Scientific Note

First report of two kdr mutations L1014F/S in natural populations of *Triatoma pallidipennis* Stal and *Triatoma picturata* Usinger vectors of Chagas disease in Mexico

Jesus Davila-Barboza, O. Karina Villanueva-Segura, Gustavo Ponce-Garcia, Beatriz Lopez-Monroy, Iram P. Rodiguez-Sanchez, and Adriana E. Flores✉

Universidad Autonoma de Nuevo Leon (UANL), Facultad de Ciencias Biologicas. Av. Universidad s/n Cd. Universitaria, San Nicolas de los Garza, N.L. 66455 Mexico, adriana.floressr@uanl.edu.mx

There have been around 5,000 reported cases of Chagas disease in Mexico in recent years (2013 – 2018), and these only represent a sample of the true situation with the disease (SS 2017). Given the possible transmission of the disease by 19 species of triatomines in the country, the maintenance of the disease is facilitated given the absence of a program for the monitoring and surveillance of these vectors, the use of insecticides for other vectors, and the lack of an evaluation of triatomines regarding their susceptibility to insecticides (Salazar-Schettino et al. 2010).

The control of vectors of Chagas disease in Mexico is regulated by the Official Mexican Standard for epidemiological vigilance, prevention, and control of vector-transmitted diseases, NOM-032-SSSA-2014, which establishes management of the environment and the use of pyrethroid insecticides of residual action such as deltamethrin.

The intensive use of insecticides is one of the main factors that lead to the emergence of resistance that can involve several physiological or behavioral changes. For example, changes in the insecticide target site that reduce its binding to insecticides is known as target site resistance and includes the phenomenon of knockdown resistance, or kdr, which the best understood mechanism of resistance (Corbel and N'Guessan 2013).

Kdr mutations are distributed in the sodium channel (*vgsc*), more specifically in domain II between the transmembrane segments IIS4–IIS6, and have been reported in more than 50 species of insects of economic or medical importance (Rinkevich et al. 2013). The first kdr mutations reported in triatomines are L1014F and L925I associated with resistance to deltamethrin in *Triatoma infestans*, and more recently the mutations K964R and A943V for *T. mazzottii* and *T. longipennis,* respectively, related to resistance to permethrin and deltamethrin (Fabro et al. 2012, Capriotti et al. 2014, Davila-Barboza et al. 2018). In the present study, the sequences of the region IIS4–IIS6 of the *vgsc* of field populations *T. pallidipennis* and *T. picturata* from Mexico were obtained to identify the presence of kdr mutations.

Triatomines were collected by an active search in the outdoor and indoor areas of Jojutla de Juarez, in the state of Morelos (*T. pallidipennis*) and San Martin de Hidalgo (*T. picturata*) in the state of Jalisco, Mexico (Figure 1). The captured insects were reared under controlled laboratory conditions. The nymphs were fed on rabbit blood (Thermo Fisher Scientific, Waltham, MA), every seven to 15 days using an artificial feeder and maintained until reaching the adult stage. Pairs were formed for breeding under controlled conditions of temperature (27 ± 2° C), relative humidity (70 ± 15%), and photoperiod (12:12). The reference strains for each of the species mentioned consisted of triatomines bred under laboratory conditions with at least five generations without contribution from wild individuals or those exposed to insecticides (WHO 1994).

The insecticides used were deltamethrin (99.5% purity) (ChemService, West Chester, PA, 250 mg) and permethrin (99.5% purity, mixture of isomers) (ChemService, West Chester, PA, 250 mg). The technique of topical application was used with a volume of 0.2 µl of acetone solution of the insecticide active ingredient, on the ventral part of the abdomen of each insect, with a microapplicator (0.5 µl Hamilton microliter syringe, Hamilton Co., Reno NV). Ten 1st instar nymphs were used per dose with a minimum of four different doses per insecticide and two repetitions per dose (WHO 1994). The insects were placed in wide-mouthed containers with folded paper inside and covered with gauze. Post-treatment environmental conditions were 25-30° C and 50-70% RH. Mortality was recorded at 24 h and the data were used to calculate LD_{50} and LD_{90} and their respective confidence intervals. All parameters were expressed in nanograms per insect (ng/i).

The mortality criterion was the inability to walk from the center to the edge of a Petri dish (100x15 mm) with filter paper inside (157-mm diameter). The control groups consisted of ten insects with the same volume of acetone used in the treatments. Abbott's correction was used in the case of mortality in the control groups, (Abbott 1925).

The results obtained in the bioassays were subjected to probit analysis using the POLO Plus program (LeOra Software 2002-2017) to determine the LD_{50} and LD_{90}. The resistance ratio (RR_{50}) was determined by dividing the LD_{50} values of the populations under study by that obtained for the susceptible strain, and resistance was established on the basis of the criteria of Zerba and Picollo (2002), where RR_{50}>2 characterizes populations considered resistant, while RR50>1 is considered indicative of an alteration in susceptibility according to D`Ávila Pessoa et al. (2014).

The sequence of domain II of sodium channel gene para of *T. infestans* (GenBank Accession No. JF761319.1) was used for the virtual amplification of this gene in *T. pallidipennis* (Morelos) (TpM) and *T. picturata* (Jalisco) (TpJ) using the

BLAST program (blast.ncbi.nlm.nih.gov), using the sequence previously described for *T. infestans* by Fabro et al. (2012) and Capriotti et al. (2014).

Total RNA was extracted for each species from groups of 15 1st instar nymphs using the 1:1 w/v TRIzol reagent (Ambion, Cat. No. 15596-026) following the insert of the provider. cDNA was synthesized using the SuperScript III First-Stand Synthesis System for RT-PCR (Invitrogen, Cat. No. 18080-051).

Gene amplification of the IIS4–IIS6 region was performed by PCR; 5 µl of total cDNA (~100 ng), GoTaq DNA polymerase (Promega, Madison, WI) and 5 µM of each primer JD5: 5'-AAATCCTGGCCAACATTGAA-3' and TiRev2: 5'-TGAACCTTGTTTCCAGCTGG-3' (Davila-Barboza et al. 2018). The amplification reaction was designed to have a final volume of 25 µl. The amplification program used consisted of 4 min at 94° C, 42 cycles of 60 s at 94° C, 45 s at 60° C, 60 s at 72° C, and 10 min at 70° C. The amplification products were electrophoresed on 2% agarose gels (Bioline, Tauton, MA). The expected fragments were ~500 bp in size, visualized and photographed under ultraviolet light (UV) using a Uvitec transilluminator (Cambridge, United Kingdom).

At least three amplifications of domain II of para were purified (Wizard SV Gel and PCR Clean Up System, Promega, Madison, WI) and sequenced by capillary electrophoresis in Macrogen, for both susceptible and wild type strains for each species.

The data obtained from the sequencing were analyzed by BLAST to determine their similarity with *T. infestans*, using the program BioEdit v7.2.5 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and GeneStudio (Professional Edition, Version 2.2.0.0, genestudio.com). We also searched for non-synonymous point mutations between the susceptible and field populations.

The population of *T. pallidipennis* had the highest LD_{50}, with a value of 295.77 ng/i for deltamethrin, and an LD_{90} of 563.29 ng/i, both significantly higher ($p<0.05$) than the LD_{50} and LD_{90} values of the susceptible strains 107.92 and 257.43 ng/I, resulting in a $RRLD_{50}$ of 2.74X. Contrary to the results with deltamethrin, in the permethrin assays no difference was observed between the values of LD_{50} and LD_{90} for the field population with respect to the susceptible strain, with an $RRLD_{50}$ value of 1.12X (Table 1).

In *T. picturata*, the difference in deltamethrin LD_{50} between the susceptible strains and the field population was significant ($p<0.05$) with values of 72.96 and 137.35 ng/i, respectively; however, $RRDL_{50}$ was 1.88X. The LD_{50} values of permethrin in *T. picturata* were significantly different ($p<0.05$) between the susceptible strains (1217.02 ng/i) and the field population (3452.83 ng/i), showing an $RRLD_{50}$ of 2.84X.

Therefore, in *T. pallidipennis*, there was resistance to deltamethrin with an $RRLD_{50}$ >2 and an alteration in susceptibility to permethrin with an $RRLD_{50}$ >1, contrary to *T. picturata*, a population in which resistance to permethrin was found ($RRLD_{50}$ >2) and only an alteration in susceptibility to deltamethrin ($RRLD_{50}$<1).

The alignment of the resulting sequences, both reference and field, with the sequence of *T. infestans* [GenBank Accession No. JF761319.1] showed various percentages (82-95%) of similarity depending on species and strains. The sequence of domain II of the vgsc in the triatomine species

Table 1. Bioassay statistics and resistance ratios for deltamethrin and permethrin in *T. pallidipennis* and *T. picturata* from Mexico.

Deltamethrin	*T. pallidipennis S*	*T. pallidipennis M*	*T. picturata S*	*T. picturata J*
N	113	329	132	120
LD_{50} (ng/i)	107.92	295.77	72.96	137.35
(95%CI)	(88.7-135.8)	(268.7-321.5)	(41.17-91.21)	(118.78-151.20)
slope ± SE	3.39±0.78	4.57±0.48	4.54±1.07	6.01±1.25
LD_{90} (ng/i)	257.43	563.29	99.23	224.36
(95%CI)	(184.87-578.34)	(501.35-662.52)	(71.78-114.48)	(195.96-298.22)
RR_{50}	-	2.74 (1.18-7.97)	-	1.88 (1.31-2.81)
Permethrin	*T. pallidipennis S*	*T. pallidipennis M*	*T. picturata S*	*T. picturata J*
N	200	158	104	122
LD_{50} (ng/i)	1573.38	1765.74	1217.02	3452.83
(95%CI)	(1291.76-1975.57)	(958.19-3004.34)	(1076.74-1375.53)	(3079.32-3842.15)
slope ± SE	2.99±0.43	2.22±0.36	3.83±0.80	8.87±2.04
LD_{90} (ng/i)	4214.95	6640.02	2156.97	4814.3
(95%CI)	(3007.56-8367.19)	(3621.49-6688.05)	(1689.68-2753.42)	(4228.30-6443.25)
RR_{50}	-	1.12 (0.77-1.34)	-	2.84 (2.50-3.60)

RR_{50}: Resistance ratio was significant ($p< 0.05$) based on the test of the lethal dose ratio (Robertson et al. 2017). LD: Lethal doses; ng/i: nanograms/insect; N: Number of nymph used for bioassays; RR: Resistance Ratio; S: Reference strain; M: Morelos; J: Jalisco.

Figure 1. Collection sites. Morelos state (Jojutla de Juarez), site of collection of *T. pallidipennis*; Jalisco state (San Martin de Hidalgo), site of collection of *T. picturata*.

was analyzed, obtaining a corresponding fragment of ~500 bp, and the sequence of their corresponding reference strains was determined for comparison. The amplification of the fragments showed a high similarity (99%) between the individuals of the reference strains and those of the field populations.

The analysis of the sequences of the field populations revealed that at least one kdr mutation was present in the species of triatomines analyzed. In both species, when compared with sequences of other vector insects (i.e., *Culex pipiens quinquefasciatus*, *Aedes albopictus*, *Anopheles gambiae,* and *Musca domestica*), the mutation kdr characterized, L1014F, previously reported in *T. infestans* by Fabro et al. (2012) was found in *T. pallidipennis*, resulting in the substitution of Leu (TTA)>Phe (TTT), while in the substitution of Leu (TTA)>Ser (TCC) , the kdr mutation L1014S was found in *T. picturata* (Figure 2).

This study widens the panorama of the possible state of resistance of populations and species of triatomines in Mexico and it demonstrates that kdr mutations L1014F/S play an important role in pyrethroid resistance in *T. pallidipennis* and *T. picturata* from Mexico. The characterization of genetic resistance mechanisms is essential in the strategic and rational management of resistance in triatomines.

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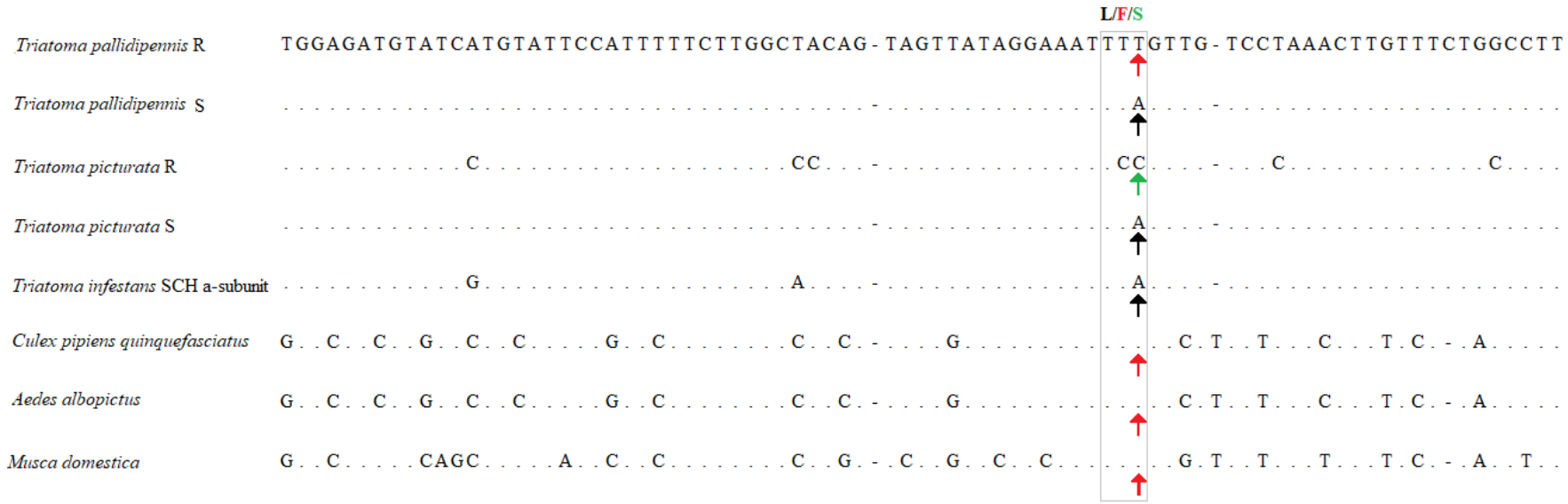
Figure 2. Partial section of the sequence of DIIS4 of the VGSC (para gene) in *Triatoma pallidipennis* susceptible (S) and resistant (R), *Triatoma picturata* susceptible (S) and resistant (R), *Triatoma infestans* voltage-sensitive sodium channel alpha-subunit (GenBank; JF761319.1), *Culex pipiens quinquefasciatus* sodium channel (GenBank: DQ408541.1), *Aedes albopictus* isolate gDNAv2 sodium channel gene (GenBank: DQ538358.1), *Musca domestica* sodium channel (kdr) (GenBank: AF461153.1); Red and Green arrows show the resistance strains, Black arrows indicate the susceptible strains.



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