Journal of the American Mosquito Control Association, 39(3):000–000, 2023

SCIENTIFIC NOTE

CDC BOTTLE BIOASSAYS FOR DETECTION OF INSECTICIDE RESISTANCE IN *CULEX PIPIENS*, *AEDES ALBOPICTUS*, AND *AEDES KOREICUS* COLLECTED ON US ARMY GARRISONS, REPUBLIC OF KOREA

CRAIG A. STOOPS,[1] MYONG SUN KIM,[1] SANJEEV MAHABIR,[1] SUNG TAE CHONG,[1] STEPHANIE S. CINKOVICH[2] AND JENNIFER B. CARDER[3]

ABSTRACT. Mosquito-borne pathogens are a threat to US troops stationed in the Republic of Korea (ROK). Insecticide resistance has been reported in mosquito vectors in the ROK, highlighting the need for a sustained ROK-wide resistance surveillance program. To address this need from April 2022 until October 2022, larvae and pupae of *Aedes albopictus*, *Ae. koreicus*, and *Culex pipiens* were collected from US Army Garrison (USAG) Daegu (Camps Carroll and Henry), USAG Yongsan-Casey (Camp Casey), and USAG Humphreys (Camp Humphreys) and screened for resistance to insecticides using the Centers for Disease Control and Prevention (CDC) bottle bioassay. No resistance to deltamethrin or chlorpyrifos was detected in *Ae. albopictus* populations, but one population showed possible resistance to permethrin. *Aedes koreicus* populations were found to be resistant to etofenprox and permethrin with possible resistance to deltamethrin but were susceptible to chlorpyrifos. *Culex pipiens* populations were found to be resistant to chlorpyrifos, permethrin, and deltamethrin. Screening using CDC bottle bioassays will continue, and efforts will be made to determine the operational impact of the assay results on military installation mosquito control programs.

KEY WORDS *Aedes albopictus*, *Aedes koreicus*, CDC bottle bioassays, *Culex pipiens*, insecticide resistance, Republic of Korea, US Army Garrisons

Mosquito-borne pathogens are a threat to US troops stationed in the Republic of Korea (ROK). Insecticides are used to control mosquitoes and prevent pathogen transmission across the ROK and on US army garrisons (USAGs) (Lee et al. 1984, Hwang et al. 2020, Park et al. 2020). For example, as part of a vector-borne pathogen prevention strategy the Army Combat Uniform currently worn by US soldiers is factory treated with permethrin and provides a first line of defense against mosquito-borne pathogens (Estep et al. 2020). Under approved integrated pest management plans, pyrethroids such as resmethrin are used in ultra-low volume (ULV) applications for targeting adult mosquitoes on USAGs (Hwang et al. 2020). The ULV applications are made based on established mosquito thresholds and vary by location, with zero or very few ULV applications during the season on USAGs Daegu and Yongsan-Casey and from zero to more than 20 applications per season on USAG Humphreys (Hwang et al. 2020). There is also historical exposure due to mosquito control programs in the 1950s–1980s using organochlorine and organophosphate insecticides (Park et al. 2020). The impact of insecticide resistance (IR) on control and prevention is currently unknown, and any loss in efficacy of these insecticides could put soldiers at a greater risk of acquiring a mosquito-borne pathogen (Estep et al. 2020, Lee et al. 2020).

Culex pipiens L. complex is one of the most abundant human-biting mosquitoes in urban areas of the ROK and is a possible vector of Japanese encephalitis virus and West Nile virus (Kim et al. 2015, Seo et al. 2021). Due to international travel to endemic countries each year dengue cases are imported into the ROK where *Aedes albopictus* (Skuse) could cause local outbreaks (Seo et al. 2021). *Aedes koreicus* (Edwards) is an abundant human-biting urban mosquito in the ROK and has an unknown role in pathogen transmission (Ryu et al. 2019, Seo et al. 2021). In the ROK, IR has been reported in *Cx. pipiens* but not in *Ae. albopictus* or *Ae. koreicus* (Shin et al. 2012, Ryu et al. 2019, Lee et al. 2020, Park et al. 2020).

To better understand the risk IR poses to mosquito control efforts and personal protection measures as outlined in Carder et al. (2023), an IR monitoring program using the Centers for Disease Control and Prevention (CDC) bottle bioassays was established on USAGs in the ROK. The main goal of the program is initially to characterize the IR of local ROK mosquito populations and then monitor chang-

[1] Public Health, Environmental Health Section, Brian Allgood Army Community Hospital, Camp Humphreys 96271, Republic of Korea.

[2] Armed Forces Health Surveillance Division, Global Emerging Infections Surveillance Branch, Silver Spring, MD 20904; Cherokee Nation Strategic Programs, Tulsa, OK 74103.

[3] Defense Centers for Public Health – Aberdeen, Entomological Sciences Division (ESD), Pesticide Use and Resistance Monitoring (PURM) Branch, Aberdeen Proving Ground, MD 21010.

Table 1. Species, month of mosquito collection, insecticide tested, number of individuals tested, percent mortality at the diagnostic time, time of and mortality at the end of the test, and resistance status. For test end time, the number in parentheses is the percent mortality when the test ended. Concentrations for technical-grade insecticides were permethrin (30.3% *cis* and 61.5% *trans* isomers) (43 µg/bottle), deltamethrin (0.75 µg/bottle), etofenprox (12.5 µg/bottle), and chlorpyrifos (20 µg/bottle).

Insecticide	Location	Month	*N*	Time of test end (min) or 100% mortality [1]	Mortality at diagnostic time (%)	Resistance status
Culex pipiens						
Chlorpyrifos	Colony	Apr	121	55	100	Susceptible
Chlorpyrifos	Carroll	Jun	94	120 (98.9)	91.9	Possible
Chlorpyrifos	Casey	Aug	95	100 (95.8)	94.7	Possible
Chlorpyrifos	Henry	Apr	97	130 (94.8)	75.3	Resistant
Chlorpyrifos	Humphreys	Jul	95	100 (95.8)	94.7	Possible
Deltamethrin	Henry	May	94	120 (94.7)	28.7	Resistant
Permethrin	Colony	Apr	121	20	100	Susceptible
Permethrin	Carroll	Jun	96	125 (99)	77.1	Resistant
Permethrin	Casey	Aug	86	40	96.5	Possible
Permethrin	Henry	Apr	98	135 (96.9)	25.5	Resistant
Permethrin	Humphreys	Oct	100	55	77	Resistant
Aedes albopictus						
Chlorpyrifos	Humphreys	Jul	90	45	100	Susceptible
Deltamethrin	Humphreys	Jul	60	35	98.3	Susceptible
Permethrin	Carroll	Aug	94	10	100	Susceptible
Permethrin	Humphreys	Jul	66	15	90.9	Possible
Ae. koreicus						
Chlorpyrifos	Carroll	Jun	102	60	97.1	Susceptible
Chlorpyrifos	Carroll	Jun	107	50	97.2	Susceptible
Chlorpyrifos	Casey	Jul	98	60	96.6	Possible
Deltamethrin	Carroll	Jun	101	40	94.4	Possible
Deltamethrin	Casey	Jul	97	40 (99)	97.8	Susceptible
Etofenprox	Carroll	Jun	81	60 (99)	88.9	Resistant
Etofenprox	Casey	Jul	91	60 (95)	70.2	Resistant
Permethrin	Carroll	Jun	101	35	99	Susceptible
Permethrin	Carroll	Jun	109	30	100	Susceptible
Permethrin	Casey	May	96	50	71.6	Resistant
Permethrin	Casey	Jul	97	30	100	Susceptible

[1] Values in parentheses represent the mortality observed at the time listed.

es in their status over time. This information will be used to determine the operational impact of IR and guide the adoption of alternative mosquito control methods. These surveillance efforts are part of a standardization initiative to detect IR on US military installations in the ROK.

Beginning in April 2022, mosquito larvae and pupae were collected from USAG Daegu (Camps Carroll, Henry), USAG Yongsan-Casey (Camp Casey), and USAG Humphreys (Camp Humphreys), returned to the entomology laboratory on Camp Humphreys, and reared to the adult stage. In the laboratory, 100 to 150 larvae were placed in 1.5 liters of water in $27 \times 6 \times 16$-cm plastic rearing trays and provided powdered fish food and ground calf feed (1:1), and kept at 26°C room temperature in 26°C water on a cycle of 12 h light and 12 h dark. Pupae and 4th-stage larvae were moved to screen cages, and adults that emerged were provided 10% sugar solution ad libitum. Following CDC guidance, male and female adult mosquitoes, aged from 3 to 7 days old, were used in the assays (McAllister and Scott 2020). A colony of *Cx. pipiens molestus* (Forskal) maintained in the Camp Humphreys entomology laboratory since 2018 was also tested. *Culex pipiens* in the ROK is a mix of *Cx. pipiens pallens* (Coquillett) and *Cx. p. molestus* (Ryu and Shik 2022). The mosquitoes tested from field collections were only identified morphologically to the *Cx. pipiens* level. All mosquitoes were identified as adults using Lee and Egan (1985).

Technical-grade permethrin and chlorpyrifos (PESTANAL® analytical standard) were obtained from Yuillabtech, Seoul, South Korea. Technical-grade insecticides mixed with acetone and 250-ml Wheaton bottles were used for the assays. Insecticide concentrations published in the CDC Manual (McAllister and Scott 2020) were used: permethrin (30.3% *cis* and 61.5% *trans* isomers) (43 µg/bottle), deltamethrin (0.75 µg/bottle), etofenprox (12.5 µg/bottle), and chlorpyrifos (20 µg/bottle).

Diagnostic times used in this work were selected based on CDC guidelines for mosquito populations in the USA (McAllister and Scott 2020). The diagnostic times for *Cx. pipiens* and *Ae. albopictus* were followed but since no diagnostic times have been published for *Ae. koreicus*, the times were chosen as conservative estimates to establish a baseline for

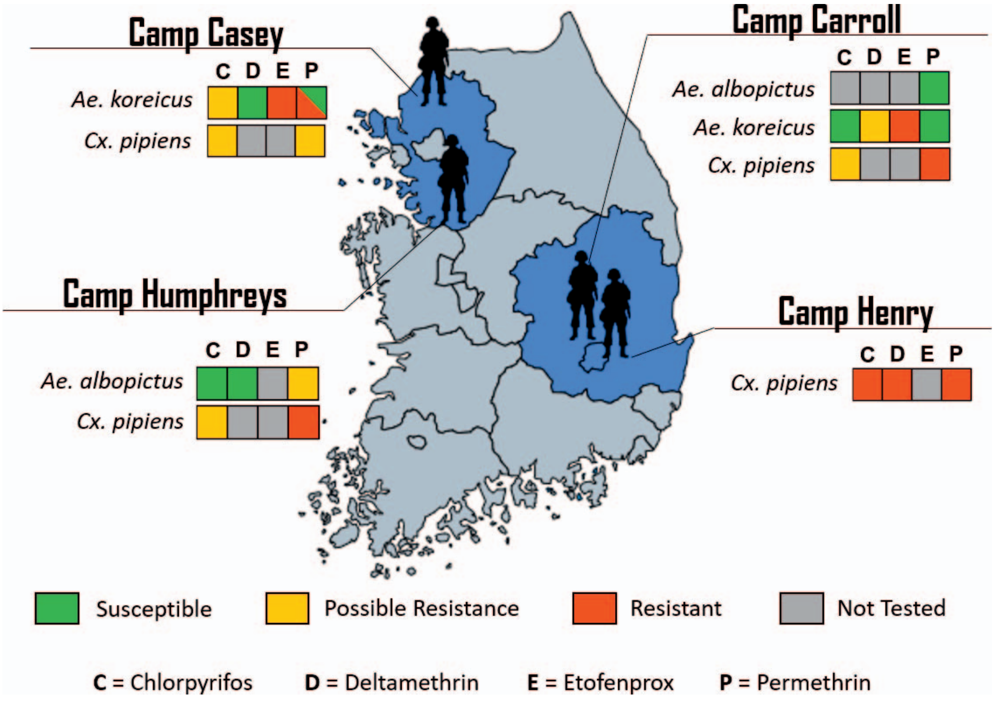

Fig. 1. Results of CDC Bioassays for *Aedes albopictus*, *Aedes koreicus* and *Culex pipien*s on U.S. Army Garrisons in the Republic of Korea in 2022 following the CDC criteria: Green = 97% to 100% mortality at the diagnostic time indicating susceptibility, Yellow = 90% to 96% mortality at the diagnostic time indicating possible resistance, Red = <90% mortality at the diagnostic time indicating confirmed resistance, and Grey = not tested. Concentrations for technical grade insecticides were chlorpyrifos (20 µg/bottle), deltamethrin (0.75 µg/bottle), etofenprox (12.5 µg/bottle), and permethrin (30.3% cis and 61.5% trans isomers) (43 µg/bottle).

future comparisons between ROK populations (McAllister and Scott 2020). Until diagnostic curves for each insecticide and species can be completed for CDC bottle bioassays in the ROK, comparing these results with other geographic areas should be done with caution. The following diagnostic times were used: *Cx. pipiens*—permethrin (30 min), deltamethrin (45 min), and chlorpyrifos (90 min); *Ae. albopictus*—permethrin (10 min), deltamethrin (30 min), and chlorpyrifos (45 min); *Ae. koreicus*—permethrin, deltamethrin, etofenprox (30 min), and chlorpyrifos (45 min).

The CDC guidance (McAllister and Scott 2020) was used to determine if resistance was present: 97% to 100% mortality at the diagnostic time indicates susceptibility, 90% to 96% mortality at the diagnostic time indicates the population is possibly developing resistance, and <90% mortality at the diagnostic time indicates resistance. If mortality was between 3% and 10% in the controls, Abbott's formula was used to correct mortality in treated bottles before resistance or susceptibility was determined (Abbott 1925). If control mortality was >10% the test was discarded.

Based on our interpretation of the assay results, no resistance to permethrin and chlorpyrifos was detected in the *Cx. pipiens* colony, which suggests that the colony represents a susceptible population and can be used in comparisons with field-collected samples (Table 1). In field-collected *Cx. pipiens*, resistance was detected to permethrin, deltamethrin, and chlorpyrifos Fig. 1 and Table 1). No resistance was detected to deltamethrin or chlorpyrifos in *Ae. albopictus*, with one population showing possible resistance to permethrin (Fig. 1 and Table 1). In *Ae. koreicus*, for chlorpyrifos all populations had mortalities at the selected diagnostic time, indicating susceptibility (Fig. 1 and Table 1). For permethrin, 3 of 4 populations had 99% to 100% mortalities at the selected diagnostic time, indicating susceptibility with one population with 71.6% mortality at the diagnostic time, indicating resistance (Table 1). One population of *Ae. koreicus* for deltamethrin had 94.4% mortality at the diagnostic time, indicating possible resistance, but at 40 min >99% mortality was observed for both populations (Table 1). Both populations for etofenprox had <90% mortality at the selected diagnostic time and >95% mortality at 60 min. Following CDC guidelines, this would indicate resistance; however, more tests for all active ingredients need to be completed to verify the results.

These results support published reports that IR in ROK mosquito populations is present and varies geographically (Shin et al. 2012). The IR in *Cx. pipiens* has been identified in the ROK, with Shin et al. (2012) reporting varying levels of phenotypic resistance to chlorpyrifos, deltamethrin, and permethrin, and Park et al. (2020) also reporting phenotypic resistance to chlorpyrifos and deltamethrin. Lee et al. (2020) and Park et al. (2020) reported the presence of L1014F genes in *Cx. pipiens* from populations across the ROK, indicating widespread pyrethroid resistance. Both reported no G119 or G119S mutations in any *Cx. pipiens* populations examined, which suggests that the resistance found in certain populations, such as the *Cx. pipiens* from Camp Henry (Fig. 1 and Table 1), may be due to a non-acetylcholinesterase resistance mechanism. Ryu et al. (2019) reported no *kdr* mutations found in *Ae. koreicus*, but the CDC bottle bioassays indicated resistance to etofenprox and permethrin, with possible resistance to deltamethrin (Table 1 and Fig. 1). Additional tests on these populations are needed to confirm the results. Park et al. (2020) found no phenotypic resistance or L1014F or G119S mutations in *Ae. albopictus* in the ROK; however, possible resistance to permethrin was detected on Camp Humphreys (Fig. 1 and Table 1), but additional confirmatory testing is required.

By establishing a program on USAGs and other US military installations using the CDC bottle bioassay, resistance levels can be tested and tracked over time in a consistent, repeatable manner. The information can be used to gain insight on the impact IR may have on the effectiveness of mosquito management programs and personal protective measures such as factory-treated uniforms.

This study was funded by the Armed Forces Health Surveillance Division, Global Emerging Infections Surveillance (GEIS) Branch, ProMIS ID P0010_22_ME. The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Defense Health Agency, Department of Defense, nor the US Government. All authors are employees of the US Government, and this work was prepared as part of their official duties.

REFERENCES CITED

Abbott WS. 1925. A method of computing the effectiveness of an insecticide. *J Econ Entomol* 18:265–267.

Carder JB, Stoops CA, Chong ST, Kim MS, Mahabir S, Cinkovich SS. 2023. Monitoring insecticide resistance in mosquitoes in U.S. Military Areas of Operations. *US Army Med Dept J* Winter Issue (in press).

Estep AS, Sanscrainte ND, Cuba I, Allen GM, Becnel JJ, Linthicum KJ. 2020. Failure of permethrin-treated military uniforms to protect against a laboratory-maintained knockdown-resistant strain of *Aedes aegypti*. *J Am Mosq Control Assoc* 36:127–130.

Hwang MJ, Kim HC, Klein TA, Chong ST, Sim K, Chung Y, Cheong HK. 2020. Comparison of climatic factors on mosquito abundance at US Army Garrison Humphreys, Republic of Korea. *PLoS One* 15:e0240363.

Kim H, Cha GW, Jeong YE, Lee WG, Chang KS, Roh JY, Yang SC, Park MY, Park C, Shin EH. 2015. Detection of Japanese encephalitis virus genotype V in *Culex orientalis* and *Culex pipiens* (Diptera: Culicidae) in Korea. *PLoS One* 10:e0116547.

Lee DE, Kim HC, Chong ST, Klein TA, Choi KS, Kim YH, Kim JH, Lee SH. 2020. Regional and seasonal detection of resistance mutation frequencies in field populations of *Anopheles* Hyrcanus Group and *Culex pipiens* complex in Korea. *Pestic Biochem Physiol* 164:33–39.

Lee KW, Egan PJ. 1985. *Illustrated taxonomic keys to genera and species of female mosquitoes of Korea (Diptera: Culicidae)*. City, ST: Department of the Army, 5th Medical Detachment, 18th Medical Command. 32 p. Available from the Defense Technical Information Center, Fort Belvoir, VA. ADA598439.pdf (dtic.mil).

Lee KW, Gupta RK, Wildie JA. 1984. Collection of adult and larval mosquitoes in U.S. Army Compounds in the Republic of Korea during 1979–1983. *Kisaengchunghak Chapchi* 22:102–108.

McAllister JC, Scott M. 2020. *CONUS manual for evaluating insecticide resistance in mosquitoes using the CDC bottle bioassay kit* [Internet]. Fort Collins, CO: US Centers for Disease Control and Prevention [accessed January 20, 2023]. Available from: https://www.cdc.gov/zika/pdfs/CONUS-508.pdf.

Park SH, Jun H, Ahn SK, Lee J, Yu SL, Lee SK, Kang JM, Kim H, Lee H, Hong SJ, Na BK, Bahk Y, Kim TS. 2020. Monitoring insecticide resistance and target site mutations of L1014 *Kdr* and G110 *ACE* alleles in five mosquito populations in Korea. *Korean J Parasitol* 58:543–550.

Ryu J, Hwang DU, Bae YJ, Lee SH, Kim YH, Lee SY, Jung HY, Choi KS. 2019. Preliminary report of knockdown resistance in *Culex pipiens pallens* and *Aedes koreicus* from Korea. *Entomol Res* 49:432–435.

Ryu J, Shik CK. 2022. Species diversity of the *Culex pipiens* complex in the Republic of Korea. *Entomol Res* 52:376–381.

Seo MG, Lee HS, Yang SC, Noh BE, Kim TK, Lee WG, Lee HI. 2021. National monitoring of mosquito populations and molecular analysis of *Flavivirus* in the Republic of Korea in 2020. *Microorganisms* 9:2085.

Shin EH, Kim NJ, Kim HK, Park C, Lee DK, Ahn YJ, Chang KS. 2012. Resistance of field-collected populations of *Culex pipiens pallens* (Diptera: Culicidae) to insecticides in the Republic of Korea. *J Asia-Pac Entomol* 15:1–4.