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SCIENTIFIC NOTE

CDC BOTTLE BIOASSAYS FOR DETECTING INSECTICIDE RESISTANCE IN *AEDES ALBOPICTUS, AEDES KOREICUS,* AND *CULEX PIPIENS* FROM U.S. ARMY GARRISONS IN THE REPUBLIC OF KOREA (2023 AND 2024)

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ABSTRACT. Mosquito-borne pathogens are a threat to US troops stationed in the Republic of Korea. From April 2023 until December 2024, larvae and pupae of *Aedes albopictus*, *Ae. koreicus*, and *Culex pipiens* collected from U.S. Army garrisons were screened for resistance to chlorpyrifos, deltamethrin, etofenprox and permethrin using the Centers for Disease Control and Prevention bottle bioassay. Colonies of *Cx. pipens* var. *molestus* from Camp Humphreys, Camp George, and the Busan Storage Facility were established and tested against permethrin and permethrin + pieronyl butoxide (PBO). No resistance to deltamethrin or chlorpyrifos was detected in *Ae. albopictus* populations, but 1 population from Camp George showed possible resistance to permethrin. *Aedes koreicus* indicated possible resistance to etofenprox from Camp Casey in 2023 and resistance to etofenprox from Camp Casey in 2024. All populations of *Ae. koreicus* tested were susceptible to chlorpyifos, permethrin, and deltamethrin. *Culex pipiens* field-collected populations were susceptible to chlorpyrifos and resistant to permethrin, etofenprox, and deltamethrin. Exposing laboratory *Cx. pipiens* var. *molestus* to PBO and then permethrin restored susceptibility to all 3 colonies. Screening will be ongoing to track changes in resistance status for these species.

KEY WORDS *Aedes albopictus*, *Aedes koreicus*, CDC Bottle Bioassay, *Culex pipiens*, Republic of Korea

Mosquito-borne pathogens are a threat to US personnel deployed to the Republic of Korea (ROK). Insecticide resistance in mosquito populations has persisted for many years in the ROK driven by public health and agricultural selection pressure (Lee et al. 2023). Insecticides such as etofenprox are used to control mosquitoes on US Army garrisons in the ROK, and US Army soldiers wear uniforms that are factory treated with permethrin to protect against vector-borne pathogens. Because of this an insecticide resistance surveillance program was established on US Army garrisons (USAG) Daegu (Camps Carroll and Henry and the Busan Storage Facility), USAG Yongsan-Casey (Camp Casey), and USAG Humphreys (Camp Humphreys) to gain a better picture of the insecticide resistance status of 3 medically important species: *Aedes albopictus* (Skuse), *Ae. koreicus* (Edwards), and *Culex pipiens* (L.) (Stoops et al. 2023). In 2022, this surveillance program found *Cx. pipiens* populations resistant to permethrin and deltamethrin, and *Ae. koreicus* was reported resistant to etofenprox and permethrin (Stoops et al. 2023). Possible resistance to permethrin was found in *Ae. albopictus* (Stoops et al. 2023). In 2023 and 2024, additional Centers for Disease Control (CDC) bottle bioassays were conducted with populations of these 3 species from the same installations from 2022 to continue to delineate insecticide resistance status on USAGs. Colonies of *Cx. pipiens molestus* Forskal were established in 2022 and 2023 from Busan Storage facility, Camp George, and Camp Humphreys and tested over time including assays using piperonyl butyl oxide (PBO) as a synergist.

Methods for mosquito collection, rearing, and CDC bottle bioassays were followed from Stoops et al. (2023) and McAllister and Scott (2020). Technical grade chlorpyrifos, deltamethrin, etofenprox, and permethrin (PESTANAL® analytical standard) were obtained from Yuillabtech, Seoul, South Korea and mixed with acetone in 250-ml Wheaton bottles for use in 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). For *Cx. pipiens* and *Ae. albopictus*, the CDC Manual diagnostic (McAllister and Scott 2020) times were used: *Cx. pipiens*: etofenprox (15 min), permethrin (30 min), deltamethrin (45 min), and chlorpyrifos (90 min; *Ae. albopictus*: permethrin (10 min), deltamethrin (30 min), and chlorpyrifos (45 min. No diagnostic times have been published for *Ae. Koreicus*, so the same diagnostic times published in Stoops et al. (2023) were used for *Ae. koreicus*: permethrin, deltamethrin, etofenprox (30 min), and chlorpyrifos (45 min). To evaluate potential metabolic resistance mechanisms, mosquitoes were preexposed for 1 h to PBO (400 µg/bottle in 1 ml absolute ethanol). After exposure, they were transferred to a holding container for an additional h before the bioassays were conducted. The CDC

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Table 1. Values in parentheses represent the mortality observed at the time listed. LCHC = Lake Camp Humphreys Colony established from larval collections on Camp Humphreys in July 2022. CGC = Camp George Colony established from larval collection in April 2023. BSFC = Busan Storage Facility Colony established from larval collections in June 2023. Only minutes of 100% mortality are reported for species without known diagnostic time.

Insecticide	Location	Month	*N*	Time of test end *or* 100% mortality (min)	Mortality at diagnostic time (%)	Resistance level
Culex pipiens						
2023						
Chlorpyrifos	LCHC	Apr	115	90	100	Susceptible
Chlorpyrifos	George	Apr	104	60	100	Susceptible
Chlorpyrifos	Busan	Jun	95	60	100	Susceptible
Deltamethrin	George	Apr	94	130 (46.5%)	24.4	Resistant
Etofenprox	George	May	94	130 (91.2%)	21.6	Resistant
Etofenprox	Busan	Jul	101	140 (95%)	46.5	Resistant
Permethrin	LCHC	Apr	97	135	74.2	Resistant
Permethrin	Henry	Apr	112	150 (99%)	80.4	Resistant
Permethrin	George	Apr	89	140 (86.5%)	28.1	Resistant
Permethrin	George	Apr	100	140 (93%)	51	Resistant
Permethrin	Busan	Jun	105	140 (98.1%)	54.3	Resistant
Permethrin	LCHC	Dec	106	85	94.3	Possible
Permethrin	LCHC	Dec	103	45	95.1	Possible
Permethrin + PBO[1]	LCHC	Dec	112	20	100	Susceptible
Permethrin + PBO	LCHC	Dec	102	20	100	Susceptible
Permethrin	CGC	Dec	108	130 (93.5%)	21.3	Resistant
Permethrin + PBO	CGC	Dec	54	35	98.1	Susceptible
Permethrin + PBO	CGC	Dec	120	40	99.2	Susceptible
Permethrin	BSFC	Dec	108	130	74.1	Resistant
Permethrin + PBO	BSFC	Dec	78	50 (96.2)	94.9	Possible
Permethrin + PBO	BSFC	Dec	98	50	92.9	Possible
2024						
Chlorpyrifos	Henry	May	99	80	100	Susceptible
Chlorpyrifos	Walker	May	105	100	99	Susceptible
Permethrin	LCHC	Feb	97	65 (100%)	87.6	Resistant
Permethrin	LCHC	Feb	102	120 (98%)	89.2	Resistant
Permethrin	LCHC	Feb	105	85 (100%)	89.5	Resistant
Permethrin	BSFC	Feb	81	150 (98.8%)	70.4	Resistant
Permethrin	CGC	Feb	103	150 (94.2%)	80.6	Resistant
Permethrin	Henry	May	100	135 (84%)	31.0	Resistant
Permethrin	Henry	May	100	120 (100%)	58.4	Resistant
Permethrin	Walker	May	100	135 (93%)	68.0	Resistant
Etofenprox	Henry	May	103	130 (87.4%)	13.6	Resistant
Etofenprox	Henry	May	101	130 (98%)	30.7	Resistant
Etofenprox	Walker	May	103	135 (91.3%)	40.8	Resistant
Deltamethrin	Henry	May	97	135 (97.9%)	64.9	Resistant
Deltamethrin	Walker	May	99	105(100%)	68.7	Resistant
Aedes albopictus						
2023						
Chlorpyrifos	Humphreys	Sep	99	35	100	Susceptible
Deltamethrin	Humphreys	Sep	59	30	100	Susceptible
Permethrin	George	Apr	66	15	92.4	Possible
Permethrin	Humphreys	Sep	97	10	100	Susceptible
Aedes koreicus						
2023						
Chlorpyrifos	Casey	May	96	55	100	Susceptible
Chlorpyrifos	Stanley	May	104	45	100	Susceptible
Deltamethrin	Casey	May	109	45	100	Susceptible
Deltamethrin	Stanley	May	109	45	100	Susceptible
Etofenprox	Casey	May	91	40	91	Possible
Etofenprox	Stanley	May	91	40	91.7	Possible
Permethrin	Casey	Apr	104	30	100	Susceptible
Permethrin	Casey	Apr	85	30	100	Susceptible
Permethrin	Stanley	May	109	35	99.1	Susceptible
Permethrin	Hovey	May	95	30	100	Susceptible

Table 1. Continued.

Insecticide	Location	Month	*N*	Time of test end *or* 100% mortality (min)	Mortality at diagnostic time (%)	Resistance level
2024						
Chlorpyrifos	Casey	May	104	45	100	Susceptible
Chlorpyrifos	Casey	May	105	45	100	Susceptible
Permethrin	Casey	May	103	35	99	Susceptible
Permethrin	Casey	May	102	30	100	Susceptible
Etofenprox	Casey	May	96	50	86.5	Resistant
Etofenprox	Casey	May	103	55	84.5	Resistant
Deltamethrin	Casey	May	103	45	97.1	Susceptible

[1] Piperonyl butoxide.

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, then 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.

As shown in Table 1, resistance in field collected *Cx. pipiens* was detected to deltamethrin, etofenprox, and permethrin in 2023 and 2024. The *Cx. pipiens* populations tested in 2022 showed possible resistance or confirmed resistance to chlorpyrifos (Stoops et al. 2023). However, chlorpyrifos resistance was not detected in any population in 2023 and 2024. No resistance was detected to deltamethrin or chlorpyrifos in *Ae. albopictus*, and as in 2022, 1 population in 2023 showed only possible resistance to permethrin. No populations of *Ae. albopictus* were tested in 2024. In 2023 and 2024, no resistance to chlorpyrifos, deltamethrin, or permethrin was detected in *Ae. koreicus*; however, in 2023, possible resistance to etofenprox was detected at Camp Casey, and in 2024, the Camp Casey population tested was confirmed resistant.

Figure 1 presents mortality against permethrin for field-collected *Cx. pipiens* and colony-reared *Cx. pipiens* var. *molestus* across 3 locations (Busan Storage Facility, Camp Humphreys, and Camp George) in 2022 to 2024. As was reported in Stoops et al. (2023), the 2018 colony of *Cx. pipiens* var. *molestus* was fully susceptible to permethrin and field collected *Cx. pipiens* from Camp Humphreys were resistant to permethrin in October 2022 (77% mortality). A colony of *Cx. pipiens* var. *molestus* was established in October 2022 from Camp Humphreys (Lake Camp Humphreys colony [LCHC]) and tested against permethrin in November 2022 (56.5% mortality), December 2022 (47.6% mortality), and April 2023 (74.2% mortality). Assays against permethrin were done again on the same LCHC in December 2023 with mortalities of 94.3% and 95.1%. At the same time, using mosquitoes from the same LCHC, assays of permethrin + PBO yielded 100% mortality at the 30-min diagnostic time. In February 2024, the LCHC colony was tested against permethrin again, yielding mortalities of 87.6%, 89.2%, and 89.5%. From Camp George 2 assays of field collected *Cx. pipiens* in April 2023 resulted in 28.1% and 51% mortalities against permethrin. In April 2023, a colony of *Cx. pipiens* var. *molestus* was established from Camp George (Camp George Colony [GCG]). In December 2023, CGC mosquitoes were tested against permethrin that gave 21.3% mortality at the diagnostic time. Two simultaneous assays of permethrin + PBO against CGC in December 2023 resulted in 98.1% mortality and 99.2% mortality. In February 2024 the CGC was tested against permethrin only with 80.6% mortality. Field-collected *Cx. pipiens* from the Busan Storage Facility were tested in June 2023 and resulted in 54.3% mortality. A colony of *Cx. pipiens* var. *molestus* established in June 2023 from the Busan Storage Facility (Busan Storage Facility Conlony [BSFC]), showed 74.1% mortality when tested against permethrin in December 2023. In 2 simultaneous assays of BSFC against permethrin + PBO resulted in 94.9% mortality and 92.9% mortality. In February 2024, BFSC was tested against permethrin only with 70.4% mortality.

All populations of the 3 species were fully susceptible to chlorpyrifos in 2023 and 2024. For field collected *Cx. pipiens* in 2023 and 2024, resistance to deltamethrin, etofenprox, and permethrin was widespread. *Aedes albopictus* were susceptible to all pyrethroids tested in 2023. *Aedes koreicus* was susceptible to deltamethrin and permethrin; however, to etofenprox it showed possible resistance in 2023 and resistance in 2024 from Camp Casey. Etofenprox is one of the most widely used insecticides in the ROK for mosquito control (Kim et al. 2024). Unlike Camp Humphreys, which applies etofenprox, no garrison-wide mosquito control program is conducted on Camp Casey using any pyrethroid, so it is possible that the resistance is coming from the surrounding community of Dongducheon. Etofenprox differs from permethrin as it is a nonester pyrethroid, but resistance has been reported to etofenprox in *Cx. pipiens* (Richards et al. 2017). The resistance in *Ae. koreicus* is at our self-established diagnostic time of 30 min, and until more in-depth mortality curves are

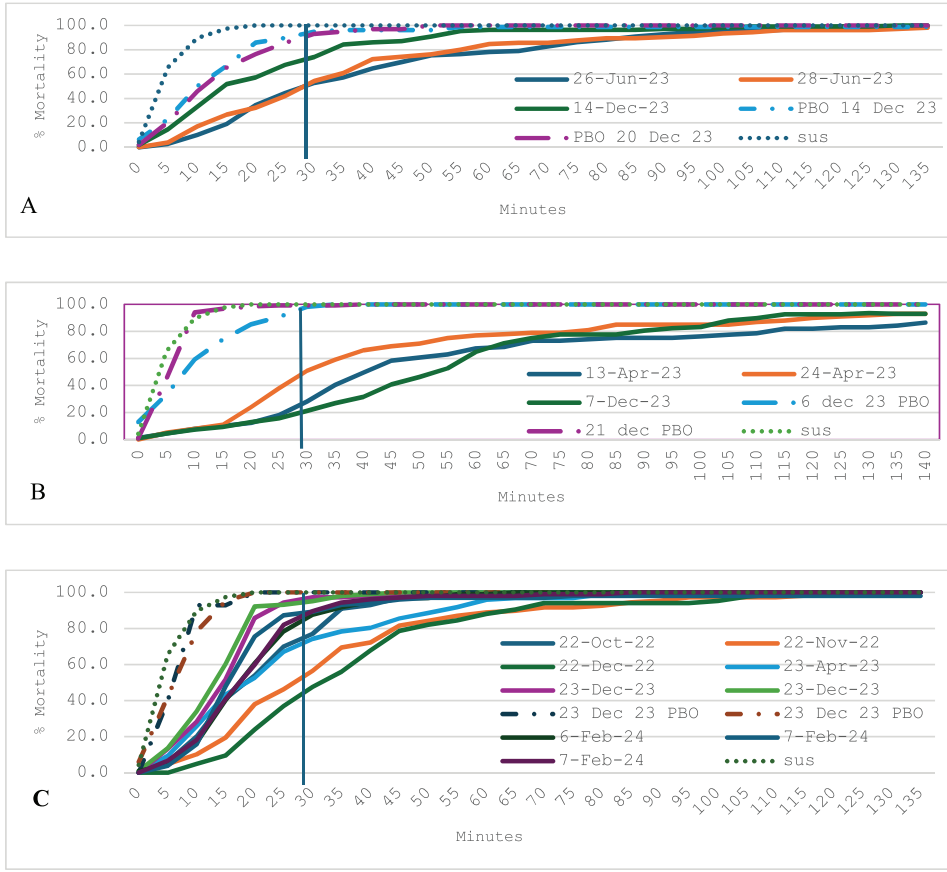

Fig. 1. Graphs represent % mortality and time for all permethrin assays for the field-collected *Culex pipiens* and permethrin and permethrin + PBO colony *Cx. pipiens* var. *molestus* from 2022, 2023, 2024 from Busan Storage Facility, Camp George, and Camp Humphreys. The dotted line labeled "sus" is the *Cx. pipiens* var. *molestus* colony established in the Camp Humphreys Entomology lab in 2018 and is provided in all three graphs for reference. Vertical line is the diagnostic time for permethrin, 30 min. (A) Assays with permethrin and permethrin + PBO for *Cx. pipiens* field collections and *Cx. pipiens* var. *molestus* from the Busan Storage Facilty Colony (BSFC). (B) Assays with permethrin and permethrin + PBO for *Cx. pipiens* field collections and *Cx. pipiens* var. *molestus* from Camp George Colongy (CGC). (C) Assays with permethrin and permethrin + PBO for *Cx. pipiens* field collections and *Cx. pipiens* var. *molestus* from Camp Humphreys (Lake Camp Humphreys Colongy [LCHC]).

calculated for *Ae. koreicus* and etofenprox, it is possible that we are either under- or overestimating the level of resistance in this population. However, our findings provide a baseline for *Ae. koreicus* to compare future results with this insecticide (Richards et al. 2017). Understanding etofenprox resistance for the US military is critical as etofenprox has an Environmental Protection Agency approval as an alternative to permethrin for treating uniforms (EPA 2016).

Because of the importance of permethrin in protecting soldiers from vector-borne pathogens, testing on the 3 colonies was focused on permethrin. No field-collected *Cx. pipens* were tested against permethrin following exposure to PBO, but PBO exposure restored susceptibility to permethrin to the 3 colonies of *Cx. pipiens* var. *molestus*, indicating metabolic resistance mechanisms may be involved.

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