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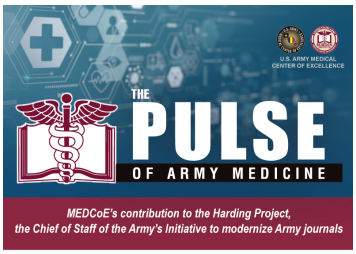

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Introduction from COL (Ret.) Mustapha Debboun

This special edition of Operational and Public Health Entomology was compiled and sponsored by Colonel (Ret) Mustapha Debboun, an Entomological Society of America Board Certified Medical and Veterinary Entomologist. Debboun has organized and assembled an outstanding collection of entomological articles featuring topics related to public health and force health protection from entomologists throughout Department of Defense (DoD). The DoD entomological community encompasses a wide range of expertise in a myriad of locations, with the ability and flexibility to provide local, national, and global entomological support to all DoD personnel wherever and whenever the need arises.

"Seasonal activity and metagenomic analysis of the introduced Asian longhorned tick, *Haemaphysalis longicorni*s at the United States military academy in West Point, New York" by LTC Silas Davidson and coauthors open this special edition with a stimulating article on this invasive tick which was first discovered in 2020 in the US military academy in West Point. They conducted intensive weekly sampling for two years to determine when the tick's different life stages were active. In addition, a metagenomic analysis was conducted to determine what bacteria were present in it to understand how the tick is a human vector and protect the health of cadets and Soldiers at West Point.

"Comparison of a mosquito cuticle penetration and feeding assays to evaluate novel water-soluble insecticides" by Dr. Craig A. Stoops and coauthors show that increasing levels of insecticide resistance to pyrethroid insecticides is a significant threat to military Force Health Protection and finding new modes of action needs to be a priority. For example, novel techniques such as RNA interference (RNAi) show promise in controlling mosquitoes and other pests.

MAJ Derek Monthei discussed a comparison of battery chemistries in the operation of CDC light traps at Aberdeen Proving Ground, Maryland, USA.

"Field evaluation of two commercial off-the-shelf spatial repellent units to prevent mosquito entry into two-person tents" by Drs. Emily McDermott and James E. Cilek showed that these devices have advantage over currently employed personal protective measures and could be incorporated into the DoD Insect Repellent System to improve Force Health Protection against vectors and vector-borne diseases.

"Monitoring insecticide resistance of mosquitoes in United States military areas of operations" by Dr. Jennifer B. Carder and coauthors show how the US military is working to monitor and address insecticide resistance in mosquito populations, which pose a significant threat to the health of military stationed throughout the world.

"Improving the vector-borne disease risk assessment to deployed forces in Djibouti: Military operational entomology in the Horn of Africa 2018-2022" by LCDR James F. Harwood and coauthors summarize the joint efforts of US Navy, Afrmy, and Air Force entomologists to better document the mosquito vectors present in Camp Lemonier and Chabelley Air Base in Djibouti, Africa.

"The entomological situation of ectoparasite-borne diseases in areas used for Cobra Gold military training exercise in Thailand between 2017 and 2022" by Dr. Piyada Linsuwanon and coauthors discuss how ectoparasite-borne diseases pose significant threats to the readiness of US and Allied military forces, especially in tropical regions like Thailand, where ectoparasite vectors and reservoir hosts thrived. Their study analyzed 1,568 rodents and 3,696 chiggers from areas supporting the "Cobra Gold" exercise in Thailand.

"Control of adult *Culex quinquefasciatus* mosquito populations in catch basins in Houston, Texas using the Provector Military Camouflage Tube with an attractant toxic sugar bait incorporating Bacillus thuringiensis and Methoprene" by LTC (Ret) Thomas M. Kollars Jr. and coauthors provide and excellent research on the Provector Military Camouflage Tube with EntobacM, a target specific and non-toxic pesticide that was tested and found to be effective in controlling *Cx. Quinquefasciatus*, the principal vector of West Nile virus. Using a camouflage device to deliver non-toxic target specific pesticides reduces the risk of detection and vector-borne diseases by deployed military personnel.

"*Vivax* malaria among US military perszonnel and civilians attributed to exposure in the Republic of Korea (ROK), 2006-2020" by Dr. Heung-Chul Kim and coauthors close this special edition of the Medical Journal with an excellent review and discussion of vivax malaria. They show how *vivax* malaria was reintroduced in the ROK in 1993 when a ROK soldier stationed along the demilitarized zone (DMZ) with no travel history was diagnosed with vivax malaria. Initially, malaria mostly occurred in the ROK Army, but rapidly spread to the civilian community and members of the United States military. After 2000, malaria decreased with the institution of malaria control practices among the ROK military, civilian communities, and US servicemen.

I hope you will enjoy reading this special edition that has a broad variety of entomological content and I'm sure you will all be as impressed as I am with what our excellent DoD military and civilian entomologists are doing every day.

Dr. Mustapha Debboun, COL (Ret.), PhD, BCE, Fellow ESA & Honorary Member

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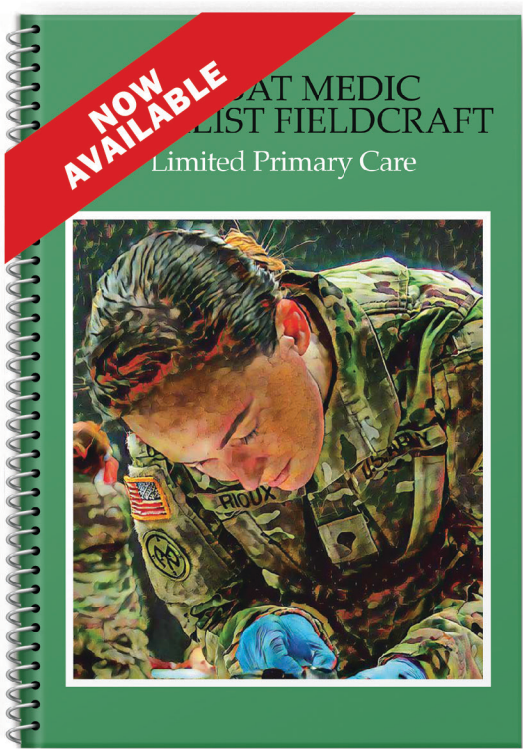

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Seasonal Activity and Metagenomic Analysis of the Introduced Asian Longhorned Tick, *Haemaphysalis longicornis*, at the United States Military Academy in West Point, New York

LTC Silas A. Davidson, PhD, 2LT Alex M. Burgess, BS, CDT Amber L. Chen, CDT Spencer C. Deremer, 1LT Dylan J. Nun, BS, 1LT Alyssa H. Chellaraj, BS,1LT Jason Y. Johnson, BS,COL (Ret) Mustapha Debboun, PhD, BCE, Fellow ESA & Honorary Member

ABSTRACT

The Asian longhorned tick (ALT), *Haemaphysalis longicornis* Neumann, is an invasive species first discovered at West Point, New York in April 2020. It is a potential disease vector because it transmits several pathogens in its native range. After its initial discovery, weekly surveillance was conducted using the tick drag method to determine the seasonal emergence of its different life stages. Degree days, representing cumulative environmental heating, were calculated for the years 2020 and 2021 based on daily maximum and minimum air temperatures. The degree day model showed when the three life stages emerged each year and was useful for tracking climate change or unusual weather patterns. In the second year, more ticks were collected at more locations indicating populations of ALT were increasing at West Point. A small number of ALT adults and nymphs collected from 2020 were sent to a company for metagenomic analysis to determine the types of bacteria present. No pathogenic bacteria were found. The microbiome of all tick samples was dominated by a *Coxiella* like endosymbiont (CLE) that is critically important for tick nutrition and behavior. This study reports the initial discovery of ALT at West Point and provides a baseline to understand how this tick may expand and adapt to its new environment. It also highlights the importance of conducting routine tick surveillance on military installations and the need to prevent tick-borne diseases among cadets and soldiers at West Point.

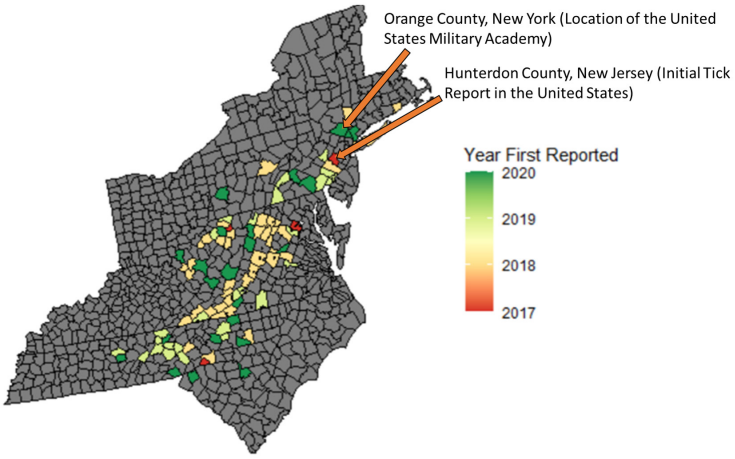
INTRODUCTION

Haemaphysalis longicornis Neumann, commonly known as the Asian longhorned tick (ALT), is an invasive tick species native to eastern China, the Korean peninsula, and Japan. It was first discovered in the United States (US) in Hunterdon County, New Jersey in 2017 feeding on sheep.[1] Immediate effort was made to contain and eradicate it but were unsuccessful. The ALT has since spread throughout the east coast and to a total of 17 states (Fig 1).[2] Retrospective studies indicate ALT may have been present in West Virginia since 2010 and New Jersey since 2013.[3]

The ALT are considered a public health threat since they are important vectors of disease to animals and humans in their native range. In Asia, they transmit pathogenic bacteria from the genera Anaplasma, Borrelia, Rickettsia, and Ehrlichia; the protozoan parasite Babesia; and the virus that causes Severe Fever with Thrombocytopenia Syndrome (SFTS).[3] There is evidence from Japan that ALT may play a role in causing the α-1,3-galactose (α-gal) red meat allergy.[4] It is unclear what pathogens ALT may transmit in North America. Laboratory studies indicate invasive ALT in the US are poor vectors of Borrelia burgdorferi (Lyme disease).[5] However, they are capable of transmitting Rickettsia rickettsia (Spotted Fever Rickettsiosis)[6] and Powassan virus (POWV).[7] Although transmission of human disease is unclear at this time, there is evidence ALT are affecting livestock and transmit the parasite, Theileria orientalis which has caused significant losses in cattle in Virginia.[8]

The ALT follows a three-host lifecycle consisting of larval, nymphal, and adult stages. Each stage requires a separate blood meal, and a wide variety of mammals and birds are fed upon.[2] The generalized life cycle in North America is that nymphs are active first in the spring, followed by adults in the summer, and larvae in the fall.[9] However, the seasonal timing of each life stage is highly dependent on the environment and varies across different locations. A key factor contributing to the rapid spread of ALT in North America

Figure 1. Choropleth map displaying the first report at the county level for Haemaphysalis longicornis (Asian longhorned tick). Data was retrieved from the USDA Situation Report.[2] Only states on the east coast are shown. The tick has also been found in Arkansas and Missouri.



is that adult females are parthenogenic and do not require males to produce fertile eggs.[3] Therefore, a single blood-fed female can start a new population when transported to a new location. All populations of ALT observed in the US have been parthenogenic. However, in their native range, both bisexual (consisting of males and females) and parthenogenic populations occur together. These populations are genetically different in that bisexual populations are diploid and parthenogenic populations are triploid.[10]

In this study, we report on the April 2020 initial discovery of ALT at the United States Military Academy (USMA) in West Point, New York. Weekly sampling was conducted over the next two years to determine the life cycle and seasonal activity for each life stage of the tick. Weather information was obtained and analyzed as degree days to determine the impact of climate on tick development. Also, some ticks were submitted for metagenomic sequencing to determine what types of bacteria they carried.

METHODS

Tick Collections

The tick drag method was used to collect ticks throughout the study (Fig. 2). This technique involved pulling a white corduroy cloth through leaves and grass and stopping every 5-10 meters to check if questing ticks had attached to the cloth.[11] Attached ticks were removed with forceps and placed in a plastic vial. Sampling was conducted weekly at a time between 0800 and 1800 for a duration of 30 minutes to an hour. The exact time and distance sampled (dragged) varied according to the individual doing the collecting. Therefore, tick abundance was not estimated per unit area or amount of time sampled.[11] The results do provide information if questing ALT were present or absent at a particular location and the dates when the different life stages were active.

After the initial discovery of ALT, weekly sampling continued through the end of 2021. Tick drags were conducted consistently at the site of initial discovery, but other locations were also sampled on the main garrison of West Point and at Camp Buckner, a large training area immediately west of the main garrison (Fig. 3). The date, location, and number of ticks were recorded after each collection event and ticks were stored alcohol-free in a -80°C freezer. Ticks were also collected from deer during hunting season, i.e., from the first week of November to mid-December at West Point to determine if ALT were present. After deer were killed by local hunters, they were weighed by the staff from the Natural Resources Branch who removed visible ticks and provided them to the authors.

Degree Day Calculations

A simple degree day model was developed to measure the effect of climate on the emergence of the different life stages of the ALT.[12]

$$\textit{Cumulative Degree Day} = \sum((Max+Min)/2) - D_T$$

The maximum and minimum air temperature in degrees Celsius for each day of the year were obtained from a weather station at the Stewart International Airport located in Orange County, New York.[13] This is the closest weather station to the West Point garrison and is approximately 24 km away. A development threshold (DT) of 0°C was selected since the threshold temperature for development of the different life stages of ALT was not known. Other degree day models for ticks have used a development threshold of 0°C.[14] Cumulative degree-day graphs were constructed for both 2020 and 2021 using Julian dates.

DNA Extraction and Metagenomic Sequencing

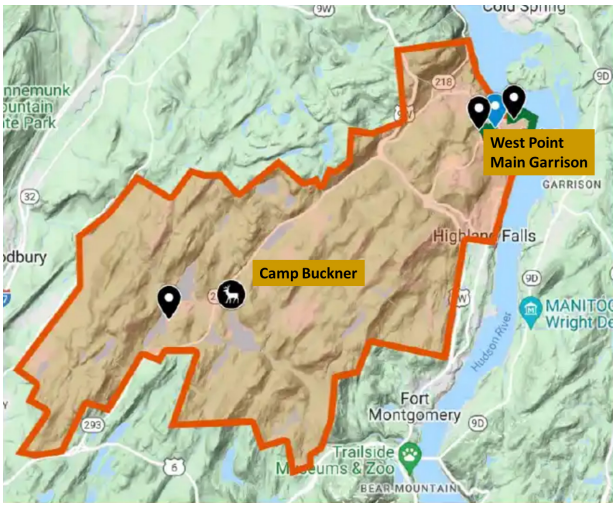
A small subset of ALT were selected for metagenomic sequencing. Five adult females collected between 8 -18 July 2020 were selected and analyzed individually. Twenty-seven nymphs collected between 10 April through 12 May 2020 were pooled into groups of three ticks (all collected on the same day and at the same location) to form 9 nymph pools.

Tick extraction began by individually washing ticks in a 5% bleach solution and rinsing with distilled water. The ticks were blotted dry with laboratory cleaning tissues and bisected along the anterior-posterior axis using a sterile razor blade that was cleaned between each use with 80% ethanol. The purpose of bisection was to open the exoskeleton and allow internal tissues to be

Figure 2. Photo of cadet performing the tick drag method of surveillance.

released during the extraction process that was performed with a Qiagen DNAEasy Blood and Tissue Kit using the MiniElute Protocol (Qiagen, Hilden, Germany). Extracted DNA was shipped on dry ice to a commercial company, Microbiome Insights (Vancouver, British Columbia), to perform metagenomic sequencing and data analysis. The 16S rRNA V4 region was targeted for amplification using primers of 515F (5′-TCGTCGGCAGCGTCAGATG TGTATAAGAGAAGGTGCCA GCGCCGCGGTAA-3′) and 806R (GTCTCGTGGGCTCGGAGAT GTGTATAAGAGACAGGGACTACGGGT TCTAAT-3′). Sequencing by synthesis was performed using an Illumina iSeq 100 sequencing system (San Diego, CA). Raw Fastq files were quality-filtered and clustered into 97% similarity operational taxonomic units (OTUs) using the Mothur software package [http://www.mothur.org]. High quality reads were taxonomically classified using the bioinformatics database Greengenes v. 13_8 (https://greengenes.secondgenome.com/). From the OTU information produced, a Shannon diversity index was determined for each sample to show the diversity of bacteria present within each tick

Figure 3. A map of the West Point Military Reservation. The blue icon represents the center of the main garrison. Black icons indicate collection locations, and the deer icon depicts the location where ticks were removed from hunted deer. The map was created using MyMaps from Google



sample. A Wilcoxon rank-sum test was used to compare differences in bacterial diversity between adult and nymphal ticks. In addition, a principal coordinate analysis (PCoA) was provided to visualize correlation between samples.

RESULTS AND DISCUSSION

On 8 April 2020, 14 nymphs of a previously undetected species of nymphal tick were collected near Delafield Pool (41.39365, -73.9672) on the main garrison of West Point. This location is within a deciduous forest comprised of old-growth beech, maple, and oak trees. *Ixodes scapularis* ticks had been commonly found at this location for many years and were the target of tick sampling at that time. The newly discovered nymphs were initially identified as *Haemaphysalis longicornis* using a key from Rutgers University.[15] Nymphs were later sent to the National Veterinary Services Laboratories (NVSL) in Ames, Iowa where they were morphologically confirmed as *H. longicornis* and added to the United States Department of Agriculture's collection. This was the first reported detection of ALT in Orange County, NY and they have since been found throughout the lower Hudson Valley in New York.[2]

After initially finding nymphs near Delafield Pool, more than 20 locations were sampled on the main garrison and Camp Buckner to determine the extent of their spread. A single nymph was found at Redoubt Number 4 (41.39031, -73.97334) and two nymphs were found near Fort Putnam (41.38989, -73.96451) on the main garrison. A single nymph was also found at Camp Buckner near Landing Zone Owl (41.34754,

Table 1. Total Asian longhorned ticks (ALT) collected at West Point, NY in 2020 and 2021

Year	Nymphs	Adult Females	Larvae	Total
2020	148	12	714	874
2021	414	92	2,010	2.516

-74.05660). No adult females were collected other than at Delafield Pool. Larvae were found at Flirty Walk (41.39588, -73.95695) in addition to Delafield Pool. In 2021, ALT were collected at many additional locations. Nymphs were found at 15 locations, adults at 8 locations, and larvae at 15 locations. Essentially, ALT were found everywhere substantial tick dragging was conducted. Tick abundance was also greater the second year. In 2020, 148 nymphs, 12 adults, and 714 larvae were collected. In 2021, 414 nymphs, 92 adults, and 2,010 nymphs were collected (Table 1). This indicated the population of ALT had grown and spread to new areas on the installation.

Sampling results of ALT in this study are indicative of a one-year cycle. This matches the typical pattern observed for ALT both in its home range and in countries where it has been introduced.[15] The seasonal timing for blood-feeding life stages of ALT showed nymphs emerged in early April and were active until late May, adults were active from May to early September, and larvae were found from mid-September through October (Table 2). These results roughly align with two other studies conducted in New York.[9,16] However, in these studies, the adults and larvae became active about a month earlier than at West Point. These studies were done at Staten Island and Yonkers, New York and higher temperatures at these locations may have contributed to earlier emergence of some stages. Both studies also observed a small number of nymphs emerging in the fall, which could be from larvae that successfully blood-fed and molted during the same year, and larvae emerging in April, which could be unfed larvae that survived from the previous fall and were still able to quest.[16] These exceptions to the normal one-year life cycle were not observed at West Point.

Degree days are an important tool to predict and understand when ticks are active. Although light and other environmental factors play a role, cumulative temperature is often the most effective predictor of life cycles.[12] Degree days provide information often not apparent using calendar dates. For example, in this study, the nymphal stages were first detected around the same date each year (Fig. 4), but the nymphs in 2021 were found at 393 degree days compared to 463 degree days in 2020. Degree days are also used to predict the range where ticks may expand.[14] This study provided a baseline for data at West Point and can be compared with other locations to understand how the ALT may continue to spread throughout North America.

Air temperature was used to determine daily maximum and minimum temperatures. This is not the optimal predictor of development since ticks spend most of their time in or near the soil. Soil temperature is a better method to determine

Figure 4. Graphical depiction of 2020 and 2021 degree days as compared to the observed life cycle stages of collected ticks atethe West Point Military reservation.

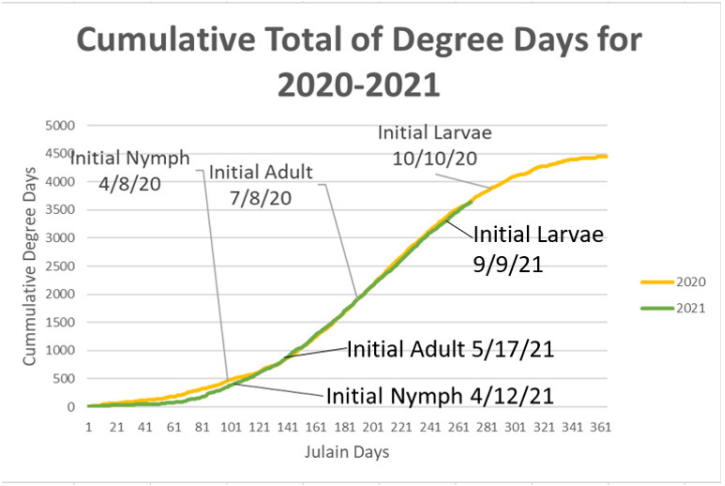
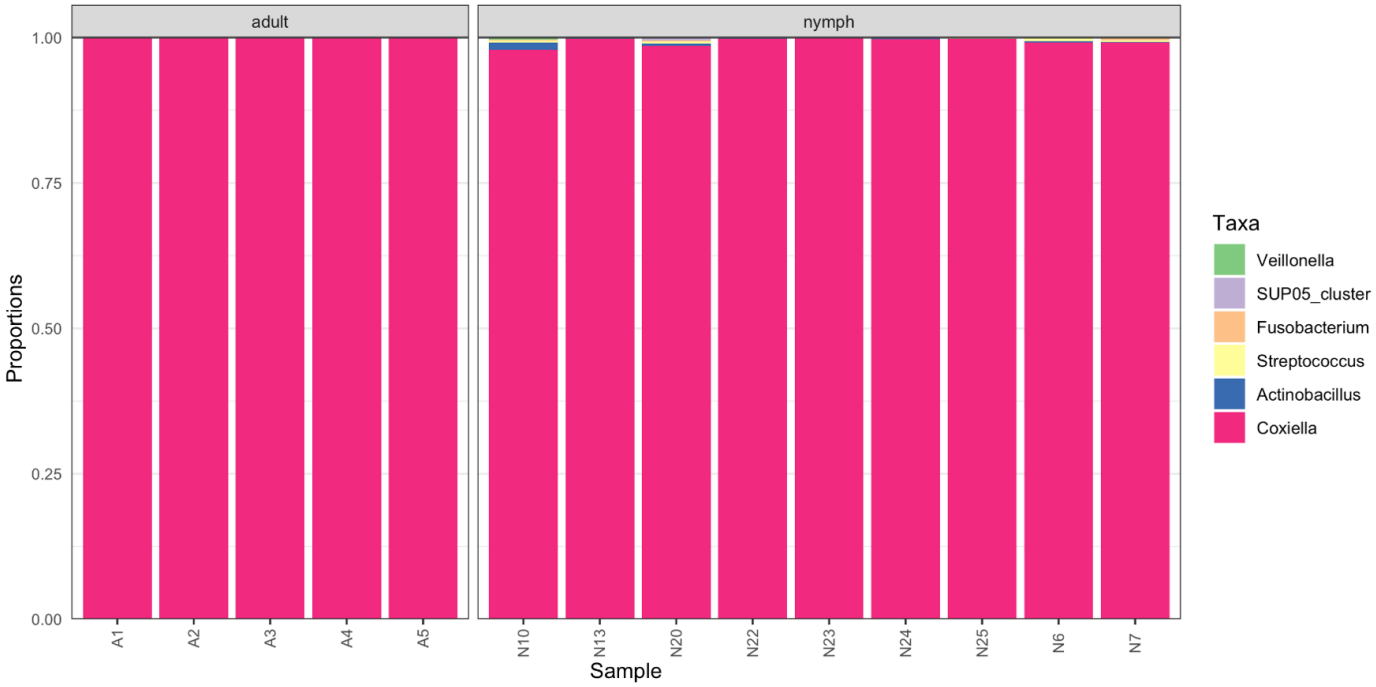


Table 2. First Detection Dates of three blood-feeding life stages of Asian longhorned ticks (ALT) observed at Westpoint, Ny in 2020 and 2021

Year	Growth Stage	Activity	Cumulative Degree Day	Julian Day	Date
2020	Nymph	Emergence	467.2	99	8-Apr
2020	Adult	Emergence	1,902.5	190	8-Jul
2020	Larva	Emergence	3,884.2	284	10-Oct
2021	Nymph	Emergence	393.9	102	12-Apr
2021	Adult	Emergence	860	137	17-May
2021	Larva	Emergence	3,300	252	9-Sep

Figure 5. The microbiome composition of each tick sample. The adult samples are for individual femaales and the nymph samples are comprised of three pooled nymphs. Only bacteria genera that comprised more than 1% of the total reads are shown.



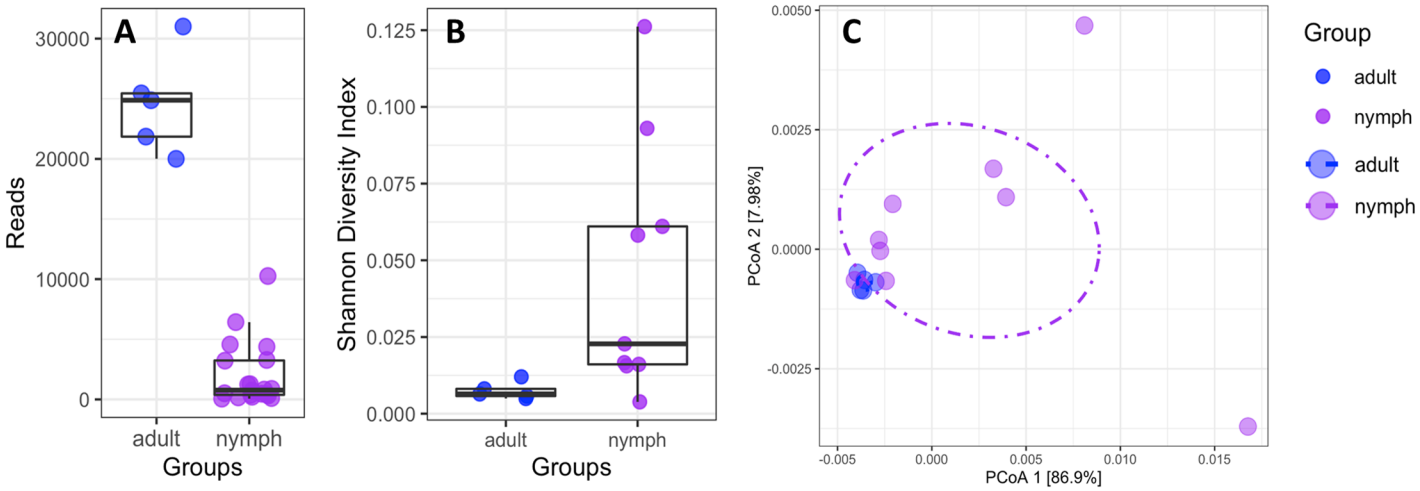
accumulative temperature effects on ticks.[18] However, not every location has access to soil probes and air temperature can serve as a rough approximation of cumulative heat during a year. Historical records of air temperature are also readily available and allow retrospective analysis of previous collection efforts.

No ALT were collected from deer during this study. Hunting season was from mid-November through December each year and other ticks (*Ixodes scapularis* Say and *Dermacentor albipictus* (Packard)) were readily collected. The reason why ALT were not found on deer is that nymphs and adults are not active in November and December. Collecting ticks from deer during hunting season is therefore not an effective method of surveillance for ALT unless it is done earlier in the year. There would be benefits to survey for ALT on animals because this would help to determine the animal hosts fed on by this tick. The results would also be useful for surveillance. The initial detection of ALT in the US was from an animal host.[1]

A small sample of ALT collected at West Point in 2020 were sent for metagenomic sequencing to determine what bacteria they carried. No human pathogens were discovered in the analysis. A few common genera of environmental bacteria (*Veillonella*, *Fusobacterium*, *Streptococcus*, *Actinobacillus*) were detected at low percentages. The low percentage of environmental bacteria is partly explained because all ticks were externally cleaned with bleach before extracting DNA. Additional bacteria would likely have been present on the exterior surface of ticks if this step was not performed.

The microbiomes of all ticks sequenced were dominated by a single type of *Coxiella* bacteria (Fig. 5). It is significant this bacterium comprised more than 98% of all sequencing pools and greatly outnumbered all other genera of bacteria. A NCBI BLAST (National Center for Biotechnology Information Basic Local Alignment Search Tool) confirmed the species as a symbiont commonly found *Haemaphysalis* and *Rhipecephalus* ticks and referred to as the *Coxiella*-like endosymbiont (CLE). This bacterium does not cause human disease although it is closely related to *Coxiella burnetii*, the causative agent of Q fever.[19] This endosymbiont plays an important role in tick nutrition because it produces B vitamins which are at low levels in vertebrate blood. If the CLE are experimentally removed with antibiotics, then ticks are unable to complete normal development.[20] The CLE are concentrated in the ovary tissues of female ALT and are passed to the next generation transovarially through the eggs.[20] This explains why adult females had higher read counts indicating a greater amount of bacteria present (Fig. 6A). The reads shown in the figure compare a single female to three pooled nymphs. The adults also had a lower Shannon diversity index indicating the microbiome was dominated by a single species (Fig. 6B). The principal coordinate analysis (PCoA) provides a visual representation showing less diversity of bacteria present in the adult females (Fig. 6C).

Figure 6. (A) Graphical depiction of the read counts per sample, (B) measure of the Shannon diversity index per sample, and (C) principal coordinate analysis (PCoA) grouping samples by similarity.



The sequencing results are not unusual. Metagenomic sequencing for bacteria in ALT has been performed at Staten Island, New York.[21] The tick microbiomes in that study were dominated by CLE with adult females containing the greatest amount of the endosymbionts. The Staten Island study also did not detect any human bacterial pathogens or viruses within the ALT samples. It should be noted their study sequenced ticks collected during the initial year they were discovered at Staten Island. This study sequenced ticks collected from the first year ALT were discovered at West Point. It is possible that as ALT become established at a new location, they will feed on a wider variety of host animals and acquire new tick-borne pathogens. The tick microbiome also plays an important role in what pathogens are transmitted.[22] The ALT may become more or less capable of transmitting certain pathogens if their microbiome becomes more diversified with other bacteria.

CONCLUSIONS

This study reports the initial discovery of ALT at West Point. Baseline information was collected on the life cycle, a degree days analysis was performed for the emergence of different life stages, and a metagenomic analysis of the tick microbiome was conducted. The ALT is now established at West Point and this baseline data will be useful to determine how the ticks adapt or change in the coming years. No pathogens were detected from the small number of ticks sampled in this study. Future studies are needed with larger pools of ticks to determine if pathogens are present in ALT or if they acquire them over time. The most medically important tick in the northeastern US is *Ixodes scapularis* because it vectors Lyme disease and many other important pathogens.[23] The distribution of ALT now overlaps with *Ixodes scapularis* at many locations, including West Point. Since the two species feed on many of the same animals at the same time of the year, it will be important to study their interactions and possible effects on disease transmission.

The ALT were first detected at West Point by faculty and cadets conducting tick dragging as part of a research course. This study highlights the need for routine tick surveillance on military installations. This important function is often ignored and knowledge about ticks from previous years can be used to determine the current threat of tick-borne diseases. In addition, this study showed the situation can quickly change in a location due to invasive species or environmental change. Current knowledge of tick activity and the diseases they carry are important to the development of the best force health protection plans to prevent tick-borne diseases among Soldiers.

ACKNOWLEDGEMENTS

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DISCLAIMER

The views expressed in this paper are those of the authors

and do not reflect the official policy or position of the United States Military Academy, Department of the Army, DOD, or the U.S. Government.

REFERENCES

1. Rainey T, Occi JL, Robbins RG, Egizi A. Discovery of *Haemaphysalis longicornis* (Ixodida: Ixodidae) parasitizing a sheep in New Jersey, United States. *J Med Entomol*. 2018 May 4;55(3):757-759.
2. United States Department of Agriculture. National *Haemaphysalis longicornis* (Asian longhorned tick) Situation Report. As of 23 August 2022. https://www.aphis.usda.gov/animal_health/animal_diseases/tick/downloads/longhorned-tick-sitrep.pdf Accessed 10 September 2022.
3. Schappach BL, Krell RK, Hornbostel VL, Connally NP. Exotic *Haemaphysalis longicornis* (Acari: Ixodidae) in the United States: biology, ecology, and strategies for management. *J Integrated Pest Management*, Volume 11, Issue 1, 2020, 21.
4. Chinuki Y, Ishiwata K, Yamaji K, Takahashi H, Morita E. *Haemaphysalis longicornis* bites are a possible cause of red meat allergies in Japan. *Allergy*. 2016. 71: 421-425.
5. Breuner NE, Ford SL, Hojgaard A, Osikowicz LM, Parise CM, Rosales Rizzo MF, Bai Y, Levin ML, Eisen RJ, Eisen L. Failure of the Asian longhorned tick, *Haemaphysalis longicornis*, to serve as an experimental vector of the Lyme disease spirochete, *Borrelia burgdorferi* sensu stricto. *Ticks Tick Borne Dis*. 2020 Jan;11(1):101311. doi: 10.1016/j.ttbdis.2019.101311.
6. Stanley HM, Ford SL, Snellgrove AN, Hartzer K, Smith EB, Krapiunaya I, Levin ML. The ability of the invasive Asian Longhorned Tick *Haemaphysalis longicornis* (Acari: Ixodidae) to acquire and transmit *Rickettsia rickettsii* (Rickettsiales: Rickettsiaceae), the agent of Rocky Mountain Spotted Fever, under laboratory conditions. *J Med Entomol*. 2020 Sep 7;57(5):1635-1639. doi: 10.1093/jme/tjaa076
7. Raney WR, Herslebs EJ, Langohr IM, Stone MC, Hermance ME. Horizontal and vertical transmission of Powassan Virus by the invasive Asian longhorned tick, *Haemaphysalis longicornis*, under laboratory conditions. *Front Cell Infect Microbiol*. 2022 Jul 1;12:923914. doi: 10.3389/fcimb.2022.923914
8. Thompson AT, White S, Shaw D, Egizi A, Lahmers K, Ruder MG, Yabsley MJ. *Theileria orientalis* Ikeda in host-seeking *Haemaphysalis longicornis* in Virginia, U.S.A. *Ticks Tick Borne Dis*. 2020 Sep;11(5):101450. doi: 10.1016/j.ttbdis.2020.101450
9. Tufts DM, VanAcker MC, Fernandez MP, DeNicola A, Egizi A, Diuk-Wasser MA. Distribution, host-seeking phenology, and host and habitat associations of *Haemaphysalis longicornis* Ticks, Staten Island, New York, USA. *Emerg Infect Dis*. 2019 Apr;25(4):792-796
10. Qiu ZX, Li Y, Li MM, Wang WY, Zhang TT, Liu JZ. Investigation of three enzymes and their roles in the embryonic development of parthenogenetic *Haemaphysalis longicornis*. Parasit Vectors. 2020 Jan 31;13(1):46. doi: 10.1186/s13071-020-3916-7
11. Egizi AM, Robbins RG, Beati L, Nava S, Vans CR, Occi JL, Fonseca DM. A pictorial key to differentiate the recently detected exotic *Haemaphysalis longicornis* Neumann, 1901 (Acari, Ixodidae) from native congeners in North America. *Zookeys*. 2019 Jan 23;(818):117-128. doi: 10.3897/zookeys.818.30448
12. Laemmlen F, Philips P, Newman J. Degree-Days: The calculation and use of heat units in pest management. University of California Extension online. http://cesantabarbara.ucanr.edu/files/75290.pdf Accessed 13 September 2022.
13. Weather Underground. Newburgh, NY Weather History: Stewart International Airport Station. Available online at https://www.wunderground.com/history/monthly/KSWF/date2020-2. Accessed August 24, 2022.
14. Leighton PA, Koffi JK, Pelcat Y, Lindsay LR, Ogden NH. Predicting the speed of tick invasion: an empirical model of range expansion for the Lyme disease vector *Ixodes scapularis* in Canada. *J Applied Ecol.* 2012; 49(2): 457-464
15. Salomon J, Hamer SA, Swei A. A beginner's guide to collecting questing hard ticks (Acari: Ixodidae): a standardized tick dragging protocol. *J Insect Sci*. 2020 Nov 1;20(6):11. doi: 10.1093/jisesa/ieaa073
16. Piedmonte NP, Vinci VC, Daniels TJ, Backenson BP, Falco RC. Seasonal activity of *Haemaphysalis longicornis* (Acari: Ixodidae) in southern New York state. *J Med Entomol*. 2021 Mar 12;58(2):676-681. doi: 10.1093/jme/tjaa203
17. Burtis JC, Yavitt JB, Fahey TJ, Ostfeld RS. Ticks as soil-dwelling arthropods: an intersection between disease and soil ecology. *J Med Entomol*. 2019 Oct 28;56(6):1555-1564.
18. Brenner AE, Muñoz-Leal S, Sachan M, Labruna MB, Raghavan R. *Coxiella burnetii* and related tick endosymbionts evolved from pathogenic ancestors. *Genome Biol Evol.* 2021 Jul 6;13(7):evab108. doi: 10.1093/gbe/evab108
19. Zhang XY, Li SS, Chen KL, Yang C, Zhou XJ, Liu JZ, Zhang YK. Growth dynamics and tissue localization of a *Coxiella*-like endosymbiont in the tick *Haemaphysalis longicornis*. *Ticks Tick Borne Dis.* 2022 Sep;13(5):102005. doi: 10.1016/j.ttbdis.2022.102005
20. Tufts DM, Sameroff S, Tagliafierro T, Jain K, Oleynik A, VanAcker MC, Diuk-Wasser MA, Lipkin WI, Tokarz R. A metagenomic examination of the pathobiome of the invasive tick species, *Haemaphysalis longicornis*, collected from a New York City borough, USA. *Ticks Tick Borne Dis.* 2020 Nov;11(6):101516. doi: 10.1016/j.ttbdis.2020.101516
21. Cross ST, Kapuscinski ML, Perino J, Maertens BL, Weger-Lucarelli J, Ebel GD, Stenglein MD. Co-Infection patterns in individual *Ixodes scapularis* ticks reveal associations between viral, eukaryotic and bacterial microorganisms. *Viruses*. 2018 Jul 22;10(7):388.
22. Eisen RJ, Eisen L. The blacklegged tick, *Ixodes scapularis*: an increasing public health concern. *Trends Parasitol*. 2018 Apr; 34(4): 295-309.

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Comparison of Mosquito Cuticle Penetration and Feeding Assays to Evaluate Novel Water Soluble Insecticides

Craig A. Stoops, PhD, Alden S. Estep III, MS, Man-Yeon Choi, PhD

ABSTRACT

The spread of insecticide resistance is a direct threat to military Force Health Protection and has prompted Department of Defense medical entomologists to consider new insecticides to control mosquito vectors. Water soluble insecticides such as molecular insecticides, including RNA interference (RNAi) technology, represent a possible revolutionary solution. Feeding currently is the best method of delivering molecular insecticides, but making their use operationally significant for the military will involve designing contact insecticides. Because molecular insecticides are highly polar molecules formulating a contact insecticide needed by the military will require an understanding of how these types of molecules penetrate the cuticle to reach susceptible tissues. To understand the difference between feeding and topical application, laboratory-reared adult *Aedes aegypti* (L.) were treated with combinations of Rhodamine B dye in a water-soluble oil, acetone or were fed Rhodamine B dye and sugar water. Untreated controls were fed sugar water only. To measure the dye uptake, relative fluorescence intensity of fecal spots was measured and whole specimens were processed and read in a microplate fluorescence reader. The order of intensity of fecal spot fluorescence for topical applications was Control < Acetone < Oil < Fed. The relative order of fluorescence intensity of specimens read in a microplate reader was Control < Oil < Acetone < Fed. Both oil and acetone were effective in disrupting the physical barrier and setting up the necessary diffusion gradient but feeding appeared to be the most effective way to deliver water soluble molecules.

INTRODUCTION

Vector-borne pathogens threaten the readiness of military personnel stationed in endemic countries. Around the world, as part of integrated vector management programs, military preventive medicine units use insecticides to kill adult mosquitoes to decrease the risk of pathogen transmission. Few new insecticide active ingredients are being developed for mosquito control, however, new biological insecticides such as RNA interference (RNAi) may play an important role in future mosquito control programs. [1-3]

Insecticides are more than just their active ingredients. Other chemicals, including stabilizers, solvents, or anti-Ultraviolet light agents are formulated along with the active ingredient to improve stability, dispersal, delivery, and efficacy of the insecticide. How the insecticide is formulated and what solvent is used to carry the active ingredient, plays an important role in passing the active ingredient through the insect epidermis and/or mouth to reach target tissues. Insecticides once were thought to penetrate mainly through membranes found in leg and abdominal joints, rather than directly through the cuticle. The use of radiolabeled isotopes, however, demonstrated that insecticides penetrate across the entire insect cuticle.[4]

A water-soluble molecule (= hydrophilic) is less able to penetrate mosquito cuticle due to the wax layer of cuticle to repel water.[5] Currently, ingestion is the best way to get water-soluble insecticides, such as RNAi into the target pest.[1] To maximize the potential of molecular insecticides such as RNAi as adulticides suitable for military use, it is critical that the insecticides are able to penetrate the cuticle and enter vulnerable tissues.[6] To understand if there is a way to get a water-soluble molecule across the mosquito cuticle barrier that is equal to feeding, we used Rhodamine B (RB) dye as a surrogate for an insecticide because fluorescence intensity can be measured and compared between treatments. By comparing the fluorescence intensity between topical applications and feeding, we can better understand how to formulate a topical insecticide with a water-soluble active ingredient that meets or exceeds an insecticide formulated for mosquito feeding.

METHODS

Fluorescence Intensity in Fecal Spots – Topical Application and Feeding

Ten-day-old adult *Ae. aegypti* (L.) females were placed in plastic cups with ten mosquitoes in each cup. All topical treatments were conducted using a 700 series syringe and a repeating dispenser. Mosquitoes were chilled on a chill table,

and in trial one, 40 Ae. aegypti females, and 50 females in trial two were subjected to each of the following treatments: topical application of Poly(ethylene glycol) methyl ether (MPEG) oil (100 nanoliterd (nL) with RB dye 0.3%) and 10% sucrose provided as food for 24h; topically applied acetone (100 nL with RB dye 0.3%) and 10% sucrose for 24h no topical application with only 10% sucrose with RB dye 0.3% for 8h with 10% sucrose only for the remaining time; and a control of no topical application and 10% sucrose without RB dye for 24 hrs. The RB dye is readily soluble in the water-soluble MPEG oil. All Ae. aegypti were obtained from the ORL1952 colony at the United States Department of Agriculture (USDA) Agricultural Research Service (ARS) Center for Medical, Veterinary and Agricultural Entomology in Gainesville, FL.

Each of the 20 cups had size two filter paper at the bottom. Following treatment and the dye-fed treatment, mosquitoes were kept in a chamber at 26°C and 80% RH. Mosquitoes were chilled on a chill table and papers were changed after 8h and collected after 24h. Specimens were frozen and stored at -20°C until use.

Ten fecal drops were randomly selected on the 24hfilter paper and measured with 50 drops total (ten drops on each of five filter papers) for the MPEG, Acetone, Dye Fed, and for the untreated control treatments. Mean intensity was measured from photographs taken of individual droplets with the one Frame time setting on a fluorescence microscope camera. Mean intensity was measured by using the mean intensity tool placed across the center of the droplet.

Preliminary assessment of normality and homoscedacity (homogeneity of variances) of the mean intensity using numerous goodness-of-fit (GOF) tests, including the Kolmogorov-Smirnov test for normality and the Bartlett test for homoscedacity indicated non-normal, non-homoscedastic behavior, even after log-transformation of the data.[7] Thus, for data analysis, the nonparametric Kruskal-Wallis (K-W) hypothesis test was used in lieu of the parametric analysis of variance (ANOVA) test at the 95% confidence level. One-way K-W test (a = 0.05) was applied to the mean intensity of the three treatments.

Detection of Dye Fluorescence - Microplate Reader

To determine the amount of dye remaining in the mosquitoes after provision of RB-Sucrose and topical application of RB dye, ten Ae. aegypti from each treatment (MPEG, Acetone, Fed, and untreated) in the fecal spot experiment were washed, and processed as the following procedure described below. The intensity of fluorescence for each mosquito was measured using a microplate reader equipped with filters (excitation: 485 nm and emission: 520 nm).

Mosquitoes first were soaked individually in a water and surfactant solution for five minutes to remove any available dye remaining on the cuticle. Following the immersion period, mosquitoes were removed from the water and dried on filter paper for a period of ten minutes with each specimen moved to the opposite side after five minutes. Each mosquito was placed in an individual microcentrifuge tube with 500µL of phosphate buffered saline (PBS). Each specimen was manually crushed using a plastic tissue disrupter, and each tube was centrifuged for two minutes at 15,000 rpm. Five hundred µL of supernatant was removed from each tube and placed in a clean microcentrifuge tube then held at -200 C until testing.

A microplate reader was used to obtain fluorescence intensity readings of each sample. The microplate reader reads the fluorescence intensity of each sample three times to calculate an average intensity. As in the fecal spot experiment, following preliminary assessment of normality and homoscedacity, a one-way K-W test (a = 0.05) was applied to the mean intensity of the three treatments.

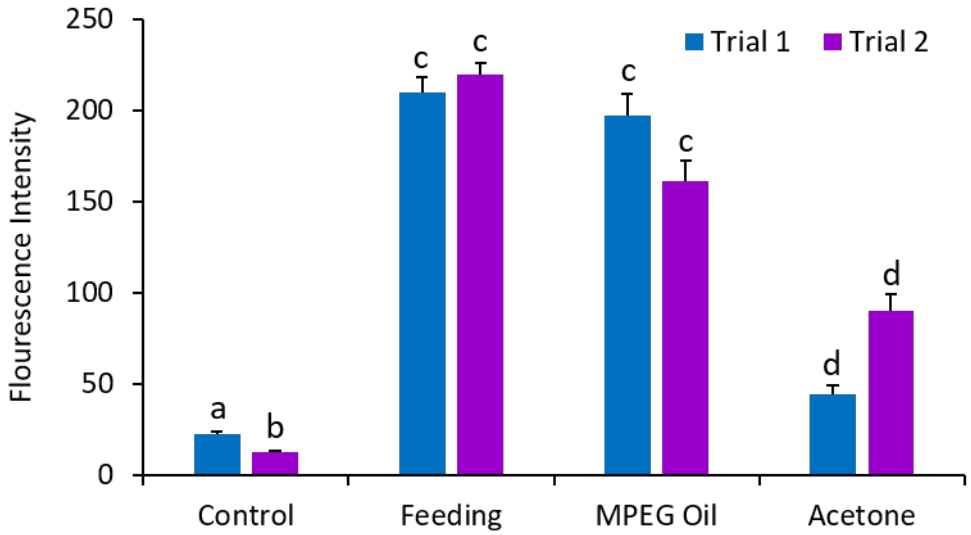
RESULTS

Flouresence Intensity in Fecal Spots – Topical Application and Feeding

For trial one, mean intensities were 22.5 (S.E. + 1.304) for the control, 210.1 (S.E + 7.931) for Dye Fed, 196.8 (S.E. + 12.095) for MPEG oil, and 44.2 (S.E. + 4.975) for acetone. For trial two, mean intensities were 12.5 (S.E. + 0.433) for the control, 210.6 (S.E + 6.258) for Dye Fed, 161.4 (S.E. + 11.082) for MPEG oil, and 90.2 (S.E. + 8.797) for acetone. At the 95% confidence level (a = 0.05), there was a significant difference in fluorescence intensity among the four treatments of trial one and trial two ($P < 0.0001$). The relative order of fluorescence intensity for both trials was: control < acetone < MPEG < Fed.

Among the N = 4 treatments in each trial, there were N*(N-1)/2 = 6 pair-wise combinations. Tukey multiple-comparisons tests identified the specific pairs of treatments that were significantly different from each other, which contributed to the overall source of variance: Trial 1 (SE = 293.0292, k = 4, Qcrit = 3.6330); trial 2 (S.E. = 409.2660, k = 4, Qcrit = 3.6330). When both trails were analyzed together all six pairs showed a significant difference in fluorescence intensity between each other, confirming the overall result of a significant difference in fluorescence intensity among the four treatments. In trial one, the only pairing that did not show a significant difference was Dye MPEG vs. Fed pair (Q = 0.3191). In trial two, the six pairs that showed a significant difference in fluorescence intensity were Dye Fed vs. Control Fed (Q = 16.6127); Dye Fed vs. Dye Acetone (Q = 8.5959); Dye Fed vs. Dye MPEG (Q = 4.5911); Dye MPEG vs. Control Fed (Q = 12.0215); Dye

Figure 1. Trial two, the six pairs that showed a significant difference in fluorescence intensity were Dye Fed vs. Control Fed.



MPEG vs. Dye Acetone (Q = 4.0047); and Dye Acetone vs. Control Fed (Q = 8.0168) (Figure 1).

Detection of Dye Fluorescence - Microplate Reader

The control mean intensity was 6,449 (S.E. + 93.981), and the Dye Fed mean intensity was >65,000, which exceeded the measurable limit of fluorescence for the reader. The MPEG oil mean intensity was 7,193.68 (S.E. + 334.220), and the Acetone mean intensity was 11,177 (S.E. + 1,450.672). At the 95% confidence level (a = 0.05), there was a significant difference in fluorescence intensity among the 4 treatments (Acetone, MPEG Oil, Control, and Fed) ($P < 0.0001$) and three of the six pairs showed a significant difference in fluorescence intensity between each other (Figure 2).

Among the N = 4 treatments, there were N*(N-1)/2 = 6 pair-wise combinations. Tukey multiple comparisons was used to identify specific pairs of treatments that differed significantly from each other and three of the six differed significantly in fluorescence intensity (SE = 36.9476, k = 4, Qcrit = 3.6330): Fed vs. Control (Q = 7.5783), Fed vs. MPEG (Q = 5.4672), and Acetone vs. Control (Q = 4.3846). At the 95% confidence level (a = 0.05), there was a significant difference in fluorescence intensity among the four treatments (Acetone, MPEG Oil, Control, and Fed) with pairs Fed-Control, Fed-MPEG, and Acetone-Control contributing to the difference. The relative order of fluorescence intensity was Control < MPEG Oil < Acetone < Fed.

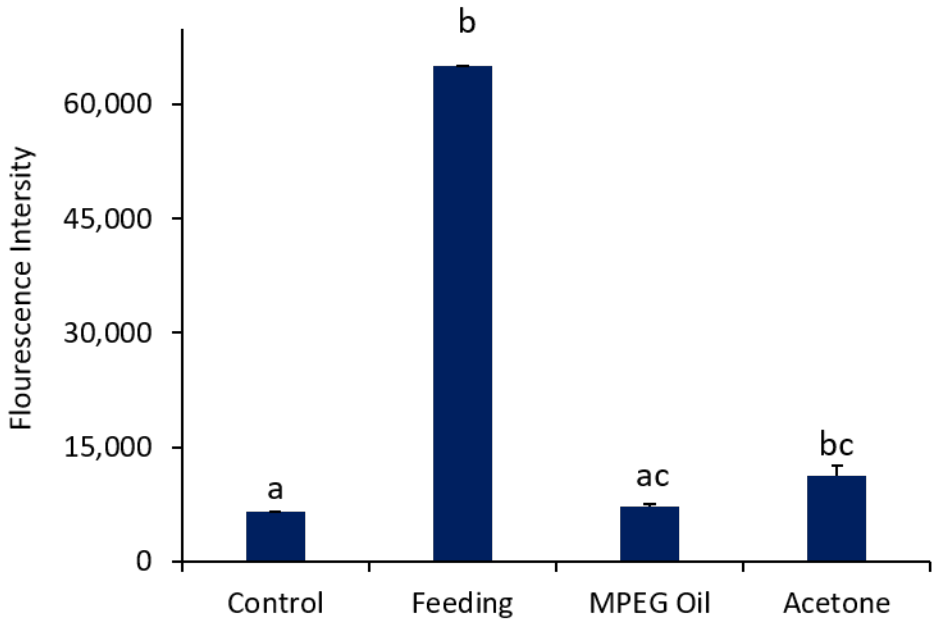
DISCUSSION

Results of the fecal dropping readings showed that a highly water-soluble molecule, Rhodamine B, moves through the cuticle and is excreted. The mixture of MPEG oil and RB had higher intensity fecal spot readings than the acetone combination indicating that the dye in oil penetrated the cuticle better than in acetone. Earlier formulations studies have found oil to improve cuticle penetration by an active ingredient.[8-9]

For the topical applications, results suggest that more dye passes out of mosquitoes without dissolving in internal or external tissue with MPEG oil solution versus acetone solution. The higher florescence readings in the acetone treatment, but lower florescence intensity in the fecal droppings, suggest that acetone may disrupt the wax layer of the cuticle in a way that binds the dye in the cuticle tissue with less dye reaching the digestive system to be excreted. Whereas the MPEG oil mixture with higher fecal florescence with the lower florescence intensity indicated that the dye in the oil penetrated the cuticle but is effectively excreted before it is absorbed into internal tissues.

Feeding double-stranded RNA (dsRNA, RNAi material) to mosquito larvae has been shown to be an effective way to inhibit adult development.[1,3] Our data support the observation that feeding is an effective mechanism to get water-soluble molecules including dsRNA and bioactive peptides into adult mosquitoes. The high relative fluorescence intensity of the fecal droppings and the highest relative intensity in the body homogenates was observed when the organisms where introduction of the dye was by feeding. The dye taken in by feeding is excreted in large amounts but may be absorbed in internal tissues in greater amounts than either of the topical applications possibly due to fewer physical barriers in the alimentary tract.

Figure 2. Significant difference in fluorescence intensity among the 4 treatments and three of the six pairs showed a significant difference in fluorescence intensity between each other



Insect detoxification mechanisms make xenobiotics more polar so that they can be more easily passed out of the body via the excretory system.[10] Two phases of detoxification exist, and a highly nonpolar molecule will go through both phases. A polar molecule, however, will go through only the first detoxification phase. The highly polar RB dye probably is easily excreted which should have similar importance for other polar molecular insecticides. Such insecticides will need to quickly reach the target site before they are metabolized, degraded in other pathways or excreted.[10-11]

CONCLUSION

For any future water-soluble molecules, particularly biologically-based active ingredients, developed as topical adulticides, penetration of the cuticle is possible despite the protective nature of the cuticle. However, formulations need to be developed that maximize the diffusion gradient and overcome detoxification mechanisms to be most effective.

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REFERENCES

1. Airs PM, Bartholomay LC. RNA interference for mosquito and mosquito-borne disease control. *Insects*. 2017;8;4;doi:10.3390/insects80100004
2. Coy MR, Sanscrainte ND, Chalaire KC, Inberg A, Maayan I, Glick E, Paldi N and Becnel JJ. Gene silencing in adult *Aedes aegypti* mosquitoes through oral delivery of double-stranded RNA. *J Appl Entomol.* 2012;136:741-748.
3. Van Ekert E, Powell CA, Shatters Jr RG, Borovsky D. Control of larval and egg development in *Aedes aegypti* using RNA interference using juvenile hormone acid methyl transferase. *J Insect Physiol*. 2014;70:143-150.
4. Schouest LP, Umetsu N and Miller TA. Solvent-modified deposition of insecticides on house fly (Diptera: Muscidae) cuticle. *J Econ Entomol*. 1983;76:973-982.
5. Benoit JB, Denlinger DL. Suppression of water loss during adult diapause in the northern house mosquito, *Culex pipiens*. *J Exp Biol*. 2007;210:217-226.
6. Bellés X. Beyond *Drosophila*: RNAi *in vivo* and functional genomic in insects. *Ann Rev Entomol*. 2010;55:11-128.
7. Zar, JH. *Biostatistical Analysis*. 1999. Fourth edition. Prentice Hall: Upper Saddle River, New Jersey. 663 pp.
8. Sundaram KMS. Cuticular penetration and in vivo metabolism of Fenitrothion in spruce budworm. *J Environ Sci Health*. 1988;23:643-659.
9. Webb JE, Green RA. On the penetration of insecticides through the insect cuticle. *J Exp Biol*. 1945; 22:8-20.
10. Yu SJ. *The Toxicology and Biochemistry of Insecticides*. 2008. CRC Press, Boca Raton, FL. 276 pp.
11. Lucas KJ, Myles KM, Raikhel AS. Small RNAs: A new frontier in mosquito biology. *Trends Parasitol*. 2013; 29:295-303

A Comparison of Battery Chemistries in the Operation of CDC Light Traps at Aberdeen Proving Ground, Maryland, USA

MAJ Derek Monthei, PhD

ABSTRACT

The CDC light trap is a common mosquito surveillance trap that utilizes a six-volt battery. A typical light trap is often powered by a lead-acid battery. The U.S. Army currently utilizes lead-acid batteries for most CDC light traps. Lead-acid batteries have some limitations, especially when needed in austere environments. They can be greatly affected by extreme temperatures, are not environmentally friendly to produce and dispose of, and the weight of the batteries limits the number that deploying units can take with them for a surveillance program. Alternative battery compositions to current lead-acid technology need to be identified and evaluated under field conditions. We evaluated batteries that are lighter in weight, more resilient to extreme high temperatures, protected from overcharging/undercharging, and provided the same amount of time of use in the field as conventional 6V lead-acid batteries when operating mosquito traps.

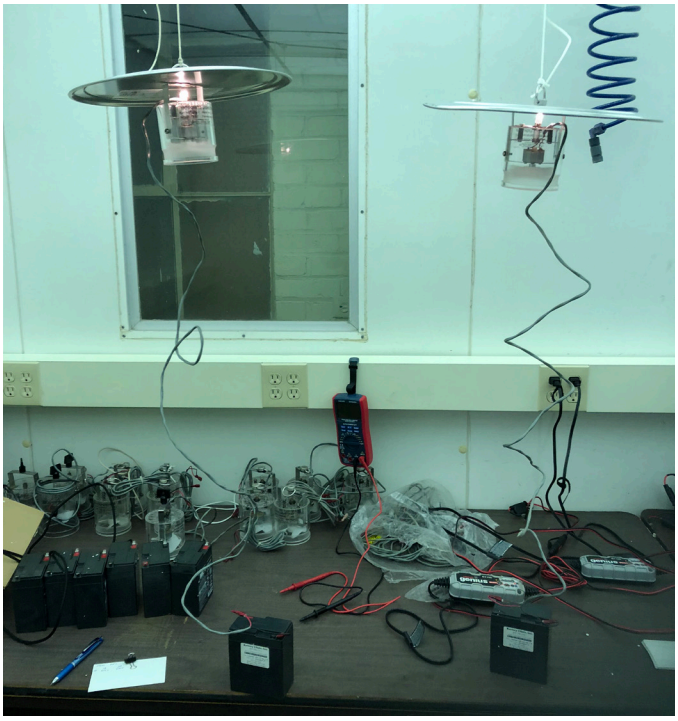
INTRODUCTION

Mosquito traps play a vital role in monitoring mosquito populations and mosquito-borne diseases.[1] Abundance of data from these traps have provided justification for implementation of vector control measures applied by several federal, state, and local agencies against mosquitoes. The New Jersey light trap, developed in the 1920s, was once the "gold standard" for mosquito surveillance.[2] Since the New Jersey light trap requires alternating current (AC) from a household, the battery-powered Center for Disease Control and Prevention (CDC) light trap became the trap of choice.[1] Many mosquito traps have been developed since the New Jersey light trap that continue to be evaluated to this day.[3-5] The CDC light trap is now commonly used and incorporates light, and can be supplemented by a source of carbon dioxide for increased effectiveness.[6] The CDC light trap was developed by the U.S. Centers for Disease Control and Prevention to provide a reliable and portable sampling device for the collection of mosquitoes and sand flies used in arbovirus and taxonomic studies.[6] These traps are small, lightweight, and battery operated. They generally run on six volts (V) supplied by 4 D-cell batteries or a rechargeable 6-volt, 10 Amp hour (Ah) gel cell battery (Figure 1). A photoelectric switch allows for the trap to automatically begin operating at dusk.[6] A rain shield can be fitted to the trap for use in damp conditions. The fan remains running until the battery is disconnected to prevent live mosquitoes from escaping through the top of the trap. A minimum of at least two rechargeable 6V batteries are needed for field use so one battery can be charged while the other is in use to operate the trap.

Figure 1. An Absorbed Glass Mat (AGM) non-spillable lead-acid battery.

The most popular and current standard battery used is the inexpensive lead-acid battery. The lead-acid battery has some limitations that include a short run time (often 24 - 48 hours of continuous usage), low capacity, and low power density per unit weight. Recent trends in battery technology have focused on developing higher efficiency while ensuring proper size, shape, and weight as well as include low maintenance requirements.[7] More recent batteries that contain nickel metal hydride (Ni-MH) and lithium-ion (Li-ion) materials have different features with fewer limitations of lead-acid batteries. Gel cell (type of lead-acid battery) batteries are considered ideal for powering entomological surveillance equipment because they are maintenance-free and spill-proof. The compact size of gel cell batteries and having relatively low weight make them highly transportable. The main issue is that multiple gel cell (lead-acid) batteries are needed for a proper surveillance program and moving multiple lead-acid batteries is heavy and a potential hazardous waste issue. Lead-acid batteries can also work for many years, however improper charging can greatly reduce their life span. In addition, high temperatures experienced in field conditions and operational environments can also reduce the life of a lead-acid battery. Therefore, alternative battery compositions to current lead-acid technology need to be identified and evaluated under field conditions. We conducted this study to evaluate batteries that are lighter in weight, more resilient to extreme high temperatures, protected from

Figure 2. CDC light traps with rainshield, light housing, and motor assembly, but without the cage assembly.



overcharging/undercharging, and provide the same amount of time of use in the field as conventional 6V lead-acid batteries when operating mosquito traps.

MATERIALS AND METHODS

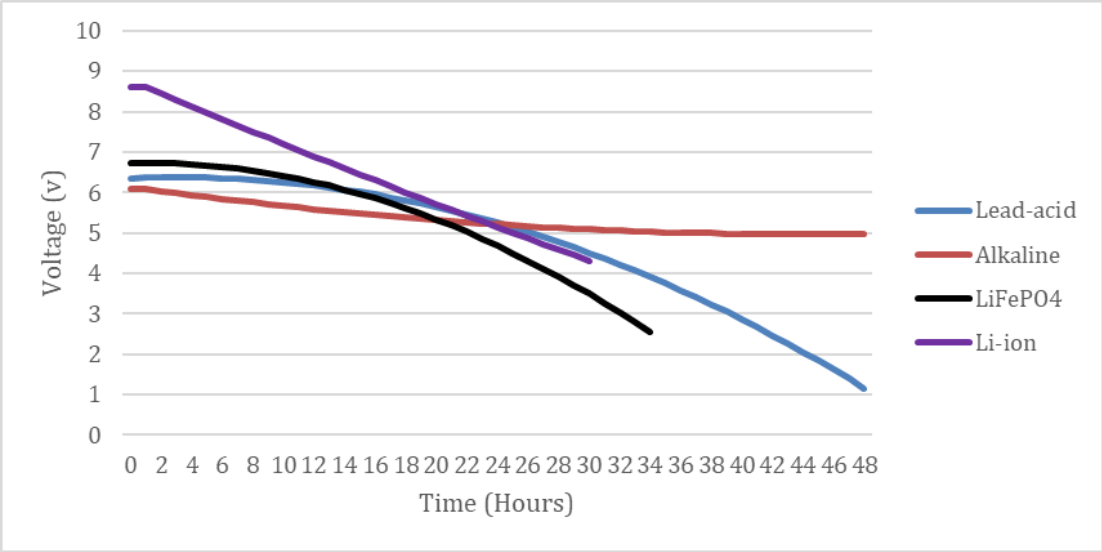
Four battery chemistries were tested: lithium iron phosphate (LiFePO4), lead-acid, lithium-ion (Li-ion), and alkaline. Studies were conducted at the Entomological Sciences Division building on Aberdeen Proving Ground, Maryland, from 21 June 2018 to 01 January 2019. In the first study, the total length of time continuously running a CDC light trap (cutoff at 48 hours) was monitored. In addition, this study used an incorrect charger to charge the lithium-ion and lithium iron phosphate batteries when operating a CDC light trap while an appropriate charger was used to charge the lead-acid batteries. The second study used the correct charger for the lithium-ion and lithium iron phosphate batteries and used those batteries to power a CDC light trap. The third study charged and stored the batteries for six months to determine if they maintained a charge. The lead-acid batteries were charged with a commercially available 6V charger for every study. In the first study, the lithium-ion batteries were charged with a commercial lithium-ion battery charger that was not from same manufacturer of the batteries and the lithium iron phosphate batteries were charged with a commercially available lithium iron phosphate charger not from the same manufacturer of the batteries. In the second study, the lithium-ion and lithium iron phosphate batteries were charged with their respective manufacturer charger. In the third study, the lithium-ion batteries and the lithium iron phosphate batteries were charged with their respective manufacturer charger. We compared the first study with second study to determine if using the wrong charger on the lithium-ion or lithium iron phosphate batteries would cause over-charging or under-charging of the batteries and shorten the life or functionality of the battery like it would for lead-acid batteries. Two sets of four alkaline batteries ran two CDC light traps on three separate occasions. Two lithium-ion and two lithium iron phosphate batteries ran CDC light traps with three replications each. There were four lead-acid batteries tested to run CDC light traps with three replications each. All batteries tested are commercially available. Battery testing was performed using a commercially available digital multimeter in ambient room temperature (approximately 23°C). Before a voltage reading was taken, the ambient air temperature was recorded using the multimeter and temperature probe. A reading was taken by turning the multimeter to the direct current voltage (DCV) region. The red probe of the multimeter was placed on the battery's positive terminal and the black probe to the battery's negative terminal. For the alkaline D-cell batteries, the positive terminal is the protruding end. The lead-acid batteries used were Absorbed Glass Mat (AGM) non-spillable batteries and are rated 6V, 7.65 Ah. Four D-cell batteries were held in an external battery holder to operate the CDC light trap. These D-cell batteries were non-rechargeable, so four new batteries had to be used per trap for each replication. The lithium iron phosphate batteries used were rated 6.4V, 7.4 Ah and the lithium-ion batteries rated 7.26V, 6.7 Ah. Batteries were given a number, labeled using masking tape, and charged until the indicator light on the respective charger indicated a full charge.

Two CDC light traps were hung approximately five feet off the ground in parallel in studies 1 and 2. The light traps had the rain shield, light, housing, and motor assembly, but did not have the cage assembly (Figure 2). The temperature was first recorded using the multimeter and then the batteries were initially tested ($t = 0$) for voltage. Batteries were tested at liberty and the times ranged from one hour to 13 hours apart. An effort was made to conduct a voltage check at least every eight hours ($t = 8, 16, 24, 32, 40, 48$).

Batteries were charged using the chargers as previously described for study 2 for study 3. On 01 August 2018, batteries from all chemistries were tested for voltage in study 3. Batteries were stored on a shelf at room temperature (approximately 23 ºC) and tested for voltage every 30 days (± 4 days) using the multimeter to determine if any of the batteries lost some of their charge during long periods of storage. Voltage was tested for six months after charging.

For statistical analysis of voltage over time for study 1, a second order polynomial was fitted to each of the data sets using a trendline. A Kolmogorov-Smirnov test (KS-test) was used to test for statistical differences ($\alpha = 0.05$) between the battery chemistries. The KS-test compares the fitted

Figure 3. Fitted curves of voltage from all battery chemistries showing voltage over time.



The lithium ion (Li-ion) and lithium iron phosphate (LiFePO4) batteries were charged with the incorrect charger. A second order polynomial was used as the fitted curve equation. Lead-acid is considered the control.

curves of the lead-acid battery to the fitted curves of the other battery chemistries to determine if there is a difference in voltage over time datasets and was performed by entering data in a KS-test program.[8] The KS-test determines if two datasets differ significantly. Data collected in one situation (running a light trap with a lead-acid battery) is compared to data collected in a different situation (running a light trap with a different battery chemistry) to examine if the first situation produces different results from the second one. The null hypothesis that the lead acid-battery and other battery chemistry curves were the same was rejected if $P \leq 0.05$.

RESULTS

Tables 1 and 2 show the length of time of the batteries ran in studies 1 and 2. In study 1, Mean (± SD) times for lead-acid, lithium-ion, lithium iron phosphate, and alkaline batteries running a CDC light trap during a 48 hour period were 21.3 ± 12.5 hours, 12 ± 8.6 hours, 13.1 ± 9.1 hours, 20.4 ± 13.9 hours, respectively. In study 2, Mean (± SD) times for lithium-ion and lithium iron phosphate batteries running a CDC light trap during a 48 hour period are 10.3 ± 7.4 hours and 14 ± 9.6 hours, respectively.

Results from the voltage testing of stored batteries are shown in Table 2. There was little change in the voltage during the six months of storage at room temperature for all battery types.

Figure 3 shows voltage over time for all battery chemistries and second order polynomial trendlines for batteries in study 1. A significant difference between curves was found at α = 0.05 using the KS- test between lead-acid batteries and alkaline batteries (D = 0.5, P = 0.001), lithium iron phosphate (D = 0.3636, P = 0.047), and lithium-ion (D = 0.5, P = 0.002). The null hypothesis is that all battery chemistries were sampled from populations with identical distributions as the lead-acid batteries. The results indicated that the other battery chemistries do not operate a CDC light trap the same as lead-acid batteries. If the trendlines for the different battery chemistries were closer in similarity it would show that they operate similarly. The different batteries produce too large of a difference in the curves from the data we collected. We observed that lead-acid and alkaline batteries can run a CDC light trap for 48 hours, however the alkaline

Table 1. Time from CDC light trap start to battery failure or 48 hour stopping point.

Battery	(Run Time [mean ± SD] h)
Lead-acid	48 (21.3 ± 12.5)
Lithium-ion	30 (12 ± 8.6)
LiFePO4	34 (13.1 ± 9.1)
Alkaline	48 (20.4 ± 13.9)

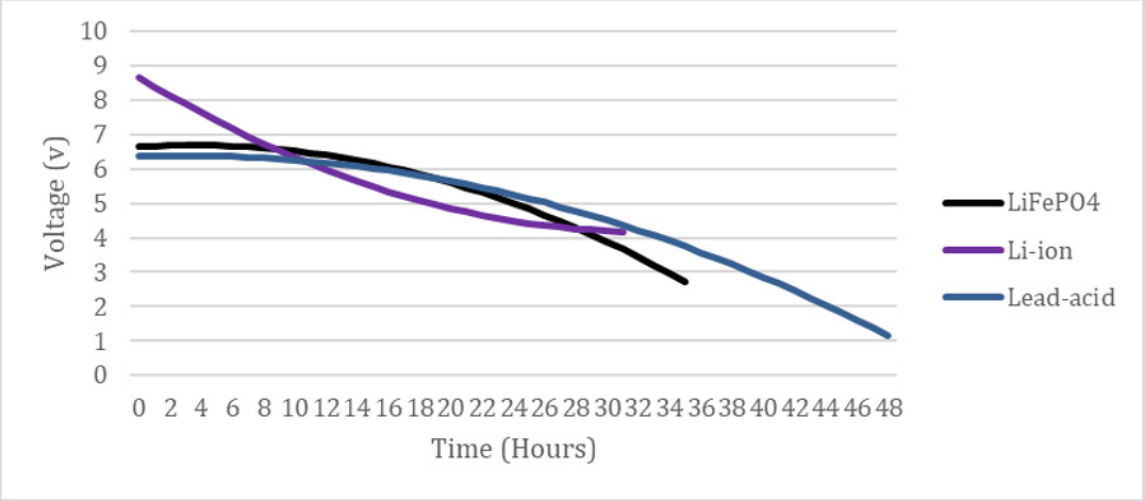
LiFePO4 = lithium iron phosphate; SD = standard deviation; h = hours

Table 2. Time from CDC light trap start to battery failure.

Battery	(Run Time [mean ± SD] h)
Lithium-ion	31 (10.3 ± 7.4)
LiFePO4	35 (14 ± 9.6)

LiFePO4 = lithium iron phosphate; SD = standard deviation; h = hours

Figure 4. Fitted curves of voltage from three battery chemistries showing voltage over time.



Fitted curves of voltage from lithium ion (Li-ion) and lithium iron phosphate (LiFePO4) batteries that were charged with the appropriate charger. A second order polynomial was used as the fitted curve equation. Lead-acid is considered the control.

batteries will have more incremental changes in voltage over time where lead-acid has larger changes. Lithium-ion and lithium iron phosphate (LiFePO4) batteries ran for 30 and 34 hours. More replications and more batteries tested could have increased the mean time and may have reduced the standard deviation as well as allowed for other statistical analysis to be conducted, however resources were limited for these studies. The results indicated the run times and voltage between battery chemistries were significantly different from lead-acid batteries.

Figure 4 shows voltage over time for lithium-ion and lithium iron phosphate batteries and their second order polynomial trendlines in study 2. A significant difference between curves was found at $\alpha = 0.05$ using the KS-test between lead-acid batteries and lithium iron phosphate ($D = 0.4286$, $P = 0.013$) and lithium-ion ($D = 0.4706$, $P = 0.009$) batteries. The results show that the different batteries do not power the CDC light trap the same over time.

DISCUSSION

The commonly used CDC light trap is used by the military because it is portable and durable enough to operate in remote areas. The 6V AGM lead-acid batteries are used by various military units and across the Armed Forces Branches to operate them. Since the lead-acid battery is commonly used as the standard battery, it was used as the control for this study and was compared against the other battery chemistries. Table 4 shows a comparison of characteristics for these batteries.

Although the batteries had similar voltage and amp hours, they all performed very differently. The lithium-ion and lithium iron phosphate batteries have a protection circuit module to prevent over/under voltage, over current, and cell balancing. The lithium-ion batteries

Table 3. Monthly battery testing for stored batteries.

Date	1-Aug-18	30-Aug-18	3-Oct-18	1-Nov-18	30-Nov-18	2-Jan-19
Battery Type						
Alkaline	5.17	5.16	5.15	5.14	5.14	5.14
Alkaline	5.46	5.46	5.47	5.47	5.48	5.48
Alkaline	6.47	6.47	6.47	6.47	6.47	6.48
Lead-Acid	6.41	6.38	6.35	6.34	6.33	6.32
Lead-Acid	6.43	6.4	6.37	6.35	6.34	6.33
Li-ion	8.55	8.38	8.34	8.31	8.3	8.28
Li-ion	8.38	8.28	8.26	8.25	8.24	8.23
LiFePO4	6.69	6.67	6.67	6.66	6.67	6.66
LiFePO4	6.8	6.69	6.68	6.67	6.67	6.67

Batteries were tested for voltage using a multimeter and stored on a shelf in an ambient temperature room (approximately 23°C). Readings are measured in volts (V). LiFePO4 = lithium iron phosphate; Li-ion = lithium ion.

were only supposed to charge to 7.26 volts; however, no matter which charger was used, they reached up to 8.41 volts at full charge at t = 0. There could have been a defect with the batteries or the protection circuit module in the batteries. Most traps will operate at 6V, and all batteries tested ran the CDC light traps. All battery chemistries have similar amp hours (Ah). The amp hour for lead-acid is 7.65 Ah while lithium iron phosphate and lithium-ion are 7.4 and 6.7 Ah, respectively. An amp hour is the amount of energy charge in a battery that allows one ampere of current to flow for one hour. The higher the amp hour rating, the longer the battery will remain at its peak under load.[9]

All battery chemistries were able to hold a charge to operate a CDC light trap for six months at room temperature. The temperatures experienced by the batteries were considered to be moderate temperatures because batteries under more extreme temperatures could show greater differences. Generally, an AGM lead-acid battery must be 2.5 times larger in capacity than a lithium-ion to get comparable life in a moderate climate. In temperatures averaging 33.3 0C, the disparity between lithium-ion and lead-acid is exacerbated as lead-acid drops to 50% of its moderate climate rating while lithium-ion will remain stable until temperatures exceed 48.9 0 C.10 Batteries will lose capacity in cold weather environments as well as high temperature environments. Lithium-ion loses significantly less capacity as the temperature drops into the -20 ºC range when compared to lead-acid batteries.[10]

Lead-acid batteries compare poorly to lithium-ion with regards to environmental friendliness because they require many times rawer material than lithium-ion to achieve the same energy storage, making a larger impact on the environment during the mining process. The lead processing industry is also very energy intensive, leading to large amounts of pollution. The major components of a lithium-ion cell require the mining of lithium carbonate, copper, aluminum, and iron ore. Lithium mining is resource intensive, but lithium is only a minor portion of the battery cell by mass, so the aluminum and copper environmental impacts are much more significant.[10] The lithium iron phosphate battery had the best battery chemistry based on the observations in this study to operate a CDC light trap out of the four tested. Lithium iron phosphate (LiFePO4) was superior to lithium-ion batteries in that LiFePO4 batteries have a longer life cycle and a very constant discharge voltage compared to lithium-ion batteries. Compared to other lithium chemistries, LiFePO4 experiences much slower degradation when stored in a fully charged state.[11] The LiFePO4 is highly resilient during oxygen loss, which typically results in an exothermic reaction in other lithium cells. As a result, lithium iron phosphate cells are much harder to ignite in the event of mishandling (especially during charge) although any fully charged battery can only dissipate overcharge energy as heat. It is also commonly accepted that LiFePO4 battery does not decompose at high temperatures.[11]

CONCLUSION

The results of the observations and data collected in this study indicated that lithium iron phosphate could be an ideal replacement for the current lead-acid battery used as an energy source to operate CDC light traps for mosquito surveillance. The LiFePO4 battery is smaller in size, weighs less, rechargeable, and environmentally friendlier than a lead-acid battery. The LiFePO4 batteries did not run a light trap continuously for 48 hours, but the expectation is for it to operate a CDC light trap for at least 12 hours and ideally 24 hours, in case the trap could not be retrieved in a 12-hour timeframe. Krieger et al.[12] found the LiFePO4 batteries were well-suited for off-grid renewable applications as the cells showed the least degradation under various charging protocols as well as the best voltage performance compared to lead-acid and lithium-based battery technologies.[12] A battery technology like lithium iron phosphate can be arranged in such a way that it takes fewer cells to operate a device than a lead-acid battery. For example, four cells of a lithium iron phosphate battery can be placed in a series for a nominal voltage of 12.8V which would be similar to the nominal voltage of six-celled lead-acid batteries. This means a lithium iron phosphate battery would be lighter and smaller in size yet perform the same function as a lead-acid battery. Another possibility is examining an alkaline battery system that is rechargeable. Alkaline batteries worked well in these studies, but the cost and environmental impact does not make it sustainable for a mosquito surveillance program. A rechargeable alkaline system may exist that meets a surveillance program's needs

Table 1. Comparison of battery characteristics.

Technology	Size mm (LxWxH)	Weight (lbs)	Holds Charge ≥ 6 Months	Voltage	Rechargeable?	48 h of Continuous power	Disposal Hazard
Alkaline (4 x 1.5 V D-Cells)	34.2 x 61.5 individually	1.5 with holder	Yes	1.5 each	No	Yes	No
Lead-Acid	102 x 56 x 120	3.5	Yes	6	Yes	Yes	Yes
Lithium Iron Phosphate	53.3 x 26.9 x 133.4	1	Yes	6.4	Yes	No	No
Li-ion	37.6 x 21.1 x 133.4	0.5	Yes	7.26	Yes	No	Yes

V = volts; mm = millimeters; L = length; W = width; H = height; lbs = pounds; Li-ion = lithium ion.

but is sustainable. Further testing might be needed, but the Armed Forces should consider using a lithium iron phosphate battery in place of lead-acid batteries for mosquito and vector surveillance equipment.

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REFERENCES

1. Kline DL. Mosquito population surveillance techniques. *Tech Bull Florida Mos Con Association.* 2006; 6: 2-8.
2. Reinert WC. The New Jersey light trap: an old standard for most mosquito control programs. *Proc. Annu. Meet. N. J. Mosqu. Exterm. Assoc.* 1989; 76: 17-25.
3. 3. Moore SJ, Zunwei D, Hongning Z, et al. The efficacy of different mosquito trapping methods in a forest-fringe village, Yunnan Province, Southern China. *Southeast Asian J Trop Med Public Health*. 2001; 32: 282-289.
4. Ritchie SA, Zborowski P, Banks D, et al. Efficacy of novel updraft traps for collection of mosquitoes in Cairns, Australia. *J Am Mosq Control Assoc.* 2008; 24: 520-527.
5. Li C, Smith ML, Fulcher A, et al. Field evaluation of three new mosquito light traps against two standard light traps to collect mosquitoes (Diptera: Culicidae) and non-target insects in northeast Florida. *Florida Entomologist.* 2015; 98: 114–117.
6. *Technical Guide 48: Pest and Vector Surveillance.* Washington DC: Armed Forces Pest Management Board. November 2013.
7. Anuphappharadorn S, Sukchai S, Sirisamphanwong C, Ketjoy, N. Comparison the economic analysis of the battery between lithium-ion and lead-acid in PV stand-alone application. *Energy Procedia*. 2014; 56: 352-358.
8. KS-test program, http://www.physics.csbsju.edu/statsKS-test.html. Accessed August 18, 2018.
9. Cope E, Smith J. Maintenance for "maintenance-free" batteries. *Wing Beats.* 2010; 21(2): 23-30.
10. AllCell Technologies LLC. A comparison of lead acid to lithium-ion in stationary storage applications. March 2012. Accessed 24 June, 2021. https://www.batterypoweronline.com/wp-content/uploads/2012/07/Lead-acid-white-paper.pdf
11. Rechargeable Lithium Batteries. (n.d.). In Electropaedia- Battery and Energy Technologies. Accessed September 5, 2018. https://www.mpoweruk.com/lithiumS.htm#lto .
12. Krieger EM, Cannarella J, and Arnold CB. A comparison of lead-acid and lithium-based battery behavior and capacity in off-grid renewable charging applications. *Energy.* 2013; 60: 492-500.

Field Evaluation of Two Commercial off-the-Shelf Spatial Repellent Units to Prevent Mosquito Entry Into Two-Person Tents

Emily McDermott, PhD, James E. Cilek[1], PhD

ABSTRACT

We evaluated two off-the-shelf mosquito spatial repellent units to reduce entry of host-seeking mosquitoes into two-person tents. Patio Shield® (21.97% d-cis/trans allethrin) and Radius® Zone Mosquito Repellent (4.0% metofluthrin) reduced the number of host-seeking mosquitoes recovered from inside of tents baited with dry ice in field trials by about 95% and 88%, compared with untreated controls when placed in front of two-person tents. These devices have advantages over currently employed personal protective measures and could be incorporated into the Department of Defense (DoD) Insect Repellent System to improve Force Health Protection against vectors and vector-borne diseases.

INTRODUCTION

Insects have carried disease onto the battlefield for more than two thousand years. For example, Napoleon's Grande Armeé was infamously laid low by louse-borne typhus during the 1812 Russian campaign.[1] However, the bloodiest of American conflicts, the Civil War, marked a culmination of insect prowess in this arena. Of the ~620,000 soldiers who perished in those battles, two-thirds died of disease, with insect-borne pathogens among the primary killers.[2] Even in modern times, the United States Military has suffered from vector-borne diseases in actively deployed service members. As many as 2% of troops deployed to the Middle East during Operations Enduring Freedom and Iraqi Freedom suffered from leishmaniasis,3 with cases peaking at a maximum of 40.[6] reports per 100,000 person-years in 2003.[4] As a result, protecting military personnel against arthropod-borne diseases remains critical for mission readiness and success. Because Service Members are routinely deployed in areas where mosquito and other vector-borne diseases are endemic, the Department of Defense (DoD) supports the application of repellents to exposed skin in combination with properly worn permethrin-treated uniforms and permethrin-treated bed nets as part of the DoD Insect Repellent System5 with regard to personal protective measures. Even though considerable emphasis is placed on personal application of topical repellents to minimize contact between mosquitoes as well as other vectors, compliance by military personnel continues to be a serious issue within deployed units, especially when treated uniforms are not worn. Sanders et al.[3] reported that about 51% of personnel returning from Iraq and Afghanistan in 2004 did not use topical repellents although most knew that N,N-diethyl-3-methyl-benzamide (DEET) was available. Additional anecdotal reports of non-compliance for service members include personal discomfort due to perceived greasy and/or uncomfortable feelings with some repellent formulations. Moreover, some lotions can cause dirt and sand to stick to the skin and clothes where it is applied. Therefore, there is continued need (especially for deployed military personnel) for protection from disease- carrying arthropods, as well as in and around encampments, to ensure mission readiness as it relates to Force Health Protection. Localized area spatial repellents may provide this protection with minimal effort. Several commercially available products are available in the civilian marketplace.[6] These devices function by theoretically creating an envelope of volatile active ingredient that is continuously released into the air column. We evaluated two commercially available spatial repellent products in field trials to determine the amount of protection each provided against local populations of host-seeking mosquitoes in a semi-tropical environment.

Figure 1. Commercial spatial repellent units evaluated in this study (L) Thermacell Patio Shield® with 21.97% d-cis/trans allethrin-treated pad and (R) Radius® Zone Mosquito Repellent with 4.0% metofluthrin reservoir cartridge and USB charging cord.

Methods

Evaluations were conducted from 19-23 August 2019 in northeastern Florida near a freshwater swamp with a history of adult mosquito production on the grounds of Camp Blanding Joint Training Facility, near Starke, FL. We were interested in operationally determining if the Patio Shield® (21.97% d-cis/trans allethrin) and Radius® Zone Mosquito Repellent (4.0% metofluthrin) (both manufactured by Thermacell Repellents, Inc., Bedford, MA) would reduce host-seeking mosquitoes from entering two- person tents (Figure 1). Both units are commercially avail- able to the public. The Patio unit features a 12-hour liquid butane cartridge to provide a heat source to vaporize the active ingredient impregnated in a 5.1 x 3.5 cm wafer (for 4 hours of vaporization). The Radius unit consists of a USB rechargeable Li-Ion battery (6.5 hours of operation per charge) that provides the heat for vaporization. The unit contains a 3.3 ml cartridge of liquid metaflu- thrin (for 12 hours of vaporization according to the manufacturer). Three, two-person, 2 door-Camp Dome-2 tents (Model #893-927-0011, Overhang Forest Floor, Recreational Equipment, Inc. Co-op, Kent, WA) were placed in a linear fashion 300 m apart along the edge of grassy road (Figure 2). Tents were assembled with the factory-provided rainflies. Entrance doors to the tents remained closed until the start of evaluations. A tent without a repellent device was used as a control. At the start of testing, one door of each tent remained open and a Centers for Disease Control and Prevention incandescent light suction trap (John W. Hock Company, Gainesville, FL) with light on and collection bag was previously suspended from the middle top of each tent (Figure 3). A 1.9 L Igloo cooler that contained 1 kg of dry ice pellets was also suspended next to the light trap provided carbon dioxide as an attractant. Repellent devices were immediately turned on at the time of opening each tent and placed ~0.5 ft from the entrance. Units were turned on 1 hour before sunset (1930) and turned off two hours later. During this period, no wind was experienced by the authors in the study area. At 2130, doors to all tents were quickly closed at approximately the same time and only one investigator re-entered each tent to mechanically remove all mosquitoes using a 12 V battery powered Prokopack aspirator (Model 1419, John W. Hock Company, Gainesville, FL) in addition to the light trap collection. Tent rotations followed a 3x3 Latin square design. To prevent cross contamination of repellent active ingredients and controls, tents and associated treatments were rotated together at each location throughout the transect. Treated wafers and reservoir cartridges in each repellent unit were replaced daily. Mosquitoes were identified to species using the taxonomic key of Darsie and Morris.[7] Pooled mean abundance of all species per treatment was subjected to a paired *t*-test to determine differences ($P<0.05$) between spatial repellent units compared with controls.

Figure 2. Linear transect showing tent locations (300 m apart) and general scrub habitat where spatial repellent evaluations were conducted in northeastern Florida. Freshwater swamp was located to the left of the dirt road within 150 m of tents.

Figure 3. Placement of CDC light trap (with collection container) baited with dry ice pellets in 1.9-L Igloo cooler suspended inside each of the two-person tents during this study.

RESULTS AND DISCUSSION

During the five nights of this study, 376 mosquitoes were collected from the control tent, 46 from the Radius tent, and 20 from the Patio Shield tent. Depending on treatment, 8-14 species were collected from each tent (Table 1) with the four most abundant species generally being *Coquilletidia perturbans* (Walker) (29.8% of the total collections), *Aedes mitchellae* (Dyar) (27.4%), *Anopheles crucians* Wiedemann (22.6%), and *Culex nigripalpus* Theobald (13.8%). Relative species abundance in tents were similar among treatments and controls with the exception that *Ae. atlanticus* Dyar & Knab was more abundant than *An. crucians* in the Radius-treated tent. Both mosquito repellent units significantly reduced the relative abundance of host-seeking mosquitoes entering tents compared with the control (Table 2).

In previous work by Lloyd et al.[4], the MR150 portable ThermaCELL Mosquito Repeller (20% d-allethrin) provided the best reduction (76%) against host-seeking *Ae. albopictus* (Skuse) entering a BG Sentinel-1 trap during a 12hperiod compared with traps without repellents. The OFF! Clip On units (percent metofluthrin content proprietary) delivered the next best reduction at 64%. Unfortunately, both units evaluated in that study were designed to be used in close proximity to the user, so that the individual may be directly exposed to the active ingredients. Although the MR150 created a spatial repellency zone similar to the Patio Shield and Radius, the OFF! Clip On unit provided only personal protection. Another potential drawback of the OFF! Clip On unit is that it uses a circulating fan to dispense the repellent that creates a noise, which may adversely affect the safety of military personnel (depending on the local level of threat) in deployed situations and may otherwise be undesirable for overnight use. Later, McPhatter et al.[9] evaluated the Raid Dual Action Insect Repellent and Home Freshener (0.4% transfluthrin) unit and reported that this product decreased entry of *Ae. aegypti* (L.) into 3-man tents by 66% in semi-field trials conducted in northeastern Florida.

We also note that the two spatial repellent units evaluated in this study also have their drawbacks. The Patio Shield relies on non-refillable, liquid butane cartridges for operation.

Table 1. Mosquito species collected with dry ice baited CDC traps placed in two-person tents with and without commercial mosquito spatial repellent units at Camp Blanding Joint Training Facility, 2010.

Mosquito species	% of collection Control	% of collection ThermaCELL Patio Shield	% of collection Radius Zone Mosquito Repellent
Aedes atlanticus	4.3	5.0	17.4
Aedes mitchellae	22.3	25.0	30.4
Anopheles crucians	21.0	15.0	8.7
Coquilletidia perturbans	25.2	30.0	23.9
Culex erraticus	7.2	0	8.7
Culex nigripalpus	14.6	10.0	2.1
Culiseta melanura	0	5.0	0
Mansonia dyari	3.2	0	4.4
Mansonia titllitans	0.8	0	0
Psorophora ciliata	0.8	5.0	4.4
Uranotaenia lowii	0.3	0	0
Uranotaenia sapphirina	0.3	5.0	0
Total collected	376	20	46

Table 2. Mean ± SE and (percent reduction) of host-seeking mosquitoes entering two-person tents with and without commercial mosquito spatial repellent units, Camp Blanding Joint Training Facility, 2010.1Thermacell Patio Shield (21.97% d-cis/trans allethrin), Radius® Zone Mosquito Repellent (4.0% metofluthrin).

Treatment[1]	N	Mean mosquitoes entering tent (% reduction)	*t*-value
Thermacell Patio Shield	5	4.0 ± 0.4 (94.7)	0.019*
Radius Zone Mosquito Repellent	5	9.2 ± 3.1 (87.8)	0.023*
Control	5	75.2 ± 16.6	---

*Means significantly different when compared with control, paired t-test (P<0.05).

Currently, the DoD is pivoting away from the use of liquid fuels, especially with regard to arthropod pest management materiel, as overseas cargo transport of these items can be a fire risk. Because these canisters are "single-use" (12hof operation), the Patio Shield would require procurement and shipment of large amounts of canisters to supply an operation and would generate significant waste. Moreover, the manufacturer states that the impregnated pads be replaced at 4- hour intervals. Given this context, we believe the best option to reduce host-seeking mosquitoes from entering two-person tents is the rechargeable Li-ion Radius Zone Mosquito Repellent unit (with a 12- hour cartridge replacement requirement). Even though mosquito REDUCTION ACHIEVED BY this unit (88%) was slightly below the criteria developed by the World Health Organization8 threshold of ≥90%, the use of a spatial repellent, in combination with the other components of the DoD Insect Repellent System, could provide important redundancy to compensate for lack of compliance with topical repellent, uniform, or bed net usage. Although the electricity requirement to charge the battery for the Radius could present a problem in austere environments, portable solar charging panels could allow personnel to operate the unit when mainstay electricity is not available.

However, it should be noted that the effectiveness of either device may be adversely affected by the same lack of compliance issues as other DoD personal protective measures if they are not applied consistently with best practices (i.e., not charging the battery completely or placing the device too far from the tent). Just like other personal protective measures, personnel will need to be properly trained on how to operate and maintain these spatial repellent devices in order to achieve their maximum protective potential. In any event, identification and evaluation of additional volatile compounds that act as area spatial repellents that meet the World Health Organization protection criteria is warranted in order to sustain protection of military personnel against arthropod-borne disease transmission for mission readiness and success. In addition, further research on alternative rechargeable fuel sources for these devices will also increase their operational use.

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References

1. Peterson, KD. Insects, disease, and military history. Am Entomol. 1995; 41: 147-161.
2. Miller, GL. Historical natural history: insects and the civil war. Am Entomol. 1997;43: 227-245.
3. Sanders JW, Putnam SD, Frankart C, et al. Impact of illness and noncombat injury during operations Iraqi Freedom and Enduring Freedom (Afghanistan). Am Trop Med Hyg. 2005;73: 713-719.
4. Sthalman S, Williams VF, Taubman SB. Incident diagnoses of leishmaniasis, active and reserve components, U.S. Armed Forces, 2001-2016. Med Surv Monthly Rept. 2017;24(2): 2-7.
5. Armed Forces Pest Management Board. Personal Protective Measures Against Insects and Other Arthropods of Military Significance, Technical Guide No. 36;2009.
6. Lloyd AM, Farooq M, Diclaro JW, Kline DL, Estep AS. Field evaluation of commercial off-the-shelf spatial repellents against the Asian tiger mosquito, Aedes albopictus (Skuse), and the potential for use during deployment. US Army Med Dept J. April-June 2013;80-86.
7. Darsie Jr RF, Morris CD. Keys to the Adult Females and Fourth Instar Larvae of the Mosquitoes of Florida (Diptera: Culicidae). Techn Bull Florida Mosq Control Assoc. 2003;v. 1.
8. WHO (World Health Organization). Guidelines for Efficacy Testing of Spatial Repellents. Geneva: World Health Organization; 2013.
9. McPhatter LP, Mischler PD, Webb MZ, Chauban K, Lindroth EJ, Richardson AG, Debboun M. Laboratory and semi-field evaluations of two (transfluthrin) spatial repellent devices against Aedes aegypti (L.) (Diptera: Culicidae). US Army Med Dept J. January-June 2017;13-22.

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Monitoring Insecticide Resistance of Mosquitoes in United States Military Areas of Operations

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ABSTRACT

Vector-borne diseases kill 700,000 people annually. Mosquito-vectored diseases have impacted missions of United States (US) Forces globally for decades. To minimize or decrease the risk of vector-borne diseases, mosquito populations must be controlled, often requiring the use of insecticides. Frequent use of insecticides can result in resistance which reduces or removes their effectiveness. To preserve the few classes of insecticides available for public health interventions, insecticide resistance characterization and monitoring in mosquitoes are conducted by the US Military personnel around the world. The knowledge obtained from this work supports informed pest/vector management decision makers and allows for use of appropriate personal protective measures.

Keywords: mosquito, insecticide, resistance, vector-borne disease, integrated pest management, CDC bottle bioassay, US Army, force health protection

INTRODUCTION

"People are our Soldiers - Regular Army, National Guard, and Reserve - their Families, Civilians, and Soldiers for Life Retirees and Veterans... We win through our people, and people will drive success in our Readiness, Modernization and Reform priorities. We must take care of our people."[1] -General James McConville, 40th Chief of Staff of the Army

The United States (US) Army Preventive Medicine programs work toward the mission of taking care of the Army's people as they identify and address health threats.[2] Vector-borne diseases are responsible for more than 700,000 deaths per year worldwide.[3] The US Forces live and serve worldwide and therefore risk immediate and long-term health impacts if infected. Mosquito-borne pathogens have historically had a dramatic impact on US Forces stationed stateside and deployed around the world. During World War II, vector-borne diseases such as malaria and dengue severely affected US Forces in the Pacific Theater. Outbreaks of Plasmodium falciparum malaria among Marines deployed to Liberia in 2003 and P. vivax malaria in a rotational unit deployed to the Republic of Korea in 2015 highlight the continuing vulnerability of Service Members (SM) stationed globally.[4-5] Such incidents are expected to continue and worsen into the future due to rapid landscape disruption and climate change. The emergence of chikungunya and Zika viruses, their spread to the Western Hemisphere, and the recently extended distribution of Anopheles stephensi, a competent malaria vector, place both garrisoned and deployed troops at increased risk.[6] Mosquito-borne disease events that pose a significant threat to Force Health Protection (FHP) need to be mitigated through personal protection measures and integrated vector and pest management programs.

The US military installations worldwide, in accordance with Department of Defense (DoD) Instruction, employ integrated pest management (IPM) principles to control the size of vector populations.[7] Mosquito management includes cultural and mechanical control methods which emphasize the removal of breeding sites (i.e., water sources) to impact the ability of mosquitoes to increase population sizes. Fewer mosquitoes results in less biting and a decreased potential for pathogen transmission to people on an installation. Unfortunately, the proximity to local communities where IPM practices are not followed and where pathogens occur in the population, increase the risk to SMs. When non-chemical control measures (i.e., breeding sites and resources removal) do not curb vector populations, deployment of chemical control measures (i.e., pesticides) occurs.

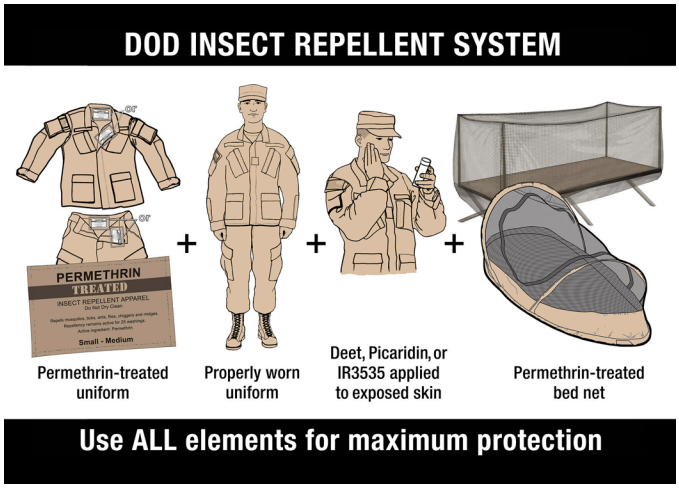
Four classes of insecticides are commonly used to control mosquito populations: pyrethroids (permethrin, lambda-cyhalothrin), organophosphates (malathion, naled, chlorpyrifos), carbamates (propoxur, bendiocarb), and organochlorines (dieldrin, DDT). Insecticides within the same class share common chemical structures and modes of action. Insecticide resistance (IR) surveillance programs can prioritize monitoring widely used insecticides from these classes. As more insecticides from the same class confer resistance in mosquito populations, priority must shift to a control strategy that leverages a different class

of insecticide to minimize pathogen transmission threats from those vectors.

The Insecticide Resistance Action Committee (IRAC) defines IR as 'a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species'.[8] The World Health Organization (WHO) Global Report on Insecticide Resistance in Malaria Vectors, 2010-2016 reports the observance of resistance to at least one of the four main insecticide classes in 73 of the 81 malaria-endemic countries.[9] Likewise, widely observed resistance of Aedes aegypti (L.) to permethrin has limited the effectiveness of routine preventive measures in military operations.[10-11] US Army installations closely monitor pesticide use, however the use of pesticides off the installation falls outside the jurisdiction of installation personnel. Consequently, off-installation pesticide use could result in increased exposure of vectors to pesticides that over time drives the development of IR.

Permethrin-treated uniforms are the primary defense that the military has against vectors and vector-borne diseases, secondary to insecticide treated nets and topical insect repellents (Figure 1). However, many mosquito populations have adapted to have at least partial resistance to pyrethroids, including permethrin. A recent study showed that pyrethroid-resistant mosquitoes can negate the efficacy of permethrin-treated uniforms and put SMs at risk of infections from resistant vector populations.[11] Resistance to pesticides that the military relies on to protect SMs must be tracked in operational locations worldwide as emerging resistance to fundamental preventive measures further amplifies the risk for SMs to contract mosquito-borne illnesses.

Figure 1. Permethrin-treated components are foundational to the DoD Insect Repellent System but become points of potential failure in vector-borne disease Force Health Protection and Readiness measures in areas where insecticide resistance is present.



METHODS

The Centers for Disease Control and Prevention (CDC) Bottle Bioassay12 and WHO Susceptibility test13 are the standard approaches to establish baseline IR information for the spectrum of insecticides available. These assays are used worldwide to assess the level of IR in mosquito populations based on comparisons to documented mortality levels that coincide with degrees of susceptibility. Not all insecticides and mosquito species have the same diagnostic times. The combination of pesticide, diagnostic time, and mosquito species sets the threshold that indicates susceptibility or resistance in the tested population. Additionally, molecular approaches to detect knockdown resistance (kdr) mutations in the voltage-gated sodium channel, one of the mechanisms of resistance to pyrethroids and DDT, have been established.[14] Military laboratories are uniquely positioned to drive the characterization and monitoring of IR in mosquito populations (Figure 2).

Military Organizations Driving this Work

The military strives to keep military populations healthy and mandates public health initiatives that civilian public health authorities cannot.[15] Unfortunately, in terms of mosquito IR monitoring, action by the military is comparable to that of the public. A 2017 survey conducted by the National Association for City and County Health Officials (NACCHO) found that 86% of civilian health official respondents did not perform mosquito IR testing.[16] Fortunately, the US Army Public Health Center (APHC) has conducted IR studies on various public health pests, to include mosquitoes, since the 1970s. Mission adjustments resulted in a shift away from IR testing, however, in response to the military's IR information gap and limited insecticide mode of actions available, the APHC revived IR testing in 2019 for Continental United States (CONUS) installations with a focus on mosquitoes. The goal of testing at APHC is to provide a gauge as to the potential for IR in local Culex and Aedes mosquito populations. Application of acquired IR information can be used to update pest management plans to include design of resistance management strategies for vector and pest species so that Soldiers, families, civilians, and Soldiers for Life who live, work, and train on CONUS installations are protected from vectors and ineffective pesticide usage.

Several Outside the Continental United States (OCONUS) installations and operational areas have produced evidence that IR is present in local populations, overlapping with where mosquito-borne diseases are prevalent. In response to the risk associated with this situation, the Global Emerging Infections Surveillance (GEIS) Branch of the Armed Forces Health Surveillance Division (AFHSD) is driving the adoption of insecticide resistance testing into existing surveillance efforts. Because of the potential breakdown in the ability to protect the warfighter with existing measures, the GEIS Program has increased their

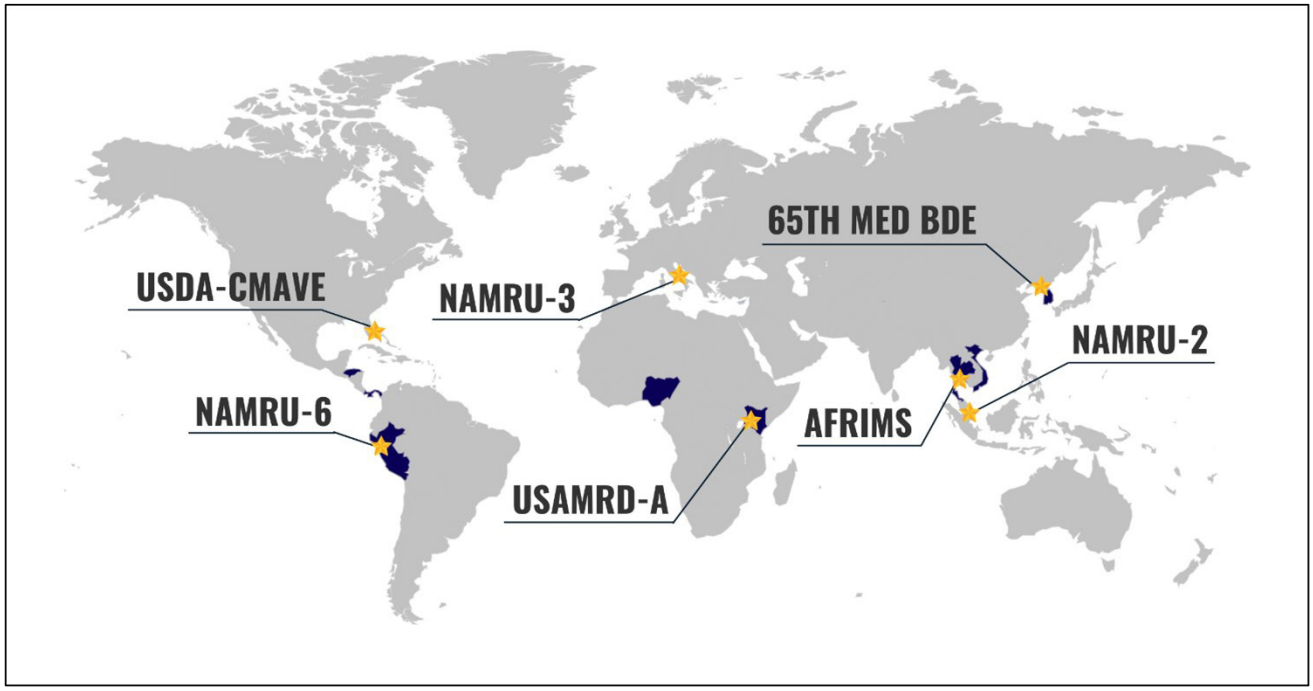
Table 1. The CDC bottle bioassay results for insecticide resistance to select pyrethroid active ingredients with a 30-minute diagnostic time for *Culex quinquefasciatus*.

Site	Pesticide (Dose)	No. Tested	Result
Aberdeen Proving Ground – North (August 2021)			
1	Permethrin (43 µg/mL)	92	Susceptible
2		114	Susceptible
3		134	Susceptible
Aberdeen Proving Ground – South (August 2021)			
1	Permethrin (43 µg/mL)	156	Susceptible
2		159	Susceptible
3		75	Susceptible
4		154	Susceptible
Fort Campbell (June 2021)			
1	Resmethrin (30 µg/mL)	142	Susceptible[a]
2		57	Susceptible
3		156	Susceptible
4		110	Susceptible
Fort Meade (September 2021)			
1	Permethrin (43 µg/mL)	148	Susceptible
2		140	Susceptible
3		34	Susceptible
4		82	Susceptible

funding towards surveillance projects to close these knowledge gaps where US military SMs are operating globally. The GEIS-Network currently funds seven different military laboratories to conduct IR surveillance to include the 65th Medical Brigade (65th MED BDE) in South Korea, the Armed Forces Research Institute of Medical Sciences (AFRIMS) in Thailand, and the US Army Medical Research Directorate-Africa (USAMRD-A) in Kenya (Figure 2).

In OCONUS areas of operation, such as the Republic of Korea (ROK) where US Army Medical Department Activity-Korea (MEDDAC-K) supports the 65th MED BDE, mosquito control using insecticides occurs for both pathogen vectors and nuisance mosquitoes in civilian areas and on US Army Garrisons. In the ROK, mosquito exposure to pyrethroids and other active ingredients is also possible via agriculture, especially for the species that use rice paddies and associated irrigation canals for breeding sites. Studies over the last 50 years have examined insecticide resistance and presence of resistance genes in ROK mosquitoes. Resistance has been reported in *Culex tritaeniorhynchus* Giles (vector of Japanese encephalitis) to the organochlorines DDT and lindane, and pyrethroids.[17,18] Phenotypic resistance has been reported in *Anopheles* spp.(vectors of malaria) to organophosphates, such as chlorpyrifos and pyrethroids such as deltamethrin 19-21 with several populations across the ROK also found to have genes suspected of conferring resistance to these insecticides.[22-23] *Cx. pipiens pallens* L, a member of the *Cx. pipiens* group, with many insecticide resistant populations found worldwide, has been observed to be resistant to

Figure 2. Partner locations and country coverage of insecticide resistance surveillance being conducted by the GEIS-Network. Blue countries represent where IR surveillance is being conducted, while yellow stars show the physical locations of the GEIS-Network laboratories



organophosphates and pyrethroids with many populations across the ROK having suspected resistance conferring genes.[19-20,22-25] The presence of IR in ROK mosquito vector populations increases the risk for SMs in that area of operation to contract mosquito-borne illnesses.

RESULTS AND DISCUSSION

Table 2. The CDC bottle bioassay results for mosquito insecticide resistance to select active ingredients on select installations in USAG Korea.

Mosquito Species	Location	Insecticide	Diagnostic Concentration (µg/bottle)	No. Tested	Percent Mortality at Select Times
Culex pipiens pallens	Camp Henry	Permethrin	43	98	10 min 4.1% 30 min 25.5%
		Chlorpyrifos	20	93	10 min 6.2% 30 min 11.3% 55 min 36.1% 90 min 76.3%
Aedes albopictus	Camp Humphreys	Permethrin	43	66	10 min 90.9% 15 min 100%
		Chlorpyrifos	20	90	10 min 7.8% 30 min 80% 45 min 100%

Note: Diagnostic times for Cx. pipiens are: permethrin, 30 minutes and chlorpyrifos, 90 minutes. Diagnostic times for *Ae. albopictus* are: permethrin, 10 minutes and chlorpyrifos, 45 minutes.

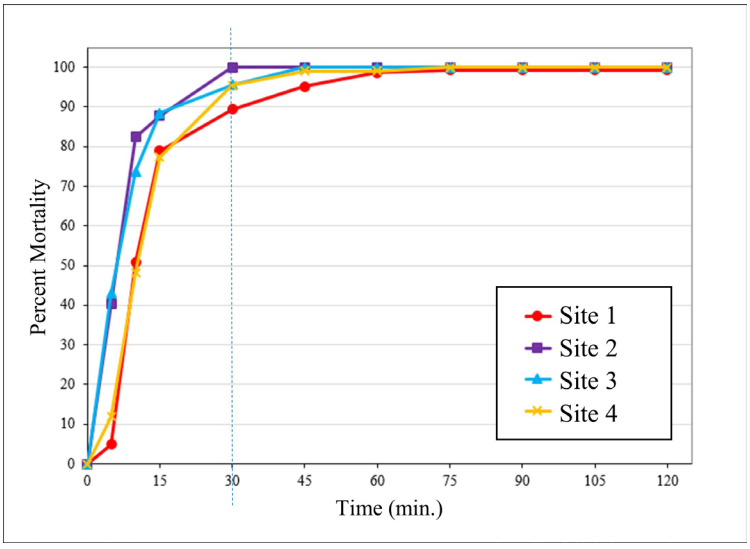
Operationalizing Insecticide Resistance

CONUS. The APHC began to address mosquito IR in 2021 by working in conjunction with US Army CONUS installations in the Atlantic region. Before the collection time period, APHC personnel consulted with IPM and Environmental Health (EH) personnel. Consultation with IPM personnel resulted in selection of an active ingredient for use in CDC bottle bioassays. Sampling for this assessment occurred at locations where EH personnel conduct routine mosquito surveillance in accordance with Army Regulation (AR) 40-5.26 Mosquito egg samples were sent to the APHC, where they were reared to the adult stage. The CDC bottle bioassay procedure was used to screen 3-5 day old adult male and female mosquitoes for IR from each contributing site on an installation.

Table 1 provides an example of "bottom line up front" results using installation sites that were tested with the CDC bottle bioassay in 2021. These results suggest susceptibility of mosquitoes at the installation sites sampled. Results are visualized using time-mortality plots, like the one displayed in Figure 3 for Fort Campbell, and show when mosquitoes from a site reach pre-determined percent mortality markers. Attention to the trajectory of the plot line past the threshold can provide information on the degree of susceptibility. For example, Figure 3 shows that *Cx. quinquefasciatus* Say mosquitoes (vectors of St. Louis encephalitis, Western equine encephalitis, and West Nile fever) from three sites at Fort Campbell, KY exhibited greater than 95% mortality at the 30-minute diagnostic time, whereas samples of the same species from the fourth site only reached 90% mortality by 45 minutes, but did not reach 100% before the test concluded. This fourth site was labeled as susceptible but with the recommendation to monitor management strategies and sample the following season to track possible decreases in mortality times. Mosquitoes from this fourth site are candidates for testing of enzyme levels and/or kdr genes to determine the resistance mechanism present. Susceptibility testing results and resistance mechanism data inform pest management programs which in turn contributes to maintaining force health protection (FHP) standards.

OCONUS. Mosquito surveillance and control on US Army Garrisons (USAG) across the ROK have been conducted routinely since the Armistice between North and South Korea in 1953, however, regular monitoring for IR IN mosquito populations has not. As noted previously, mosquito vectors in Korea are documented to be resistant to select active ingredients. In 2022, with the support of APHC and GEIS, Medical Activity Korea personnel established an IR surveillance program to build baseline information regarding mortality in vector and pest mosquito species present on Army installations.

Figure 3. Time mortality graph for 3-5 day old Culex quinquefasciatus adults collected from designated mosquito surveillance sites at Fort Campbell, KY in June 2021 and tested against resmethrin (30 µg/bottle). Susceptible mosquitoes display greater than 90% mortality at the diagnostic time of 30 minutes



Mosquito larvae and adults were collected on USAG Humphreys, USAG Daegu, and USAG Yongsan-Casey. When reared from larvae, three to five days old male and female mosquitoes were tested. Collected adults were held until sufficient individuals were collected to run the assay. All were tested using the CDC bottle bioassay. Species tested included *An. sinensis s.l. Wiedemann*, *Ae. albopictus* (Skuse), *Ae. koreicus* (Edwards), *Ae. vexans* (Meigen), *Cx. pipiens pallens*, *Mansonia uniformis* (Theobald), and *Armigeres subalbatus* (Coquillett).

Table 2 provides data from CDC bottle bioassays for a population of *Cx. pipiens pallens* (West Nile virus/Japanese encephalitis vector) collected on Camp Henry in Daegu, South Korea and a population of *Ae. albopictus* (dengue/Zika vector) collected on Camp Humphreys in Pyeongtaek, South Korea. Based on the guideline that less than 90% mortality at the diagnostic time for an active ingredient suggests possible resistance in a population, *Cx. pipiens pallens* is possibly resistant to both permethrin and chlorpyrifos while *Ae. albopictus* is susceptible to both permethrin and chlorpyrifos at these respective locations. Status of IR in the other vector species listed is still being evaluated.

With few new active ingredients on the horizon for mosquito control and as climate change advances with the potential to shift the distributions of mosquito vectors, the need to monitor IR in local mosquito populations increases. By monitoring for IR, pest control and public health professionals can use the IR status of the vector or pest species as a key factor in the design of vector management programs to conserve pesticide efficacy and maintain confidence that the pesticides will provide the desired level of control.

Mosquitoes are not the only vectors with IR concern. Tick-borne diseases are on the rise27 and concern about resistance development is widespread.[28,29] Kissing bug species found in the US are vectors of *Trypanosoma cruzi*, the causative agent of Chagas disease.30 The IR in these vectors has been reported but further characterization studies are needed.[31] Cockroaches and bed bugs are public health pests that do not transmit human pathogens of concern, however, they have been documented to develop IR.32, 33 These pests can serve as fomites, instigate allergic reactions, cause anxiety, and disrupt sleep which is an essential element of the Performance Triad.2

Employment of methodologies used by non-military public health entomologists across the realm of mosquito programs provides an opportunity for the US Army to routinely contribute information on the global status of IR. The focal spatial and temporal nature of IR requires regular monitoring particularly in areas where Army personnel serve. Knowledge of IR in an area affords Soldiers information regarding personal protective measures, as well as information required to select efficacious chemical treatment.

The US military personnel stationed around the world are working to determine the IR status of mosquitoes using multiple methods of IR surveillance. With partnerships such as APHC and GEIS these efforts will result in increased awareness of the threat of IR and provide the information needed for informed pest management and personal protection decisions that contribute to Force Health Protection.

REFERENCES

1. *The Army People Strategy*. US Department of the Army. October 2019. Accessed 29 August 2022. https://www.army.mil/e2/downloads/rv7/the_army_people_strategy_2019_10_11_signed_final.pdf.
2. US Department of the Army. *Army Techniques Publications (ATP) 4-02.8 Force Health Protection*. March 2016.
3. World Health Organization. *Vector-borne Diseases (Fact Sheet)*. March 2020. Accessed 9 September 2022. https://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases.
4. Whitman, TJ, Coyne, PE, Magill, AJ, et.al. An outbreak of *Plasmodium falciparum* malaria in US Marines deployed to Liberia. *Am J of Trop Med Hyg.* 2010; 83(2): 258-265. doi: 10.4269/ajtmh.2010.09-0774.
5. Mace, KE, Arguin, PM, Tate, KR. *Malaria Surveillance – United States, 2015*. MMWR, 2018; 67(No. SS-7): 1-28.
6. Sinka, ME, Pironon, S, Massey, NC, Willis, KJ. A new malaria vector in Africa: Predicting the expansion range of *Anopheles stephensi* and identifying the urban populations at risk. *PNAS*. 2020; 117(40): 24900-24908. doi: 10.1073/pnas.2003976117.
7. Under Secretary of Defense for Acquisition and Sustainment. *DoD Pest Management Program* (DoD Instruction 4150.07). December 2019.
8. Insecticide Resistance Action Committee. *The IRAC register* (https://irac-online.org/). 2022. Accessed 29 August 2022. https://irac-online.org/training-centre/resistance/.
9. World Health Organization. *Global Report on Insecticide Resistance in Malaria Vectors, 2010-2016*. 2018. Geneva: World Health Organization.
10. Hamid PH, Ninditya VI, Prastowo J, Haryanto A, Taubert A, Hermosilla C. Current Status of *Aedes aegypti* insecticide resistance development from Banjarmasin, Kalimantan, Indonesia. *Biomed Res Int*. 2018; 2018:1735358. doi:10.1155/2018/1735358
11. Estep AS, Sanscrainte ND, Cuba I, Allen GM, Becnel JJ, Linthicum KJ. Failure of permethrin-treated military uniforms to protect against a laboratory-maintained knockdown-resistant strain of *Aedes aegypti*. *J Am Mosq Control Assoc*. 2020 Jun 1;36(2):127-130. doi: 10.2987/19-6906.1.
12. Centers for Disease Control and Prevention. *CONUS Manual for Evaluating Insecticide Resistance in Mosquitoes Using the CDC Bottle Bioassay Kit*. 2020. Accessed 29 August 2022. https://www.cdc.gov/zika/pdfs/CONUS-508.pdf.
13. World Health Organization. *Test Procedures for Insecticide Resistance Monitoring in Malaria Vector Mosquitoes, 2nd edition*. June 2018.
14. Estep AS, Sanscrainte ND, Waits CM, Bernard SJ, Lloyd AM, Lucas KJ, et al. Quantification of permethrin resistance and kdr alleles in Florida strains of Aedes aegypti (L.) and Aedes albopictus *(Skuse)*. 2018. *PLoS Negl Trop Dis.* 12(10): e0006544. doi: 10.1371/journal.pntd.0006544.

15. Marble, S. *Why the Military Makes Public Health a Priority.* Foreign Policy Research Institute. July 2010. Accessed 27 August 2022. https://www.fpri.org/article/2010/07/why-the-military-makes-public-health-a-priority/

16. National Association of City and County Health Officials (NACCHO). *Mosquito Control Capabilities in the US* October 2017.

17. Self, LS, Shim, JC, Jolivet, P. Susceptibility of *Culex tritaeniorhynchus* and six other mosquitoes to insecticides in Korea. *Cah. O.R.S.T.O.M., ser Ent med. Et parasitol.* 1974;12(2):81-92.

18. Yoo, DH, Shin, EH, Lee, DK, Ahn, YJ, Chang, KS, Kim, HK, Kim, SY, and Park C. Insecticide susceptibility of field-collected populations of *Culex tritaeniorhynchus* in the Republic of Korea. *J Insect Sci.* 2013;13:2. doi:10.1673/031.013.0201.

19. Kim, HC, Rueda, LM, Wilkerson, RC, et al. Distribution and larval habitats of *Anopheles* species in northern Gyeonggi Province, Republic of Korea. *J Vector Ecol* 2011;36(1):124-134.

20. Chang, KS, Kim, HC, Klein, TA, and Ju, YR. Insecticide resistance and cytochrome-P450 activation in unfed and blood-fed laboratory and field populations of *Culex pipiens pallens. J Pest Sci*. 2017;90:759-771.

21. 21. Park, SH, Jun, H, Ahn, SK, et. al. Monitoring insecticide resistance and target site mutations of L1014 kdr and G110 Ace alleles in five mosquito populations in Korea. *Korean J Parasitol*. 2020;58:543-550.

22. Kang, S, Jung, J, Lee, S, et. al. The polymorphism and the geographical distribution of the knockdown resistance (kdr) of *Anopheles sinensis* in the Republic of Korea. *Malaria J*. 2012;11:151.

23. Lee, DE, Kim HC, Chong, ST, et al. Regional and seasonal detection of resistance mutation frequencies in field populations of *Anopheles* Hyrcanus Group and *Culex pipiens* complex in Korea. *Pestic Biochem Phys.* 2020;164:33-39.

24. Shin EH, Kim NJ, Kim HC, et al. Resistance of field-collected populations of *Culex pipiens pallens* (Diptera: Culicidae) to insecticides in the Republic of Korea. *J Asian Pac Entomol.* 2012;15:1-4.

25. Scott JG, Yoshimizu, MH, Kasai S. Pyrethroid resistance in *Culex pipiens* mosquitoes. *Pestic Biochem Physiol.* 2015;120:68-76.

26. Army Regulation 40-5. *Army Public Health Program*. May 2020. Headquarters, Department of the Army.

27. Beard,CB, Visser, SN, and Petersen, LR. The need for a national strategy to address vector-borne disease threats in the United States. *J Med Ent.* 2019;56(5):1199-1203.

28. Kaplan, ZD, Richardson, EA, Taylor, CE, et. al. Determination of the discriminating concentration towards permethrin for surveying resistance in *Amblyomma americanum. J. Med Entomol. 2022;59(3):922.929.*

29. Burtis, JC, Poggi, JD, Payne, B, et al. Susceptibility of Ixodes scapularis (Acari: Ixodidae) to Permethrin under a long-term 4-poster deer treatment area on Shelter Island, NY. *J Med Entomol*. 2021;58(4):1966-1969.

30. Rodriguez, F, Luna, BS, Calderon, O, et al. Surveillance of *Trypanosoma cruzi* infection in Triatomine vectors, feral dogs and cats, and wild animals in and around El Paso County, Texas, and New Mexico. *PLoS Negl Trop Dis.* 2021;15(2): e0009147. doi:10.1371/journal.pntd.0009147.

31. Pessoa, GCD, Vinãs, PA, Rosa, ACL, Diotaiuti, L. History of insecticide resistance of Triatominae vectors. *Rev Soc Bras Med Trop*. July-August 2015;48(4):380-389. doi: 10.1590/0037-8682-0081-2015.

32. Lee, S-H, Choe, D-H, Rust, MK, Lee, C-Y. February. Reduced susceptibility towards commercial bait insecticides in field German cockroach (Blattodea: Ectobiidae) Populations from California. *J Econ Entomol*. 2022;115(1): 259–265. doi:10.1093/jee/toab244.

33. 33Cho, S., Kim, HC, Chong, ST, et al. Monitoring of pyrethroid resistance allele frequency in the common bed bug (*Cimex lectularis*) in the Republic of Korea. *Korean J Parasitol*. 2020;58:99-102.

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Improving the Vector-borne Disease Risk Assessment to Deployed Forces in Djibouti: Military Operational Entomology in the Horn of Africa 2018-2022

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ABSTRACT

The U.S. Forces deployed to Camp Lemonnier, Djibouti and other installations in the Republic of Djibouti are at risk of being exposed to a variety of vector-borne disease threats. Following local dengue fever outbreaks in 2018 and reports of increased seasonal prevalence of the invasive urban malaria vector, *Anopheles stephensi* Liston, Department of Defense medical entomologists, coordinated by the U.S. Naval Medical Research Unit No. 3, began increased surveillance on military installations in Djibouti. Entomologists conducted multiple technical assist visits and provided training to deployed preventive medicine and medical staff, conducted supplemental larval and adult mosquito surveillance, and began molecular testing of field-collected mosquitoes for pathogens. The improved surveillance activities documented the seasonality and distribution of significant disease vectors and recorded a previously undocumented mosquito species, *Ae. vexans* (Meigen). Additionally, the entomology teams demonstrated that *An. stephensi* and *Aedes aegypti* (L.) were actively breeding on the installation, which posed a significant risk to force health. Initial molecular testing of these vectors failed to detect any pathogens in the vectors, but cases of malaria and dengue are reported annually. Despite these improvements, there continues to be a lack of critical ecological information on mosquitos and other disease vectors due to limited organic surveillance capacity and subject matter expertise. Vector-borne diseases are a year-round threat to force health protection in Djibouti, which can be mitigated by consistent entomology support.

Keywords: *Anopheles stephensi*, *Aedes aegypti*, *Aedes vexans*, Camp Lemonnier, Chabelley Air Base

INTRODUCTION

The Republic of Djibouti is a geostrategically important nation located in the Horn of Africa at the junction of the Red Sea and the Gulf of Aden. China, France, Germany, Italy, Japan, Saudi Arabia, Spain, and the United States each have national interests in the region and a military presence in Djibouti.[1,2] The U.S. Marine Corps established the Combined Joint Task Force-Horn of Africa (CJTF-HOA) at Camp Lemonnier, Djibouti (CLDJ) in 2003 following the increased international terrorism threats cascading from the September 11th attack in 2001. The U.S. Navy assumed responsibility of CLDJ in 2006 and continues to support approximately 25 joint tenant commands pursuing a wide array of missions in this highly competitive strategic space.

Multiple vector-borne diseases (VBDs) are common in both urban and rural environments of East Africa, including Djibouti, and pose serious force health protection (FHP) risks for U.S. Forces.[3] Mosquito-borne diseases are the greatest threat. The malaria parasites, *Plasmodium falciparum* and *P. vivax*, are reported annually by the Djibouti Ministry of Health and these infections have increased, particularly in urban areas, since the introduction of the invasive Asian malaria mosquito, *Anopheles stephensi* Liston in 2012.[4,5] The *Aedes*-associated Dengue and Chikungunya viruses have been circulating in Djibouti since 1991[6,7] and 2010[6], respectively. West Nile virus (WNV) positive pools of *Culex* mosquitoes in Djibouti were first detected in 2010[8] and the virus' circulation in the area was further confirmed by serosurveillance studies.[6] These studies reported that 20% of the WNV antibody-positive individuals corresponded to the 2010 *Culex* surveillance study areas.[6] Notably, one of the WNV positive areas (Ambouli) reported in each of these studies is the same district where CLDJ is located. Emerging mosquito-borne fevers, caused by Sindbis, Rift Valley, and O'nyong-nyong viruses, are also suspected of circulating in this region.[9] Phlebotomine sand fly-associated pathogens causing fevers and leishmaniasis are of great concern to FHP as well. Sand Fly Fever viruses belonging to the Naples serocomplex, to include Toscana virus, have been widely circulating in

the region for decades.[10] Recent serological surveillance studies in Djibouti found Toscana-related viral infections had the second highest incidence among seven arboviruses screened.[6] Cutaneous and visceral leishmaniasis have been reported since at least the early 1970s,[3,11] but many uninvestigated questions remain about the transmission cycle in this region.[12] Tick-borne diseases such as Crimean-Congo hemorrhagic fever, Alkhurma hemorrhagic fever, and spotted fever rickettsioses have not been reported in Djibouti, but there have been a few studies investigating components of their transmission cycles. The few studies that have been completed in the country have detected human pathogens in ticks[13,14] and one human exposure to Alkhurma Hemorrhagic Fever virus.[6]

Amplifying these VBD risks, host nation public health has limited preventive medicine programs and surveillance capabilities for early pathogen detection to confidently assess the probability of direct disease transmission to U.S. Forces.3,5 Thus, to protect the health of deployed U.S. Forces, it is imperative that the organic military health service support infrastructure can provide a robust preventive medicine program that includes the surveillance, identification, and control of VBDs. The current force design excludes the permanent assignment of operational entomologists at CLDJ. Instead, CLDJ relies on contracted vector and pest management services to supplement the Navy's Expeditionary Medical Facility Preventive Medicine Department (EMF PMD), which is responsible for the camp's public health and preventive medicine programs. Recognizing this technical knowledge gap and following the Zika epidemic and a dengue fever outbreak on CLDJ in 2018, the U.S. Naval Medical Research Unit No. 3 (NAMRU-3) secured funding from the Global Emerging Infections Surveillance (GEIS) Branch of Armed Forces Health Surveillance Division to assist mosquito-borne disease surveillance in Djibouti. Additionally, entomologists from the Navy Environmental and Preventive Medicine Unit-7 (NEPMU-7) and Navy Entomology Center of Excellence (NECE) have been providing ad hoc assistance to NAMRU-3 and the EMF PMD at both CLDJ and Chabelley Airfield, Djibouti (CADJ), a small Air Force Base located 13 km from the capital Djibouti City. Military entomologists from the Air Force 4th Medical Group 4th Fighter Wing and the Army Public Health Command Europe (PHCE) provided further support during their deployments as the Combined Joint Task Force-Horn of Africa (CJTF-HOA) Force Health Protection Officer (2019-2020) and as the Environmental Science Officer for a Civil Affairs Battalion (2021-2022), respectively.

This paper summarizes the findings from the coordinated mosquito surveillance efforts from 2019 to early 2022 and examines the VBD burden for U.S. personnel assigned to Djibouti. Ultimately, it aims to provide a broader context of the VBD risks to military forces in Djibouti and set the foundation for future force health design.

METHODS

Vector Surveillance:

Vector surveillance on U.S. military installations in Djibouti was conducted through pest management contracts, which were augmented by deployed and visiting uniformed entomologists (Table 1). Contracted vector surveillance was conducted once per week from January 2019 to March 2022 using CO2 (dry ice)-baited Center for Disease Control and Prevention (CDC) light traps (MOSQUITO LIGHT TRAP, NSN 740-00-134-9229) with incandescent bulbs and Mosquito Magnet (MOSQUITO MAGNET EXEC USA, Contract No. GS-21F-0044W) traps with lure at set points in CLDJ. Traps were set around 1800 and collections ended at 0700 the next day. Each week, 3-4 Mosquito Magnets and 4-6 CDC light traps were rotated between one of several set surveillance locations based on EMF PMD recommendations, biting pressure complaints from personnel, or contractor observations of potential larval habitats. This weekly surveillance was only conducted on CLDJ and did not cover installations in more remote areas like CADJ. Augmented vector surveillance was conducted by U.S. Army, Air Force, and Navy entomologists at CLDJ and at CADJ through targeted larval surveillance, adult mechanical aspirations, and use of Biogents Sentinel (Trap, Mosquito, Biogents (BG) BG-1 Sentinel Mosquito Trap, NSN 3740-01-628-9326, Contract No. 47QSWA20D002D) and CDC light traps baited with lures (Lure, BG-Lure, human skin odor, non-toxic, for use with BG Sentinel mosquito trap, NSN 3740-01-628-9325). The augmented traps were placed for 18 to 24 hours per trapping period (trap night) in areas based on the anticipated threat of mosquito-transmitted disease at each installation. Specific trap locations and trapping periods were dependent on the timeline and area of operations of the visiting entomology team. During each assistance visit, the military entomology teams provided training on mosquito larval surveillance, trap placement, mosquito identification, and data management to newly deployed preventive medicine and medical personnel.

Surveillance Data Analysis:

The monthly collection data from the contracted surveillance on CLDJ was totaled by adding the cumulative number of mosquitoes collected in each trap and each day, for a gross number of mosquitoes per genus and species collected per month. A log-transformation was completed for the number of mosquitoes per genus captured in a trap per day and the median number of mosquitoes per species captured in a trap day (m) with 1 being added to this number to ensure that

Table 1. Mosquito surveillance efforts by contracted and military entomologist augmented mosquito surveillance conducted at Camp Lemonnier, Djibouti (CLDJ) and Chabelley Air Base, Djibouti (CADJ). Trap night refers to the number of surveillance periods that were conducted during the date range and trap hours indicates how long traps were active each trap night. The number of each trap/collection method representing the number each was implemented placed during the dates of surveillance.

Installation	Surveillance Effort	Dates	Trap Nights	Trap Hours	CDC[1]	MM[2]	BGS[2]	ASP[3]	LS[4]
CLDJ	Contracted	04-25 Jan 2019	4	13	21	12	-	-	-
		01-22 Feb 2019	3	13	17	11	-	-	-
		08-22 Mar 2019	3	13	8	4	-	-	-
		04-26 Apr 2019	4	13	23	16	-	-	-
		03-31 May 2019	6	13	23	16	-	-	-
		07-28 Jun 2019	4	13	21	5	-	-	-
		19-26 Jul 2019	2	13	11	5	-	-	-
		02-30 Aug 2019	5	13	23	12	-	-	-
		06-27 Sep 2019	4	13	21	8	-	-	-
		04-25 Oct 2019	4	13	24	10	-	-	-
		01-29 Nov 2019	4	13	20	9	-	-	-
		06-28 Dec 2019	5	13	24	12	-	-	-
		03-31 Jan 2020	5	13	22	9	-	-	-
		07-28 Feb 2020	8	13	20	28	-	-	-
		06-27 Mar 2020	5	13	12	23	-	-	-
		03-24 Apr 2020	4	13	16	20	-	-	-
		01-29 May 2020	5	13	22	22	-	-	-
		05-26 Jun 2020	4	13	17	13	-	-	-
		03-31 Jul 2020	4	13	13	11	-	-	-
		07-28 Aug 2020	4	13	16	11	-	-	-
		11-25 Sep 2020	3	13	12	9	-	-	-
		02-30 Oct 2020	5	13	20	15	-	-	-
		06-26 Nov 2020	4	13	17	8	-	-	-
		03-25 Dec 2020	4	13	17	10	-	-	-
		03-28 Jan 2021	5	13	19	17	-	-	-
		04-25 Feb 2021	4	13	12	20	-	-	-
		04-18 Mar 2021	3	13	12	12	-	-	-
		08-29 Apr 2021	4	13	12	12	-	-	-
		06-27 May 2021	4	13	16	12	-	-	-
		05-26 Jun 2021	4	13	16	12	-	-	-
		09-30 Jul 2021	4	13	15	15	-	-	-
		05-26 Aug 2021	4	13	9	11	-	-	-
		02-30 Sep 2021	5	13	18	21	-	-	-
		08-29 Oct 2021	4	13	14	13	-	-	-
		05-26 Nov 2021	5	13	16	19	-	-	-
		02-23 Dec 2021	5	13	15	18	-	-	-
		06-27 Jan 2022	3	13	7	10	-	-	-
		04-24 Feb 2022	4	13	11	12	-	-	-
		03-30 Mar 2022	5	13	16	19	-	-	-
CLDJ	Entomologist Augmented	19 Dec 2019	1	24	-	-	1	1	2
		03 Mar 2020	1	24	-	-	1	1	1
		21 Nov 2020	1	24	-	-	2	1	1
		05-26 Apr 2021	10	24	-	-	21	-	-
		29 Apr – 24 May 2021	11	24	7	-	15	-	-
		10 Dec 2021	1	24	-	-	2	-	-
		20-26 Feb 2022						1	1
CADJ	Entomologist Augmented	12 Sep 2019	1	18	4	-	2	-	-
		12 Oct 2019	1	18	4	-	2	-	-
		13-17 Dec 2019	3	18	4	-	-	-	-
		29 Feb-04 Mar 2020	4	18	4	-	-	-	1
		23-25 Nov 2020	1	24	2	-	4	-	-
		18 Dec 2020	1	18	2	--	2	-	-
		29 Apr 2021	1	18	4	-	6	-	-
		07 May 2021	1	18	-	-	5	-	-
		27 Nov 2021	1	24	1	-	2	-	-
		02-20 Dec 2021	2	24	2	-	5	-	-
		03-07 Jan 2022	2	24	-	-	5	-	-

[1]Centers for Disease Control and Prevention Light Trap; [2]Mosquito Magnet; [3] Biogents Sentinel Trap; [4]Mechanical Aspiration; [5] Number of water sources with larvae detected during Larval Survey

Figure 1. The average number of mosquitoes collected per trap per month from each of the three genera present on CLDJ (log m+1).

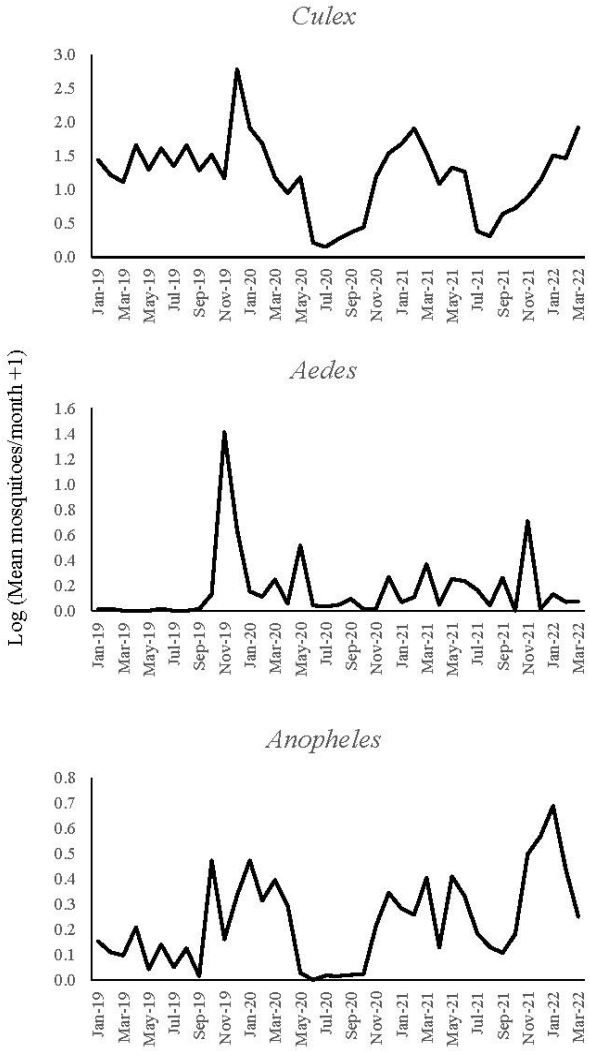
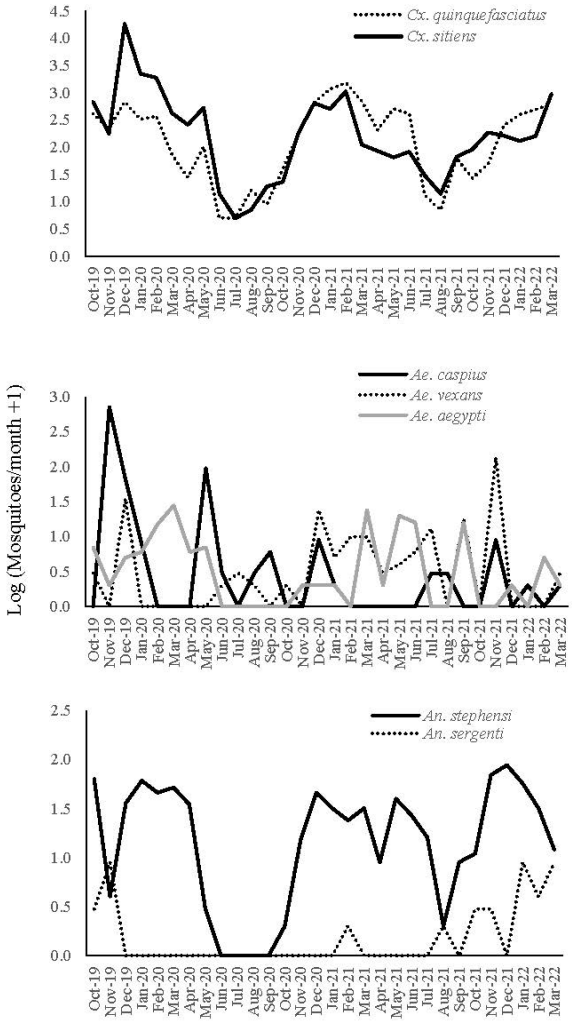


Figure 2. Total number of each mosquito species collected per month (log m+1).



records with no catches did not return errors (i.e., log(m+1)). This allowed for the differing number of trap nights conducted each month to be corrected for so general patterns in density over time could be made. For the augmented surveillance, the number of traps used and the trapping period duration varied between each surveillance event, so raw numbers were provided to highlight the species abundance/diversity that were identified during the assistance visits.

Spatial Distribution:

Trap distribution was recorded for the surveillance locations in decimal degrees and datum World Geodetic System 1984 (WGS 84) using Garmin 12 XL (GPS TIPO GARMIN 12X NSN 6605-15-215-3722) receiver with the fix recorded to the fifth decimal digit, then plotted using ArcGIS (SOFTWIRE ARCGIS 3D, NSN 7030-01-559-8475). The total number of individual mosquitoes collected from each species at each surveillance location was compiled and presented as a genus, then the average number of each genus was calculated for each location. Locations on CLDJ with the highest mosquito abundances per genus was estimated using an inverse distance weighting technique to interpolate the value of the variable into the unmeasured sites and to identify the presence and density hotspots.[14,15]

Molecular Testing:

Anopheles stephensi collected from 25 October 2019 to 28 February 2020 were tested for *P. falciparum*. The DNA was extracted from mosquito head and thorax with the QIAamp DNA mini kit (DNA Analysis Test Kit, NSN 6550-12-376-1330) using the standard protocol. Quantitative Real-time PCR was performed on an ABI 7500 (SVC MFC RL TIME PCR SYS 7500, Contract No. GS-07F-0636W) following Rougemont et al., 2004.[17] Non-template and positive reference controls were used to detect any variation between runs. *Aedes aegypti* collected from 19 March 2021 to 21 May 2021 were tested for Dengue virus using a mobile PCR thermocycler, using a Pan-Dengue test kit, following the manufacturer's standard protocol.

Figure 3. Confirmed larval habitat on CLDJ (a-e) and CADJ (f) from augmented military entomologist surveillance. Aedes aegypti larvae confirmed on CLDJ from plastic container holding a discarded water pump stored under a counter in an outdoor space of the Wardroom (a). Anopheles stephensi larvae confirmed from CLDJ from a large water-filled tire used for physical fitness training at an outdoor exercise facility (b) and from a discarded food tin filled with water in a garden kept in the HAZMAT storage area (c). Culex sitiens larvae confirmed from marsh habitat along the flight line (d). Culex quinquefasciatus larvae confirmed from drainage system on CLDJ (e). Anophelese stephensi larvae confirmed from CADJ from damaged underground water tank.

Table 2. Vector-borne disease case report search criteria based on International Classification of Diseases, 9th or 10th Edition, Clinical Modification (ICD-9-CM and ICD-10-CM) codes.

Vector-borne Disease Nomenclature	ICD-9-CM Codes	ICD-10-CM Codes
Malaria	084	B50, B51, B52, B53, B54
Dengue	061	A0, A91
Other mosquito-borne fevers (includes Chikungunya, West Nile Virus, Rift Valley Fever)	066.2, 066.3, 066.4	A92
Mosquito-borne viral encephalitis	062	A83
Other arthropod-borne fevers (includes Sand Fly fever)	066.0, 066.1, 066.8	A93
Leishmaniasis	085	B55

Vector-borne Disease Burden:

The VBDs of interest were limited to only include encounters or medical event reports between January 2018 and March 2022 based on the International Classification of Diseases, 9th or 10th Edition, Clinical Modification (ICD-9-CM and ICD-10-CM) codes in Table 2. Medical encounter data from the Theater Medical Data System (TMDS), Comprehensive Ambulatory Patient Encounter Record (CAPER), and Military Health System (MHS) GENESIS were compiled with medical event reports from Disease Reporting System internet (DRSi). The TMDS records were included if the encounter occurred in Djibouti. The CAPER and MHS GENESIS records were included if the patient also had a TMDS encounter in Djibouti (with any diagnosis) within 90 days prior to the CAPER or MHS GENESIS encounter to approximate travel-related VBD cases that were diagnosed outside of Djibouti. Data from DRSi was extracted for the following reported medical event diagnoses: malaria, dengue, leishmaniasis, chikungunya, Rift Valley fever, Zika virus and arboviral diseases, a category which includes West Nile virus, mosquito-borne viral encephalitis, and other arthropod-borne fevers. Records were limited to confirmed, suspected, or probable cases reported in Djibouti. Medical event report data was combined with medical encounter data, and the first encounter per VBD per person within the analysis timeframe was included. If a record for the same person and for the same VBD was identified in both data sources within 30 days of each other, this was considered to reflect the same case, and all information from both sources was included. Four additional malaria cases were provided to the authors by the medical staff of the contracted pest management team that were not reported in the systems indicated earlier and included in the total numbers.

RESULTS

Vector Surveillance

The contracted vector surveillance on CLDJ collected a total of 47,503 mosquitoes from three genera with the majority as *Culex* species (95.8%). The *Culex* collections were comprised of two species: *Cx. sitiens* Wiedemann (66.9%) and *Cx. quinquefasciatus* Say (22.0%). The remaining mosquitoes were *Aedes* (2.7%) or *Anopheles* (1.5%) species. *Aedes* was the predominant genus, with four species: *Ae. caspius* (Pallas) (2.1%), *Ae. vexans* (0.6%), *Ae. aegypti* (0.3%), and *Ae. vittatus* (Bigot) (0.004%). *Anopheles* collections included *An. stephensi* (1.9%) and *An. sergenti* (Theobald) (0.1%). The general monthly collections showed temporal variation across all three genera in the average number of mosquitoes collected per trap (Figure 1). Species-specific collection patterns varied, with total number of each mosquito species collected per month presented in Figure 2. The highest number of any mosquito collected during the entire surveillance period was *Cx. sitiens* with a total of 18,289 collected in December 2019.

The augmented vector surveillance resulted in the collection of an additional 2,479 mosquitoes from the two installations (Table 3). *Culex quinquefasciatus* (49.9%) and *Cx. sitiens* (31.2%) were the predominant species on CLDJ followed by *An. stephensi* (6.8%), *Ae. aegypti* (5.2%), *Ae. vexans* (1.8%) and *Ae. caspius* (0.2%). The remaining 2.9% of mosquitoes were unidentified *Anopheles* species. Similarly, *Cx. quinquefasciatus* (96.4%) was the most collected mosquito on CADJ followed by *Ae. vexans,* (1.6 %) and *Ae. vittatus* (0.9%). All other mosquitoes collected on CADJ represented less than 0.1% of the collection and consisted of *Ae. caspius*, *Cx. sitiens*, *An. stephensi*, and *An. sergenti* (Table 3).

Larval surveillance targeted a variety of artificial containers (including tires, buckets, and various piles of refuse), the installation drainage system, and areas of standing water near structures and air conditioning units. While a diversity of artificial containers holding water were surveyed during the augmented surveillance periods, larvae were not always collected. *Aedes aegypti* were collected in December 2019 from a water-filled plastic container discarded in the officers' recreation facility on CLDJ (Figure 3a). *Anopheles stephensi* larvae were collected in December 2019 on CLDJ from a water-filled tire used for physical fitness training at the outdoor exercise area (Figure 3b) and from a water-filled tin can located in the hazardous material (HAZMAT) storage area in March 2020 (Figure 3c). *Culex sitiens* larvae were collected from brackish marsh area at the east end of the CLDJ runway (Figure 3d). *Culex quinquefasiatus* larvae were collected from the underground drainage system in November 2020 and in February 2022, which were accessed through grates near the installation

Table 3. Total number of each mosquito species collected through military entomologist augmented surveillance from Camp Lemonnier, Djibouti (CLDJ) and Chabelley Air Base, Djibouti (CADJ) with various surveillance methods and effort.

Installation	Assistance Visit	Mosquito Species										
		Cx. quinquefasciatus	*Cx. sitiens*	*Culex spp.*	*Ae. aegypti*	*Ae. vexans*	*Ae. caspius*	*Ae. vittatus*	*An. sergenti*	*An. stephensi*	*Anopheles spp.*	Total
CLDJ	19 Dec 2019	18	53	0	4	0	0	0	0	1	8	84
	03 Mar 2020	7	0	4	10	0	0	0	0	0	0	21
	21 Nov 2020	49	8	0	0	0	1	0	0	0	0	58
	05-26 Apr 2021	49	29	0	6	4	0	0	0	12	1	101
	29 Apr – 24 May 2021	63	30	4	0	3	0	0	0	13	2	115
	10 Dec 2021	6	0	0	0	0	0	0	0	0	0	6
	SUB-TOTAL	192	120	8	20	7	1	0	0	26	11	385
CADJ	12 Sep 2019	0	3	0	0	0	0	0	0	0	0	3
	12 Oct 2019	0	0	0	0	0	2	0	0	0	0	2
	13-17 Dec 2019	70	0	0	0	31	0	14	0	0	0	115
	29 Feb-04 Mar 2020	12	0	0	0	1	0	3	0	0	0	16
	23-25 Nov 2020	311	2	0	0	0	0	0	2	0	0	315
	18 Dec 2020	388	10	0	0	2	0	2	0	0	0	402
	29 Apr 2021	44	2	0	0	0	0	0	0	0	0	46
	07 May 2021	48	0	0	0	0	0	0	0	0	0	48
	27 Nov 2021	84	1	0	0	0	0	0	1	1	0	85
	02-20 Dec 2021	361	0	0	0	0	0	0	0	0	0	361
	03-07 Jan 2022	701	0	0	0	0	0	0	0	0	1	701
	SUB-TOTAL	2,019	18	0	0	34	2	19	3	1	1	2,094
TOTAL		2,211	138	8	20	41	3	19	3	27	12	2,479

perimeter (Figure 3e). On CADJ, *An. stephensi* were collected in March 2020 from a damaged, water-filled septic tank that was no longer holding sewage water (Figure 3f).

Spatial Distribution

The highest density of *Culex* spp. occurred on the eastern edge of the installation nearest the salt marshes that flow to the sea, and along the southern perimeter where the drainage system directs water off base. The highest density of *Aedes* spp. were near liberty clubs, the HAZMAT storage site, wastewater treatment site, and by the largest concentration of living quarters. For *Anopheles* spp., the densities were highest along the northern and southern perimeters near living quarters and by the Defense Logistics Agency Disposition Services and HAZMAT storage sites.

Molecular Testing

There were no positive detections of P. falciparum or Dengue Virus from the 129 An. stephensi and 40 Ae. aegypti samples screened.

Vector-borne Disease Burden

A total of 38 VBDs, with an average of 9.5 cases per annum, were reported from personnel in Djibouti: dengue fever (20), malaria (16), chikungunya (1), and leishmaniasis (1) (Figure 4a). No VBD cases were reported within the investigation period for 2022 (January-March). The VBD cases were reported in every month except October and November, with bimodal peaks in January and May (Figure 4b).

DISCUSSION

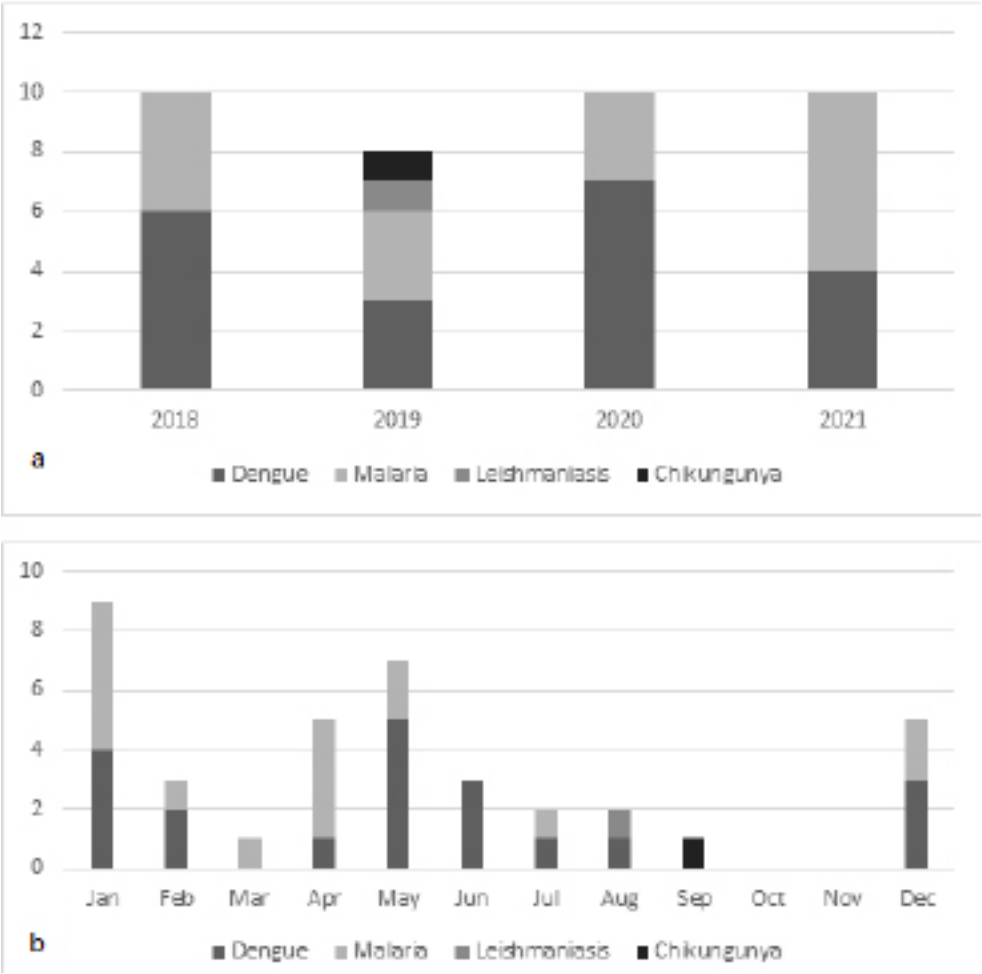
The threat to deployed forces from VBD varies according to the prevalent endemic diseases at the deployed locations, the availability of protective vaccines, and the implementation of individual and group preventive measures.[18] Personnel deployed to Djibouti are directed to arrive in theater with uniforms treated with permethrin, chemoprophylactic medications, and skin repellents to prevent VBDs.[19] Despite these efforts, roughly ten VBD cases are reported in the U.S. Forces in Djibouti annually. Anecdotal evidence compiled by the visiting entomology teams during this period indicated that conformity with FHP orders were similar to non-compliance rates historically documented in other military locations[20-22] and was likely a contributing factor to the cases of dengue, malaria, chikungunya, and leishmansisis reported here. Military commanders are recommended to assume non-compliance in highly endemic areas and maximize other preventive strategies as a safeguard to force health. Military entomologists are trained

to implement advanced vector surveillance techniques that focus on understanding the biological and ecological factors critical to an integrated vector management (IVM) program. At this time many military installations rely on contracted pest and vector management services for daily operations, as in Djibouti, and rely on "reach-back" support of military entomologists, which may delay the timeliness of responses to VBD threats. Although the CLDJ contracted pest management team is trained in IVM, they have restricted access to certain secured sites on the installations and their actions are still dependent on the EMF PMD authorization to work on issues involving vectors.

The EMF PMD is staffed by an Environmental Health Officer (EHO) and two Preventive Medicine Technicians (PMTs). The EHO and PMT skills vary, but generally the specialties only maintain rudimentary vector surveillance and control skill sets. This technical limitation requires frequent assistance visits by entomologists to provide on-the-job training in vector biology, surveillance, and identification. In fact, all mosquitoes collected by CLDJ pest management contractors prior to September 2019 could only be identified to genus by the EMF PMD because of the limited technical skill sets. This lack of species-level identification represented a significant gap in the ability to accurately determine the seasonal risk of VBD and inform the proper mitigation and control strategies. For example, pesticide applications were being conducted when mosquito numbers exceeded 10 females per trap, regardless of the mosquito genus or species. This created the potential for over application of pesticides that could have impacted non-target species and ultimately resulted in insecticide resistance in the mosquito populations. The NAMRU-3 entomologists developed a mosquito pictorial key, first based on the historic records and then modified to reflect actual collection data, and provided mosquito identification training to the EMF PMD. This training allowed the EMF PMD to better understand the mosquito diversity, population distribution, and adjust pesticide application thresholds to species-specific levels to mitigate disease threats while also ensuring pesticides were not being over applied. Still, mosquito identification remains a challenge for EMF PMD personnel.

In addition to better informing pesticide application strategies, the improved information derived from the identification training resulted in a better understanding of the diversity of CLDJ and CADJ mosquitoes and an improved risk assessment posed by each mosquito species. By far, the most represented mosquito genera were *Culex*, with *Cx. sitiens* comprising 64.0% of all collected mosquitoes on CLDJ. While it is a potential vector of diseases endemic to the Asia-Pacific, this species is currently not known to vector diseases in Djibouti; however, it represents a significant nuisance biter during the night.[9] Larvae were collected on the installation from marsh water near the flight line and adults were collected at high frequencies on the eastern side of the base toward the end of the flight line near a salt marsh. It is likely that the high population at this location was attributed to the vast larval habitat adjacent to CLDJ, where brackish wetlands surround the eastern portion of the installation. On CADJ, *Cx. sitiens* was rarely collected and was typically found only after the surrounding area had experienced significant rains that would have provided substantial water sources off the installation. The potential WNV vector, *Cx. quinquefasciatus,* represented 18.5% of all collected mosquitoes on CLDJ and poses a threat of chikungunya and Rift Valley virus transmission.[23-24] Like *Cx.*

Figure 4. Vector-borne diseases reported by a) year and b) month from U.S. military and contractor personnel in Djibouti, 2018-2022. No cases have been reported in 2022.



sitiens, it also represents a significant nuisance biter during the night. *Culex quinquefasciatus* larvae have been collected on base from standing water in tires and in the installation's drainage system, specifically in areas where the water pools near the perimeter walls. The underground drainage system runs throughout the base, with no active larviciding activities occurring to lower the populations. However, the installation does flush the system on a regular basis but it does not appear to be completely effective. On CADJ, *Cx. quinquefasciatus* is the most collected mosquito, suggesting that it is also the most abundant on the installation. This is most likely due to the sewage system being comprised of septic tanks, which often are not properly sealed and have an off-gassing ventilation system that is open to the environment. This creates an ideal mosquito larval habitat with no physical barrier preventing oviposition of gravid mosquitoes.

The spatial distribution analysis demonstrates that *Aedes* mosquitoes are most abundant near areas where most of the camp living quarters and recreational clubs are located. The HAZMAT storage area and water treatment sites also have higher abundance of *Aedes* compared to other areas on the installation. Importantly, these areas are most likely to generate artificial water sources, which are ideal habitat for larval *Ae. aegypti*. The abundance of *Aedes* mosquitoes at the living and liberty areas demonstrate a very high risk to FHP, as personnel spending time at these sites will be out of uniform and less likely to be protected by the DOD Insect Repellent System. Prior to September 2019, all IVM decisions against *Aedes* mosquitoes were approached without regard to the ecological, behavioral, and differences between larval habitat requirements between the species. Additionally, there was not the full understanding of what could be vectored on CLDJ. *Aedes aegypti* comprised only 0.3% of mosquitoes collected by contracted surveillance, but this low collection rate may be the result of a critical limitation due to the traps used by the pest management contractors and the trapping times (overnight as opposed to daylight hours). The augmented surveillance suggest that *Ae. aegypti* may be more abundant on the installation (accounting for 5.3% of mosquitoes) since the entomologists incorporated time appropriate collection periods with *Aedes*-specific traps (BGS traps with BG lures). As the principal vector of Yellow Fever, Dengue, Chikungunya, and Zika viruses, *Ae. aegypti* is a significant threat to FHP on CLDJ. Larvae have been collected once from a water-filled container, but the larval sources have been difficult to identify most times of the year. Traditional water containers that *Ae. aegypti* uses for larval habitats have been observed to often evaporate quickly on CLDJ and are not frequently encountered during technical assistance visits. Moreover, the environment immediately adjacent to the installation does not provide suitable larval or adult habitat and are not within the flight range of this mosquito.[25] A*edes aegypti* may have adapted to use the drainage system that runs through the base as has been documented in other *Ae. aegypti* populations in arid environments.[26] This should be explored for CLDJ and vector management practices must be adjusted accordingly. A single specimen of *Ae. vittatus* was collected on CLDJ, but has been collected with more frequency on CADJ. It is a known vector of Yellow Fever, Dengue, Chikungunya, and Zika viruses.[27] During the study period, the larval habitat of *Ae. vittatus* was not confirmed, but this mosquito is known to share larval habitat preferences with *Ae. aegypti* (tires, plastic containers, etc.), but also breeds in natural water sources such as water-filled rock holes.[27] Both of these larval habitats are widely available in the environment surrounding CADJ. *Aedes vexans* was first detected by NAMRU-3 on CLDJ in October 2019 and subsequent collections on CLDJ and CADJ suggest it is established in Djibouti. The mosquito is primarily a nuisance biter to humans, but is a potential vector of several pathogens, including WNV and Zika.[28] Its larval habitats include flood pools, water-filled ditches, marshlands, and stormwater facilities.[28] *Aedes caspius* comprised 2.1% of collected mosquitoes, and is a vector of Rift Valley Fever and WNV.[29] Its larvae are typically collected in both fresh and saline marshes, a habitat that is present along the flight line on CLDJ.

As with the other genera, all *Anopheles* mosquitoes were considered to be homogeneous in their biology and control strategies due to improper identification by the EMF PMD. This resulted in most being incorrectly assumed to be *An. gambaie* Giles, despite this species not being identified by entomologists during the surveillance period. What is most significant from this improved surveillance is that the most common *Anopheles* mosquito on CLDJ is the recently introduced, urban malaria vector, *An. stephensi,* which was not known to be present on CLDJ prior to this surveillance. *Anopheles stephensi* has a unique biology relative to the other *Anopheles* species in Djibouti, as it is able to utilize a variety of artificial water sources as larval habitat, making it well adapted to urbanized environments.[30] It is a competent vector of *P. falciparum* and *P. vivax*[31], bringing these malaria parasites into urban areas. The discovery of both larvae and adult *An. stephensi* on CLDJ demonstrates that the continuous infrastructure improvements[32-33] has made the installation a more favorable environment for this mosquito to survive all year, regardless of the typical seasonal variation that impacts other malaria vectors in the region.

The collection of *An. stephensi* adult and suspected larvae from an exposed underground water tank from CADJ demonstrates it is not only a FHP risk in urbanized settings in Djibouti (i.e., CLDJ), but also an increase malaria threat to all military throughout the country. Despite assumptions that adult An. gambaie were being collected on CLDJ, the only other Anopheles that was collected was An. sergenti which was also collected on CADJ. It is also a competent vector of P. falciprium and P. vivax and is typically associated with rural environments, with larval habitats typically being slow moving or stagnant water with high amounts of vegetation to include irrigation canals.34 Neither CLDJ nor CADJ have this type of land-use immediately nearby. Moreover, this mosquito preferentially feeds on livestock34, which are not

found on or near either base. This mosquito may become a higher threat should land-use practices change around the installations.

In general, mosquito collections are highest during the period between October and April, which is considered the rainy season in Djibouti. Average temperatures range between 27°C and 33°C, and the area experiences an average of one precipitation event per month causing between 13-36 mm of rainfall. The highest mosquito collections occurred following significant flood events in November 2019[35], which resulted in the highest number of *Ae. caspius* and *Cx. sitiens* collected at CLDJ. This period was also associated with increased collections of *Ae. vexans* and *Ae. vittatus* due to the flooding in the area around CLDJ. Importantly, similar peaks were not observed in *Ae. aegypti*, *Cx. quinquefasciatus,* or the *Anopheles* species at either installation, nor was there an increase in reported human diseases. Mosquito collections drop substantially from May until September, when temperatures range from 35°C and 38°C with rainfall between 3-41mm from an average of 1.8 precipitation events per month. While the number of mosquitoes reduces the amount of manpower required to process and identify the collections, it does not demonstrate a period of reduced VBD risk, because *Ae. aegypti* and *An. stephensi* are collected at similar rates during months with low precipitation and high temperatures. Both of these mosquito species are able to opportunistically exploit a variety of artificial water habitats that are present all year. These water-filled habitats are cryptic on CLDJ, with surveillance efforts only successfully collecting larvae of these two species in four water sources during all of the augmented surveillance periods. This demonstrates that these mosquitoes are not necessarily only migrating onto the installation from habitats outside of the perimeter, but are actively breeding on CLDJ and CADJ. A more robust, longitudinal mosquito larval survey should be conducted across the installation to better understand the vectors' ecology and distribution to implement engineering and policy controls to eliminate their habitats.

CONCLUSION

The data reported here greatly improved the overall FHP risk assessment for U.S. Forces deployed to Djibouti and was shared with the Armed Forces Pest Management Board to update the DoD's Disease Vector Ecology Profile for Djibouti.[9] However, as discussed earlier, much work is still needed to better characterize the local transmission factors found on these installations and even more work is needed to support the FHP of the U.S. Forces operating in the greater region. For example, coordinated tick and sand fly surveys have not been pursued on CLDJ, despite a reported case of leishmaniasis. Moreover, forward operating areas in CJTF-HOA area of responsibility have very limited pest and vector management support, and even less vector surveillance data has been collected at these locations. Importantly, insecticide resistance should be a prime concern where adult mosquito control is the overriding vector control method being used, however, the pest management contractors and EMF PMD lack the technical expertise and supplies required to do this type of monitoring. All of this work could be completed by a military entomologist assigned to the EMF PMD rather than relying on the ad hoc Navy entomological support currently provided by NEPMU-7 (Spain), NAMRU-3 (Italy), and NECE (USA). These augmented teams require lead time, significant coordination, and may reduce mission effectiveness in other regions while also causing delayed expert on-site management for vector-borne disease outbreaks or emergent vector transmission cycles.

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REFERENCES

1. The World Bank. The container port performance index 2020. 2021. World Bank, Washington DC. License: Creative Commons Attribution CC BY 3.0 IGO
2. Sun D, Zoubir YH. The Eagle's nest in the horn of Africa: US military strategic deployment in Djibouti. *Afr Spectr*., 2016 Apr;51(1):111-24.
3. National Center for Medical Intelligence. Djibouti Infectious Disease Risk Assessment. 2022. National Center for Medical Intelligence
4. Faulde MK, Rueda LM, Khaireh BA. First record of the Asian malaria vector *Anopheles stephensi* and its possible role in the resurgence of malaria in Djibouti, Horn of Africa. *Acta Trop*., 2014;139 (May 2013):39–43.
5. Seyfarth M, Khaireh BA, Abdi AA, Bouh SM, Faulde MK. Five years following first detection of *Anopheles stephensi* (Diptera: Culicidae) in Djibouti, Horn of Africa: populations established—malaria emerging. *Parasitol Res*., 2019 Mar;118(3):725-32.
6. Andayi F, Charrel RN, Kieffer A, Richet H, Pastorino B, Leparc-Goffart I, Ahmed AA, Carrat F, Flahault A, De Lamballerie X. A sero-epidemiological study of arboviral fevers in Djibouti, horn of Africa. *PLoS Negl Trop Dis.,* 2014 Dec 11;8(12):e3299.
7. Kodier GR, Gubler DJ, Cope SE, Cropp CB, Soliman AK, Polycarpe D, Abdourhaman MA, Parra JP, Maslin J, Arthur RR. Epidemic dengue 2 in the city of Djibouti 1991–1992. *Trans. R Soc Trop Med Hyg.,* 1996 May 1;90(3):237-40.
8. Faulde MK, Spiesberger M, Abbas B. Sentinel site-enhanced near-real time surveillance documenting West Nile virus circulation in two *Culex* mosquito species indicating different transmission characteristics, Djibouti City, Djibouti. *J EgyptSoc Parasitol*., 2012 Aug 1;42(2):461-74.
9. Armed Forces Pest Management Board. 2021. *Disease Vector Ecology Profile: Djibouti*. Armed Forces Pest Management Board, Washington, DC.
10. Tesh RB, Saidi S, Gajdamovič SJ, Rodhain F, Vesenjak-Hirjan J. Serological studies of the epidemiology of sandfly fever in the Old World. *Bull World Health Organ*., 1976;54(6):663.
11. Pratlong F, Debord T, Garnotel E, Garrabe E, Marty P, Raphenon G, Dedet JP. First identification of the causative agent of visceral leishmaniasis in Djibouti: *Leishmania donovani*. *Ann Trop Med Parasitol*., 2005 Jan 1;99(1):21-5.
12. Jones CM, Welburn SC. Leishmaniasis Beyond East Africa. *Front Vet Sci*. 2021:65.
13. Socolovschi C, Matsumoto K, Marie JL, Davoust B, Raoult D, Parola P. Identification of rickettsiae, Uganda and Djibouti. *Emerg Infect Dis*., 2007 Oct;13(10):1508.
14. Horton KC, Fahmy NT, Watany N, Zayed A, Mohamed A, Ahmed AA, Rollin PE, Dueger EL. Crimean Congo hemorrhagic fever virus and Alkhurma (Alkhumra) virus in ticks in Djibouti. *Vector Borne and Zoonotic Dis*., 2016 Oct 1;16(10):680-2.
15. Omar, K., Khafaga, O., Elkholy, M.A. Geomatics and plant conservation: *GIS for best conservation planning*. LAP LAMBERT Academic Publishing; 2013, 312 pp.
16. Habib, M. A., Basuki, T., Miyashita, S., Bekelesi, W., Nakashima, S., Phoungthong, K, Techato, K. Distribution of naturally occurring radionuclides in soil around a coal-based power plant and their potential radiological risk assessment. *Radiochim Acta*, 2019, 107(3), 243-259.
17. Rougemont, M., Van Saanen, M., Sahli, R., Hinrikson, H.P., Bille, J. and Jaton, K., Detection of four *Plasmodium* species in blood from humans by 18S rRNA gene subunit-based and species-specific real-time PCR assays. *J Clin Microbiol.,* 2004, 42(12), pp.5636-5643.
18. O'Donnell FL, Fan M, Stahlman S. Surveillance for vector-borne diseases among active and reserve component service members, US Armed Forces, 2016-2020. *MSMR*. 2021 Feb 1;28(2):11-5.
19. United States Africa Command. *Force Health Protection Requirements and Medical Guidance for Entry into the U.S. Africa Command Theater*, 2017, ACI 4200.09.
20. Wanja EW. Observed Noncompliance with Implementation of Vector-Borne Disease Preventive Measures Among Deployed Forces. *US Army Med Dept J*., 2010 Apr 1.
21. Lalani T, Yun H, Tribble D, Ganesan A, Kunz A, Fairchok M, Schnaubelt E, Fraser J, Mitra I, Kronmann KC, Burgess T. A comparison of compliance rates with anti-vectorial protective measures during travel to regions with dengue or chikungunya activity, and regions endemic for *Plasmodium falciparum* malaria. *J Travel Med*., 2016, Sep 1;23(5).
22. Saunders DL, Garges E, Manning JE, Bennett K, Schaffer S, Kosmowski AJ, Magill AJ. Safety, tolerability, and compliance with long-term antimalarial chemoprophylaxis in American soldiers in Afghanistan. *Am J Trop Med Hyg*., 2015 Sep 2;93(3):584.
23. Lutomiah, J., Mulwa, F., Mutisya, J. et al. Probable contribution of *Culex quinquefasciatus* mosquitoes to the circulation of Chikungunya virus during an outbreak in Mombasa County, Kenya, 2017–2018. *Parasit Vectors.,* 2021 14, 138 ().
24. Ndiaye el H, Fall G, Gaye A, Bob NS, Talla C, Diagne CT, Diallo D, B A Y, Dia I, Kohl A, Sall AA, Diallo M. Vector competence of *Aedes vexans* (Meigen), *Culex poicilipes* (Theobald) and *Cx. quinquefasciatus* Say from Senegal for West and East African lineages of Rift Valley fever virus. *Parasit Vectors*., 2016 Feb 20;9:94.
25. Moore TC, Brown HE. Estimating *Aedes aegypti* (Diptera: Culicidae) Flight Distance: Meta-Data Analysis. *J Med Entomol*., 2022 Jul 13;59(4):1164-1170.
26. Wang X, Zhou G, Zhong D, Li Y, Octaviani S, Shin AT, Morgan T, Nguyen K, Bastear J, Doyle M, Cummings RF, Yan G. Impact of underground storm drain systems on larval ecology of *Culex* and *Aedes* species in urban environments of Southern California. *Sci Rep*., 2021 Jun 16;11(1):12667.
27. Walter Reed Biosystematics Unit, 2022. *Aedes vittatus* species page. Walter Reed Biosystematics Unit Website, http://wrbu.si.edu/vectorspecies/mosquitoes/ae_*vittatus*, accessed on 14 July, 2022
28. Walter Reed Biosystematics Unit (2022). *Aedes vexans* species page. Walter Reed Biosystematics Unit Website, http://wrbu.si.edu/vectorspecies/mosquitoes/*vexans*, accessed on 14 July 2022
29. Walter Reed Biosystematics Unit (2022). *Aedes caspius* species page. Walter Reed Biosystematics Unit Website, http://wrbu.si.edu/vectorspecies/mosquitoes/*caspius*, accessed on 14 July, 2022
30. akken W, Lindsay S. Increased Threat of Urban Malaria from *Anopheles stephensi* Mosquitoes, Africa. *Emerg Infect Dis*., 2019 Jul;25(7):1431–3.
31. Walter Reed Biosystematics Unit (2022). *Anopheles stephensi* species page. Walter Reed Biosystematics Unit Website, http://wrbu.si.edu/vectorspecies/mosquitoes/*stephensi*, accessed on 14 July 2022
32. Kelly, T. Good things come in small packages: The US-Djiboutian Security Partnership. *Am Amb Rev.,* 2015 Fall.
33. Congressional Research Service. 2021. *In Focus: Djibouti*. IF11303. https ://crsreports.congress.gov
34. Walter Reed Biosystematics Unit (2022). *Anopheles sergentii s*pecies page. Walter Reed Biosystematics Unit Website, http://wrbu.si.edu/vectorspecies/mosquitoes/*sergentii*, accessed on 15 July 2022
35. Djibouti: Flash Floods Final report - DREF n° MDRDJ003. *International Federation of the Red Cross and Red Crescent Societies (IFRC)*. 2020.

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The Entomological Situation of Ectoparasite-Borne Diseases in Areas Used for Cobra Gold Military Training Exercise in Thailand Between 2017 and 2022.

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ABSTRACT

Ectoparasite-borne diseases have a long history of adverse impacts on United States (US) and allied military operations, particularly in tropical and subtropical areas where the environment and climate are favorable for ectoparasite vectors and reservoir hosts. Knowledge on the disease epidemiology and geographic distribution of many ectoparasite and rodent-borne pathogens remains largely under-determined, and these limitations consequently reduce the Total Forces Readiness in support of the Geographic Combatant Command and limits our ability to understand the circulating infectious disease threats and develop timely, actionable, and effective countermeasures to mitigate those threats. In this study, we investigated ectoparasite-borne rickettsioses in the areas used to support multinational exercise "Cobra Gold" in Thailand, primarily focusing on chigger-borne diseases. A total of 1,568 rodents and 3,696 chiggers were analyzed for the presence of *Orientia* and *Rickettsia* agents. The positivity rate for *Orientia* and *Rickettsia* in chiggers was 0.7% and 3.8%, respectively, implicating that not only *Orientia* but *Rickettsia* infection are possible via chigger bites when Service Members are deployed to Thailand or other regions known to be endemic for chigger mites. At least three *Rickettsia* spp. were identified in chiggers based on the analysis of 17-kDa genus-specific antigen protein including *Rickettsia felis*, *R. akari* and *R. typhi*. The role of chiggers as a vector for rickettsial pathogens and the significance of chigger control and bite prevention in inducing spotted fever group rickettsioses remains understudied and further work is needed.

Keywords: Chigger-borne pathogen, scrub typhus, spotted fever rickettsiosis, murine typhus, Orientia tsutsugamushi, Rickettsia felis, Rickettsia typhi, Cobra Gold. Military training exercise

INTRODUCTION

Thailand is one of the strategic locations in the United States Indo-Pacific Command (USINDOPACOM) routinely used for multinational military exercises such as Cobra Gold (CG; the largest joint military training of its kind established in 1982) and Tropic Lightning Joins Hanuman Guardian where more than 13,000 US Service Members and participating nations join the training annually. Although this region is recognized as endemic for many vector-borne diseases, epidemiological data regarding the circulating etiological agents, vector species, and geographic distribution are still scarce to date. Our understanding of the infectious disease risks associated with ectoparasites and rodents in strategic locations remains largely underestimated and could place immune-naïve military personnel at risk of disease exposure and may even result in suspension or cancellation of operations.

Ectoparasite is a specific term used to define an external parasite that lives on a vertebrate host's skin or body surface. Ectoparasites consist of diverse taxonomic categories including fleas, sucking lice, chewing lice, flies, ticks, and several types of mites. The majority of ectoparasite-borne diseases are zoonotic, and the perpetuation of the pathogen depends on vector parasitism of the vertebrate host. Comprehensive entomological risk assessments in the disease endemic areas suggest that the abundance and infection prevalence of the ectoparasite populations are influenced by the presence of hosts and host presence may consequently increase the probability of transmitting the disease to humans, especially murine rodents that are ecologically widespread and serve as reservoir hosts for bacteria, viruses, and protozoa. [1,2] Among the diverse groups of ectoparasites, chiggers serve as a prominent vector transmitting pathogens of public health and military significance.[3].

The trombiculid (Acari: Acariformes) mite, or chigger, is a unique ectoparasite as the larval form of its lifecycle feeds preferentially on the vertebrate host, while other life stages are predatory, free-living in soil and feed on insect eggs. Chigger mites are a competent vector of the intracytosolic bacteria *Orientia tsutsugamushi-Otsu* of the family *Rickettsiae*, causative agent of scrub typhus. Scrub typhus is a life-threatening disease causing an average of 1.5% to 6% mortality rate with millions of cases occurring annually and over a billion people living in or traveling to the disease endemic areas. The traditional geographic distribution of scrub typhus, known as the 'Tsutsugamushi Triangle', covers approximately 13 million square kilometers of Asia, northern Australia, and the Pacific Islands, mirroring USINDOPACOM's evolving strategic priorities. During the last decades, evidence in China indicates that the incidence of scrub typhus rose by 1.8-times and is associated with a geographic expansion from rural to urban areas.[4] A similar rapid increase of scrub typhus incidence of over 3.8-times between 2001 and 2013 and the urbanization trend was also observed in Korea.[5] In the military context, recent outbreaks of scrub typhus were reported among Thai (9.8% and 9.1% attack rate in 2002 and 2013) and Australian deployed forces (suspected 36% attack rate in 2011) that were on active duty in the endemic areas of their countries. Several cases also occurred in the US military forces training at Camp Fiji, Japan (1.1% and 0.9% attack rate in 2000 and 2001).[6-9] The outbreaks threatened Soldiers' health and simultaneously resulted in the suspension of their missions.

Chiggers are under-recognized and under-studied compared to the other arthropod vectors that cause equivalent disease prevalence and severity, in part due to belief in the geographic restriction within the "Tsutsugamushi Triangle". However, culture-confirmed cases of scrub typhus caused by infection of new *Orientia* species as well as the detection of infected chiggers have recently been reported outside the endemic area, i.e., in locations as distant as the United Arab Emirates, Kenya, and Chile, signifying scrub typhus as an emerging global health threat.[10-12] Besides the potential for climate change to increase chigger population numbers and range, the biology of chiggers involving low host specificity and ability to adapt to a variety of environments and climates, are key factors driving the expansion to new areas, which in turn, allows them to be more efficient in the spread of diseases.[13] In addition to its well-recognized role in transmitting scrub typhus, our work over the past few years and new findings from research communities throughout the world have revealed that chiggers are suspected vectors of multiple medically important pathogens, including *Rickettsia*, *Bartonella*, *Borrelia*, and *Anaplasma*.[14-17] Evidence from research groups in China has also implicated chiggers as potential vectors of lethal viruses including Hantaan virus, the agent of hemorrhagic fever with renal syndrome, and Dabie bandavirus, the pathogen that causes severe fever with thrombocytopenia syndrome.[18,19] Many of these pathogens are of concern to the military.

In this study, we report the past and present status of circulating pathogens, diversity of vectors, reservoir hosts, and areas at high risk by focusing on ectoparasite-borne disease in locations used for the Cobra Gold (CG) military training exercise in Thailand from 2017 to 2022. The vector-borne diseases of interest include scrub typhus, murine typhus, and spotted fever group rickettsiosis.

MATERIALS AND METHODS

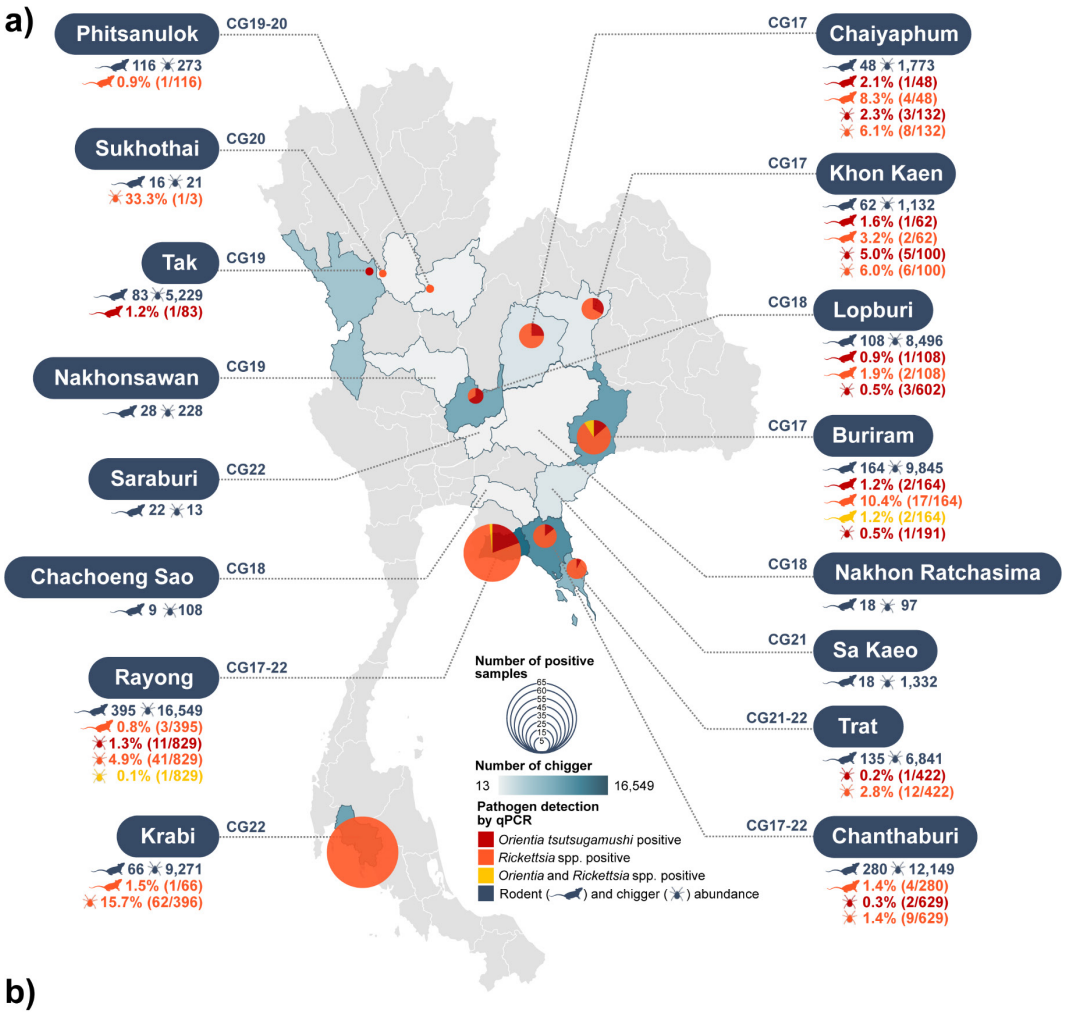
Study sites

Entomological and rodent surveillance was conducted from October to February of each year to cover the period of at least one month before the training exercise took place in January to March. The CG annual joint exercise consists of two missions: (1) field exercise that combines air, land, maritime training under numerous tropical terrain operations and (2) humanitarian assistance to enhance regional military strategy and relationship between allies, host nation communities, and the US. In contrast to the field exercise that is concentrated in restricted areas of the Thai military bases, the humanitarian assistance is typically conducted in 5 to 8 locations per year in rural and suburban areas across Thailand. Activities for the humanitarian assistance include construction of multi-purpose school buildings, community outreach, and capability building where Service Members spend at least one month at the training site and requires extensive cooperation between the Joint team and local villagers. Therefore, surveillance sites under this study included areas that were used to support the mission, surrounding areas for military (military installations and recreational areas), and potential risk areas for rodent hosts and ectoparasite vectors such as nearby residential areas, agro-ecological areas, and the edge of the forests near the training sites. A summary of the study sites is shown in Figure 1. Partial investigation results during the period between 2017 and 2018 were reported previously[20] and the data were combined in this report to provide a more complete status of the current ectoparasite-borne disease threats affecting military exercises and for effective medical planning.

Sample size determination

Information on the prevalence of ectoparasite-borne and rodent-borne diseases in Thailand especially in vector and reservoir host remains largely undetermined in many locations. Therefore, this study used an average of the *Otsu* infection prevalence from previous reports as the representative value for sample size calculation and a precision rate of 5%. The following formula for sample size calculation was used: Minimum number of captured rodents = $z^2 (p)(1-p)/d^2$, where z = 1.96 for a confidence level of 95%, d =

Figure 1: a) Thailand map showing locations used for Cobra Gold humanitarian mission in Thailand between 2017 and 2022, and distribution of pathogen positivity rate in rodents and chiggers in each location based on molecular detection of the pathogen of interest. Colored pie charts and size of the chart represent pathogen positivity rates in rodents and chigger samples based on molecular assay (red-*Orientia tsutsugamushi* positive, orange-*Rickettsia* spp. positive, yellow-*Orientia tsutsugamushi* and *Rickettsia* spp. positive) b) Environment surrounding the training areas and study sites.



desired level of precision (5%), and p = prevalence of disease of interest (16%). An estimated sample size of free-roaming rodents to be sampled was 207 rodents and used for animal use protocol submission and trap setting. All procedures involving animals were conducted in compliance with the animal use protocol (Protocol number 18-04 and 21-10: Field Sampling of Small Mammals (Orders: Erinaceomorpha; Soricomorpha; Scandentia; Macroscelidea and Rodentia) approved by the Armed Forces Research Institute of Medical Sciences (AFRIMS) Institutional Animal Care and Use Committee as well as with local and national laws regarding the use of animals for research purposes.

Collection of small reservoir hosts and their ectoparasites

Rodents

Small rodents were live-trapped from various locations across Thailand following the previously described methodology.[21] Sherman live traps were set around 1600-1800 pm with the maximum number of 100 traps/night for three consecutive nights/trap session. Rodents were baited with bananas or other food recommended by villagers or local hunters such as palm seed and fruit as appropriate. When possible, rodent traps were set at 10 m intervals along transects following the protocol for field and laboratory rodent collection.[22] Rodent traps were inspected and collected the following morning before 0800. New clean traps were replaced if rodents were found to maintain a constant total number of traps per site and enhance capture efficiency. The geographic location of the individual collection sites was recorded using a Global Positioning System (GPS) tracker and trapping locations were initiated at the center of each village, as local villagers assisted with trap set up on their private properties.

Trapped rodents were euthanized at the field sites using compressed carbon dioxide gas followed by cardiocentesis to collect whole blood and serum samples. Rodents were identified to species based on morphology following the Asia rodent taxonomic keys.[23] Rodent tissue samples including lung, liver, spleen, intestine, and kidney were collected and stored on dry ice as stock tissue samples. Following the institutional guidelines for biosafety and biosecurity, small portions of tissues were dissected and preserved in a stabilization solution to inactivate infectious pathogens and materials before being stored in dry ice container and transported to the AFRIMS laboratory and used for nucleic acid purification.

Ectoparasites collection

Infesting ectoparasites (chigger, flea, tick, louse) on rodent bodies were thoroughly inspected using a head magnifier. Chiggers are usually clustered as a group of the same or mixed species in rodent ears or abdominal areas where they are difficult to dislodge by the host. Some portions of chiggers were collected with a thin layer of host skin using sterile tweezers and placed in a charcoal vial to allow them to detach from the skin and prevent damage to the mouthparts, while other chiggers were collected and placed into 70% ethanol for further morphological identification and pathogen screening. Other ectoparasites were repelled from the rodent bodies using the ether fumigation method in close plastic zipped lock bags and preserved in either 70% ethanol or 5% ethanol before being substituted with 70% ethanol as recommended by the American Veterinary Medical Association Guidelines for the Euthanasia of Animals (2020 Edition). All ectoparasites were stored according to vector type, host, collection site, and collection trip. Representative chiggers were sampled from each rodent host and identified to genus using published standard taxonomic keys.[24] Chiggers from the same cluster on the same rodent host were identified and grouped as the same genus based on morphology characterization results and a representative sample of up to 10% of pre-identified chiggers was processed for total nucleic acid extraction.

Purification of nucleic acid from chigger and rodent tissue samples

Total nucleic acid was purified from the individual chiggers using a commercial nucleic acid extraction and purification kit following the manufacturer's instructions in combination with the challenge to lyse bacterial cells. Briefly, individual chiggers were allowed to air-dry at room temperature to remove ethanol residues and surface sterilized using nuclease-free water, before transferring into a 2 mLxx micro-centrifugal tube containing 100 µL tissue lysis buffer, 20 µL proteinase K and approximately five of 5 mm zirconium beads or ½ scoop of 3 mm glass beads to enhance lysis efficiency. Chiggers were homogenized by the beat-beating method with 25-30 Hz oscillation frequency for 30-sec x 2 times. Chiggers were checked under the microscope to ensure complete homogenization of the exoskeleton and incubated at 56°C for 1 hour by using a thermomixer with the temperature setting at 105°C and shaking at 200-rpm speed. After completion of the lysis step, chigger lysates were centrifuged at 14,000 xg at room temperature and the clear homogenates were used for total nucleic acid purification. Nucleic acid was eluted in 100 µl elution buffer before concentrating at 45°C to approximately 50 µl using a vacuum concentrator. Molecular-grade nuclease-free water and pathogen-free chiggers from the colony were processed in parallel in every extraction batch as blank and negative control to ensure that the extracted samples were free from sample-to-sample cross-contamination. Purified nucleic acid samples were used for pathogen detection based on the molecular technique described in the pathogen detection section.

Lung, spleen, and liver samples from all rodent hosts, whether they had ectoparasite infestation or not, were processed for total nucleic acid extraction following the methodology described above with some modifications. Approximately 3 mm^3 of each tissue was transferred to 2 mL micro-centrifugal tube containing 200 µl tissue lysis buffer, 20 µl proteinase K, and approximately 10 beads or one scoop of 5 mm zirconium beads, followed by homogenized using beat-beating method. After incubation at 56°C for 3 hours, tissue lysates were centrifuged at 14,000 xg for 1 minute, and 200 µl of clear tissue lysates were collected and used for nucleic acid purification using a commercial kit. Nucleic acid was eluted using 150 µl elution buffer followed by concentration to reduce the total volume to approximately 100 µl final volume and stored at -80°C until further processing for pathogen detection.

Detection of the *Orientia* and *Rickettsia* spp. in chiggers and rodents

The presence of pathogens of interest in rodent tissue was tested as an individual sample, while pathogen detection in chigger nucleic acid samples were processed as pooled samples of up to 12 samples per pool (5 µl/ sample) according to genus of chigger, host, collection site, and trip. *Otsu* and *Rickettsia* spp. detection was performed using multiplex real-time polymerase chain reaction assay targeting 47-kDa high-temperature requirement A family protein (*htr*A) gene for *Orientia* and 17-kDa genus-specific antigen protein for pan-*Rickettsia* (17-kDa) with a real-time PCR machine.[25,26] *Otsu* str. Karp-related were purified from the AFRIMS laboratory colony of *Leptotrombidium* chiggers and *R. typhi* str. Wilmington from cell culture were used as positive control for *Otsu* and *Rickettsia*. If a pooled chigger sample was positive for the pathogen of interest, individual samples were re-analyzed to determine the infection prevalence and genetic diversity in correspondence to the amplification signals using nested PCRs: *Otsu* - 56-kDa type specific antigen protein (*tsa56*) for *Orientia*, *Rickettsia*-partial 17-kDa, 120 to 135-kDa outer membrane protein (*omp*B), 190-kDa outer membrane protein (*omp*A), and citrate synthase (*glt*A) as previously described.[27-30] The PCR products were purified using a commercial gel extraction kit according to the manufacturer's recommendations and sequencing was conducted bi-directionally using a local sequencing service provider. To investigate the possibility of detection of *candidatus* O. chuto in Thailand, 100 each of pooled chigger samples sampling from all CG locations and archive samples from previous surveillance in endemic areas in Thailand were investigated for the presence of *ca.* O. chuto using PCR targeting for 16s rDNA gene following in-house protocol provided by the Naval Medical Research Center in Silver Spring, MD. Positive control for *ca.* O. chuto str. Dubai was kindly provided by Prof. Stuart Blacksell, Mahidol-Oxford Research Unit, Thailand.

Molecular identification of chiggers

Chiggers with *Orientia* or *Rickettsia* positive status plus an additional 5% of chiggers from the same rodent hosts were further analyzed for the genetic identification markers using PCR analysis based on partial sequence of mitochondrial cytochrome oxidase subunit I (*cox*I) (31). Briefly, 3 µl nucleic acid sample was used in a total reaction volume of 25 µl containing 0.3 µM of each primers, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.02 U/ µM Platinum tag DNA polymerase, and 1x PCR buffer and nuclease-free water. The reaction mixture was attempted for PCR detection by incubation at 94°C for 1 minute followed by five cycles of denaturation at 94°C for 1 minute, annealing at 45°C for 1.3 minute, extension at 72°C for 1 minute, then 35 cycles of the similar PCR condition with the exception of changing annealing temperature to 50°C. The PCR amplification was finished with final elongation at 72°C for 5 min. DNA extracts of the morphologically confirmed *Leptotrombidium deliense* chiggers from institutional chigger colonies were used as a positive control to determine the *cox*I sequence relatedness. The DNA templates were replaced with nuclease-free water in negative controls. All PCR products were visualized using a commercial gel stain on 1.5% (w/v) agarose gel and purified using a commercial gel extraction kit. In summary, the total number of 140 *cox*I amplicons were directly sequenced and nucleotide sequences without primer binding sites were used for phylogenetic analysis.

Serological analysis

Seropositivity of rodent hosts against the causative agents of scrub typhus, murine typhus, and spotted fever group rickettsiosis were determined using whole-cell antigen-based immunofluorescence staining assays as previously described.[2]

Data analysis

Nucleotide sequences from both DNA strands were assembled into single overlapping DNA fragment (contig) using commercially available DNA sequence analysis software. Obtained sequences were aligned with those sequences available in GenBank database utilizing Basic local alignment search tool for nucleotide sequence (BLASTn). Phylogenetic analyses were performed using Neighbor-Joining method and 1,000 bootstrapping replicates for the individual genes using freely available phylogenetic inference software software. The evolutionary distances were computed using Kimura 2-parameter with pairwise distances estimated using maximum composition likelihood approach. The GPS coordinates of the individual rodent collection sites, data on morphologically based identification of rodents and ectoparasites, and pathogen detection results (when available) were deposited as eVoucher data to support VectorMap in collaboration with the Walter Reed Biosystematics Unit.

Statistical analysis

The 95% confidence interval and the chi-square analysis were performed for the chigger infestation rate, rodent species, chigger genera and pathogen infection rate related with location study sites ($p < 0.05$). Correlation of chigger abundance and rodents infested with chiggers was analyzed by a simple linear model using commercially available statistical analysis software. The CG surveillance map was generated by using open-source geographic information system software.

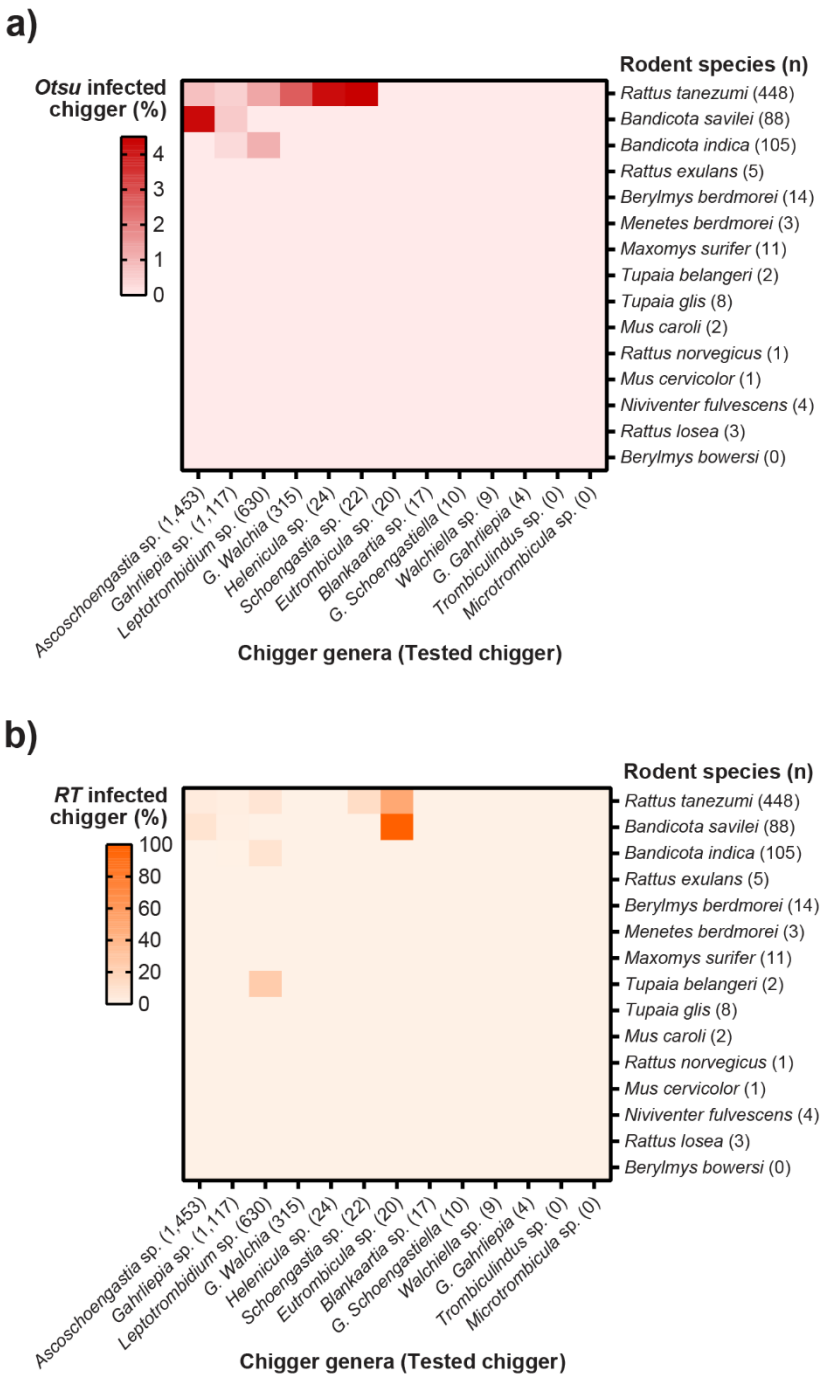
RESULTS

Rodent diversity and infection prevalence

As part of the pre-exercise vector-borne and rodent-borne disease surveillance program to promote Total Forces Readiness, our team conducted entomological risk assessment in all locations used for the CG humanitarian mission with an addition of one military base in Chanthaburi province (Camp Baan Chan Khrem, Chanthaburi province in 2017 and

2018). In summary, the risk assessment was conducted in 61 sites located in 22 districts of 16 provinces. A majority of the training exercises were held in the rural lowland (approximately 500 m in elevation) and suburban areas of eastern and central regions of the country with sporadic exercises in other regions. A total number of 1,568 rodents of 15 species were trapped during the collection period with the top three highest rodent infestation rate in eastern (57.1 % (397/695)), followed by northeast (19% (132/695)) and central (9.8% (68/695)). There was a significant difference in the chigger infestation rate with rodent species ($p < 0.0001$). Of which, 695 rodents had chigger infestation, accounting for overall 44.3% infestation. During the study period, some fleas (n = 21), mange mites (n = 67) and ticks (n = 26) were observed on the rodent bodies. Therefore, we only included the results of pathogen investigation in chiggers and capitalized results in parallel to infection prevalence in rodent hosts to correlate the possible role of chiggers as the primary vector of the pathogen of interest.

Figure 2: Correlation analysis between *Orientia tsutsugamushi* (*Otsu*), *Rickettsia* spp. (*RT*) infection, chigger genera, and rodent host.



Although the sites for CG humanitarian mission are varied annually, there are some key sites that are typically used for the exercise every year including Rayong and Chanthaburi. Therefore, the majority of rodents that were mainly collected from these sites reflect the frequency of exercise and site use. Chiggers were found in all CG sites with infestation rates on rodent hosts varied from 7.8% to 77.3% (Table 1). Chigger infestation on rodents relied on the ecology of the collection sites, which reflected the diversity of rodent species - *Rattus losea* had highest chigger infestation rate of 100% (3/3), followed by *Niviventer fulvescens* (66.7% (4/6)), *Maxomys surifer* (57.9% (11/19)), and *R. tanezumi* (56.5% (448/793)) (Table 2). Regarding the primary host for chiggers in Thailand, the majority of chiggers were predominantly recovered from *R. tanezumi* (67%), *Bandicota indica* (16.7%), and *B. savilei* (12.2%). Although chiggers have low host specificity and could be found on a wide variety of hosts, we did not observe chigger infestation on *Berylmys bowersi*.

Identification of *Otsu* infection prevalence in chiggers and rodents

Eleven chigger genera were collected during the study period. Evidence from multiplex qPCR analysis for *htr*A and 17-kDa of 3,696 chigger samples was conclusive that the overall prevalence of *Otsu* infection in chiggers was 0.7% (26/3,696, range 0.2% to 5.0%) and 0.4% (6/1,568, range 0.9% to 2.1%) in wild-rodent hosts. Rickettsemic or persistent infection of *Otsu* in wild rodents is inconsistently observed when compared to the seroprevalence in rodents, infection prevalence in chiggers, and chigger abundance. This finding potentially confirms the recent hypothesis that the vertebrate host only

Table 1. Seroprevalence and infection prevalence of *Orientia* and *Rickettsia* spp. in rodents and chiggers.

Province	Total rodent collected	Rodent			Seroprevalence						%Chigger infestation	Total chigger retrieved	Chigger	
		Pathogen positivity rate based on multiplexed qPCR											Pathogen positivity rate based on multiplexed qPCR	
		%Otsu	%Rickettsia	%Otsu +Rickettsia	No. serum tested	Otsu	MT	SFG	Otsu +SFG	MT +SFG			%Otsu	%*Rickettsia*	%Otsu +Rickettsia
Buriram	164	1.2% (2)	10.4% (17)	1.2% (2)	160	6.9%	0%	0%	0%	0%	37.8% (62)	9,845	0.5% (1/191)	0% (0/191)	0% (0/191)
Khon Kaen	62	1.6% (1)	3.2% (2)	0%	58	15.5%	3.4%	0%	1.7%	1.7%	21.0% (13)	1,132	5.0% (5/100)	6.0% (6/100)	0% (0/100)
Chaiyaphum	48	2.1% (1)	8.3% (4)	0%	44	13.6%	0%	0%	0%	0%	75.0% (36)	1,773	2.3% (3/132)	6.1% (8/132)	0% (0/132)
Chanthaburi	280	0%	1.4% (4)	0%	268	10.4%	1.5%	3.0%	0%	0.4%	32.1% (90)	12,149	0.3% (2/629)	1.4% (9/629)	0% (0/629)
Lopburi	108	0.9% (1)	1.9% (2)	0%	108	0%	0.9%	0%	0%	0%	55.6% (60)	8,496	0.5% (3/602)	0% (0/602)	0% (0/602)
Nakhon Ratchasima	18	0%	0%	0%	18	0%	0%	0%	0%	0%	44.4% (8)	97	0% (0/41)	0% (0/41)	0% (0/41)
Sa Kaeo	18	0%	0%	0%	18	5.6%	0%	0%	0%	0%	61.1% (11)	1,332	0% (0/140)	0% (0/140)	0% (0/140)
Trat	135	0%	0%	0%	133	5.3%	0%	5.3%	0%	0%	61.5% (83)	6,841	0.2% (1/422)	2.8% (12/422)	0% (0/422)
Phitsanulok	116	0%	0.9% (1)	0%	114	6.1%	0%	0%	0%	0%	7.8% (9)	273	0% (0/20)	0% (0/20)	0% (0/20)
Sukhothai	16	0%	0%	0%	15	0%	0%	0%	0%	0%	25.0% (4)	21	0% (0/3)	33.3% (1/3)	0% (0/3)
Tak	83	1.2% (1)	0%	0%	79	10.1%	1.3%	0%	0%	0%	56.6% (47)	5,229	0% (0/115)	0% (0/115)	0% (0/115)
Nakhonsawan	28	0%	0%	0%	28	0%	0%	0%	0%	0%	14.3% (4)	228	0% (0/30)	0% (0/30)	0% (0/30)
Saraburi	22	0%	0%	0%	21	4.8%	0%	0%	0%	0%	18.2% (4)	13	0% (0/7)	0% (0/7)	0% (0/7)
Chachoeng Sao	9	0%	0%	0%	7	0%	0%	0%	0%	0%	44.4% (4)	108	0% (0/39)	0% (0/39)	0% (0/39)
Rayong	395	0%	0.8% (3)	0%	391	6.1%	0.3%	3.6%	0%	0%	52.9% (209)	16,549	1.3% (11/829)	4.9% (41/829)	0.1% (1/829)
Krabi	66	0%	1.5% (1)	0%	53	18.9%	0%	17.0%	0%	0%	77.3% (51)	9,271	0% (0/396)	15.7% (62/396)	0% (0/396)
Total	1,568	0.4% (6)	2.2% (34)	0.1% (2)	1,515	7.4%	0.6%	2.5%	0.1%	0.1%	44.3% (695)	73,357	0.7% (26/3,696)	3.8% (139/3,696)	0.02% (1/3,696)

Otsu indicates Orientia tsutsugamushi, agent of scrub typhus; MT indicates Rickettsia typhi, causative agent of murine typhus; SFG indicates Rickettsia spp., causative agent of spotted fever rickettsiosis.

serves as the conduit host to provide a source of tissue fluid for chiggers for completion of their lifecycle rather than as an amplifying host in rapid horizontal transmission of *Otsu*.

The largest number of *Otsu* positive chiggers was determined in the genus *Ascoschoengastia*, while the smallest number of *Otsu* positive chiggers was found in *Gahrliepia* (*Walchia*), *Schoengastia* and *Helenicula* genera. Figure 2 provides an overview of the correlation between infection prevalence and the genus of chigger studied. Field-collected chiggers had a bacterial load, as determined by qualifying copy number of *htr*A gene per individual chigger extract, ranging from 9.8 to 1,801.3 copies/chigger, with a median of 49.8 copies/chigger. A significant difference in the mean of *Otsu* load in field-collected chiggers was observed when compared to the partially fed chiggers from laboratory-reared colonies (4,207 ± 532.8 copies/ chigger) in which an average of 2.3-times lower was found in field-collected chiggers. *Otsu* positive chiggers were found in 7 of 16 provinces with the highest positivity rate in the CG site located in Khon Kaen (5.0% (5/26)), followed by Chayaphum (2.3% (3/26)), and Rayong province (1.3% (11/26)) (Table 1). When combining the serological analysis results of rodent hosts and the pathogen detection in rodents and chigger vectors, only four CG sites were negative for *Otsu* in all assays, indicating that scrub typhus still presents as a widespread infectious disease threat to Service Members and should not be neglected.

In addition to the entomological risk assessment to support CG humanitarian mission, our team has conducted nation-wide surveillance for chigger-borne diseases in scrub typhus endemic areas in Thailand, and 35 archived partial *tsa*56 sequences were combined in

Table 2. The positivity rate of Rickettsia and Orientia detected in chiggers collected from live-captured rodents in corresponding to genus and species

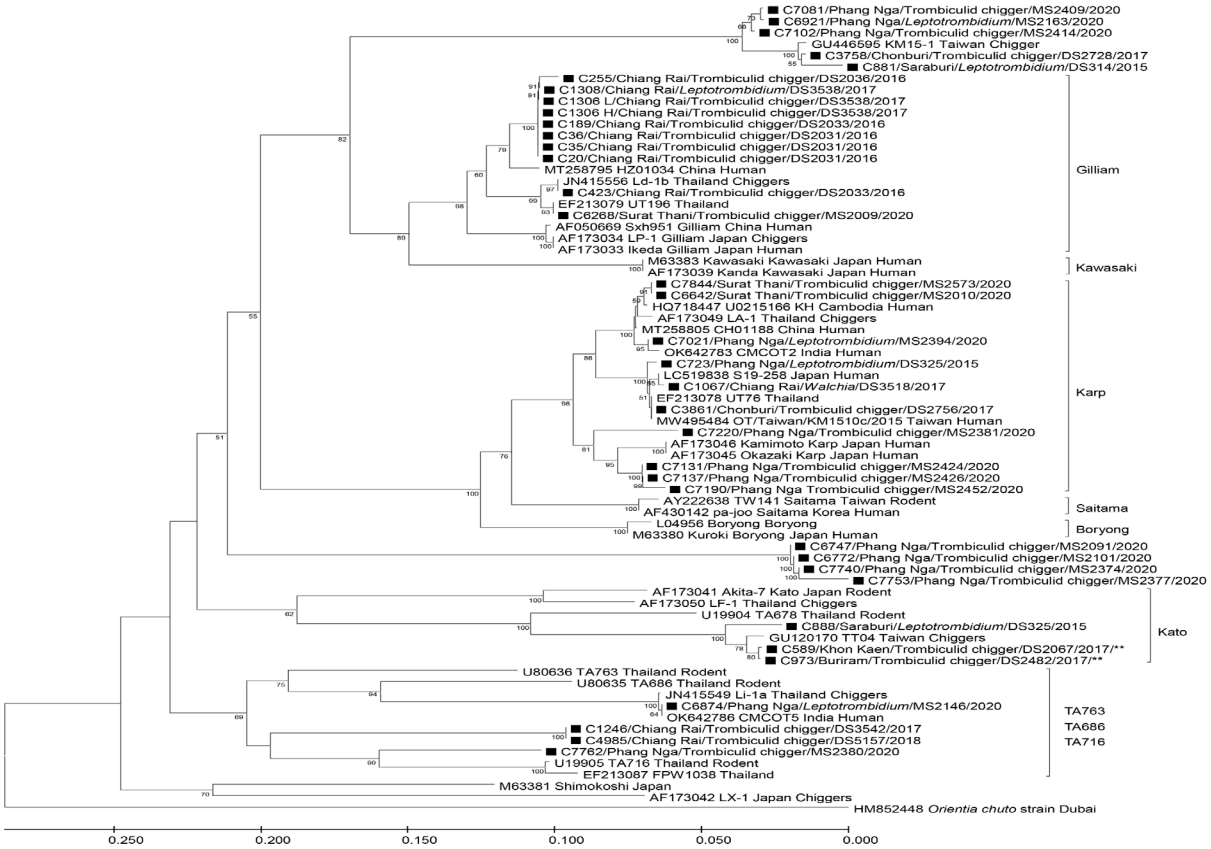
Rodent species	Total rodent collected	Rodent									%Chigger infestation	Total chigger retrieved	Chigger		
		Pathogen positivity rate based on multiplexed qPCR			Seroprevalence								Pathogen positivity rate based on multiplexed qPCR		
		%*Otsu*	%*Rickettsia*	%*Otsu* +*Rickettsia*	No. serum tested	*Otsu*	*MT*	*SFG*	*Otsu* +*SFG*	*MT* +*SFG*			%*Otsu*	%*Rickettsia*	%*Otsu* +*Rickettsia*
Rattus tanezumi	793	0.4% (3)	2.3% (18)	0.1% (1)	789	8.0%	0.6%	4.4%	0.1%	0.3%	56.5% (448)	49,184	0.9% (21/2,213)	5.2% (115/2,213)	0% (0/2,213)
Bandicota savilei	229	0.9% (2)	0.4% (1)	0%	229	7.0%	0%	0%	0%	0%	38.4% (88)	8,973	0.4% (3/744)	1.1% (8/744)	0.1% (1/744)
Bandicota indica	213	0%	1.4% (3)	0%	212	7.5%	0.5%	0.5%	0%	0%	49.3% (105)	12,282	0.3% (2/584)	2.1% (12/584)	0% (0/584)
Rattus exulans	175	0%	3.4% (6)	0.6% (1)	172	5.2%	0.6%	1.2%	0%	0%	2.9% (5)	156	0% (0/20)	0% (0/20)	0% (0/20)
Berylmys berdmorei	34	0%	0%	0%	34	11.8%	5.9%	0%	0%	0%	41.2% (14)	908	0% (0/46)	0% (0/46)	0% (0/46)
Menetes berdmorei	31	0%	3.2% (1)	0%	22	0%	0%	0%	0%	0%	9.7% (3)	451	0% (0/17)	0% (0/17)	0% (0/17)
Maxomys surifer	19	0%	0%	0%	19	5.3%	0%	0%	0%	0%	57.9% (11)	135	0% (0/19)	0% (0/19)	0% (0/19)
Tupaia belangeri	15	0%	0%	0%	0	NA	NA	NA	NA	NA	13.3% (2)	144	0% (0/8)	25.0% (2/8)	0% (0/8)
Tupaia glis	15	0%	13.3% (2)	0%	0	NA	NA	NA	NA	NA	53.3% (8)	662	0% (0/27)	7.4% (2/27)	0% (0/27)
Mus caroli	13	0%	15.4% (2)	0%	9	0%	0%	0%	0%	0%	15.4% (2)	51	0% (0/3)	0% (0/3)	0% (0/3)
Rattus norvegicus	11	9.1% (1)	0%	0%	11	18.2%	0%	0%	0%	0%	9.1% (1)	86	NA	NA	NA
Mus cervicolor	10	0%	0%	0%	8	0%	0%	0%	0%	0%	10.0% (1)	30	0% (0/1)	0% (0/1)	0% (0/1)
Niviventer fulvescens	6	0%	0%	0%	6	0%	0%	0%	0%	0%	66.7% (4)	63	0% (0/11)	0% (0/11)	0% (0/11)
Rattus losea	3	0%	33.3% (1)	0%	3	0%	0%	0%	0%	0%	100% (3)	232	0% (0/3)	0% (0/3)	0% (0/3)
Berylmys bowersi	1	0%	0%	0%	1	100%	0%	0%	0%	0%	0% (0)	0	NA	NA	NA
Total	1,568	0.4% (6)	2.2% (34)	0.1% (2)	1,515	7.4%	0.6%	2.5%	0.1%	0.1%	44.3% (695)	73,357	0.7% (26/3,696)	3.8% (139/3,696)	0.02% (1/3,696)

Otsu indicates *Orientia tsutsugamushi*, agent of scrub typhus; *MT* indicates *Rickettsia typhi*, causative agent of murine typhus; *SFG* indicates *Rickettsia* spp., causative agent of spotted fever rickettsiosis.

the recent study to demonstrate the genetic diversity of chigger-borne *Otsu* in Thailand. Phylogenetic analysis and BLASTn searches for *tsa*56 genes amplified from chiggers (646 nucleotides in the final dataset) identified numerous sequences closely related to *Otsu* previously amplified from scrub typhus patients, chiggers, and rodents from multiple countries. The majority of the chigger-borne *Otsu* clustered within the same clades of four genogroups including Gilliam, Karp, TA, and Kato (Figure 3), suggesting that the variants were common in the endemic regions. Other less common *Otsu* strains that formed a clade separated from the original *Otsu* genogroups were also observed. The divergent strains of chigger-borne *Otsu* were closely related to *Otsu* str. KM15-1 from Taiwan (accession number GU446595) (92.6% to 99.6% nucleotide similarity) previously defined as the JG-divergent and *Otsu* str. Lep.D021.1h from Thailand (accession number MH290196-data not shown) (98.5% to 100% nucleotide similarity).

Based on the analysis of partial *coxI* sequences amplified from *Otsu* positive chiggers, 23 *coxI* sequences showed matched results for the genetic marker and morphological analysis at the genus level (supplement figure). *Otsu* was presumably detected in the three genera including the genus *Leptotrombidium*, *Gahrliepia* (*Walchia*), and *Ascoschoengastia*.

Figure 3: Genetic diversity of *Orientia tsutsugamushi* based on neighbor joining phylogenetic analysis of the partial 56-kDa type specific antigen gene amplified from chigger samples (607 nucleotides). The percentage of 1,000 bootstrap replicates to support the association of taxa are shown below the branches. *Candidatus Orientia chuto* str. Dubai was used as an outgroup. (*) indicates sample collected during Cobra Gold pre-exercise surveillance. GenBank accession numbers of nucleotide sequences identified in this study are OP562410-OP562445.



Genetic markers appear to be a useful tool for family and genus identification and confirmation, but a detailed comparison based on a more comprehensive dataset such as utilization of complete mitochondrial genome and improved datasets for the reference species remains a gap that urgently needs to be filled before genetic markers may routinely be used for accurate species identification.

Orientia spp. pathogens are species-specific in certain geographical regions

Historically, chiggers were recognized as the primary vector of *Otsu* with geographic distribution encompassing 13 million square kilometers of the Asia-Pacific region- "Tsutsugamushi triangle". However, recent findings of the infected vector outside the endemic area demonstrated that *Otsu* is not only the sole member of the genus *Orientia*, but there are two novel species, *Candidatus* Orientia chuto and *Ca.* Orientia chiloensis, discovered from the Middle East, Africa, and South America. To determine whether these potential novels *Orientia* species are also present in the historical scrub typhus endemic area, we tested pooled chigger nucleic acids sampled from all CG sites and archived chigger nucleic acids samples collected from endemic areas in 20 locations in 8 provinces of Thailand from 2017-2021 using specific nested PCR for *Ca.* Orientia chuto. The results revealed that none of the chigger samples showed an amplification signal for *Ca.* Orientia chuto, indicating that the distribution of *Orientia* is potentially geographically specific and relies on the availability of a competent vector. Further epidemiological studies in areas with different levels of scrub typhus or chigger endemicity are warranted to better answer our hypothesis.

Chigger abundance in CG training sites in Rayong province

Rayong province is one of two key sites routinely used to support CG exercise, making this a target area for gap analysis and in-depth entomological risk assessment to help protect military personnel from pathogen exposure. The relative abundance of chiggers, their rodent hosts, and the infection prevalence of *Otsu* were monitored over five consecutive years in Rayong province to provide a more accurate assessment of the human health risks. From 2017-2021, we supported rodent and entomological borne disease risk assessment in 6 sites located at 4 districts of Rayong province.

In total, 395 rodents, and 16,549 chiggers were collected and analyzed. Collected rodents had an average of 52.9% chigger infestation rate. Based on morphological identification of representative chiggers, those from Rayong could be classified into eight genera comprising 58.4% *Ascoschoengastia*, 31.9% *Leptotrombidium*, 4.1% *Gahrliepia*, 2.2% *Walchiella*, 1.7% *Gahrliepia* (*Walchia*), 1.3% *Eutrombicula*, 0.3% *Gahrliepia* (*Schoengastiella*) and 0.1% *Microtrombicula*. We observed that the chigger abundance, density, and infestation rate on rodent hosts have continuously increased over the study period and more than doubled during the last five years (Figure 4). *Otsu* infection prevalence in chiggers increased from 1.1% (1/94) in 2020 to 2% (8/446) in 2021 in parallel with the abundance of chigger population, which had a linear trend of infestation rate on rodent host rising from 49.4% (41/83) in 2020 to 56.4% (114/222) in 2021. In addition, the relative abundance of chigger vectors, infection prevalence in chiggers, and seroprevalence of the rodent hosts for *Otsu* infection positively correlated with the increased incidence of scrub typhus cases in Rayong (Data from the Ministry of Public Health of Thailand).

Rickettsia infection prevalence in chiggers

Rickettsia positivity rates were identified in 3.8% (139/3,696) of representative chiggers and 2.2% (34/1,568) of rodent hosts based on qPCR analysis. Of which, rickettsial DNA was solely detected in 117 chiggers without *Otsu* co-infection. The infection prevalence of *Rickettsia* in chiggers was 5.4 times-higher than the prevalence of *Otsu* (3.8% (139/3,696) vs 0.7% (26/3,696)). We observed that 41.2% (14/34) of the *Rickettsia*-positive rodents had chigger infestations. None of the *Rickettsia* positive rodents were infested with other ectoparasites. *Rickettsia* positive chiggers were widely distributed in the CG training sites at a rate similar to *Otsu* (7 of 16 provinces) (Table 2). Of all *Rickettsia* positive chiggers from the CG pre-exercise (n = 107) and archive samples (n = 117), 94 samples showed matching results for genetic marker and morphological identification in which 55 were identified as members of *Leptotrombidium*, 24 *Ascoschoengastia*, and 15 *Gahrliepia* (*Walchia*). These findings led us to question whether chiggers could potentially serve as a vector in transmitting *Rickettsia* spp. or play a role in the maintenance of the zoonotic cycle.

For *Rickettsia* species identification, two conserved fragments of 17-kDa and *glt*A genes and two variable fragments of *omp*A and *omp*B gene were amplified from all *Rickettsia* positive chiggers. However, attempts to amplify the *omp*A, *omp*B and *glt*A fragments were unsuccessful for some *Rickettsia* positive chigger samples and there was no sample positive for all genes which hindered generation of concatenated sequences for phylogenetic analysis. Based on the PCR results, the highest positivity rate was observed when using a primer set specific for 17-kDa. Overall, we identified at least three *Rickettsia* species from chiggers based on the analysis of 17-kDa gene. A majority of the chigger-borne *Rickettsia* were closely related to *R. felis*, sharing nucleotide sequence similarity of 99.6% to 100% with previously identified strains from ticks, chiggers, or patients (Figure 5), followed by *Rickettsia* strains closely related to *R. akari* (97.8% nucleotide similarity) and *R. typhi* (99.2% to 100% nucleotide similarity). Of 26 *Otsu*-positive chiggers, one chigger sample (3.8% (1/26)) retrieved from rodents collected from the CG20-Rayong exhibited co-infection with *Rickettsia* and *Otsu*. The results showed that seven of the *Rickettsia*-positive chiggers were retrieved from four positive rodents. These *Rickettsia*-positive rodents were found in four training sites used for CG17-Chaiyaphum (1/4), CG18-Rayong (1/4), CG20-Rayong (1/4), and CG22-Krabi (1/4). Correlation analysis for *Rickettsia* positivity rates between tick, flea, chigger, and rodent was not implemented due to the very low numbers of vectors retrieved and lack of availability of the pathogen detection data.

Figure 4. Correlation of chigger abundance and rodents infested with chiggers in the Cobra Gold training sites located at Rayong province of western Thailand.

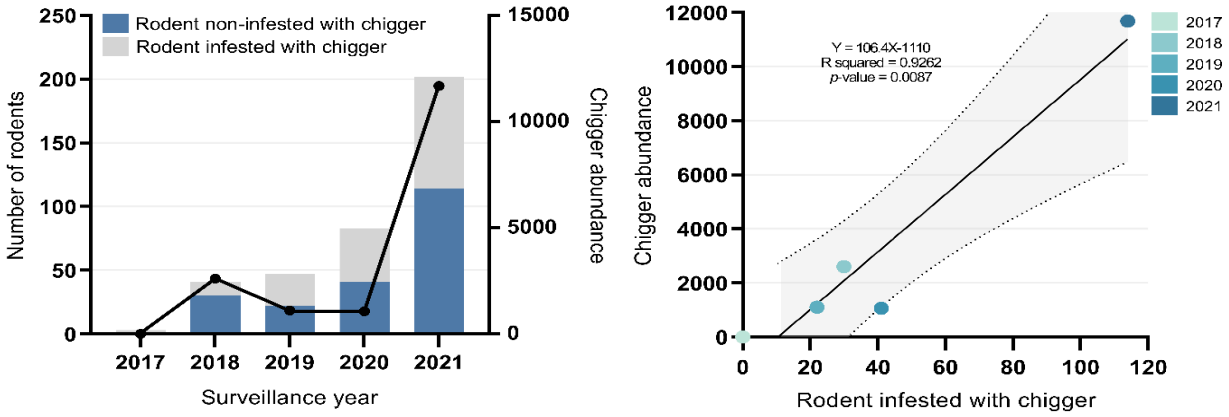
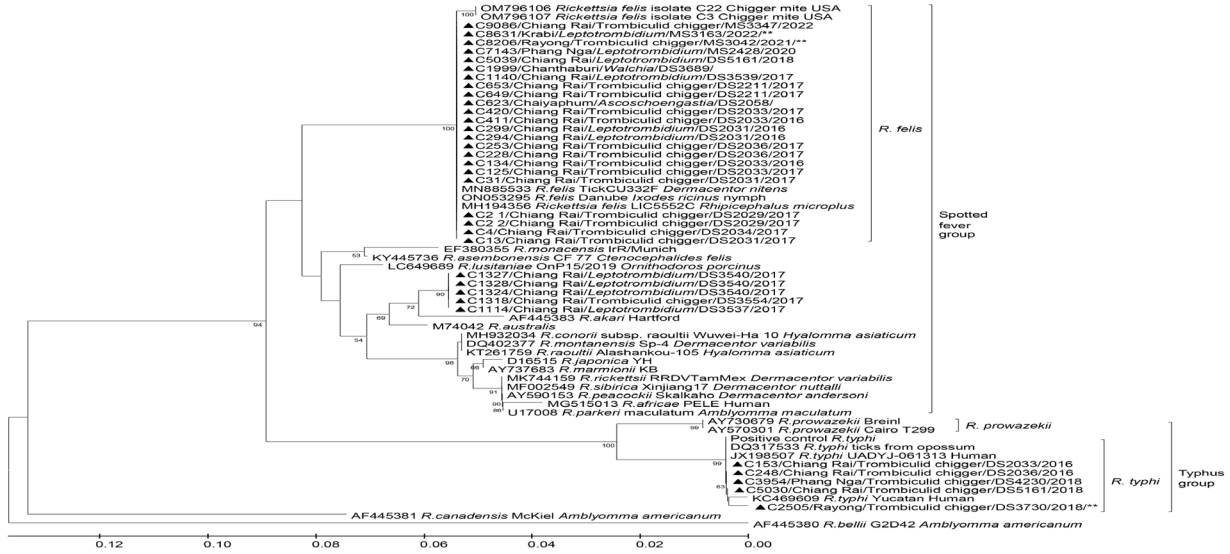


Figure 5: Genetic diversity of chigger-borne Rickettsia spp. based on neighbor joining phylogenetic analysis of the partial 17-kDa type specific antigen gene (285 nucleotides). The percentage of 1,000 bootstrap replicates to support the association of taxa are shown below the branches. (*) indicates sample collected during Cobra Gold pre-exercise surveillance. GenBank accession numbers of nucleotide sequences identified in this study are OP562446-OP562478.



DISCUSSION

Our surveillance study revealed that while the trombiculid chigger is currently primarily recognized as a vector for *Otsu*, the presence of *Rickettsia* in field-collected chiggers is more prevalent than *Otsu* in some chigger populations. Unlike *Orientia*, *Rickettsia* has a wide range of hosts and is responsible for human and zoonotic diseases transmitted by blood-feeding ectoparasites, including soft and hard ticks, mesostigmatid mites, body lice, and fleas. Potential for novel *Rickettsia* as well as *R. felis* transmission through mosquito and flea vectors have been regularly reported and implicated that the route of delivery may govern pathogenicity and disease.[32-35] Currently, at least five *Rickettsia* species have been molecularly confirmed in patients and several species of ticks in Thailand including (a) agents of spotted fever group rickettsiosis-*R. honei*, *R. japonica*, *R. felis*, *R. raoultii*, and (b) agent of epidemic typhus-*R. typhi*.[36-40] Some of these *Rickettsia* species have been identified in chiggers using molecular and sequence-based methods including *R. felis* and *R. typhi* in chiggers from China, Taiwan, and USA [17,41,42] in parallel with the identification of (c) rickettsialpox- *R. akari* in chiggers from USA, indicating chiggers may serve as a vector transmitting spotted fever group and epidemic typhus rickettsial pathogens. Findings from this study agree with previous reports and suggested that the presence of at least three species of chigger-borne *Rickettsia* in Thailand including *R. felis. R. akari* and *R. typhi.* We found that the majority of *Rickettsia*-positive chiggers were retrieved from non-rickettsemic hosts. It is plausible that *Rickettsia* in chiggers are endosymbionts or horizontally acquired during feeding on the same spot with an infected donor similar to the proposed transmission route and maintenance cycle of *R. felis* in the cat flea (*Ctenocephalides felis*) and rat flea (*Xenopsylla cheopis*) .[43]

Rickettsia positive chiggers are widely distributed in Thailand with a positivity rate 4.7 times-higher than the rate of *Orientia*, suggesting that the risk of exposure to *Rickettsia* spp. for Service Members may be a larger burden than previously thought. This report also adds new information on the list of chigger genera that harbor Rickettsiae. The recent potential competent vectors transmitting Rickettsiae may include genus *Leptotrombidium* (Thailand, Taiwan, and United States), *Eutrombicula* (Thailand and United States), *Ascoschoengastia* (Thailand), *Schoengastia* (Thailand), *Helenicula* (Thailand), and *Gahrliepia* (Thailand). In addition, our study demonstrated that a single chigger species/genus is capable of hosting more than one pathogenic Rickettsiae.

Results from this study reflect the actual infection prevalence of *Rickettsia* in field-collected chiggers (3.2%) as the individual chiggers were used after initial screening rather than the minimum infection rate (number of positive pools/total number of specimens tested) described by

other studies . Figure 4. Vector-borne diseases reported by a) year and b) month from U.S. military and contractor personnel in Djibouti, 2018-2022. No cases have been reported in 2022. However, the lower positivity rate may be due to (a) *Rickettsia* DNA presence in low copy numbers that were potentially below the detection limit of 17-kDa as a consequence of pooling samples from individual chigger extracts or (b) because the genes of chigger-borne *Rickettsia* may be too divergent for amplification utilizing primers and conditions previously optimized for *Rickettsia* from other ectoparasites. Analysis of *Rickettsia* positivity rate by using other genes in combination with primers designed for chigger-borne Rickettsiae and multilocus sequence typing are warranted for a better understanding of the epidemiology of *Rickettsia* in chiggers, particularly in areas with high chigger prevalence. Further studies are needed to determine the genetic diversity, evolution, as well as potential pathogenicity of chigger-borne *Rickettsia* in comparison with previously identified *Rickettsia* from other arthropod vectors. So far, the ability of chiggers to successfully transmit rickettsial pathogens remains poorly understood, and there are no registered epidemiological data on human infections or geographic distribution of potential vectors in the reportable system of the Ministry of Public Health of Thailand. Experiments to demonstrate whether the *Rickettsia* found in chiggers occurred due to accidental ingestion from rickettsemic hosts or are endosymbionts and can vertically transmit and be maintained through multiple generations of chiggers are warranted to examine the vector capability of chiggers for *Rickettsia* transmission. Identification of other autochthonous and non-autochthonous chigger species that function as vectors are also needed for a better understanding of the modes of transmission.

CONCLUSION

We report a wide geographic distribution of *Otsu* and *Rickettsia* positive chiggers and rodents collected from multiple CG training exercise sites in Thailand, with the possibility of medium to high risk of pathogen exposure to Service Members deployed to these areas. Continuous monitoring of rodent and ectoparasite populations is warranted for disease risk monitoring for Force Health Protection. In the absence of effective vaccines for chigger-borne diseases, performing pre-exercise rodent control in combination with use of Environmental Protection Agency (EPA)-approved repellents and treated uniforms remain the primary means of disease mitigation.

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DISCLAIMER

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REFERENCES

1. Elliott I, Pearson I, Dahal P, et al. Scrub typhus ecology: a systematic review of *Orientia* in vectors and hosts. *Parasites Vectors.* 2019;12(1):513.
2. Linsuwanon P, Auysawasdi N, Wongwairot S, et al. Assessing scrub typhus and rickettsioses transmission risks in the Chiang Rai province of northern Thailand. *Travel Med Infect Dis.* 2021;42:102086.
3. Pages F, Faulde M, Orlandi-Pradines E, et al. The past and present threat of vector-borne diseases in deployed troops. *Clin Microbiol Infect*. 2010;16(3):209-224.
4. Li Z, Xin H, Sun J, et al. Epidemiologic Changes of Scrub Typhus in China, 1952-2016. *Emerg Infect Dis*. 2020;26(6):1091-1101.

5. Jeung YS, Kim CM, Yun NR, et al. Effect of Latitude and Seasonal Variation on Scrub Typhus, South Korea, 2001-2013. *Am J Trop Med Hyg*. 2016;94(1):22-5.
6. Rodkvamtook W, Kuttasingkee N, Linsuwanon P, et al. Scrub Typhus Outbreak in Chonburi Province, Central Thailand, 2013. *Emerg Infect Dis*. 2018;24(2):361-5.
7. Harris PNA, Oltvolgyi C, Islam A, et al. An outbreak of scrub typhus in military personnel despite protocols for antibiotic prophylaxis: doxycycline resistance excluded by a quantitative PCR-based susceptibility assay. *Microbes Infect*. 2016;18(6):406-11.
8. Rodkvamtook W, Ruang-Areerate T, Gaywee J, et al. Isolation and characterization of *Orientia tsutsugamushi* from rodents captured following a scrub typhus outbreak at a military training base, Bothong district, Chonburi province, central Thailand. *Am J Trop Med Hyg*. 2011;84(4):599-607.
9. Jiang J, Marienau KJ, May LA, et al. Laboratory diagnosis of two scrub typhus outbreaks at Camp Fuji, Japan in 2000 and 2001 by enzyme-linked immunosorbent assay, rapid flow assay, and Western blot assay using outer membrane 56-kD recombinant proteins. *Am J Trop Med Hyg*. 2003;69(1):60-6.
10. Masakhwe C, Linsuwanon P, Kimita G, et al. Identification and Characterization of *Orientia chuto* in Trombiculid Chigger Mites Collected from Wild Rodents in Kenya. *J Clin Microbiol.* 2018;56(12).
11. Izzard L, Fuller A, Blacksell SD, et al. Isolation of a novel *Orientia* species (*O. chuto* sp. nov.) from a patient infected in Dubai. *J Clin Microbiol*. 2010;48(12):4404-9.
12. Abarca K, Martinez-Valdebenito C, Angulo J, et al. Molecular Description of a Novel *Orientia* Species Causing Scrub Typhus in Chile. *Emerg Infect Dis*. 2020;26(9):2148-56.
13. Wei Y, Huang Y, Li X, et al. Climate variability, animal reservoir and transmission of scrub typhus in Southern China. *PLoS Negl Trop Dis*. 2017;11(3):e0005447.
14. Loan HK, Cuong NV, Takhampunya R, et al. *Bartonella* species and trombiculid mites of rats from the Mekong Delta of Vietnam. *Vector Borne Zoonotic Dis*. 2015;15(1):40-47.
15. Literak I, Stekolnikov AA, Sychra O, et al. Larvae of chigger mites *Neotrombicula* spp. (Acari: Trombiculidae) exhibited *Borrelia* but no *Anaplasma* infections: a field study including birds from the Czech Carpathians as hosts of chiggers. *Exp Appl Acarol*. 2008;44(4):307-314.
16. Chaisiri K, Gill AC, Stekolnikov AA, et al. Ecological and microbiological diversity of chigger mites, including vectors of scrub typhus, on small mammals across stratified habitats in Thailand. *Anim Microbiome*. 2019;1(1):18.
17. Huang Y, Zhao L, Zhang Z, et al. Detection of a Novel *Rickettsia* From *Leptotrombidium scutellare* Mites (Acari: Trombiculidae) From Shandong of China. *J Med Entomol*. 2017;54(3):544-549.
18. Yu XJ, Tesh RB. The role of mites in the transmission and maintenance of Hantaan virus (*Hantavirus: Bunyaviridae*). *J Infect Dis*. 2014;210(11):1693-1699.
19. Gu XL SW, Zhou CM, Fang LZ, et al. SFTSV infection in rodents and their ectoparasitic chiggers. *PLoS Negl Trop Dis*. 2022;16(8):e0010698.
20. Linsuwanon P, Krairojananan P, Rodkvamtook W, et al. Surveillance for Scrub Typhus, Rickettsial Diseases, and Leptospirosis in US and Multinational Military Training Exercise Cobra Gold Sites in Thailand. *US Army Med Dep J*. 2018;(1-18):29-39.
21. Aplin KP, Jacob J, Krebs CJ, et al. Field methods for rodent studies in Asia and the Indo-Pacific. *Australian Centre for International Agricultural Research*. 2003.
22. Auffray BK, Bordes F, Chabe M, et al. Protocols for field and laboratory rodent studies. Bangkok (Thailand). *Kasetsart University Press*. 2011.
23. Francis C. A field guide to the mammals of South-East Asia (2nd Edition). *Bloomsbury Wildlife*. 2019.
24. Nadchatram M. A pictorial key to the subfamilies, genera and subgenera of Southeast Asian chiggers (Acari, Prostigmata, Trombiculidae). *Ins Med Res*. 1974.
25. Jiang J, Paris DH, Blacksell SD, et al. Diversity of the 47-kD HtrA nucleic acid and translated amino acid sequences from 17 recent human isolates of *Orientia*. *Vector Borne Zoonotic Dis*. 2013;13(6):367-375.
26. Blair PJ, Jiang J, Schoeler GB, et al. Characterization of spotted fever group rickettsiae in flea and tick specimens from northern Peru. *J Clin Microbiol.* 2004;42(11):4961-4967.
27. Fournier PE, Dumler JS, Greub G, et al. Gene sequence-based criteria for identification of new *Rickettsia* isolates and description of *Rickettsia heilongjiangensis* sp. nov. *J Clin Microbiol.* 2003;41(12):5456-5465.
28. Roux V, Raoult D. Phylogenetic analysis of members of the genus *Rickettsia* using the gene encoding the outer-membrane protein rOmpB (*omp*B). *Int J Syst Evol Microbiol.* 2000;50(4):1449-1455.
29. Jiang J, Blair PJ, Felices V, et al. Phylogenetic analysis of a novel molecular isolate of spotted fever group Rickettsiae from northern Peru: *Candidatus* Rickettsia andeanae. *Ann N Y Acad Sci.* 2005;1063:337-342.
30. Jiang J, Maina AN, Knobel DL, et al. Molecular detection of *Rickettsia felis* and *Candidatus* Rickettsia asemboensis in fleas from human habitats, Asembo, Kenya. *Vector Borne Zoonotic Dis.* 2013;13(8):550-558.
31. Kumlert R, Chaisiri K, Anantatat T, et al. Autofluorescence microscopy for paired-matched morphological and molecular identification of individual chigger mites (Acari: Trombiculidae), the vectors of scrub typhus. *PloS one*. 2018;13(3):e0193163.
32. Angelakis E, Mediannikov O, Parola P, et al. *Rickettsia felis*: The Complex Journey of an Emergent Human Pathogen. *Trends Parasitol.* 2016;32(7):554-564.
33. Maina AN, Klein TA, Kim HC, et al. Molecular characterization of novel mosquito-borne *Rickettsia* spp. from mosquitoes collected at the Demilitarized Zone of the Republic of Korea. *PloS One*. 2017;12(11):e0188327.
34. Zhang J, Lu G, Li J, et al. Molecular Detection of *Rickettsia felis* and *Rickettsia bellii* in Mosquitoes. *Vector Borne Zoonotic Dis*. 2019;19(11):802-809.
35. Brown LD, Macaluso KR. *Rickettsia felis*, an Emerging Flea-Borne Rickettsiosis. *Curr Trop Med Rep*. 2016;3:27-39.
36. Jiang J, Sangkasuwan V, Lerdthusnee K, et al. Human Infection with *Rickettsia honei*, Thailand. *Emerg Infect Dis*. 2005;11(9):1473-1475.
37. Gaywee J, Sunyakumthorn P, Rodkvamtook W, et al. Human infection with *Rickettsia* sp. related to *R. japonica*, Thailand. *Emerg Infect Dis*. 2007;13(4):671-673.
38. Edouard S, Bhengsri S, Dowell SF, et al. Two human cases of *Rickettsia felis* infection, Thailand. *Emerg Infect Dis*. 2014;20(10):1780-1781.
39. Assarasakorn S, Veir JK, Hawley JR, et al. Prevalence of *Bartonella* species, hemoplasmas, and *Rickettsia felis* DNA in blood and fleas of cats in Bangkok, Thailand. *Res Vet Sci.* 2012;93(3):1213-1216.
40. Doornbos K, Sumrandee C, Ruang-Areerate T, et al. *Rickettsia* sp. closely related to *Rickettsia raoultii* (Rickettsiales: Rickettsiaceae) in an *Amblyomma helvolum* (Acarina: Ixodidae) tick from a *Varanus salvator* (Squamata: Varanidae) in Thailand. *J Med Entomol*. 2013;50(1):217-220.
41. Ponnusamy L, Garshong R, McLean BS, et al. *Rickettsia felis* and other *Rickettsia* species in chigger mites collected from wild rodents in North Carolina, USA. *Microorganisms.* 2022;10(7):1342.
42. Kuo CC, Lee PL, Wang HC. Molecular identification of *Rickettsia* spp. in chigger mites in Taiwan. *Med Vet Entomol*. 2022;36(2):223-229.

43. Brown LD, Christofferson RC, Banajee KH, et al. Cofeeding intra- and interspecific transmission of an emerging insect-borne rickettsial pathogen. *Mol Ecol*. 2015;24(21):5475-5489.

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Control of adult *Culex quinquefasciatus* Mosquito Populations in Catch Basins in Houston, Texas using the ProVector® Military Camouflage Tube™ with an Attractant Toxic Sugar Bait incorporating *Bacillus thuringiensis israelensis* and Methoprene

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ABSTRACT

West Nile virus (WNV) is the principal mosquito-borne pathogen threat to public health in the United States (US). The southern house mosquito (*Culex quinquefasciatus* Say) is the primary vector of WNV throughout the southern US. Controlling adult *Cx. quinquefasciatus* within catch basin breeding sites using fogging can cause damage to the environment and is often ineffective. The development and testing of new pesticides and delivery methods are key to mitigating adaptation by vectors and is important in reducing environmental impact of pesticides. The ProVector® Military Camouflage Tube (MCT™) incorporating ProVector Entobac M™ (active ingredients, *Bacillus thuringiensis israelensi*s and methoprene) was tested to determine the effect on adult mosquito populations inhabiting catch basins in Houston, Texas. After an initial two-week increase, the population of *Cx. quinquefasciatus* was controlled in the test sites during the study period, whereas, the population was not controlled at the control site. Deployment of ProVector Military Camouflage Tubes with Entobac M in one-month and three-month formulations was effective in significantly reducing the population of adult *Cx. quinquefasciatus*. The ProVector MCT™ applicator provides housing for slow delivery of eco-friendly pesticides, target specificity, with low visibility to reduce the risk of detection of deployed military personnel. Since this study, a biodegradable, camouflaged, and thermally invisible device was developed to house ProVector pesticides.

INTRODUCTION

The southern house mosquito, *Culex quinquefasciatus* Say, is a vector of several viruses and parasites, including St. Louis encephalitis virus (SLEV) and West Nile virus (WNV), which is the most common mosquito-borne pathogen in the continental US, and Texas reported its highest number of WNV cases during the 2012 epidemic.[1] In 2014, Harris County and the City of Houston, Texas, reported their highest number of WNV human cases: 139 cases and two deaths.[2] *Culex quinquefasciatus* prefers to oviposit in water sources rich in organic materials, such as sewage overflow, containers with organic debris, septic tanks, and catch basins. It is the most collected mosquito species inhabiting catch basins in Houston, Texas.[3] There is an ongoing challenge for public health and integrated vector management (IVM) professionals to counter the development of pesticide resistance in mosquito populations. In order to reduce the risk of pesticide resistance developing in *Cx. quinquefasciatus*, the Harris County Public Health Mosquito and Vector Control Division (HCPH-MVCD) is actively engaged in implementing the best practice strategy of rotating pesticides.[4] The ProVector pesticide technology was tested as a viable method to be used in pesticide rotation for mosquito control operations that target catch basins in Houston.

Bacillus thuringiensis israelensis (*Bti*) has been effective in reducing mosquito larvae and it can also be effective in reducing adult mosquito populations when used with an attractive toxic sugar bait (ATSB). The susceptibility of adult *Cx. quinquefasciatus* to *Bti* in sucrose solutions was first described in 1984.[5] The adulticidal activity of ProVector® Entobac™ pesticide composed of *Bti* in a nectar-like Mosquito Attractant Bait (MAB™) against *Aedes aegypti* (L.) and *Anopheles dirus* (Peyton & Harris) mosquitoes was evaluated in Thailand.[6] The *Bti* treated mosquitoes died within three to seven days post treatment. The ProVector Flower pesticide applicators with Entobac were utilized in Kenya to successfully reduce mosquito populations of several medically important mosquito

Figure 1. Catch Basin Sites in Houston, Texas with photo of ProVector Military Camouflage Tube hanging below a manhole cover.

species, including *Cx. quinquefasciatus*.[7] The delayed lethality of *Bti* in adult mosquitoes may also create opportunities for auto-dissemination (indirect-transfer) of *Bti* to oviposition sites, reducing larvae. Another formulation of ProVector Entobac which incorporates methoprene to target adult and larval *Cx. quinquefasciatus* (ProVector Entobac M™) was developed and tested for efficacy against adult mosquitoes inhabiting catch basins in Houston, TX with the goal of reducing mosquito populations by affecting fecundity and morbidity of adults and development and morbidity in larvae.

MATERIALS AND METHODS

The HCPH-MVCD evaluated ProVector Military Camouflage Tube (MCT™) applicators with ProVector Entobac M in catch basins in Houston, TX to target underground breeding mosquitoes such as *Cx. quinquefasciatus*. Mosquitoes can detect the colors on the ProVector MCT applicator and are attracted to the MAB containing pesticides, where they come into contact and feed through the holes in the base of the tube. Three catch basin sites were randomly selected from the Mosquito Operation Control area #65 (Figure 1) for testing. Five manholes were randomly chosen for mosquito collection from each site. One CDC light trap with 0.5 kg dry ice was placed under each manhole cover from 1300 CST and mosquitoes were serviced the following morning at 0900 CST during each trap period. Pre-test and post-test samples were collected. At the conclusion of the pre-test sampling on 28 May 2021, two of the three sites were randomly chosen to receive treatments (Figure 1). At the conclusion of the pre-test period, no pesticide was applied to the negative control site; and 10 manhole covers on each storm drain received a ProVector MCT with a ProVector Entobac M pad at the two treatment sites. One treatment site received 10 ProVector MCTs each with an Entobac M 1-Month Pad (E1M) with 2g Entobac M, and the other treatment site received 10 ProVector MCTs each with an Entobac M 3-Month Pad (E3M) with 12g Entobac M. Entobac M active ingredients are 7% *Bti* and 0.7% methoprene within an attractant toxic sugar bait, Mosquito Attractant Bait (MAB™). After ProVector MCT placement, mosquitoes were sampled every two weeks from 18 June to 28 August 2019, for a total of six sampling days during each treatment period. An additional sampling was conducted on September 12, post removal of the ProVector MCTs, to determine residual effect in the catch basins. The collected mosquitoes were stored in -80° freezer until enumeration and identification to species using Centers for Disease Control and Prevention pictorial keys.[8] The treatment sites were monitored once per week for potential removal or possibility of being washed away by flooding after heavy rains. During the study, 10 of the deployed tubes were either damaged or lost but were immediately replaced with new tubes and baits. Analysis of variance (ANOVA), with Fishers least significance (LSD) test, was used to analyze biweekly collection data using Statistica v13.3 (Tibco, Inc.).

RESULTS AND DISCUSSION

Analysis of the mosquito data collected from the catch basins revealed the dominance of *Cx. quinquefasciatus,* a primary mosquito vector of WNV and SLEV in Harris County and Houston. A total of 3,253 mosquitoes were collected and identified to species; nearly 98.5% (3,204) were *Cx. quinquefasciatus*. The remaining 1.5% included eight *Cx. nigripalpus* (Theobold), 31 *Ae. aegypti*, 12 *Ae. albopictus* (Skuse) and four *Ae. vexans* (Meigen). Although the

Figure 2. Percent of mean populations of *Cx. quinquefasciatus* within study sites compared to those from a long-term study in Houston conducted by *Dennett et al.3 which showed the yearly population pattern in Houston, TX (Entobac 1-Month Pad, E1M; Entobac 3-Month Pad, E3M).

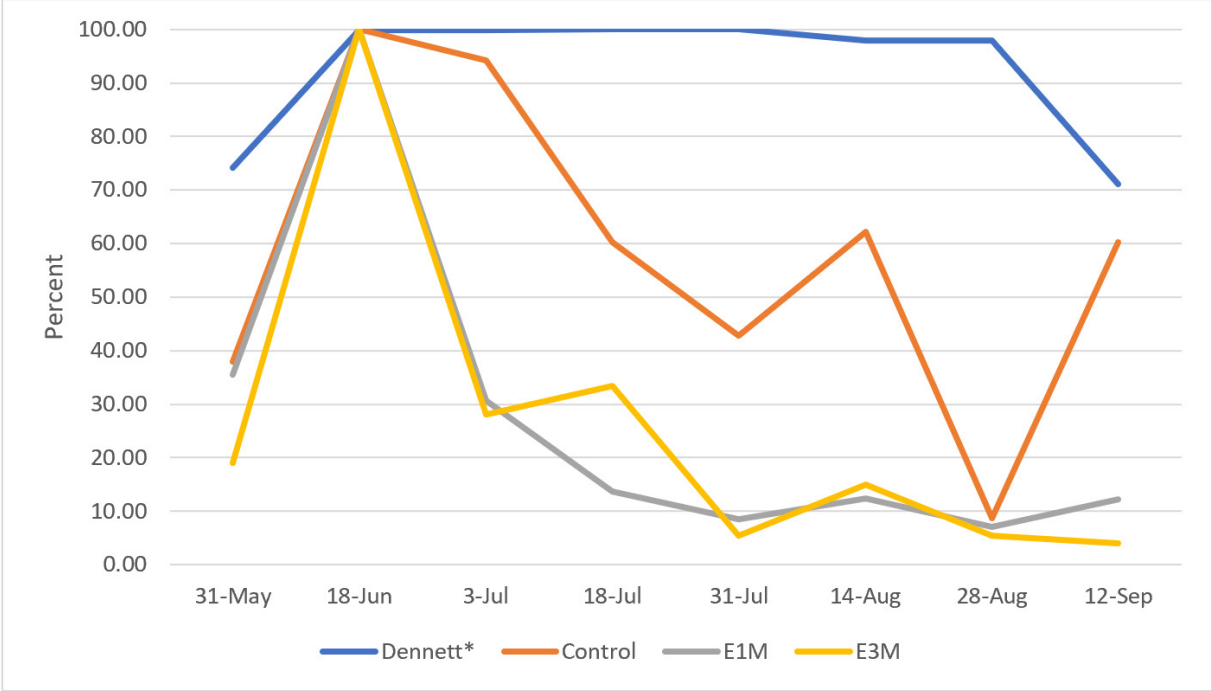

Table 1. Comparison of mean number of *Cx. quinquefasciatus* captured at the control and treatment sites with the ProVector Military Camouflage Tubes with Entobac M 1-Month pads and 3-Month pads, includes standard error below the mean.

Site	Pre-Sample	2wks	4wks	6wks	8wks	12wks	16wks	18wks
Control	7.8 4.5	20.6 4.5	19.4 4.5	12.4 4.5	8.8 4.5	12.8 4.5	1.8* 4.5	12.4 4.5
1MonthPad	69.6 52.9	196.0 52.9	60.0 52.9	26.8* 52.9	16.6* 52.9	24.2* 52.9	14.0* 52.9	24.0* 52.9
3MonthPad	10.5* 12.5	55.0 11.9	15.4* 11.9	18.4* 11.9	3.0* 11.9	8.2* 11.9	3.0* 11.9	2.2* 11.9

*Denotes significance at $p \leq 0.05$ between periods and week 2 using ANOVA with Fisher's LSD test, MS=1309.7, df=457.00). Control 4.5, 1-Month Pad 52.93, 3-Month Pad 12.51, and 11.19 post treatment measured at 18 weeks.

numbers collected were low, the findings of *Ae. aegypti* and *Ae. albopictus* from catch basins were noteworthy considering these species normally inhabit above-ground container habitats. The local population of *Cx. quinquefasciatus* inhabiting catch basins in Houston is usually over 95%.[9]

Culex quinquefasciatus populations normally increase rapidly after May and remain at a high level for the rest of the summer until November.[3] The number of mosquitoes initially increased two weeks after the ProVector MCTs were deployed, and then declined rapidly and stayed lower than the initial increase. Five of the subsequent bi-weekly population means were significantly lower than the week two means at the ProVector MCT with EM1 Pad site, six were significantly lower at the ProVector MCT with EM3 Pad site, and one was lower at the control site (Table 1). The number of months of reduced *Cx. quinquefasciatus* after the initial two-week increase was significantly lower in the E1M Pad site than the Control site (Chi-square=5.33, $p \leq 0.05$) and lower in the E3M Pad vs Control (Chi-square=8.57, $p \leq 0.05$). There was not a significant difference in the number of reduced months between the E1M Pad and E3M Pad sites (Chi-square=0.92, $p \geq 0.05$)

Historical data from Dennett et al.[3] was used as a historical comparison, using the four years of data, to determine whether the mean *Cx. quinquefasciatus* was historically high or low in Houston during the 2019 test period. Using the historical data and the data from this study, the *Cx. quinquefasciatus* population rapidly increased after May. The mean percent of the total *Cx. quinquefasciatus* population for each site during the 8-week trap period was determined by dividing the mean of the highest capture number, occurring during the second period where rapid population increase occurs, and plotted against the mean percent of captures each month from the multi-year captures (Figure 2). The mean percent of the total population of the Control (58.3, sd 29.7), E1M Pad (27.5, sd 31.1), and E3M Pad (26.3, sd 31.7) sites were significantly lower than the historical mean percent (92.6, sd 10.5) from Dennett et al.[3], (MS=760.95, df 28.0, $p \leq 0.05$). The mean percent of the total population of the Control site was significantly higher than that of the E1M Pad and E3M Pad sites during the study period, ($p \leq 0.05$). The mean percent of the E1M Pad and E3M Pad sites were not significantly different during the study period ($p \leq 0.05$).

The ProVector Military Camouflage Tube with Entobac M had a significant impact on the *Cx. quinquefasciatus* population during the 18th week, even after ProVector MCTs were removed at the end of the 16th week (Table 1). Having a method to target *Cx. quinquefasciatus* within their breeding sites should prove effective in reducing populations and WNV transmission because approximately 90% of *Cx. quinquefasciatus* females stay within 3km of their larval habitat.[10] Thermal fogging pesticides in catch basins in Houston was ineffective in controlling *Cx. quinquefasciatus* and the method was discontinued in 2010.[9] In contrast with the abandoned adulticides used historically, both the E1M and E3M pads were effective in reducing *Cx. quinquefasciatus* populations during the study period, with the E3M pad having the added benefit of only needing placement at three-month intervals. There was also a measured effect in reduction after removal of the ProVector MCT from the catch basins (Table 1). The ProVector MCT with Entobac M provided control of *Cx. quinquefasciatus* in catch basins and is an additional tool for IVM with the additional benefit of being environmentally friendly.

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REFERENCES

1. Rosenberg R, Lindsey NP, Fischer M, Gregory CJ, Hinckley AF, Mead PS, Paz-Bailey G, Waterman SH, Drexler N, Kersh GJ, Hooks H, Partridge SK, Visser SN, Beard CB, Petersen LR. Vital Signs: Trends in reported vector-borne disease cases - United States and Territories, 2004–2016. *Morb Moral Wkly Rep.* 2018; 67(17):496-501.
2. Martinez D, Murray KO, Reyna M, Arafat RR, Gorena R, Shah UA, Debboun M. West Nile virus outbreak in Houston and Harris County, Texas, USA, 2014. *Emerg Infect Dis.* 2017; 23(8):1372-1376.
3. Dennett JA, Bala A, Wuithiranyagool T, Randle Y, Sargent CB, Guzman H, Siirin M, Hassan HK, Reyna-Nava M, Unnasch TR, Tesh RB, Parsons RE, Bueno R Jr. Associations between two mosquito populations and West Nile virus in Harris County, Texas 2003-2006. *J Am Mosq Control Assoc.* 2007; 23(3): 64-75.
4. Stark PM, Fredregill CL, Nolan MS, Debboun M. Field cage insecticide resistance tests against *Culex quinquefasciatus* Say (Diptera: Culicidae) in Harris County, Texas, U.S.A. *J Vect Ecol.* 2017; 42(2):279-288.
5. Klowden MJ, Bulla LA. Oral toxicity of *Bacillus thuringiensis* subsp. *israelensis* to adult mosquitoes. *J Vect Ecol.* 1984; 30(3):155-162.
6. Alongkot P, Jaichapor B, Kollars TM. Susceptibility of adult *Aedes aegypti* and *Anopheles dirus* to bait containing *Bacillus thuringiensis israelensis*. *IOSRJ. Pharma* 2016; 6(2):1-3.
7. Yalwala, S, Kollars JW, Kasembeli G, Chrisostim BW, Senessie C, Kollars PG, Kollars TM. Preliminary report on the reduction of adult mosquitoes in housing compounds in western Kenya, using Entobac biopesticide and the ProVector Flower. *J Med Ent.* 2016; 83(5): 1252-1254.
8. Centers for Disease Control and Prevention. Pictorial keys to arthropods, reptiles, birds and mammals of public health significance. www.cdc.gov/nceh/ehs/docs/pictorial_keys; 1966.
9. Fredregill CL, Motl GC, Dennett, JA Flat KL, Bueno RJr. Thermal fog efficacy tests against mosquitoes in storm drains in Harris County, Texas 2008–09. *J Am Mosq Control Assoc.* 2011; 27(1): 61-68.
10. Hamer GL, Anderson TK, Donovan DJ, Brawn JD, Krebs BL, Gardner AM, Ruiz MO, Brown WM, Kitron UD, Newman CM, Goldberg TL, Walker ED. Dispersal of adult *Culex* mosquitoes in an urban West Nile virus hotspot: a mark-capture study incorporating stable isotope enrichment of natural larval habitats. *PLoS Negl Trop Dis.* 2014; 8(3):e2768.

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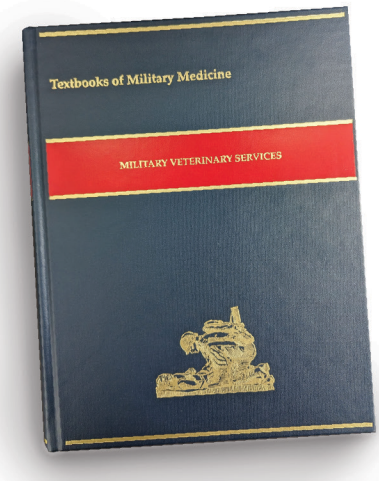

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Vivax Malaria among US Military Personnel and Civilians Attributed to Exposure in the Republic of Korea, 2006-2020

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ABSTRACT

Vivax malaria cases have declined from a high of >4,000 cases (2004) among Republic of Korea (ROK) military/civilian populations and >55 cases (1999) among US military personnel assigned to the ROK to about 500 cases (2012-2020) and 1-14 cases (2009-2020), respectively. However, *vivax* malaria continues to be a major health threat to both military and civilian communities in the ROK as demonstrated by an outbreak in 2015 that resulted in 11 malaria cases over three days. United States (US) military personnel diagnosed with malaria were reported in the Department of Defense (DoD) Disease Reportable Systems internet (DRSi), 65th Medical Brigade, and subsequently interviewed to determine the most likely site of infection, preventive medicine measures that were implemented, and reporting and diagnosis of *vivax* malaria. A total of 93/127 US Army (87) and Korean Augmentation to the US Army (KATUSA) (4) Soldiers and US civilians (2) were interviewed, while 32 US Army Soldiers were not interviewed. Nearly all malaria infections (95.6%; 87) among US Army and KATUSA Soldiers and US civilians interviewed were attributed to exposure at US/ROK operated training sites and installations located at/near the demilitarized zone (DMZ). One malaria case was most likely attributed to exposure at Humphreys US Army Garrison (south of Seoul), while it could not be determined for 3 (3.3%) US Army Soldiers. Only 61.5% of the US Army and KATUSA Soldiers stated that they used insect repellent. Except for one respondent, all reported typical malaria paroxysms of chills, followed by high fever, intense sweating, and mild-severe headaches and myalgia, while lower numbers reported nausea, vomiting, and diarrhea. The mean number of days from the onset of symptoms to reporting at a health clinic was 3.7 days, while the mean number of medical visits prior to diagnosis was 2.1 days. Most US Army and KATUSA Soldiers were treated with chloroquine (65.6%), while 18.3%, 14.0%, and 2.2% were treated with hydroxychloroquine, Malarone, and other (mefloquine and Artemether-lumefantrine) antimalarial drugs. The majority of US Army and KATUSA Soldiers were treated with 15 mg primaquine base x 14 days (51, 54.8%), while 42 (45.2%) were treated with 30 mg primaquine base x 14 days. Among the 91 US Army and KATUSA Soldiers interviewed, two US Army Soldiers relapsed due to low dosage of primaquine (15 mg base x 14 days). *Vivax* malaria continues to pose a significant health threat to US and Korean Forces in the ROK. Vigilance, malaria prevention, vector control, use of insect repellent and early treatment are paramount to reduce human-mosquito-human transmission.

INTRODUCTION

While malaria-like illnesses were recorded as early as the 12th century in Korea, with outbreaks reported in 1152 and 1283, *vivax* malaria was not officially documented in Korea until 1886.[1-3] *Vivax* malaria in Korea and northern Asia was designated as "temperate zone" malaria due to latent liver stage parasites (hypnozoites) that emerged 6-18+ months after infection.[4,5] Due to collaborative efforts between the World Health Organization (WHO) and the Republic of Korea (ROK) in 1979, the WHO designated the ROK as malaria free.[6,7] Despite the introduction of imported malaria by Korean travelers returning from malaria endemic areas following its eradication in 1979, only one case of autochthonous transmission was reported in 1981.[8] However, in & H1993, a ROK Army Soldier deployed near the demilitarized zone (DMZ) who had no related travel history outside of the ROK was diagnosed with *vivax* malaria.[9,10] Subsequently, the number of malaria cases rapidly increased to 4,142 cases by 2000, and while it was hypothesized that it would spread throughout the ROK, reported cases remained localized near the DMZ.[11,12] *Vivax* malaria cases declined following 2000, but continued to pose a significant health threat to both ROK military, Korean civilian populations, and US personnel deployed to the ROK. The purpose of this review is to provide a summary of malaria among the United States Forces Korea (USFK) personnel attributed to exposure in the ROK and malaria prevention measures, diagnosis, treatment, and continued health threat risks.

METHODS

Base Camp and Training Activities

US military personnel, family members, and civilians deployed to the ROK reside at US military installations and surrounding towns/cities throughout the ROK. Unaccompanied and accompanied military personnel deploy for one or two years, while civilians are assigned for one to three years, but both groups may request extensions. Arrivals are through Osan Air Base, Pyeongtaek-si (city) or Incheon International Airport, after which they travel to their respective units where they in-process.

Military units often conduct training for periods of a few days to several weeks at US/ROK operated training areas within 3-20 km south of the DMZ where *vivax* malaria risks are greatest. Training areas range in size from <1 km^2, e.g., Local Training Areas (LTA) to >20 km^2, e.g., Twin Bridges Training Area (TBTA), and are often partially bordered by water sources, e.g., rivers, streams, rice paddies, and/or low-lying grassy areas, e.g., Dagmar North Training Area (DNTA) that partially floods during the rainy season. Many LTAs are not secured (fenced), while most primary modernized training sites are secure to prevent unauthorized entry. However, civilians occasionally enter training areas to fish along streams/ponds (e.g., DNTA), while other training areas are proximal or interspersed among small villages or farming communities consisting of wetland (rice) cultivation, other crops, and dairy/beef farming (e.g., TBTA). Training areas vary from modernized, e.g., Rodriguez Live Fire Complex, (RLFC) to unimproved training areas interspersed with dirt trails and dirt/gravel roads, e.g., DNTA and TBTA.

Malaria Case Definition and Detection

Vivax malaria is characterized by periodic paroxysms that includes rapid onset of chills (shakes), followed by a high temperature (> 39.4°C), and then intense sweats that may last for 2-6 hours (h), followed by a period of malaise until the next paroxysm.[13] Initially, *vivax* malaria paroxysms occur at intervals of 24-48 h, but as the malaria parasites become synchronized, paroxysms occur at 48-h intervals (tertian malaria). Mild to severe headaches and myalgia (muscle aches) are commonly reported and may occur prior to and continue after the paroxysms, while nausea, vomiting, and diarrhea are reported to a lesser degree. Thrombocytopenia (*Plasmodium vivax* invades reticulocytes, 1-2% of the red blood cells) and splenomegaly are characteristic, especially in long-term infections.

Febrile patients suspected of having malaria in Korea, e.g., periodic paroxysms, were admitted to US military medical clinics/hospitals or local hospitals where blood was drawn. Similarly, malaria patients suspected of having malaria in the US were admitted to military medical clinics/hospitals, or occasionally to civilian hospitals when on leave. Clinical diagnosis of *Plasmodium* spp. was accomplished using a malaria antigen test that distinguishes *P. falciparum* from non-*falciparum* infections, examination of blood films for malaria parasites (species not determined), using species-specific polymerase chain reaction (PCR) tests, or a combination as described by Klein et al.[14]

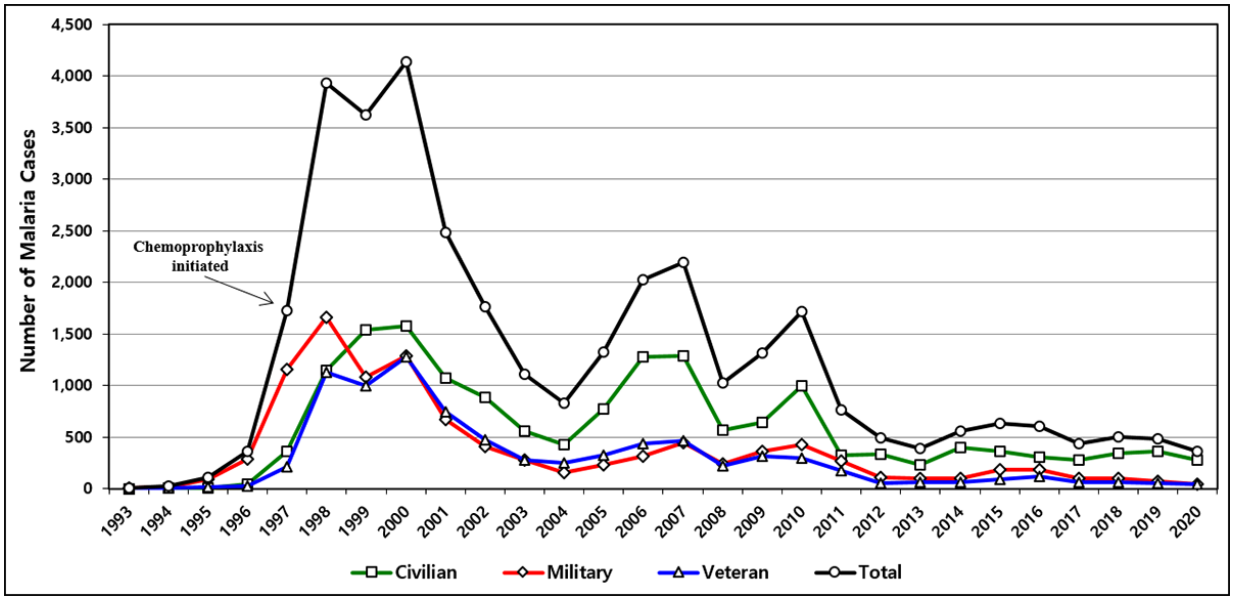
Vivax Malaria Case Reporting and Epidemiological Investigations for US Service Members and Civilians

US personnel diagnosed with malaria attributed to exposure in the ROK from 2006-2020 were reported to the Chief, Public Health Nurse (PHN), Force Health Protection and Preventive Medicine (FHP&PM), 65th Medical Brigade, through the DRSi and the Armed Forces Health Surveillance Division (AFHSD) as described by Klein et al.[14] Korean Augmentation to the US Army (KATUSA) Soldiers diagnosed with malaria in the ROK were reported to the PHN, FHP&PM. Malaria patients were interviewed to determine the most likely site of infection and asked a series of questions related to training activities and exposure, e.g., base camp location, dates and conditions of training in field environments, use of insect repellents, implemented preventive medicine measures (PMM), perceived numbers of mosquitoes present and bites received, and other malaria related questions. When possible, telephonic and face-to-face patient interviews were conducted and medical records screened to determine the onset of symptoms, first and subsequent admission to Aid Stations/medical clinics/centers, method of diagnosis, number of days to diagnosis, patient symptoms, and other related factors in accordance with USFK Reg 40-2.[15] Except for years 2006-2007, telephonic interviews were conducted for US military personnel diagnosed with non-*falciparum* malaria at medical clinics and civilian hospitals in the US for malaria cases that were suspected to be attributed to exposure in the ROK. Written epidemiological reports were submitted to the Commanders, 65th Medical Brigade and Brian D. Allgood Army Community Hospital (BDAACH), USFK, Eighth Army, and 2nd Infantry Division Surgeons, Chief, FHP&PM, and others as appropriate.

Korea Disease Control and Prevention Agency (KDCA) Disease Surveillance and Prevention Malaria Case Reporting for ROK Civilian, Military, and Veteran Populations

The KDCA at Osong, ROK, provided data on the number of malaria cases among Korean civilians, veterans (ROK service members discharged from the Army ≤2 years), and ROK active duty military service members.[16] The annual number of ROK Army Soldiers placed on malaria chemoprophylaxis was provided by the KDCA and the ROK Ministry of National Defense.[16,17] ROK Army Soldiers assigned to designated malaria high-risk areas were administered: (1) 400 mg hydroxychloroquine weekly during the malaria season followed by terminal prophylaxis with primaquine

Figure 1. Number of malaria cases reported by the Korea Disease Control and Prevention Agency (KDCA) for ROK military, veterans (discharged <2 yrs), and civilians from 1993-2020.



(15 mg base x 14 days) at the end of the malaria season, or (2) only administered primaquine (15 mg base x 14 days) at the end of the malaria season (Supplemental Fig. 1).

Results

Annual Prevalence of *Vivax* Malaria in ROK Civilian, Military, and Veteran Populations

The KDCA reported 34,890 autochthonous *vivax* malaria cases among ROK civilians (16,357), veterans (8,194), and military (10,339) populations, and an additional 1,356 imported cases from 1993-2020 (Fig. 1).[16] Following the rapid increase in the number of malaria cases from one case in 1993 to 4,142 cases in 2000, the annual number of malaria cases decreased to a low of 826 by 2004. Subsequent variations in annual numbers (high 2,192 cases in 2007; low 356 in 2020) of malaria cases were attributed to environmental factors, e.g., rainfall patterns and flooding of low-lying areas with abundant vegetation, and the chemoprophylaxis program established for selected ROK units residing in designated malaria high-risk areas.[12,17]

Vivax Malaria in US Personnel Attributed to Exposure in the ROK

The following information is based on reports from the DRSi, patient medical records, and interviews of US Army and KATUSA Soldiers and US civilians diagnosed with vivax malaria attributed to exposure in the ROK from 2006-2020 (Fig. 2). Vivax malaria cases among US military personnel who trained near the DMZ were similar to those reported for ROK populations from 1993-2007. No vivax malaria cases were reported among USFK personnel during 2008, and the following years, as the number of annual cases ranged from 1-13 (Table 1).

US Army personnel accounted for 94.9% (119/125) of vivax malaria cases attributed to exposure in the ROK from 2006-2020 (reported through the DRSi or Public Health Nurses, FHP&PM, 65th Medical Brigade), followed by KATUSA Soldiers (3.2%, 4), and US civilians (1.6%, 2) (Table 1). Recrudescent cases or true relapse, defined as infections due to liver stage hypnozoites after the clearance of blood stage parasites were reported for two patients in 2006 and 2016, two and 10 months after treatment for both blood and liver stage parasites. Both patients were initially treated with 15 mg primaquine base x 14 days for liver stage parasites, instead of 30 mg recommended by US Centers for disease Control and Prevention (CDC).[18] Following the relapse and treatment with chloroquine, one Soldier was treated with 15 mg primaquine base x 14 days, while the other was treated with 30 mg x 14 days. Neither US Soldier relapsed a second time.

Most of the US Army vivax malaria patients (29) who were not interviewed, were diagnosed with malaria in the US or other countries where they were deployed following their departure from the ROK in 2006 (7/24; 29.2%) and 2007 (22/33; 66.7%). Nearly all (93.5%, 87) US Army Soldiers (94.3%, 82), KATUSA Soldiers (100%, 4), and US civilians (50.0%, 1) who were interviewed were male (Table 1). Most were Caucasian (75.3%, 70), followed by Asian (9.7%, 9), Hispanic (8.6%, 8), Pacific Islander (3.2%, 3), Black (2.2%, 2), and American Indian (1.1%, 1), while it was not determined for 32 (25.6%) of US Army Soldiers (Table 1). In addition, there were 9 imported malaria cases diagnosed in the ROK that were from South America (Honduras, US Soto Cano military base, 1), Africa (Nigeria, 4; Ghana, 2; Kenya, 1), and Southwest Asia (Afghanistan, 1) (Supplemental Table 1). For the US Army and KATUSA malaria patients interviewed, the majority of the cases were

Table 1. Summary of annual number of *vivax* malaria patients among US Army and KATUSA Soldiers and US civilians, including military and civilian status, gender, and race, that were attributed to exposure in the Republic of Korea from 2006-2020.

Year	Number of Malaria Cases					Gender		Race[6]						
	US Army[1]	KATUSA[2]	Civilian[3]	N/D[4]	Total[5]	Male	Female	Caucasian	Hispanic	Black	Asian	Pacific Islands	American Indian	Total
2006	17	0	0	7	24	17	0	13	2	0	2	0	0	17
2007	11	1	1	22	35	11	2	11	0	0	2	0	0	13
2008	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2009	1	1	0	1	3	2	0	0	0	0	2	0	0	2
2010	4	0	0	1	5	4	0	3	0	0	0	0	1	4
2011	1	0	0	0	1	1	0	1	0	0	0	0	0	1
2012	1	0	0	1	2	1	0	1	0	0	0	0	0	1
2013	3	0	0	0	3	3	0	3	0	0	0	0	0	3
2014	11	2	0	0	13	13	0	9	2	0	2	0	0	13
2015	6	0	0	0	6	6	0	4	1	0	0	1	0	6
2016	12	0	1	0	13	10	3	8	1	2	1	1	0	13
2017	4	0	0	0	4	4	0	3	0	0	0	1	0	4
2018	9	0	0	0	9	9	0	8	1	0	0	0	0	9
2019	1	0	0	0	1	1	0	1	0	0	0	0	0	1
2020	6	0	0	0	6	5	1	5	1	0	0	0	0	6
Total (%)	87 (69.6)	4 (3.2)	2 (1.6)	32 (25.6)	125	87 (93.5)	6 (6.5)	70 (75.3)	8 (8.6)	2 (2.2)	9 (9.7)	3 (3.2)	1 (1.1)	93

Total number of US Army personnel interviewed (87) and not interviewed (32), excluding 2 *vivax* malaria relapse cases.

[2] KATUSA Soldiers = Korean Augmentation to the US Army Soldiers; KATUSA Soldiers discharged from the ROK Army, but may have developed malaria after departing the military not included: KATUSA Soldiers diagnosed with malaria in 2009 were attributed to exposure during 2008. KSC = Korean Service Corp working at Warrior Base (1) not included.

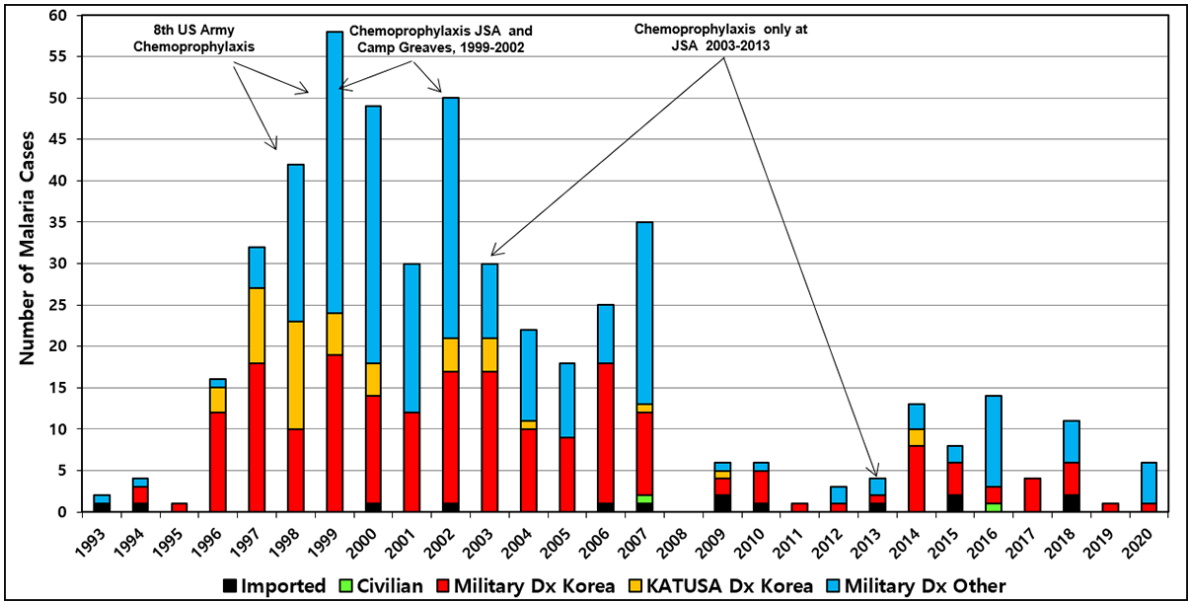
[3] Civilians = USFK employee (1, 2007) and family member (1, 2016).

[4] N/D = Not determined for US Army Soldiers who were not interviewed.

[5] Does not include two (2) *vivax* malaria cases among US Army personnel who relapsed 2 and 10 months following the primary attack.

[6] Only reported for US Army Soldiers, KATUSA Soldiers, and US civilians who were interviewed.

Figure 2. Number of malaria cases attributed to exposure in the Republic of Korea (ROK). Malaria cases were diagnosed among US Army and KATUSA Soldiers and civilians while deployed/assigned to the ROK and up to 18 months after returning to the US or other areas of assignment. JSA = Camp Bonifas adjacent to the DMZ.



most likely attributed to exposure at DNTA (41.8%), followed by Warrior Base (25.3%), Camp Bonifas (12.1%), RLFC (9.9%), New Mexico Range (2.2%), and Joint Camps Casey/Hovey (2.2%) (Supplemental Table 2, Fig. 3). In addition, 1 malaria case each was attributed to exposure at LTA 130 (1.1%), TBTA (1.1%), Monkey 7 TA (1.1%), Humphreys US Army Garrison (USAG) (1.1%), and Incheon (1.1%). Two malaria cases among US civilians, one US employee and one family member were most likely acquired at Paju-si (city) near Warrior Base and Dongducheon-si adjacent to Joint Camp Casey/Hovey. Before 2008, most malaria cases were attributed to exposure at Warrior Base, a malaria high-risk area, in part due to ill kept tents that did not preclude the entry of mosquitoes during the evening hours. However, from 2008-2020, Soldiers were housed in air-conditioned barracks that greatly reduced exposure to biting mosquitoes. A combination of factors led to increased and variable number of malaria cases reported from 2014-2020, e.g., an outbreak of 11 malaria cases over a 3-day period at DNTA.[14]

Preventive Medicine Measures (PMM)

US Army (87) and KATUSA (4) Soldiers were interviewed to determine PMM that were instituted to reduce exposure to biting mosquitoes and transmission of malaria (Table 2). Only 9.9% (9/91) remembered receiving a medical threat brief prior to traveling to Korea, shortly after their arrival, or prior to conducting training exercises, while 23.1% (21) and 67.0% (61) stated that they did not receive a medical threat brief or could not remember receiving one.

US Army soldiers diagnosed with malaria who resided at Camp Bonifas located in the Joint Security Area (JSA) near Panmunjeom and Joint Camp Casey/Hovey slept in barracks during the evening hours (Table 2). Similarly, nearly all military personnel who trained at the Warrior Base range complex and RLFC slept in barracks that greatly reduced exposure to biting mosquitoes. However, malaria patients stated that while they slept in barracks, they often went outdoors at night in their Army Physical Fitness Uniform (APFU) when going outdoors to the restroom, smoking and talking with friends, or making telephone calls without using insect repellents when mosquitoes were present. Military personnel who trained at other training sites near the DMZ, e.g., DNTA and TBTA, slept in tents (33.0%) in their APFUs (often without secure screens), outdoors in their sleeping bags/ponchos or on the ground/cots without screened nets (8.8%). Military personnel who slept in/on tracked and wheeled vehicles (25.3%) (e.g., operators and crewmembers of tracked Abrams tanks, Bradley Fighting Vehicles, and Light Medium Tactical Vehicles (LTMVs) slept in their military uniforms to maintain security and readiness, but often removed their boots (Table 2). Although it was often hot and humid during training exercises during the summer months, military personnel reported that they protected themselves from biting mosquitoes by using their sleeping bags, ponchos, or other covers, often without applying repellent on exposed skin areas.

Most military personnel used battle dress uniforms (BDUs), which transitioned to all climate uniforms (ACUs) and operational camouflage pattern (OCPs) uniforms or fire retardant uniforms during duty hours or while training in field environments. Only 44.0% of the US Army and KATUSA Soldiers indicated that they had self-permethrin-treated uniforms [applied using space sprays or the Army Individual Dynamic Absorption (IDA) kits] or factory-permethrin-treated uniforms, while 56.0% indicated that their uniforms were not treated, or they were not aware if their uniforms were treated

Table 2. Personal protective measures taken by US Army (87) and KATUSA Soldiers (4) diagnosed with *vivax* malaria attributed to exposure in the Republic of Korea from 2006-2020.

Year	No. Military Respondants[1]	No. (%) Sleeping Conditions[2]				No. (%) Uniforms-Treated[3]		Personal Protective Measures				Mosquitoes[8]	
		Barracks	Tent	Vehicles (tracked/ wheeled)	Ground/ Cots	Treated w/ Permethrin	Untreated/ Unknown	No. (%) Repellent Usage[4]	Unit (%) Provided Repellent[5]	No. (%) Uniform Proper Wear[6]	No. (%) Pro-phylaxis[7]	Present Range (Mean)	Bites Range (Mean)
2006	17	7 (41.2)	10 (58.8)	0	0	2 (11.8)	15 (88.2)	10 (58.8)	2 (11.8)	6 (35.3)	1* (5.9)	2-5 (4.2)	0-5 (3.4)
2007	12	2 (1.7)	8 (66.7)	2 (1.7)	0	3 (25.0)	9 (75.0)	10 (83.3)	4 (33.3)	4 (33.3)	1* (8.3)	4-5 (4.7)	1-5 (3.3)
2008	0	0	0	0	0	0	0	0	0	0	0	0	0
2009	2	1 (50.0)	1 (50.0)	0	0	2 (100)	0	2 (100)	0	2 (100)	0	0-4 (2.0)	0-2 (1.0)
2010	4	1 (25.0)	1 (25.0)	2 (50.0)	0	2 (50.0)	2 (50.0)	1 (25.0)	0	4 (100)	0	1-5 (1.8)	0-5 (1.5)
2011	1	1 (100)	0	0	0	1 (100)	0	0	0	0	1* (100)	5 (5.0)	3 (3.0)
2012	1	1 (100)	0	0	0	0	1 (100)	1 (100)	1 (100)	0	0	1 (1.0)	3 (3.0)
2013	3	0	0	3 (100)	0	0	3 (100)	3 (100)	1 (33.3)	0	0	5 (5.0)	4-5 (4.7)
2014	13	7 (53.8)	0	3 (23.1)	3 (23.1)	7 (53.8)	6 (46.2)	7 (53.8)	3 (23.1)	10 (76.9)	1 (7.7)	2-5 (3.8)	0-5 (3.0)
2015	6	2 (33.3)	1 (16.7)	3 (50.0)	0	2 (33.3)	4 (66.7)	3 (50.0)	1 (16.7)	2 (33.3)	0	3-5 (4.7)	2-5 (3.2)
2016	12	2 (16.7)	4 (33.3)	6 (50.0)	0	3 (25.0)	9 (75.0)	6 (50.0)	0	12 (100)	0	0-5 (4.4)	0-5 (4.0)
2017	4	2 (50.0)	1 (25.0)	0	1 (25.0)	2 (50.0)	2 (50.0)	3 (75.0)	3 (75.0)	3 (75.00)	0	2-5 (3.0)	0-5 (1.5)
2018	9	3 (33.3)	2 (22.2)	1 (11.1)	3 (33.3)	9 (100)	0	6 (66.7)	1 (11.1)	7 (77.8)	0	0-5 (3.7)	0-5 (2.9)
2019	1	1 (100)	0	0	0	1 (100)	0	0	0	0	0	5 (5.0)	3 (3.0)
2020	6	0	2 (33.3)	3 (50.0)	1 (16.7)	6 (100)	0	4 (66.7)	0	1 (16.7)	0	3-5 (3.8)	1-4 (2.8)
Total (%)	91	30 (33.0)	30 (33.0)	23 (25.3)	8 (8.8)	40 (44.0)	51 (56.0)	56 (61.5)	16 (17.6)	51 (56.0)	4 (4.4)	0-5 (4.0)	0-5 (3.1)

1 Number of US Army and KATUSA malaria patients interviewed who were attributed to exposure in the Republic of Korea.
2 Number (%) of malaria patients interviewed who slept during the evening hours in barracks/buildings, tents (often not secured), on/in tracked and wheeled vehicles, or outdoors on the ground or cots.
3 Uniforms treated with permethrin uniforms, e.g., battle dress uniforms (BDU), all climate uniforms (ACU), operational camouflage pattern (OCP), and fire-retardant uniforms worn by tracked vehicle operators and crew.
4 Number of US and KATUSA Soldiers who used arthropod repellents while training in the field when mosquitoes were present.
5 Number of military units that provided arthropod repellents to Soldiers while conducting field exercises when mosquitoes were present.
6 Number of Soldiers who wore sleeves down and trouser legs tucked into boots; many wore the Army Physical Fitness Uniforms (APFU) during the evening.
7 Number of US Soldiers placed on chemoprophylaxis at Camp Bonifas.
8 On a scale of 0 (none) to 5 (too many to count)

.Table 3. Symptoms reported for US Army *vivax* malaria patients attributed to exposure in the Republic of Korea from 2006-2020.

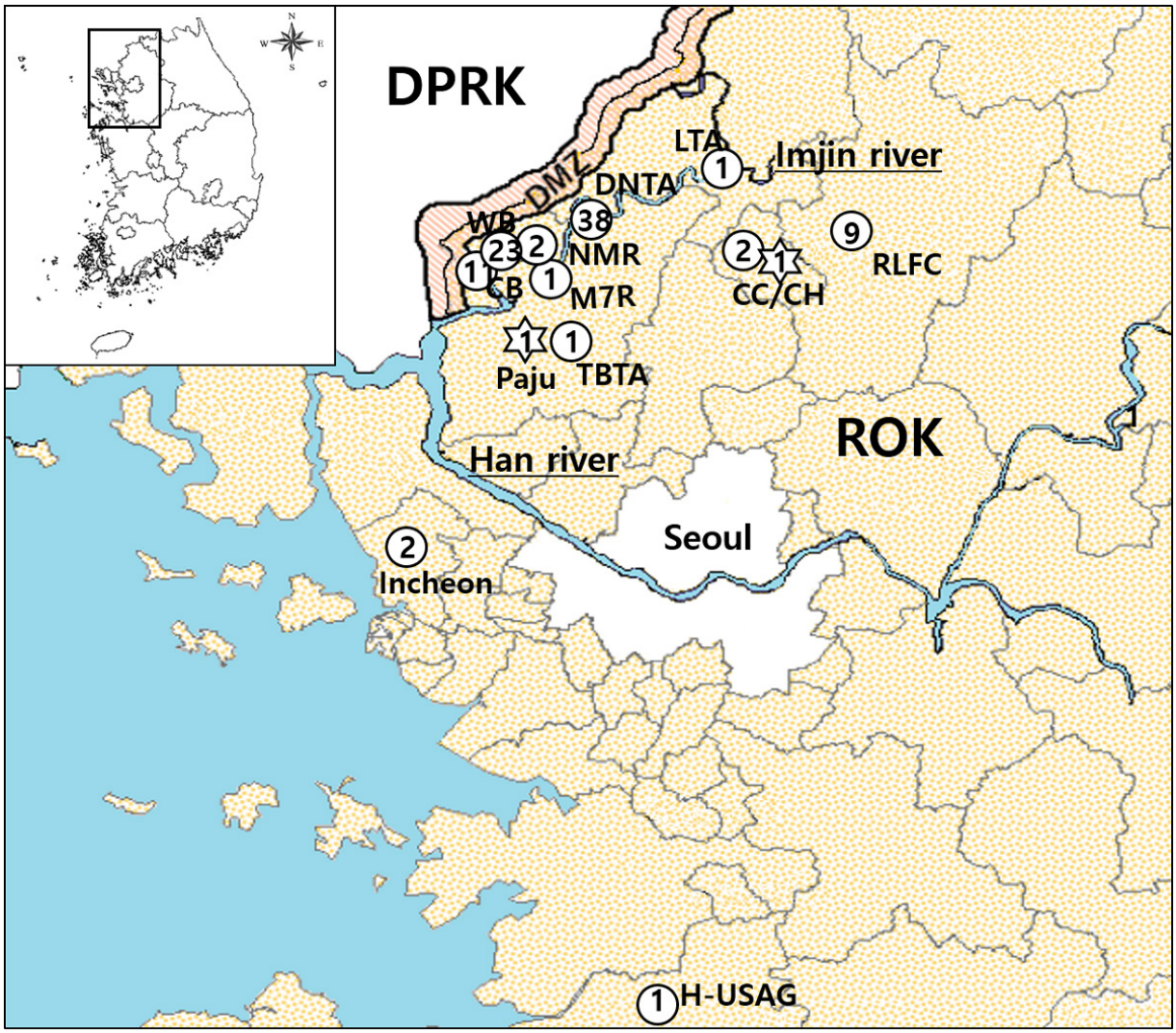
Year	Number Malaria Cases[1]	Number Relapses[2]	Medical Records Available	Symptoms															
				Fever (Range)[3]	Chills (%)		Sweats (%)	Malaise (%)		Headache (%)		Myalgia (%)		Nausea (%)	Vomiting (%)		Diarrhea (%)	
2006	24	1	17	97.0- 105.0°F	17	(100)	17 (100)	17	(100)	17 (100)		17	(100)	9 (52.9)	2 (11.8)		3 (17.6)	
2007	34	0	12	99.5- 105.6°F	12	(100)	12 (100)	12 (100)		12	(100)	12 (100)		9 (75.0)	7 (58.3)		0	
2008	0	0	0	0	0		0	0		0		0		0	0		0	
2009	3	0	3	102.8- 103.0°F	3 (100)		3 (100)	3 (100)		3	(100)	3 (100)		2 (66.7)	1 (33.3)		1 (33.3)	
2010	5	0	4	103.0- 104.0°F	4	(100)	4 (100)	4 (100)		4	(100)	4 (100)		2 (50.00)	0		0	
2011	1	0	1	101.2°F	1	(100)	1 (100)	1	(100)	1 (100)		1	(100)	1 (100)	1	(100)	1	(100)
2012	2	0	1	101.0°F	1	(100)	1 (100)	1	(100)	1 (100)		1	(100)	1 (100)	1	(100)	1	(100)
2013	3	0	3	101.2- 104.0°F	3 (100)		3 (100)	3 (100)		3	(100)	3 (100)		1 (33.3)	1 (33.3)		0	
2014	13	0	13	100.4- 106.0°F	13 (100)		13 (100)	13 (100)		13	(100)	12 (92.3)		12 (92.3)	8	(61.5)	5	(38.5)
2015	6	0	6	98.7- 104.6°F	6 (100)		6 (100)	6 (100)		6	(100)	6 (100)		5 (83.3)	3 (50.0)		3 (50.0)	
2016	12	1	12	98.5- 105.4°F	12	(100)	12 (100)	12 (100)		12	(100)	12 (100)		8 (66.7)	6	(50.0)	5 (41.7)	
2017	4	0	4	102.5- 105.1°F	4	(100)	3 (75.0)	4 (100)		4	(100)	4 (100)		2 (50.0)	1 (25.0)		2 (50.0)	
2018	9	0	9	96.3- 105.6°F	9 (100)		9 (100)	9 (100)		7	(77.8)	7 (77.8)		3 (33.3)	2	(22.2)	4 (44.4)	
2019	1	0	1	104.9°F	1	(100)	1 (100)	1	(100)	1 (100)		1	(100)	0	0		0	
2020	6	0	6	102.0- 104.9°F	6	(100)	6 (100)	6 (100)		6	(100)	6 (100)		4 (66.7)	2 (33.3)		3 (50.0)	
TOTAL	123	2	92 (74.8)	96.3- 106.0°F	92	(100)	91 (98.9)	92 (100)		90	(97.8)	89 (96.7)		59 (64.1)	35 (38.0)		28 (30.4)	

[1] Only US military and civilian personnel. KATUSA Soldiers not included.

[2] Two relapse cases reported not included.

[3] Patients often reported at the clinic following the paroxysm when temperatures were near normal.

Figure 3. The most likely sites of exposure and number of military and civilian personnel (circle = military; star = civilian) diagnosed with Plasmodium vivax cases attributed to exposure in the Republic of Korea. DNTA (41.8%) = Dagmar North Training Area, LTA 130 (1.1%) = Local Training Area, RLFC (9.9%) = Rodriguez Live Fire Complex, TBTA (1.1%)= Twin Bridge Training Area, WB (27.5%) = Warrior Base Training Area, NMR (2.2%) = New Mexico Range, CC/CH (2.2%) = Combined Camp Casey/ Camp Hovey, CC/CH (star) (1.1%) = Dongducheon city, M7R (1.1%) = Monkey 7 Range, CB (12.1%) = Camp Bonifas (Joint Security Area), Paju (star) (1.1%) = Paju city, and H-USAG (1.1%) = Humphreys US Army Garrison.



(Table 2). However, some malaria patients reported that they used older permethrin-treated uniforms that exceeded their effective wear date or >50 washings while training in field environments.

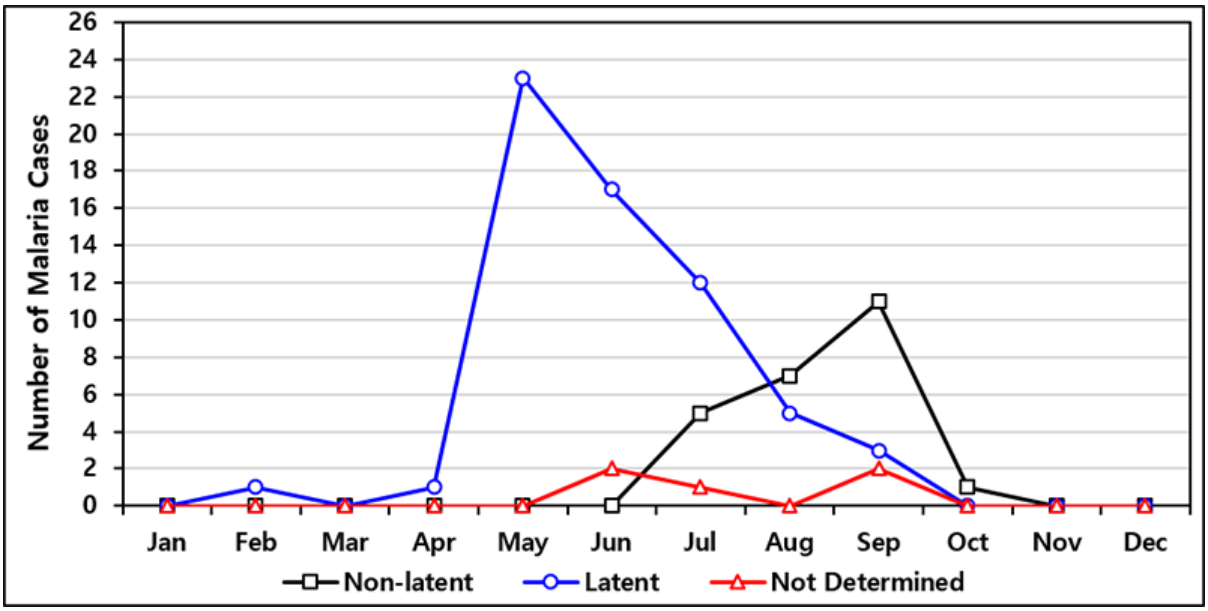
Soldiers were often not aware of the abundance of biting mosquitoes prior to moving to areas where they conducted training exercises. Although it is the unit responsibility to provide arthropod repellents to military personnel when conducting field-training exercises when mosquitoes and other vectors are present, only 17.6% of the respondents stated that the unit provided repellents. Therefore, military personnel either purchased their own repellents, borrowed repellents from team members, or did not use them. Of the US Army and KATUSA Soldiers who purchased or borrowed various types of repellents from other Soldiers, 61.5% reported using some form of arthropod repellent while conducting field-training exercises when mosquitoes were present. Of the 56 individuals who used repellents, 88.5% stated they used 15-33% N,N-diethyl-3-methyl benzamide (DEET) formulations, while the remaining 11.5% used some other form of repellent, e.g., creams or sprays of unknown formulations. A total of 56.0% of the respondents stated that they properly wore their uniforms (sleeves rolled down and trouser legs tucked into their boots) in accordance with command policy while residing near the DMZ or training in field environments when mosquitoes were biting. Others (44.0%) wore APFUs after duty hours, while sleeping (e.g., in non-secured tents), smoking, talking with friends, or making telephone calls outdoors during the evening hours. Pop-up permethrin-treated bed nets were not issued while training in field environments. However, one US Army Soldier (1/87, 1.1%) stated that he

Table 4. Number (%) of latent malaria cases among US Army and KATUSA Soldiers, mean number of days from onset of symptoms to 1st medical visit, mean number of medical visits until diagnosis, mean number of days from the 1st medical visit to diagnosis, blood stage anti-malarial drugs administered, and proportion of patients prescribed 15 or 30 mg base primaquine phosphate for liver stage parasites from 2006-2020.

Year	No. Malaria Cases[1]	No. (%) Relapses[2]	No. (%) Latent[3]	Range (Mean) Months[4]	No. (%) Non-Latent[5]	No. (%) Not Determined[6]	Range (Mean) Days to 1st Medical Visit[7]	Range (Mean) Number Medical Visit	Range (Mean) No. Days from 1st Visit to Diagnosis[8]	Blood Stage Treatment: Number Treated (%)9				No. (%) Liver Stage Treatment10	
										CHL	HCHL	Mal	OTH	15 MG	30 Mg
2006	24	1	9	9 - 13 (10.2)	8	0	0 - 5 (2.6)	1 - 5 (1.9)	1 - 8 (5.9)	17	0	0	0	17	0
2007	34	0	8	9 - 13 (11.1)	3	1	0 - 7 (2.9)	1 - 4 (2.0)	1 - 15 (3.8)	11	1	0	0	12	0
2008	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2009	3	0	3	10 (10.0)	0	0	5 - 6 (5.7)	2 (2.0)	2 - 7 (4.3)	2	1	0	0	3	0
2010	5	0	4	7 - 11 (8.7)	0	1	0 - 7 (3.2)	1 - 6 (3.5)	1 - 33 (13.5)	4	0	1	0	3	2
2011	1	0	0	NA	0	1	6	1	1	1	0	0	0	0	1
2012	2	0	0	NA	1	0	3	1	2	1	0	0	0	0	1
2013	3	0	2	10 - 11 (10.5)	1	0	1 - 5 (3.3)	2 - 3 (2.3)	7 - 12 (9.3)	3	0	0	0	1	2
2014	13	0	9	4 - 11 (8.9)	4	0	0 - 14 (4.5)	1 - 5 (2.5)	0 - 23 (6.5)	7	4	2	0	7	6
2015	6	0	3	9 (9.0)	2	1	0 - 5 (3.5)	1 - 4 (2.2)	0 - 10 d (5.3)	1	2	3	0	3	3
2016	12	1	12	8 - 10 (8.6)	0	0	0 - 24 (5.7)	1 - 3 (2.1)	0 - 9 (4.6)	9	0	2	1	2	10
2017	4	0	1	6 (6.0)	3	0	0 - 13 (5.0)	2 - 4 (3.0)	3 - 6 (4.2)	1	2	1	0	2	2
2018	9	0	6	9 - 12 (9.6)	2	1	1 - 8 (3.8)	1 - 8 (2.6)	0 - 19 (4.3)	2	5	1	1	1	8
2019	1	0	1	9 (9.0)	0	0	5	2	4	0	1	0	0	0	1
2020	6	0	6	10 - 11 (10.2)	0	0	1 - 12 (5.3)	1 - 2 (1.7)	0 - 13 (3.8)	2	1	3	0	0	6
TOT	123	2 (2.2)	64/93 (68.8)	4 - 13 (9.5)	24/93 (25.8)	5/93 (5.4)	0 - 24 d (3.7 d)	1 - 8 (2.1)	0 - 33 (4.7 d)	61 (65.6)	17 (18.3)	13 (14.l)	2 (2.2)	51 (54.8)	42 (45.2)

1 Two patients who relapsed 2 and 10 months after the first episode were not included.
2 Two patients who were only administered 15 mg base primaquine x 14 days for the primary attack relapsed at 2 and 10 months.
3 Latent malaria cases among patients interviewed (93) were reported 6-13 months following the first exposure in malaria high-risk areas; most cases were diagnosed following the patients return to the US.
4 Number of months following the period of the most likely exposure and the onset of symptoms.
5 Non-latent vivax malaria cases among patients interviewed (93) were determined if a patient had been in a malaria high-risk area for only 1 mosquito season.
6 Latency could not be confirmed for US Army and KATUSA Soldiers, and US civilians interviewed (93) who trained/resided in malaria high-risk areas for 2 or more mosquito seasons.
7 Number of days from the onset of symptoms reported by the patient to the first visit at medical facilities.
8 Number of days from the first visit at medical facilities to diagnosis.
9 CHL = chloroquine, HCHL = hydroxychloroquine, MAL = Malarone, Other includes: mefloquine (1), artemether-lumefantrine (1), and doxycycline (1). One patient was initially treated with mefloquine, then changed to chloroquine (2011); one initially treated with chloroquine, then changed to Malarone (2014); one initially treated with doxycycline and then changed to Malarone (2015).
10 Patients were administered 15 mg or 30 mg base primaquine phosphate (base) x 14 days.

Figure 4. Monthly distribution of latent and non-latent vivax malaria cases among US Army and KATUSA Soldiers from 2006-20201.1 Medical records for 93 US military members (87), KATUSA Soldiers (4), and 2 civilians (2). Latent vivax malaria cases accounted for 68.8% (64/93) malaria cases attributed to exposure the previous year. Nearly all latent malaria cases were diagnosed in the US. Non-latent (<21 days after exposure) vivax malaria cases accounted for 25.8% (24/93) of the malaria cases. It could not be determined if 5 (5.4%) of the vivax malaria cases were latent or non-latent due to exposure over two malaria seasons. Data was not included for the US civilian (1) and family member (1) since it could not be determined where they were exposed or if they demonstrated latent or non-latent infections. Two additional vivax malaria cases (data not included) were due to a relapse 2 and 10 months after the primary infection



used a bed net (type unknown) while training at LTA 130. Most respondents reported seeing numerous mosquitoes (on a scale of 0 to 5, with 0 = none, to 5 = too many to count) when training in field environments (range 0-5, mean 4.0) (Table 2). Some of the military personnel who trained during the mosquito season reported that they did not see mosquitoes (0), while others training at the same site during the same period reported too many to count (5). Similarly, the range for Soldiers receiving mosquito bites ranged from 0-5 (mean 3.1).

Symptoms, Diagnosis, Treatment, Chemoprophylaxis

Medical records were available for all vivax malaria patients (91) who were interviewed telephonically or face-to-face (excluding relapse cases) and one additional patient (Table 3). Except for one respondent, all stated that they experienced typical malaria paroxysms of chills, followed by high fevers and then intense sweats. Recorded temperatures at medical clinics and hospitals (range: 35.7oC – 41.1oC oral) were often within normal limits since patients frequently reported to the medical facilities following a paroxysm. Nearly all respondents reported moderate to severe headaches (97.8%), followed by myalgia (96.7%), nausea (64.1%), vomiting (38.0%), and diarrhea (30.4%).

Due to the low number of vivax malaria cases (<0.1/1,000) and extremely low mortality rate (no deaths among US personnel from 1993-2020), chemoprophylaxis was not recommended for US Army and KATUSA Soldiers (Fig. 2). Thus, it was recommended that military personnel apply PMM, e.g., proper wear of appropriate clothing (e.g., permethrin-treated uniforms with the sleeves rolled down, pants tucked into the boots) and arthropod repellents (e.g., DEET 20-33% or Picaridin) purchased through the Defense Logistics Agency (DLA) and provided through unit supply to military personnel while training in field environments when mosquitoes are present (Table 2). The US Army Soldiers at Camp Bonifas were placed on mandatory chemoprophylaxis from 1998-2013 due to their high operational tempo, proximity to the DMZ, and conducting activities in the JSA at Panmunjeom adjacent to the military demarcation line (MDL) that separates North and South Korea.12 Chemoprophylaxis included chloroquine (500 mg weekly from mid-May through October), followed by terminal chemoprophylaxis with 30 mg primaquine base x 14 days, as recommended by the US CDC.18,19 The Commander at Camp Bonifas discontinued malaria chemoprophylaxis in 2014 due to low numbers of malaria cases for previous years and low mortality. Although chemoprophylaxis was administered to US Army Soldiers at Camp Bonifas prior to 2014, two US Army Soldiers were diagnosed with vivax malaria (2011 and 2013), one of which was determined to be non-latent (diagnosed while in Korea) and the other latent (diagnosed in the US). The latter likely being due to the elimination of blood stage parasites while taking chloroquine and incomplete terminal chemoprophylaxis. From 2014 to 2018, the number of malaria cases attributed to exposure at Camp Bonifas remained low (Supplemental Table 2).

Based on malaria patient interviews and medical records where exposure periods were determined, 68.8% of the vivax malaria cases were defined as latent, with most diagnosed in the US 4-13 months after exposure (Table 4, Fig. 4). Non-latent malaria (onset of symptoms <21 days after exposure) accounted for 25.8%, while latency could not be determined for 5.4% of the malaria cases diagnosed in the ROK since they had trained or resided at malaria high-risk training sites/installations for >2 mosquito seasons.

While some respondents reported to medical facilities (Army medical treatment facilities and local civilian hospitals) at the onset of symptoms (day 0), others did not report to medical treatment facilities until 1-24 days (mean 3.7 days) after the onset of symptoms (Table 4). The number of medical visits until diagnosis ranged from 1-8 visits (mean 2.1). Diagnosis from the first visit to a medical facility ranged from 0-33 days (mean 4.7 days) following the first visit. Following diagnosis, the majority of patients were treated for blood stage parasites with chloroquine phosphate (65.6%), followed by hydroxychloroquine (18.3%), Malarone (atovaquone/proguanil hydrochloride) (14.0%), and 1 each for mefloquine (1.1%) and artemether/lumefantrine (1.1%). One patient was initially treated with mefloquine (discontinued) and then treated with chloroquine, while a second treated with chloroquine (discontinued) and then treated with Malarone, and a third treated with doxycycline (discontinued) and then treated with Malarone (Table 4). Most vivax malaria patients (54.8%) were treated with 15 mg primaquine base (26.3 mg salt) x 14 days for liver stage parasites (hypnozoites), while the remainder (45.2%) were treated with 30 mg primaquine base (52.6 mg salt) x 14 days.

DISCUSSION

Vivax malaria is an insidious disease that posed a serious health threat to civilian and military personnel in Korea for decades that continues today. It often remains hidden as hypnozoites in the liver for 6-18+ months after infection, making it well adapted to survive undetected during the harsh winter months.[4] While outbreaks of malaria-like illnesses were recorded in Korea as early as the 12th century, it was not identified until 1886.[1] Following the annexation of Korea by Japan in 1910, the incidence of malaria gradually decreased with the implementation of an improved medical system. Malaria continued to be a major health threat following WWII, and was further impacted during the Korean War, with increased number of cases among ROK populations and US service members who were deployed to Korea. [6] In the first year alone, there were >6,000 cases of malaria among US service members, with >12,000 cases reported the following year in the US as service members rotated back due to latent malaria.[20] With the use of chloroquine to eliminate blood stage parasites and the introduction of primaquine that eliminated latent liver stages (hypnozoites), the number of cases among US service members who rotated back to the US was greatly diminished.[21] Following the Korean War from 1950-1953, malaria continued to pose a major health threat in the war torn nation. To combat malaria, the ROK government, with the assistance of WHO, established malaria vector and malaria control programs that reduced the incidence of malaria until 1979 when WHO declared the ROK to be malaria free.[6,7] Although unsubstantiated, shortly thereafter the Democratic People's Republic of Korea (DPRK) also declared that it was malaria free.

In 1993, a ROK Soldier stationed near the DMZ with no record of foreign travel developed malaria.[6,9] Due to a slow response by the ROK government, the number of malaria cases rapidly increased, peaking to >4,000 annual cases in 2000. From 1997-1998, the US military established a malaria chemoprophylaxis program due to increasing numbers of malaria cases among US military personnel. However, decreased number of cases were not indicated, while a higher proportion of cases were reported among US Army Soldiers returning to the US.[22] Thus, the USFK malaria chemoprophylaxis program was abandoned, except for personnel assigned to Camp Greaves (now closed) and Camp Bonifas (JSA) due to their proximity to the DPRK and high operation tempo. However, in 2003 and 2014, malaria chemoprophylaxis was discontinued at Camp Greaves and Camp Bonifas, respectively, due to decreased numbers of malaria cases among US and ROK military populations.

While it was thought that malaria would spread from the DMZ to the southern tip of the Korean Peninsula, nearly all the cases were attributed to exposure within approximately 10 km from the DMZ. It was not until 2005, when members of the *Anopheles* Hyrcanus Group were identified that this phenomenon was identified.[23] Later it was determined that the primary vectors, *An. kleini* Rueda and *An. lesteri* Baisa & Hu, were recorded in much higher numbers near the DMZ and low numbers south of Seoul.[24,25]

In healthy nonimmune young adults, *P. vivax* of Korean origin causes a debilitating, but usually not life threatening acute febrile illness that reduces military effectiveness. Due to decreased numbers of *vivax* malaria cases and extremely low mortality, the USFK established a malaria control policy. However, the policy did not recommend the use of chemoprophylaxis, but instead only the use of PMM [e.g., permethrin-treated uniforms, proper wear of military uniforms, and effective repellents (e.g., 20-33% DEET and Picaridin) on exposed skin areas] when training in field environments to provide maximum protection against biting mosquitoes and transmission of malaria.[15] Additionally, the use of permethrin-treated pop-up bed nets should have been considered for Soldiers when sleeping in tents (often unsecured that allowed mosquitoes to enter), cots on the ground, or on/in vehicles to provide additional protection from biting mosquitoes.

When training in field environments, Soldiers report to the unit medic (Aid Station) during field operations or associated medical clinic when at their home base location. Malaria paroxysms often occur during off-duty evening hours, with patients reporting to sick call the following morning during the "malaise" phase when oral temperatures are within normal limits. From medical records, malaria patients frequently were not familiar with the symptoms of malaria and incompletely reported symptoms to the provider (written medical records). In addition, providers frequently failed to request patient history of the Soldier when deployed to the ROK and the field training locations, e.g., near the DMZ. Thus, for many malaria patients, the initial diagnosis was often "flu-like" or "body aches," even when they reported characteristic malaria symptoms of chills (often shaking), followed by high fever (> 39.4oC oral), and then intense sweats that generally lasted for 2-6 hours, which was then followed by malaise, in combination with body aches and mild to severe headache, and in some cases nausea, vomiting, and diarrhea. As a result, malaria often was not considered in the algorithm until after two or more visits following recurrent paroxysms or when the patients presented at the clinic during a paroxysm. The late presentation (latency) of vivax malaria emphasizes the required vigilance for medical providers to review patient history for potential exposure at malaria high-risk areas, in addition to a knowledge of the signs and symptoms of malaria. From the medical records, it is apparent that the malaria patients often provided incomplete details of their symptoms and often did not volunteer information that they were in the ROK where malaria is endemic. Therefore, it is critical that providers at all levels be cognizant of the signs and symptoms of malaria, as well as review travel/training history to determine if patients, especially after returning to the US, were deployed or trained in malaria high-risk areas.

The US Soldiers diagnosed with *vivax* malaria at ROK medical hospitals are administered hydroxychloroquine for blood stage parasites, followed by primaquine (15 mg base x 14 days) after treatment with hydroxychloroquine. However, there is an estimated 10% relapse rate among US personnel only administered 15 mg primaquine base x 14 days. At US military and civilian hospitals, chloroquine and Malarone (250 mg Atovaquone/100 mg Proguanil hydrochloride) are commonly used for US military personnel diagnosed with *vivax* malaria, followed by 30 mg primaquine base x 14 days (recommended by US CDC) to reduce the potential for relapse.[18] Two of 51 (3.9%) US Army Soldiers treated at Korean hospitals that were only administered 15 mg primaquine base x 14 days relapsed two and 10 months in 2005 and 2015 after treatment for blood and liver stage parasites. The US medical personnel should review terminal treatment regimens to ensure that *vivax* malaria patients are provided 30 mg primaquine base x 14 days to prevent relapses due to latent hypnozoites as recommended by US CDC. Additionally, primaquine should be given concurrently with chloroquine when glucose-6-phosphate dehydrogenase (G6PD) levels are known to reduce potential human-mosquito transmission as studies have shown that *vivax* malaria patients are infective to mosquitoes up to 36h after treatment with chloroquine, while when treated with chloroquine + primaquine, patients were not infective to mosquitoes 2h after the initial dose.[26] Treatment with Tafenoquine, as a single dose, for both blood and liver stage parasites should be considered as it also would likely render *vivax* malaria patients non-infective to mosquitoes.[27]

Commanders and leaders must be aware of command policy and health issues that affect units while training in field environments, especially as witnessed by explosive outbreaks, such as the one observed in 2015 where 11 US Soldiers were infected over a 3-day period.[14] Individual US military personnel are responsible for implementing PMM to reduce the vector-borne disease risks. In many cases, interviews showed that US military personnel, including medical personnel, are ill informed of the risks that *vivax* malaria poses near the DMZ, or the relative abundance of mosquitoes present at military training sites located within 10 km of the DMZ during the mosquito season. However, interviews also showed that nearly all malaria patients deployed near the DMZ or while training at field environments near the DMZ were not well informed of malaria risks, including: (1) signs and symptoms of malaria, (2) presence of malaria in the ROK, and (3) that there were increased transmission risks associated with training near the DMZ. Thus, health care providers often failed to request patient history of potential exposure in malaria-risk areas in the ROK. The lack of information provided to US Army Soldiers in the ROK, and especially those who returned to the US that subsequently developed malaria, often delayed patients reporting to the medical clinics.[14] This not only delayed diagnosis and increased morbidity, but also increased risks for infecting competent *Anopheles* vectors and subsequent autochthonous transmission to civilian and military communities in the ROK and US, or other countries where US Soldiers were subsequently deployed. Although epidemiological surveillance of malaria cases in the US were attributed mostly to imported cases and immigrants, the potential for autochthonous transmission due to military personnel returning to the US and subsequently developing malaria symptoms cannot be overlooked.[28]

Malaria rates in the DPRK have significantly decreased and there is a WHO initiative to eliminate *vivax* malaria from the DPRK by 2025.[29] The elimination of malaria from the DPRK would eliminate cross-border infections from the North to the South. Malaria rates continue to decrease in the ROK, with the potential for elimination among civilian and military communities in the near future. The US military must also play an important role in the elimination of malaria from the ROK through education of military and civilian personnel, implementation of PMM, and rapid treatment of malaria patients to reduce the potential for human-mosquito transmission.

CONCLUSION

Vivax malaria poses a significant health threat to local populations and ROK and US military personnel that reside and/or train near the DMZ. A combination of factors that included training activities which exposes Soldiers to biting mosquitoes, unavailability and non-usage of arthropod repellents, lack of permethrin-treated uniforms, absence of mosquito nets, and sleeping unprotected in/on vehicles or in ill-kempt/unsecured tents, increases the potential for malaria transmission. Malaria transmission may be explosive as evidenced by an outbreak of 11 cases of malaria over a 3-day period at DNTA. A lack of education among US military personnel and medical providers of potential malaria risks, especially near the DMZ led to delayed diagnoses. Leaders and Soldiers should use all PMM available to ensure a healthy and ready fighting force. Medical providers, from the lowest echelon must be well informed of the signs and symptoms of malaria. Effective malaria control in the ROK depends on the US military's efforts to detect, assess, rapidly diagnose, and rapidly treat malaria to reduce morbidity and human-mosquito-human malaria transmission. Case reporting is necessary to determine sources of infection that are critical for identifying malaria high-risk areas on the Korean Peninsula. The identification of the seasonal and geographical distributions and relative proportion of members of the *Anopheles* Hyrcanus Group should be conducted to provide information for the delineation of malaria high-risk areas in the ROK.

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REFERENCES

1. Allen HN, Heron JW. First annual report of the Korean government hospital, Seoul. R. Meiklejohn 1886, p. 8.
2. Chai JY. Re-emerging *Plasmodium vivax* malaria in the Republic of Korea. *Korean J Parasitol.* 1999; 37:129-143.
3. Yeo I. A history of malaria in modern Korea 1876-1945. *Korean J Med Hist.* 2011; 20:53-82. (In Korean)
4. Garnham PCC, Bray RS, Bruce-Chwatt LJ, Draper CC, Killick-Kendrick R, Sergiev PG, Tiburskaja NA, Shute PG, Maryon M. A strain of *Plasmodium vivax* characterized by prolonged incubation: morphological and biological characteristics. *Bull WHO.* 1975; 52:21-32.
5. Tiburskaja NA, Vrublevskaja OS. The course of infection caused by the North Korean strain of *Plasmodium vivax*. *WHO/MAL/77.* 1977; 895:1-19.
6. Ree HI. Unstable *vivax* malaria in Korea. *Korean J Parasitol*. 2000; 38:119-138.
7. WHO (World Health Organization): Synopsis of the world malaria situation, 1979. *Wkly Epidemiol Rec*. 1981; 56:145-149.
8. Ahn MH, Shin HJ, Im KI, Soh CT. Imported malaria cases in Korea. *Yonsei Rep Trop Med.* 1982; 13:23-29.
9. Chai IH, Lim GI, Yoon SN, Oh WI, Kim SJ, Chai JY. Occurrence of tertian malaria in a male patient who has never been abroad. *Korean J Parasitol.* 1994; 32:195-200.
10. Lee JS, Lee WJ, Cho SH, Ree HI. Outbreak of vivax malaria in areas adjacent to the demilitarized zone, South Korea, 1998. *Am J Trop Med Hyg.* 2002; 66:13-17.
11. Park JW, Jun G, Yeom JS. *Plasmodium vivax* malaria: status in the Republic of Korea following reemergence. *Korean J Parasitiol*. 2009; 47(Suppl):S39-S50.
12. Kim HC, Pacha LA, Lee WJ, Lee JK, Gaydos JC, Sames WJ, Lee HCS, Bradley K, Geung GG, Tobler SK, Klein TA. Malaria in the Republic of Korea, 1993-2007. Variables related to re-emergence and persistence of *Plasmodium vivax* among Korean populations and U.S. Forces Korea. *Mil Med.* 2009; 174:762-769.
13. Oh MD, Shin H, Shin D, Kim U, Lee S, Kim N, Choi MH, Chai JY, Choe K. Clinical features of vivax malaria. *Am J Trop Med Hyg.* 2001; 65:143-146.
14. Klein TA, Seyoum B, Forshey BM, Ellis KK, McCall H, Jackson K, Tucker C, Yi SH, Kim MS, Kim HC. Cluster of vivax malaria in U.S. soldiers training near the demilitarized zone, Republic of Korea during 2015. *MSMR*. 2018; 25:4-9.
15. USFK Reg 40-2. Prevention, surveillance, diagnosis, treatment and reporting of vivax malaria in the Republic of Korea. 9 Dec 2009, pp. 22.
16. KDCA (Korea Disease Control and Prevention Agency). Protocol for malaria control, 2020. KDCA, 18 May 2021, pp. 167.
17. 1Im JH, Huh KM, Yoon CG, Woo HT, Lee JS, Chung MH, Klein TA, Jung JH. Malaria control and chemoprophylaxis policy in the Republic of Korea Armed Forces for the previous 20 years (1997-2016). *Malaria J.* 2018; 17:295.
18. CDC. Treatment Guidelines: Treatment of Malaria (Guidelines for Clinicians) 2017. Available at: https://www.cdc.gov/malaria/resources/pdf/clinicalguidance.pdf. Accessed November 24, 2021.
19. Hill DR, Baird JK, Parise ME, Lewis LS, Ryan ET, Magill AJ. Primaquine: report from CDC expert meeting on malaria chemoprophylaxis. *Am J Trop Med Hyg.* 2006; 75: 402-415.

20. Fritz RF, Andrews JM. Imported and indigenous malaria in the United States, 1952. *Am J Trop Med Hyg.* 1953; 2:445-456.

21. Alving AS, Arnold J, Robinson DH. Status of primaquine. 1. Mass therapy of subclinical vivax malaria with primaquine. *JAMA.* 1952; 149:1558-1562.

22. Klein TA, Pacha LA, Lee HCS, Kim HC, Lee WJ, Lee JK, Jeung GG, Sames WJ, Gaydos JC. Plasmodium vivax malaria among U.S. Forces Korea in the Republic of Korea, 1993-2007. Mil Med. 2009; 174:412-418.

23. Rueda LM. Two new species of Anopheles (Anopheles) Hyrcanus Group (Diptera: Culicidae) from the Republic of Korea. Zootaxa. 2005; 941:1-26.

24. Kim HC, Klein TA, Lee WJ, Collier BW, Chong ST, Sames WJ, Lee IY, Lee DK. Mosquito species distribution and larval breeding habitats with taxonomic identification of anopheline mosquitoes in Korea. Entomol Res. 2007; 37:29-35.

25. Rueda LM, Brown TL, Kim HC, Chong ST, Klein TA, Foley HD, Anyamba A, Smith M, Pak EP, Wilkerson RC. Species composition, larval habitats, seasonal occurrence and distribution of potential malaria vector and associated species of Anopheles (Diptera: Culicidae) in the Republic of Korea. Malar J. 2010; 9:55.

26. Klein TA, Tada MS, Lima JBP, Katsuragawa TH. Infection of *Anopheles darlingi* fed on patients infected with *Plasmodium vivax* before and during treatment with chloroquine in Costa Marques, Rondonia, Brazil. *Am J Trop Med Hyg.* 1991; 45:471-478.

27. Fukuda MM, Krudsood S, Mohamed K, Green JA, Warrasak S, Noedl H, Euswas A, Ittiverakul M, Buathong N, Sriwichai S, Miller RS, Ohrt C. A randomized, double-blind, active-control trial to evaluate the efficacy and safety of a three day course of tafenoquine monotherapy for the treatment of *Plasmodium vivax* malaria. *PLoS One*. 2017, Nov 9; 12(11):e0187376.

28. Zucker JR. Changing patterns of autochthonous malaria transmission in the United States: a review of recent outbreaks. Emerg Inf Dis. 1996; 2:37-43.

29. WHO (World Health Organization). Zeroing in on malaria elimination: final report of the E-2020 initiative. Global Malaria Programme. Meeting Report 2021, WHO, 21 April 2021; p.20.

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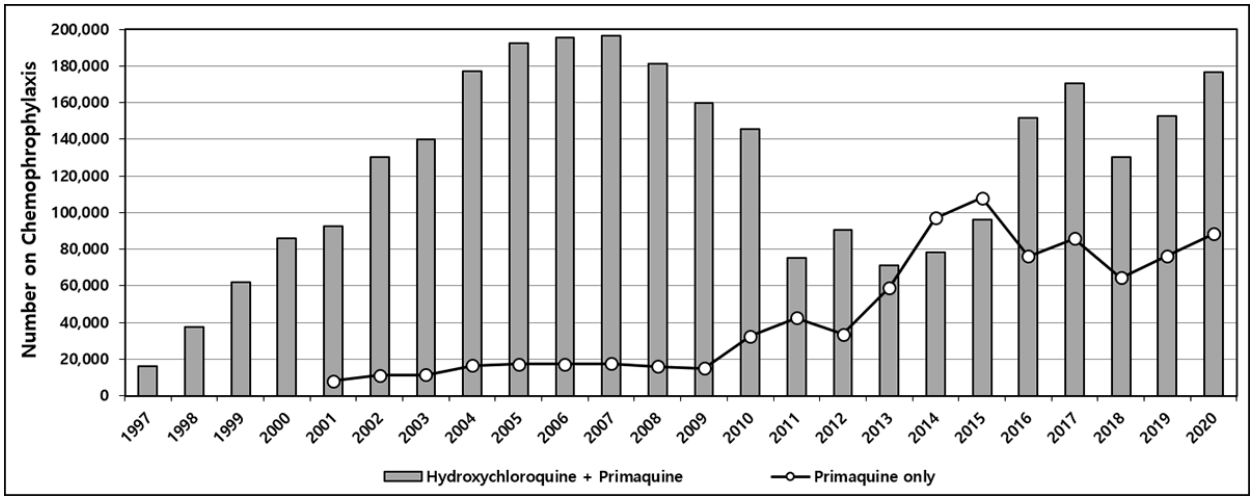
Supplemental Figure 1. Number of ROK Army Soldiers placed on hydroxychloroquine chemoprophylaxis + 15 mg base primaquine for terminal chemoprophylaxis or only 15 mg base primaquine at the end of the malaria season (terminal chemoprophylaxis). Figure 1. Number of ROK Army Soldiers placed on hydroxychloroquine chemoprophylaxis + 15 mg base primaquine for terminal chemoprophylaxis or only 15 mg base primaquine at the end of the malaria season (terminal chemoprophylaxis).



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Supplemental Table 1. Number and country of origin of imported malaria cases among US service members deployed to the Republic of Korea from 2006-2018.

Year	Gender	Country of Exposure	Conditions
2006	Female	Honduras[1]	Military Operations
2009	Male	Nigeria	Leave
2010	Male	Ghana	Leave
2013	Male	Ghana	Leave
2015	Male	Nigeria	Leave
	Male	Kenya	Leave
2017	Male	Nigeria	Leave
2018	Male	Nigeria	Leave
	Male	Afghanistan[2]	Military Operations

[1] Patient arrived to the Republic of Korea from Honduras, a *vivax* malaria endemic country, in September 2006 and was stationed at Camp Henry (Daegu), a malaria low-risk area, and did not participate in any field exercises prior to developing symptoms. Patient was non-compliant for malaria chemoprophylaxis and did not use bed nets or repellents while in Honduras.

[2] Patient developed onset of *vivax* malaria less than 7 days after arriving to the ROK. Patient was on malaria chemoprophylaxis for blood stage parasites but was not provided primaquine (30 mg base x 14 days) for liver stage parasites upon departing Afghanistan.

Supplemental Table 2. Annual number (%) of US Army and KATUSA Soldiers who conducted military training at ROK/US operated training sites or were assigned to installations near the demilitarized zone in the ROK where they most likely contracted *Plasmodium vivax* infections[1].

Year	Training Areas/ Installations							TOTAL	
	Dagmar North TA	Warrior Base	Camp Bonifas	Rodriguez LFC	New Mexico Range	Other[2]	N/D[3]	TOTAL (Military)[1]	Civilian[4]
2006	3	7	2	5	0	0	7	17	0
2007	3	6	1	2	0	0	22	12	1
2008	0	0	0	0	0	0	0	0	0
2009	0	2	0	0	0	0	1	2	0
2010	0	1	0	1	0	2	1	4	0
2011	0	0	1	0	0	0	0	1	0
2012	0	1	0	0	0	0	1	1	0
2013	3	0	0	0	0	0	0	3	0
2014	5	3	2	0	1	2	0	13	0
2015	4	2	0	0	0	0	0	6	0
2016	9	1	0	0	1	1	0	12	1
2017	1	0	2	0	0	1	0	4	0
2018	6	0	2	0	0	1	0	9	0
2019	0	0	1	0	0	0	0	1	0
2020	4	0	0	1	0	1	0	6	0
TOTAL (%)	38/91 (41.8)	23/91 (27.5)	11/91 (12.1)	9/91 (9.9)	2/91 (2.2)	8/91 (8.8)	32/125 (25.6)	91/125 (72.8)	2/125 (1.6)

[1] Includes US Army Soldiers (87) and KATUSA Soldiers (4) who trained with the US Army where interviews were conducted to determine the most likely site of malaria infections. Two *vivax* malaria cases that relapsed 2 months (2006; Twin Bridges) and 10 months (2016; Dagmar North) after the primary diagnosis were not included.

[2] Other includes: LTA 130 (1; 2010); Camp Casey (2; 2010, 2014); Twin Bridges TA (1; 2014); Incheon Area (2; 2016, 2020), Monkey 7 TA (1; 2017), and Camp Humphreys USAG (1; 2018).

[3] N/D = Not Determined; the most likely site of infection from a total of 32/125 patients who were diagnosed with *vivax* cases attributed to exposure in the ROK from 2006-2020 were not determined.

[4] US civilian cases (2) were most likely acquired in Paju-si (2007) and Dongducheon-si, adjacent to Joint Camps Casey/Hovey (2016).