SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen). The procedure was performed according to the Centers for Disease Control and Prevention rRT-PCR protocol for detection and characterization of influenza (version 2007)[19] with the addition of 5 units of RNaseOUT (Invitrogen, Carlsbad, California). The rRT-PCR reactions were performed separately using specific primers and probes to detect influenza A and B matrix gene and influenza A hemagglutinin gene for typing human seasonal H1, H3, and swine H1.[19,20] The amplification reaction was performed in the Rotor-Gene 3000 rRT-PCR thermocycling instrument (Corbett Research, Sydney, Australia) under the following condition: 30 minutes at 50°C, 2 minutes at 95°C, 45 cycles at 95°C for 15 seconds, and 30 seconds at 60°C. An assay was considered positive when the threshold cycle (C_T) of the amplified product for a pathogen was less than 40 cycles.

Detection of RNA and DNA Viral Respiratory Pathogens by MassTag PCR (Respiratory Samples)

MassTag PCR was performed following the methods described elsewhere.[21] The viral[20] and bacterial nucleic acids were extracted from respiratory specimens using NucliSens Magnetic Nucleic Acid Isolation Kit (BioMérieux), and the procedure was performed following the manufacturers' instructions. Viral RNA was reverse transcribed with random hexamers using Superscript III (Invitrogen) in a 20-µL reaction. Five microliters of cDNA were used as a template for MassTag PCR by using primers coupled to Masscode tags (Qiagen Masscode technology; Qiagen, Hilden, Germany). A 12-plex assay, which included 24 primers, was used to detect RNA viruses including influenza A and B; respiratory syncytial virus groups A and B; human coronavirus-229E and human coronavirus-OC43; human metapneumovirus; enterovirus; and human parainfluenza viruses (HPIVs) 2, 3 and 4a. Five microliters of viral and bacterial DNA were added directly to the mixture of the 7-plex assay including 14 primers to detect DNA pathogens of human adenovirus, *Haemophilus influenzae*, *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Legionella pneumonia*, and *Mycoplasma pneumonia*. Tests for HPIV 1 and Chlamydia were attempted but were considered not valid because 1 Masscode Tag was not detected for the HPIV 1 test and a positive control was not available for Chlamydia. PCR amplification was performed using multiplex PCR kit (Qiagen) primers at 0.5 µM each, and the following cycling protocol was performed: initial heating step at 94°C for 10 minutes; first 15 cycles at 94°C for 20 seconds followed by an annealing step with a temperature reduction of 1°C increments from 65°C to 51°C; and then continuing with the next 35 cycles using the following cycling profile: 94°C for 20 seconds, 50°C for 20 seconds, and 72°C for 30 seconds using a DNA Engine PTC200 thermal cycler (MJ Research, Waltham, Massachusetts). QIAquick 96 PCR purification cartridges (Qiagen; with modified binding and wash buffers) were used to separate amplification products from unused primers. Masscode tags were decoupled from amplified products through UV light-induced photolysis in a flow cell and analyzed in a single quadrapole mass spectrometer using positive-mode atmospheric pressure chemical ionization (Agilent Technologies, Santa Clara, California).

Detection of Enterovirus by RT-PCR (Stool Samples)

Each cDNA sample was used as a template for the first-round PCR. The first-round PCR reaction used 50 µL with the final volume containing 5 µL of cDNA, 1× PCR buffer II (supplied with AmpliTaq DNA polymerase; Applied Biosystems, Carlsbad, California), 0.2 mM of dNTP, 2 mM of $MgCl_2$, 0.25 mM of forward primer EVR1, 0.25 Mm of reverse primer EVR2, 0.5 U of AmpliTaq DNA polymerase (Applied Biosystems), and RNase-free water. The first-round PCR reaction was

performed at 94°C for 2 minutes, followed by 45 cycles at 94°C for 30 seconds, 55°C for 1.3 minutes, and 72°C for 1 minute and ended at 72°C for 10 minutes using DNA Engine DYAD (MJ Research). The second-round PCR mixture contained the same ingredients as the first-round PCR mixture except for the forward and reverse primers, which were replaced with primers EVRV3 and RV-2N. Five microliters of 1:50 dilution of the first-round PCR mixture were added to 45 μL of the second-round PCR mixture. The second-round PCR step was performed at 94°C for 2 minutes, followed by 50 cycles at 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute and ended at 72°C for 10 minutes. The second-round PCR product was then detected by gel electrophoresis. A specimen containing enterovirus was identified by the detection of a DNA band of 600 and 500 base pairs in the first- and second-round PCR, respectively, corresponding to the DNA amplified from the enterovirus genome of positive controls.

Ethics

This study was determined not to be human subjects research by the Walter Reed Army Institute of Research Division of Humans Subjects Protection. Only anonymous clinical questionnaires and specimen containers were available for testing and data analysis.

RESULTS

The study was conducted from April 16 to 26, 2009. A total of 10 enteric specimens and 6 respiratory specimens were collected from 16 patients. The average age of the 10 individuals with acute diarrhea was 25 years (age range 20–43 years): 5 were U.S. Marine Corps, 4 were U.S. Navy, and the military affiliation of 1 subject was not determined. The average age of the 6 individuals with acute respiratory symptoms was 24 years (age range 19–36 years): 5 were U.S. Marine Corps and 1 was U.S. Navy. All the patients were males. A majority of the specimens came from Crow Valley with breakdown as follows: diarrheal patients (Crow Valley main camp BAS 6/10, Crow Valley forward camp BAS 2/10, and Clark BAS 2/10) and respiratory patients (Crow Valley main camp BAS 5/6 and Clark BAS 1/6). Of the 10 diarrheal cases, laboratory testing at AFRIMS showed 4 specimens positive by PCR for *C. jejuni*, 2 for *C. coli*, 1 positive for both *C. coli* and ETEC, and 1 positive for *Norovirus* genogroup II. Of the 6 stool specimens collected at Crow Valley main camp BAS, 5 were positive for an enteric pathogen (3 for *C. jejuni*, 1 for *C. coli*, and 1 for *Norovirus* genogroup II) and 1 was negative. Of the 2 stool specimens collected at Crow Valley forward camp BAS, 1 was positive for *C. coli* and 1 was negative. Of the 2 stool specimens collected at Clark BAS, both were positive (1 for *C. jejuni* and 1 for both *C. coli* and ETEC) (Table I).

Of the 6 respiratory samples, influenza PCR was negative on all 6. However, the MassTag panel resulted in 3 positive for *H. influenzae*, 1 positive for both *H. influenzae* and HPIV 3, and 2 negative for all pathogens on the MassTag respiratory panel.

DISCUSSION

The majority of the acute diarrheal illnesses were attributed to *Campylobacter*. The most likely source of this pathogen was from ingestion of contaminated food obtained from the local "cafeterias" situated adjacent to the Crow Valley camps. Of the personnel who experienced diarrhea, 9/10 (90%) reported having eaten food from the local "cafeterias." This observation is similar to other studies that correlate an increase in the incidence of diarrhea with an increase in time spent off-base, suggesting increased exposure and consumption of locally prepared food and drinks.[6] Although the number of cases was small, 5 of the 7 *Campylobacter* cases occurred on the 5th day after the start of the exercise, which most likely reflected the incubation period of the pathogen after initial exposure. Given the relatively short duration of the exercise, some individuals who were infected with an enteric pathogen may not have exhibited symptoms until after the conclusion of the military exercise and thus would not have been captured by the surveillance. Therefore, there may have been more cases than were actually captured. Surveillance continued for at least 1 week after the military exercises may have given a somewhat more complete picture of infectious disease incidence and etiology.[22]

The information gathered from this laboratory-based surveillance program could potentially be useful during future exercises to decrease morbidity from diarrheal illness. For example, providing food sanitation training to local vendors and/or restricting access to locally purchased food could potentially decrease the number of diarrheal illnesses. Sensitizing medical staff at the BAS to etiologies of acute diarrhea may allow for more targeted counseling and treatment of military personnel. The fact that 8 of the 10 diarrheal cases were positive by PCR for some enteric pathogen indicates that under the conditions of this surveillance, PCR testing on frozen stool specimens from the field is a viable approach to identifying etiological enteric agents. The limitation of this approach is that culture and, therefore, sensitivities of the pathogens are not available.

TABLE I. Etiologies and Locations of Acute Diarrhea Cases

Site	*Campylobacter jejuni*	*Campylobacter coli*	*Campylobacter coli* and ETEC	Norovirus Genogroup II	Negative	Total
Crow Valley Main BAS	3	1	0	1	1	6
Crow Valley Forward BAS	0	1	0	0	1	2
Clark BAS	1	0	1	0	0	2
Total	4	2	1	1	2	10

However, in certain situations, maintaining frozen specimens may present a viable and feasible alternative to attempting to culture enteric pathogens within a prescribed time frame.

In terms of the respiratory cases, all the nasal specimens that were collected were negative for influenza by PCR testing. A likely explanation is that the military exercise occurred in the summer months and the influenza virus was not actively circulating among the military personnel or in people within the camp vicinity during that period. However, 4 out of 6 respiratory specimens were positive for *H. influenzae*, which suggests that this pathogen was a significant cause of morbidity during this deployment. The application of an advanced multiplex laboratory assay such as MassTag on samples collected and frozen on-site was able to reveal potentially useful public health information. The lack of influenza cases in this exercise certainly does not preclude the potential impact of an outbreak of influenza under different circumstances.

CONCLUSIONS

Laboratory-based infectious disease surveillance can be useful to identify and characterize infectious disease threats in military operations. Information about disease etiologies can potentially inform more targeted countermeasures to include primary and secondary prevention efforts (e.g., education in proper food handling, hygiene, proper waste disposal, implementing early and effective treatments). Testing using advanced techniques such as PCR and MassTag of frozen diarrheal and respiratory specimens obtained under certain conditions may be used to identify enteric and respiratory pathogen threats in deployed military personnel. Development and testing of rapid and forward-based diagnostic tests for respiratory and diarrheal diseases, which can be deployed in field settings, is also recommended.

ACKNOWLEDGMENTS

We acknowledge the 31st MEU staff, especially Jonathon Solonar for sample collection and AFRIMS personnel Pimmnapar Neesanant, Kaewkanya Nakjarung, Kittinun Hussem, Wudtichai Manasatienkij, Orntipa Sethabutr, and Oralak Serichantalergs for performance of laboratory testing.

Funding for this work was partially provided by the U.S. Armed Forces Health Surveillance Center - Global Emerging Infections Surveillance and Response System, Silver Spring, Maryland. This research was conducted as a public health measure and was determined as not human subjects research by the Walter Reed Army Institute of Research Division of Human Subjects Protection. Some of the authors are military service members. This work was prepared as part of their official duties. Title 17 U.S.C. 105 provides that Copyright protection under this title is not available for any work of the U.S. Government. Title 17 U.S.C. 101 defines a U.S. Government work as a work prepared by a military service member or employee of the U.S. Government as part of that person's official duties.

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