■ ORIGINAL ARTICLE

Monitoring Insecticide Resistance and Target Site Mutations of L1014 *Kdr* And G119 *Ace* Alleles in Five Mosquito Populations in Korea

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Abstract: Mosquitoes are globally distributed and important vectors for the transmission of many human diseases. Mosquito control is a difficult task and the cost of preventing mosquito-borne diseases is much lower than that for curing the associated diseases. Thus, chemical control remains the most effective tool for mosquito. Due to the long-term intensive use of insecticides to control mosquito vectors, resistance to most chemical insecticides has been reported. This study aimed to investigate the relationship between insecticide resistance and target site mutation of L1014 *kdr* and G119 *ace* alleles in 5 species/species group of mosquitoes (*Aedes vexans*, *Ae. albopictus*, *Anopheles* spp., *Culex pipiens* complex, and *Cx. tritaeniorhynchus*) obtained from 6 collection sites. For *Anopheles* spp., the proportion of mosquitoes with mutated alleles in L1014 was 88.4%, homozygous resistant genotypes were observed in 46.7%, and heterozygous resistant genotypes were observed in 41.8%. For the *Cx. pipiens* complex and *Cx. tritaeniorhynchus* species, homozygous resistant genotypes were found in 25.9% and 9.8%, respectively. However, target site mutation of L1014 in the *Ae. vexans nipponii* and *Ae. albopictus* species was not observed. *Anopheles* spp., *Cx. pipiens* complex, and *Cx. tritaeniorhynchus* mosquitoes were resistant to deltamethrin and chlorpyriphos, whereas *Ae. vexans nipponii* and *Ae. albopictus* were clearly susceptible. We also found a correlation between the resistance phenotype and the presence of the L1014 *kdr* and G119 *ace* mutations only in the *Anopheles* spp. population. In this study, we suggest that insecticide resistance poses a growing threat and resistance management must be integrated into all mosquito control programs.

Key words: *kdr*, *ace*, insecticide resistance, mutation

INTRODUCTION

Global warming affects the survival and density of poikilothermic animals, including insects. The temperature in Korea is rising rapidly due to climate change [1]. Climate change is a major factor in mosquito ecology and provides conditions that are more favorable for the prevalence of mosquito-borne diseases along with new urban development or population growth [2]. In addition, countermeasures for infectious diseases must be prepared to prevent foreign mosquito-borne diseases from entering Korea [2-4].

In this sense, vector control of mosquitoes has become a pivotal portion of the global strategy to manage mosquito-associated diseases, especially malaria, Japanese encephalitis, West Nile encephalitis, and dengue fever. In this effort, insecticides, such as chlorpyriphos (*O*, *O*-diethyl *O*-(3,5,6-trichloropyridin-2-yl) phosphorothioate) and pyrethroids, are the most important tool. Chlorpyriphos is a broad-spectrum organophosphate insecticide widely used as a pesticide to control a variety of insects [4]. Pyrethroids, which are analogous to naturally occurring pyrethrins, are a class of insecticides com-

monly used for indoor spraying against mosquitoes due to their insecticidal potency and environmental stability as well as safety [5,6]. Although massive usage of insecticides was highly effective in controlling insect-borne diseases in the past decades, the widespread development of resistance in insects, especially mosquitoes, is now causing serious issues in many regions [7-9].

After prolonged exposure to an insecticide over several generations, mosquito populations may evolve resistance against the insecticide. Since mosquitoes can produce many generations per year, high levels of resistance can evolve very quickly. It has been shown that multiple resistance mechanisms are specific to individual mosquito species [10,11]. Overall, insecticide resistance is not only conferred via multiple mechanisms as well as reduced cuticle penetrance, but also mediated through the interaction of regulatory genes and resistance genes. However, it is not evident which and how various genes are involved.

The knockdown resistance *kdr* gene is the primary mechanism responsible for resistance to dichlorodiphenyltrichloroethane (DDT) and pyrethroids [12-14]. The enzyme synaptic acetylcholinesterase (AChE1) is the target of organophosphorus and carbamate, which are competitive inhibitors of acetylcholine [15,16]. Genomic studies commonly focus on single nucleotide polymorphisms because they can be readily identified with short region sequencing. However, it is evident that structural variants, including inversions, duplications, and deletions may be involved in adaptation and speciation within mosquitoes [17,18]. In this study, we examined sequence variations in specific genes, including knockdown resistance (*kdr*) (L1014 in domain II segment 6) and acetylcholine esterase (*ace*) (G119 region) collected from mosquitoes at various sampling locations comprising *Ae. vexans nipponii*, *Ae. albopictus*, *Anopheles* spp., *Cx. pipiens* complex, and *Cx. tritaeniorhynchus*.

MATERIALS AND METHODS

Mosquito collection

In general, mosquitoes have different insecticide resistances depending on their habitat. Thus, mosquito collection sites for this study were considered depending on the type of mosquito habitat. Adult mosquitoes were collected using unbaited black-light (BL) traps (SC-2000, Shinyeong Co., Namyangju, Korea), BG-Sentinel™ (BGS) traps (Biogents, Regenburg, Germany), or hand-held battery-powered aspirators at each sample collec-

tion site in summer 2017 and 2018. The mosquitoes were identified and classified according to morphology-based specialized taxonomic keys [19-22] and compared with standard specimens in the collection of the School of Medicine, Inha University, and the Korea Centers for Diseases Control and Prevention (KCDC). Collections were made over a minimum of 2 days at each site. The collected mosquitoes were pooled by species/species group, collection site, and time of survey, and quickly transferred to microcentrifuge tubes at 4°C where the species/species group were identified. The mosquitoes were assayed to examine target site sequence variations in specific genes, including knockdown resistance (*kdr*, L1014 in domain II) and acetylcholine esterase (*ace*, G119). For insecticide resistance bottle assays, living mosquitoes were transferred to the School of Medicine, Inha University, as quickly as possible and assayed according to the standard method described below in the section "insecticide resistance bottle assays". Table 1 lists the geographic locations, collection methods, and numbers of the collected species/species group of mosquitoes. A total of 7,720 adult mosquitoes comprising the 5 species/species group of interest (*Ae. vexans nipponii* (n=1,708), *Ae. albopictus* (n=955), *Anopheles* spp. (n=2,744), *Cx. pipiens* complex (n=1,685), and *Cx. tritaeniorhynchus* (n=628), which were identified and classified according to morphology-based specialized taxonomic keys [19,20], were collected in summer 2017 and 2018 and the target site mutations were evaluated. L1014F *kdr* and G119S *ace* mutations were analyzed in 1,010 and 1,258 mosquitoes, respectively.

Insecticide resistance bottle bioassays

Adult mosquitoes were collected, were kept in cooled cages to increase survival and provided with 10% sucrose solution ad libitum until the time of testing. The bottle bioassays followed the KCDC and World Health Organization (WHO) guidelines [23]. In brief, mosquitoes were placed in a 250 ml glass Wheaton bottle coated with 1.25 mg/ml of deltamethrin (Pyrethroid) and chlorpyrifos (Organophosphate) diluted in acetone. The negative control bottle was treated with acetone only. Bottles were swirled so that the glass bottom and inside cap were coated and rotated while rocking so that the side were evenly coated with insecticide. An aspirator was used to gently add 25 mosquitoes into each bottle. Afterward, knockdown mosquitoes were recorded every 30 min for 6 hr. The exposure time for diagnosis resistance of each insecticide was set to 30 min. Collected mosquitoes were transferred into

Table 1. Collection sites, methods, and number of mosquitoes collected in summer 2017 and 2018

Species/species group	Aedes vexans nipponii		Aedes albopictus		Anopheles spp.		Culex pipiens complex		Culex tritaeniorhynchus	
Collection site	Hogok-ri, Hwaseong-si, Gyeonggi-do (Cowshed)		Arang-dong, Jeju Special Self-Governing Province	Incheon Grand Park, Incheon Metropolitan City	Hogok-ri, Hwaseong-si, Gyeonggi-do (Cowshed)		Hogok-ri, Hwaseong-si, Gyeonggi-do (Cowshed)		Seochang-dong, Gwangju Metropolitan City	Gorang-dong, Jeju Special Self-Governing Province
Collection method	Black-light traps	Aspirator	BG-Sentinel™ traps	Aspirator	Black-light traps	Aspirator	Black-light traps	Aspirator	Black-light traps	Aspirator
No. of mosquitoes collected	1,242	465	743	212	1,543	1,201	1,102	584	417	211
Total No. of mosquitoes collected	1,707		955		2,744		1,686		628	

Wheaton bottle coated with each insecticide and incubate for 30 min. After the incubation periods, all living mosquitoes were placed in a pint-sized paper cage with constant access to a 10% sucrose solution. The mortality was assessed after 24 hr. Surviving individuals were frozen at –70°C for molecular analysis.

Isolation of genomic DNA from mosquitoes.

Mosquito genomic DNA was extracted individually using the QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Briefly, the entire body of a single mosquito was homogenized in a microcentrifuge tube containing 80 μl of PBS using a pestle for 30 sec. The homogenized samples were incubated with 20 μl of proteinase K and 100 μl of ATL buffer at 56°C for 2 hr until completely lysed with occasional vortexing during incubation. After centrifuging quickly, 200 μl of AL buffer was added to the mixture followed by incubation at 70°C for 10 min and 200 μl of absolute ethanol was added with vigorous vortexing for thorough mixing. After brief centrifugation, the mixtures were applied to the QIAamp Mini Spin column. The mixture-applied column was washed with 1 ml of AW1 buffer followed by centrifugation at $6,000 \times g$ for 1 min and then with 1 ml of AW2 buffer followed by centrifuging at maximum speed for 3 min. The column-bound DNA was eluted with 150 μl of distilled water by centrifugation at $6,000 \times g$ for 1 min. The concentration of the extracted DNA was determined by measuring ultraviolet absorbance at 260 nm using a spectrophotometer (NanoDrop™ ND-1000, Thermo Fisher Scientific, Lafayette, Colorado, USA), after which the samples were stored at –70°C before use.

Detection of L1014 and G119 mutations

The primer set sequences and reaction conditions for the amplification of *kdr* and *ace* genes for each targeted mosquito are listed in (Supplementary Table S1). The specificity of the primers was confirmed using a BLAST search in the GenBank database from NCBI. PCR parameters were as follows: a denaturation step at 94°C for 5 min and then 30 cycles of a denaturation step at 94°C for 1 min, an annealing step at 52°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis in 1.5% agarose gel. The amplified DNA fragments were cloned into TA cloning vector (Promega Corporation, Madison, Wisconsin, USA) and sequenced by Macrogen Inc.

(Seoul, Korea). The DNA sequences were analyzed using DNASTAR and the NCBI-BLAST service.

RESULTS

Genotyping of target site mutations: *kdr* and *ace* genes

In this study, we investigated the status of resistance in 5 common mosquitoes (*Ae. vexans nipponii*, *Ae. albopictus*, *Anopheles* spp., *Cx. pipiens* complex, and *Cx. tritaeniorhynchus*) collected from different locations in Korea (Table 1). L1014F *kdr* and G119S *ace* mutations were analyzed in 1,010 and 1,258 mosquitoes, respectively. The frequency of target site mutations is presented in Table 2. *kdr* target site mutations in 1,010 mosquito samples were analyzed by sequencing of the Domain II region. For the L1014F *kdr* target site mutations, homozygous resistant genotypes (L1014F/L1014F and L1014C/L1014C) were observed in 17.5% (177/1,010) and in 0.7% (7/1,010) of 1,010 tested samples, respectively. Homozygous resistant genotypes (L1014C/L1014F and L1014/L1014F) were found in 5.5% (56/1,010) and 5.2% (53/1,010), respectively. A homozygous susceptible genotype (L1014/L1014) was found in 195 (19.3%) mosquitoes from a total of 1,010 sequenced samples. For *Anopheles* spp., the rate of mosquitoes with mutated alleles in the L1014 *kdr* gene was 88.4% (199/225 sequenced samples of *Anopheles* spp.), homozygous resistant genotypes were observed in 46.7% (105/225), and heterozygous resistant genotypes (L1014/L1014F and L1014C/L1014F) were observed in 41.8% (94/225). In practice, since it is difficult to classify species group of the genus *Anopheles* by morphological differences alone and it is hard to perform the PCR experiments for each *Anopheles* subspecies mosquito, we categorized mosquitoes in this genus as simply just *Anopheles* spp. It has been reported that there are various *Anopheles* subspecies in Korea including *Anopheles lesteri*, *An. sinensis*, *An. pullus*, *An. kleini*, and *An. belenrae* [24].

For *Cx. pipiens* complex and *Cx. tritaeniorhynchus*, homozygous resistant genotypes were found in 25.9% (68/263) and in 9.8% (11/112) of samples, respectively. Heterozygous resistant genotypes were found in 13.4% (15/112) of *Cx. tritaeniorhynchus* samples (Table 2). In addition, various point mutations in the Domain II region were amplified and analyzed in this study, including M976V, L982P, C983R, W986R, N1013D, and L1025M. However, as expected, target site mutation for L1014 in the *kdr* Domain II region was not observed in the *Ae. vexans nipponii* and *Ae. albopictus* species.

Table 2. Genetic variations in *kdr* and *ace* genes, which were found in this study

Gene locus	*kdr* (L1014)					*ace* (G119)				
Species	*Aedes vexans nipponii*	*Aedes albopictus*	*Anopheles* spp.	*Culex pipiens* complex	*Culex tritaeniorhynchus*	*Aedes vexans nipponii*	*Aedes albopictus*	*Anopheles* spp.	*Culex pipiens* complex	*Culex tritaeniorhynchus*
No. of total collected mosquitoes	1,707	955	2,744	1,686	628	1,707	955	2,744	1,686	628
No. of sequenced samples	138	272	225	263	112	134	272	238	441	173
No. of mosquitoes carrying mutated alleles (%)	0(0)	0(0)	199 (88.4)	68 (25.9)	26 (23.2)	0 (0)	0 (0)	188 (80.0)	0 (0)	0 (0)
Other point mutations in same allele or variation of target site (%)	M976V L982P C983R W986R W991E V996E P1003S F1005S A1007T N1013D	F959S D960G R960G R965Q T969A F971S M972T S974L R980Q V999Q V1010L/MC1011Y L1025M D1039G I1045S5T N1050D I1063S	L1014C (3.1) L1014C/ L1014F (24.9) L1004F (43.6) L1014/ L1014F (16.9) L1014 (11.5)	L1014 (74.1) L1014F (25.9)	L1014F (9.8) L1014/ L1014F (13.4)	H103R S109C G118S	H103Y M1123L I115F G117R G123D T126A	G119 (21.0) G119S (79.0)		

For the G119 *ace* target site mutation, the individuals with the homozygous susceptible genotype (G119) comprised 21% (50/238 sequenced samples in *Anopheles* spp.) and those with the homozygous resistant genotype (G119S) comprised 79% (188/238) (Table 2). However, target site mutations of G119 in the *ace* gene in the *Ae. vexans nipponii, Ae. albopictus, Cx. pipiens* complex, and *Cx. tritaeniorhynchus* species were not observed in this study. In addition, various point mutations in the *ace* gene were amplified and analyzed, including H103R, S109C, and G118S, especially in the *Culex* genus.

Insecticide susceptibility bioassays

Insecticide resistance bottle bioassays showed that the mosquitoes collected from the study areas exhibited 0-61.0% resistance to deltamethrin and chlorpyriphos. *Ae. vexans nipponii* and *Ae. albopictus* from the study areas showed no resistance to the insecticides tested (pyrethroids (deltamethrin) and organophosphates (chlorpyriphos)), which were used as target insecticides for L1014 *kdr* and G119 *ace* genes, respectively (Table 3). However, resistance was observed in a mosquito species group, *Anopheles* spp., with mortality rates of 86.1% (537/624 tested mosquitoes) for deltamethrin and 66.6% (384/577) for chlorpyriphos. Deltamethrin resistance was also observed in the mosquito species *Cx. pipiens* complex and *Cx. tritaeniorhynchus*, with mortality rates of 78.3% (267/341) and 53.8% (50/93), respectively, but resistance to chlorpyriphos was not observed at all. After insecticide bioassays, the insecticide-resistant mosquitoes exposed to deltamethrin or chlorpyriphos were tested using PCR to identify the target site mutations for L1014 *kdr* and G119 *ace* genes (Table 3). The mosquitoes used for the bioassays with deltamethrin (pyrethroids) (204 specimens) and chlorpyriphos (organophosphate) (316 specimens) were evaluated. The species/species group with the highest frequency of insecticide-resistance was *Anopheles* spp., with 78% (68/87 resistant mosquitoes) resistance to deltamethrin, followed by the same species group with 69% (133/193) resistance to chlorpyriphos, and *Cx. pipiens* complex and *Cx. tritaeniorhynchus* with 14% (10/74, and 6/43, respectively) resistance to deltamethrin. In this sense, the diagnostic test using *kdr* and *ace* as resistance markers in 5 species/species groups of mosquitoes revealed differences between deltamethrin and chlorpyriphos, with a high specificity for both (78% and 69%, respectively) especially in *Anopheles* spp. *Cx. pipiens* complex and *Cx. tritaeniorhynchus* populations resistant to chlorpyriphos were not observed. These results indicate that the presence of the L1014 *kdr* and G119 *ace* gene mutations was significantly associated with resistance to the 2 tested insecticides in *Anopheles* spp. However, with the exception of *Anopheles* spp. resistance to the 2 insecticides tested, no significant correlation was observed between target site mutations in the L1014 *kdr* and G119 *ace* genes and the resistance phenotype in the tested mosquito species/species group.

Table 3. Mutation frequency of *kdr* and *ace* genes in the mosquito (resistant/susceptible) phenotype grouped using insecticide resistance bioassay

Insecticide	Deltamethrin (Pyrethroid, for *kdr* L1014)[a]				
Species	*Aedes vexans nipponii*	*Aedes albopictus*	*Anopheles* spp.	*Culex pipiens* complex	*Culex tritaenior-hynchus*
Susceptible	194	94	537	267	50
Resistant	0	0	87	74	43
Subtotal	194	94	624	341	93
No. of genetic variation[§]	0	0	68/87	10/74	6/43
Frequency of mutated allele	0	0	0.78	0.14	0.14
	Chlorpyriphos (Organophosphate, for *ace* G119)[b]				
Susceptible	271	118	384	192	46
Resistant	0	0	193	51	72
Subtotal	271	118	577	243	118
No. of genetic variation[c]	0	0	133/193	0/51	0/72
Frequency of mutated allele	0	0	0.69	0	0

[a]Test for insecticide resistance with L1014 *kdr* gene.
[b]Test for insecticide resistance with G119 *ace* gene.
[c]No. of mosquitoes with mutated alleles in L1014 or G119/No. of surviving mosquitoes in bioassays. Because the control mortality is <5%, this study was not corrected using Abbot's formula.

DISCUSSION

Mosquitoes are important vectors of diseases. The absence of effective vaccines forces the use of insecticides as the major tool to control vector populations [25,26]. Mosquitoes were collected from 6 locations nationwide to ensure local diversity of the mosquito species/species group, and the habitat characteristics for each mosquito species/species group were taken into consideration. In general, *Ae. albopictus* is considered a forest mosquito, and therefore has had minimal exposure to insecticides sprayed countless times in rice paddies and fields, resulting in low resistance against insecticides. However, *Anopheles* spp. mosquitoes, which are found in rice paddies and are therefore exposed to agricultural pesticides sprayed each year, are thought to be more resistant than *Ae. albopictus*. Insecticide resistance bottle bioassays showed that the mosquitoes collected from the study areas exhibited resistance to deltamethrin and chlorpyriphos. Especially, *Ae. vexans nipponii* and *Ae. albopictus* species showed 100% susceptibility to the 2 tested insecticides. However, *Cx. tritaeniorhynchus* mosquitoes showed the highest resistance level of 46.2% to deltamethrin and 61.0% to chlorpyriphos. *Anopheles* spp. exhibited a resistance level of 13.9% to deltamethrin and 33.4% to chlorpyriphos while the *Cx. pipiens* complex exhibited 21.7% resistance to deltamethrin and 21.0% to chlorpyriphos (Table 3).

Grasping the mechanisms underlying these insecticide resistances is critical to understanding the optimal method to apply these chemicals and conserve their efficacy as tools to control vector populations. In addition, we also examined the well-known and widely tested insecticide target site mutations in L1014 of domain II segment 6 in the *kdr* and G119 *ace* genes. The L1014F substitution mutation confers target site insensitivity and has been reported in *Cx. pipiens* mosquitoes [27]. Other mutations including L1014S and L1014C were also reported in various regions [28-31]. Both L1014F *kdr* and L1014H/C/S/W *kdr*-type resistances have been identified in numerous species [32]. However, it has been reported that many mosquitoes that present a resistant phenotype also carry the L1014F *kdr* allele and the *kdr* site mutation may not be a unique mechanism that confers resistance to pyrethroids and DDT [33]. G119S *ace* gene mutation is responsible for the reduction of AChE1 activity in cholinergic synapses and is one of the most common mutations detected in *Cx. pipiens* mosquitoes [34]. An obvious correlation between the degrees of resistance and the frequency of insecticide usage has been

well-established in the *Cx. pipiens* population [35]. Our findings indicated that the G119S *ace* allele was present only in the tested *Anopheles* spp. population, which presented a resistant phenotype to chlorpyriphos. Chlorpyriphos, as well as temephos and pyrimiphos-methyl, are widely used in the control of mosquito larvae [36].

There are 2 ways to control mosquito-borne diseases; the first is by administering medicine to infected individuals and the second is by controlling vector mosquitoes. Chemical control for mosquito elimination is still the most efficient primary tool. However, due to the large scale and intensive application of insecticides to control mosquito vectors over many years, resistance to most chemical insecticides has been reported. After the US military was stationed in the Southern part of the Korean Peninsula in 1940's, DDT solution was sprayed in the region to prevent the outbreak of diseases carried by flies and mosquitoes in US military stations [37]. After this period, extensive use of agricultural pesticides also contributed greatly to the decrease in disease incidence through the control of the mosquito vector of the disease. Due to the nature of their habitat environment, *Ae. vexans nipponii* and *Ae. albopictus* are thought to have a lower probability of exposure to insecticides than other species. The habitats of these species are usually forests, parks, or quarantine regions, and they generally live out of reach of people and are therefore more susceptible to insecticides. In contrast, other species of mosquitoes examined in this study are continuously exposed to various insecticides. This continuous and intensive exposure to insecticides is responsible for the high levels of resistance. The resistance levels are not only due to intensive prior use but also due to the acquisition of cross-resistance [36]. Sinegre et al. [35] reported that in *Cx. pipiens* treated with chlorpyriphos, prolonged treatment led to the appearance of resistance to other organophosphates. In addition, it was confirmed that prolonged exposure to organophosphates always leads to the appearance of cross-resistance to other organophosphates and sometimes to certain carbamate-family insecticides [38]. In addition, it is suggested that the high rates of resistance could be explained by the appearance of cross-resistance, especially when the area was treated only with temephos for larvae and pyrethroid for adults [36,39].

Our study demonstrated some correlations between the resistance phenotype and the presence of the L1014 *kdr* and G119 *ace* mutations in the *Anopheles* spp. population. However, there was no clear correlation between the insecticide resistance

phenotype and target site mutation in the other 4 mosquito species tested. Target site mutation coupled with enzyme detoxification has been reported in *An. gambiae* and *Cx. quinquefasciatus* from Benin [40]. Moreover, the involvement of metabolic resistance such as carboxylesterase, cytochrome P450 monooxygenase, and glutathione S-transferase requires further investigation to elucidate the mechanisms of resistance to the insecticides currently used in mosquito-vector control.

Insecticide-resistance bottle assays and molecular identification of target-site mutation of L1014 *kdr* and G119 *ace* alleles clearly indicated that *Anopheles* spp., *Cx. pipiens* complex, and *Cx. tritaeniorhynchus* mosquitoes were resistant to 2 tested insecticides, deltamethrin and chlorpyriphos, but *Ae. vexans nipponii* and *Ae. albopictus* were clearly susceptible. The frequencies of resistant *kdr* and *ace* alleles containing L1014F or L1014C and G119S substitution mutations are high for *Anopheles* spp. populations collected at the monitoring sites. A correlation between the resistance phenotype and the presence of the L1014 *kdr* and G119 *ace* mutations was found only in the *Anopheles* spp. population. Thus, it is clear that insecticide resistance poses a growing threat and resistance management must be integrated into all mosquito control programs.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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