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Kdr mutations in *Triatoma infestans* from the Gran Chaco are distributed in two differentiated foci: Implications for pyrethroid resistance management

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ABSTRACT

Point mutations in the voltage-gated sodium channel, the primary target of pyrethroid insecticides, have been associated with the resistance in *Triatoma infestans*, an important vector of Chagas' disease. Hence, the sustainability of vector control programs requires the implementation of resistance management strategies.

We determined the sensitivity of the molecular assays previously designed for early resistance detection to be used in pooled samples from a wide area of the endemic region, and validated them for their routine use in control campaigns for the monitoring of insecticide resistance in *T. infestans*. Consequently, we used these methods to examine the distribution of resistance-associated mutations in the sodium channel gene in populations of *T. infestans* from the Argentinean and Bolivian Gran Chaco.

The PASA and REA assays tested proved sensitive enough to detect *kdr* SNPs in pooled samples, indicating these assays are suitable for routine screening in insecticide resistance surveillance. Two geographically differentiated foci were detected in *T. infestans* populations from the Argentinean and Bolivian Gran Chaco, with populations on the Bolivian-Argentinean border carrying L1014F mutation, and those from the Argentinean Chaco carrying L925I mutation. In all highly resistant populations analyzed, one of both *kdr* mutations was present, and toxicological assays determined that all pyrethroid resistant populations analyzed herein were sensitive to fenitrothion.

The principal cause of pyrethroid resistance in *T. infestans* from the Gran Chaco ecoregion is *kdr* mutations in the sodium channel. Different levels of resistance occur in different populations carrying identical mutation, suggesting the existence of contributory mechanisms.

1. Background

Eight million people in Latin America are infected with Chagas' disease, and a fifth of the population of the region remains at risk. Thirty–forty percent of those infected develop cardiomyopathy and/or digestive syndromes (Rassi et al., 2010). The causative agent of the disease, the protozoan *Trypanosoma cruzi*, is primarily transmitted to humans through the feces of triatomine bugs, and enters broken skin as their itchy bites are scratched. The most important Chagas' vector in South America is *Triatoma infestans*. Given the absence of vaccines and efficient treatments in the chronic stage, reduction of triatomine population is the method of choice for the control of disease transmission. In 1991, the Southern Cone Initiative (SCI), established by the authorities of the Southern Cone

Abbreviations: *kdr*, knockdown resistance; PASA, PCR amplification of specific alleles; REA, restriction endonuclease assay; SCI, southern cone initiative; SNP, single nucleotide polymorphism; TiNaV, *T. infestans* sodium channel; M, madrejones; LE, la esperanza; LG, la gerónima; EJ, el juramento; EM, el malá; PG, Pampa Grande; TN, Tierras Nuevas; VC, Villa El Carmen; Y, Yacuiba.

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Nations, successfully reduced Chagas' transmission by the elimination of triatomines from dwellings, and enforced Chagas' screening of blood donors (Dias, 2007; Gurtler, 2009).

Gran Chaco ecoregion covers 1.1 million km^2 distributed in Argentina, Paraguay and Bolivia (Cabrera, 1971). In this large semiarid region, the vectorial transmission of Chagas' disease has not yet been stopped, even though the vector control strategies with the spraying of pyrethroid insecticides were similar to those used in other areas (Gurtler, 2009; Gurtler et al., 2007). SCI recognized as a priority the determination of the causes for the failures in the elimination of *T. infestans* from the Gran Chaco (Dias, 2007). Even though it would be a multicausal problem, including socioeconomic factors, resistance to pyrethroid insecticides in populations of *T. infestans* from the Gran Chaco contributes to the failures in spraying with pyrethroids (Picollo et al., 2005).

An initial focus of high pyrethroid resistance in *T. infestans* was identified on the Argentinean-Bolivian border (Picollo et al., 2005; Santo-Orihuela et al., 2008) and more recent findings documented another focus of high resistance in the Argentinean Chaco Province, at the center of the Gran Chaco (Carvajal et al., 2012). Toxicological, biochemical and genetic evidence accumulated to date points to multiple origins of the resistance, rather than the spread of a single point resistant population (Capriotti et al., 2014; Fabro et al., 2012; Germano et al., 2012).

Pyrethroids are neurotoxins that target the voltage-dependent sodium channel (Na_v), a membrane protein involved in the transmission of the action potential. Single nucleotide polymorphisms (SNPs) in the Na_v gene generate amino acid changes in the protein, which reduce the sensitivity of the molecule to pyrethroids. These resistance-associated mutations are called knockdown resistance (*kdr*) alleles (Dong et al., 2014). Although several mechanisms of insecticide resistance have been described in *T. infestans* and other insects (Santo-Orihuela et al., 2008; Capriotti et al., 2014; Fabro et al., 2012; Pedrini et al., 2009), sodium channel mutations occur whenever the highest levels of resistance are observed (Dong et al., 2014). Our group recently verified the existence of two different *kdr* SNPs in *T. infestans* populations in Argentina, L1014F in a population near the Bolivian border (Salta Province) (Fabro et al., 2012), and L925I in a population from the center of the Argentinean Chaco Province (Capriotti et al., 2014). Furthermore, molecular assays to test the presence of *kdr* mutations in the field were developed (Capriotti et al., 2014; Fabro et al., 2012). As was observed in a range of insect species, an intron in *TiNa$_v$* gene is located very close to the L1014F mutation site (Fabro et al., 2012). We have also observed that this region of the gene shows a degree of variation in non-coding positions (silent mutations) between different individuals and strains (Capriotti et al., 2014). These facts would affect the performance of any assay that uses primer binding sites within the region, as is the case of the methods developed recently. Hence, a validation for its use in different population is required.

The main objectives of the present work are to analyze the presence and distribution of the two *kdr* SNPs in resistant populations from the Gran Chaco; if both mutations co-exist in the same area, or if they are distributed in differentiated foci. Furthermore, we validated molecular assays for their use in resistant management strategies, with pooled samples from a range of populations of *T. infestans*.

2. Methods

2.1. Ethics statement

Pigeons were housed, cared for, fed and handled in accordance with resolution 1047/2005 (CONICET) regarding the national reference ethical framework for biomedical research with laboratory, farm, and nature collected animals. This is in agreement with the standard procedures of the Office for Laboratory Animal Welfare, Department of Health and Human Services, NIH, and the Directive of the European Parliament (2010/63/EU), in relation to the use of animals for scientific research. Biosecurity considerations are in accordance with CONICET (Res 1619/2008) and WHO Biosecurity Handbook (ISBN 92 4 354 6503). According to those rules, approval from the CONICET Ethic Committee was not required for this work. The collection of insects in dwellings was performed in agreement with the Argentinean National Health Ministry's ethical requirements. The inhabitants of each domicile were informed about the objectives, and the protocol of the procedures was used. Collections were not performed unless they expressed their consent. Vector Control Program officers familiarized with the population participated as mediators and collaborated with insect sampling.

Fig. 1. Map of Northern Argentina and Southern Bolivia showing the small villages where *T. infestans* were collected. A: Bolivian–Argentinean border: LE: La Esperanza; LG: La Gerónima; EM: El Malá; EJ: El Juramento; PG: Pampa Grande. B: Chaco Province. M: Madrejones; VC: Villa El Carmen; TN: Tierras Nuevas; Y: Yacuiba. Scale Bar: 100 km.

2.2. Insect populations and strains

A susceptible strain (CIPEIN strain) raised since 1975 with no exposure to insecticides was used as a reference, as its response to deltamethrin had been repeatedly verified with susceptible field strains (Toloza et al., 2008; Germano et al., 2010; Castro et al., 1976). The field *T. infestans* specimens were collected in the Argentinean localities of Madrejones (M) (22°02′ S, 63°37′ W) (Salta Province), La Esperanza (LE) (26°03′ S, 60°27′ W) (Chaco Province), La Gerónima (LG) (26°04′ S, 60°16′ W) (Chaco Province), El Juramento (EJ) (25°54′ S, 60°24′ W) (Chaco Province), El Malá (EM) (25°56′ S, 60°27′ W) (Chaco Province), Pampa Grande (PG) (27°06′ S, 60°59′ W) (Chaco Province), and the Bolivian localities of Tierras Nuevas (TN) (21°44′ S, 63°33′ W) (Santa Cruz Department), Villa El Carmen (VC) (21°47′S, 63°34′ W) (Santa Cruz Department), and Yacuiba (Y) (22°01′ S, 63°40′ W) (Tarija Department) (Fig. 1). Insects were collected inside dwellings, and further generations of the field-collected insects were raised as previously described (Fabro et al., 2012). Table 2 specifies the year of collection from each population and the number of generations raised in the laboratory for the individuals used in this work.

Table 1
Sequence of the primers used in this study.

Primer name	5′-3′ Sequence
Ti Fwd	TGGCCAACATTGAATTTATTGATATC
Ti Rev 1	TGTTACGATTTGATGATAACCGGGATA
Ti Rev 2	GCTGAGCAACAAGGCCAGAAACAAG
Cnt Rev	TTAACCCGAACAAGAATATA
Res Fwd	CAACAGTAGTTATAGGAAATTTT
Sen Rev	AAATATATAAAGTACTTACAACT

2.3. Topical application bioassay and determination of resistance rate

The F_1 of the field-collected insects were evaluated for their response to deltamethrin (PG, LG and EJ) and fenitrothion (TN, VC, PG, LG and EJ). The CIPEIN strain was used as the reference population in the insecticide assays. Insecticide susceptibility was determined according to the standard WHO protocol (1994), as detailed in (Fabro et al., 2012). Briefly, first instars of *T. infestans* were topically treated in the abdomen with technical grade deltamethrin (Ehrenstorfer, Augsburg, Germany) or technical grade fenithrotion (Dr. Ehrenstorfer GmbH, Augsburg, Germany) diluted in acetone (J.T. Baker, Mexico City, Mexico). Dose-mortality data were subjected to probit regression analysis (Litchfield and Wilcoxon, 1949) in order to estimate the dose-response curve. Study populations were considered resistant when RRs were significantly higher than 1.00 (i.e., when the 95% confidence limit of the RR was higher than 1 and did not include the number 1.00) (Robertson et al., 2007).

2.4. Extraction of genomic DNA and analysis of infestans sodium channel (TiNaV) gene fragments

Genomic DNA was extracted from 10 insects from each population with a commercial kit (Promega, Madison, USA), following the manufacturer instructions. Seminested PCR reactions were performed for the amplification of a fragment of the TiNaV gene. A detailed protocol of the seminested PCR is presented in (Capriotti et al., 2014). The primers used are listed in Table 1. The amplicons of PCRs 1 and 2 are more than 3 kb in length and include two introns (for a schematic view of the segment of the TiNaV gene amplified, see Ref. (Fabro et al., 2012)). For direct sequencing, DNA from pooled nymph 1 insects (n = 10) belonging to the same population was used to amplify a region of $TiNa_V$ as described above. PCR products were directly sequenced at Macrogen (Seoul, Korea) (each reaction was performed in duplicate). The primers used for direct sequencing were TiFwd1, TiRev2 and CntRev for every sample, in order to span the whole fragment with the objective of investigating the nucleotide diversity of the targeted $TiNa_V$ region from the natural populations, including the molecular sites where the *kdr* mutations were previously found (Capriotti et al., 2014; Fabro et al., 2012).

2.5. Genotyping molecular reactions

Each sample was genotyped for the *kdr* sites 925 (L or I) and 1014 (L of P). For the validation of methods in different areas, we analyzed genomic DNA from pooled samples (n = 10 nymph/sample), each pool being composed of individuals from the same population. For genotype frequency determinations, genomic DNA was individually extracted from each specimen. The number of individuals analyzed from each population is specified in Table 2.

2.5.1. PCR1

This is the first PCR, using primers that are common to Restriction Endonuclease and PCR Amplification of Specific Allele assays.

Table 2
Deltamethrin resistance ratio (RR) and KDR allele from *Triatoma infestans* populations in the Gran Chaco.

Population	Province/Department	Country	Year of collection	Laboratory generation	Deltametrin RR	Fenitrothion RR	Genotype frequencies						Total (N)
							SS	SR1	SR2	R1R1	R1R2	R2R2	
Madrejones	Salta	Argentine	2008	F3	35.7 (27.03–47.62)[a]	ND	0.00	0.00	0.00	0.00	0.00	1	10
Tierras Nuevas	Tarija	Bolivia	2006	F12	541.6 (260.2–1127.3)[b]	0.42 (0.23–0.76)	0.27	0.00	0.64	0.00	0.00	0.18	11
Villa del Carmen	Tarija	Bolivia	2006	F10	438.0 (147.5–1300.6)[b]	0.95 (0.75–1.21)	0.00	0.00	0.00	0.00	0.00	1	9
Yacuiba	Tarija	Bolivia	2005	F11	154.4 (121.2–169.0)[c]	0.79 (0.62–1.02)[c]	ND	ND	ND	ND	ND	ND	0
Pampa Grande	Chaco	Argentine	2012	F2	2.08 (1.27–3.39)	1.45 (0.95–2.21)	1	0.00	0.00	0.00	0.00	0.00	10
La Esperanza	Chaco	Argentine	2011	F2	233.42 (116.8–466.6)[d]	1.00 (0.75–1.33)[d]	0.00	0.00	0.00	1	0.00	0.00	10
La Gerónima	Chaco	Argentine	2012	F2	5.51 (3.57–8.52)	1.2 (0.87–1.54)	1	0.00	0.00	0.00	0.00	0.00	5
El juramento	Chaco	Argentine	2013	F2	> 2000	0.84 (0.58–1.20)	0.07	0.00	0.00	0.93	0.00	0.00	14
El Malá	Chaco	Argentine	2011	F3	1031 (909.1–1213.3)[e]	2.75 (2.47–3.12)[e]	0.18	0.18	0.00	0.73	0.00	0.00	11

ND: Not determined because extintion of the colony. *S*: L925 + L1014 allele. R1: I925 + L1014 allele. R2: L925 + F1014 allele.

[a] Data from Fabro et al. (2012).
[b] Data from Germano et al. (2010).
[c] Data from Santo-Orihuela et al. (2008).
[d] Data from Germano et al. (2014).
[e] Data from Carvajal et al. (2012).

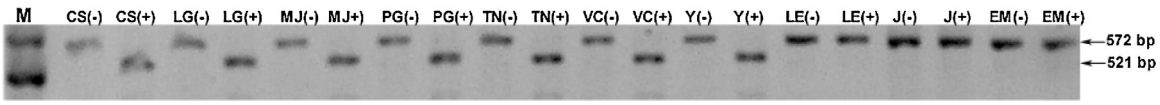

Fig. 2. REA assay confirmed the results of sequencing for every population under study. The 521 bp fragment indicates susceptible associated SNP; 572 bp fragment indicates the resistance-associated SNP. CS: Control Sensitive; LG: La Gerónima; MJ: Madrejones; PG: Pampa Grande; TN: Tierras Nuevas; VC: Villa El Carmen; Y: Yacuiba; LE: La Esperanza; J: Juramento; EM: El Malá. –: Sample not treated with SacI. +: Sample after SacI treatment.

TiFwd and CntRev primers (Table 1) were used to amplify a 572 bp fragment of *TiNaV* gene. This PCR was performed with the same component and conditions as those of PCR 1 described previously (Fabro et al., 2012).

2.5.2. PCR2

One μl of PCR1 was used as template for a secondary seminested PCR using the primers Ti Fwd and *KDR* Cont Rev. The reaction volume, composition and cycling conditions are similar to those of PCR1, with an annealing temperature of 52 °C. PCR products were digested with SacI endonuclease (Fermentas, Maryland, USA). The products of digestions were analyzed in a 2% agarose gel. For a more detailed description of REA, see (Capriotti et al., 2014).

2.5.3. PCR 3

In the PCR mix (similar volume, composition and cycling parameters to those of PCR1) primers Ti Fwd and Ti Rev1 were used (Table 1).

2.5.4. PCR4

Two μl of PCR3 product was used as template for PASA reaction (detailed in (Fabro et al., 2012)). The products of PASA reactions were analyzed in a 2% agarose gel stained with ethidium bromide (1:10.000). Different sized fragments are observed in the gel, depending on whether L1014F and/or L1014L SNPs are present in the sample (476 bp for L1014L; 130 bp for L1014F).

2.6. Sensitivity determination for PASA and REA

The genomic DNA fragment of $TiNa_V$ encompassing either the L925I or L1014F mutation site was PCR amplified from genomic DNA templates extracted from pools of 10 insects from VC, EJ or PG populations, as described above. The PCR products were inserted in the plasmid pGEM-T (Promega, USA). Plasmid DNA encoding the $TiNa_V$ fragment was extracted from transformed bacteria. The genotype of 5 clones/population was confirmed using REA and PASA assays, as described above. Once the genotype was identified, 1 clone/population was quantified and selected to be used as standard DNA templates. The standard DNA templates were mixed in the following molar ratios: 0:10, 0.25:9.75, 0.5:9.5; 1:9, 2:8, 3:7, 5:5, and 10:0 (resistant allele: susceptible allele at each mutation site). To determine the sensitivity (detection limit) of PASA and REA in these conditions, mixes of known molar composition were used as template for each test. Plasmid DNA was used in order to ensure the genetic composition of the sample and to obtain enough material for the several replicas and dilutions required. However, interference with the efficiency of the PCRs cannot be discarded if genomic DNA is used.

Using samples of known genotype, either from plasmid or genomic DNA, we assayed the rate of false positives for PASA and REA for homo- and heterozygote samples. Once PCR conditions were optimized as described above, no false positive or false negative results were obtained, after more than 30 replicas for each test. However, the conditions for PASA (annealing temperature, number of cycles, quality of DNA polymerase, etc.) are crucial to avoid unspecific amplification, and should probably be adjusted before their implementation in different laboratories.

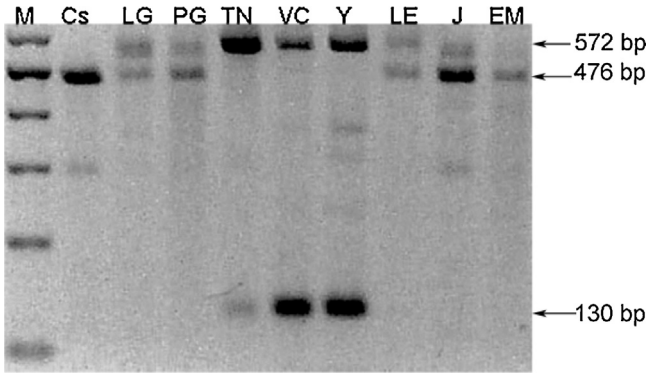

Fig. 3. PASA assay confirmed the results of sequencing for every population under study. The 572bp is a control fragment. The 476bp indicates the presence of susceptible SNP. The 130bp fragment is amplified when the resistant allele is present. M: standard size marker. CS: Control Sensitive; LG: La Gerónima; PG: Pampa Grande; TN: Tierras Nuevas; VC: Villa El Carmen; Y: Yacuiba; LE: La Esperanza; J: Juramento; EM: El Malá.

3. Results

3.1. Toxicological results

The resistance ratios for deltamethrin and fenitrothion for the populations of *T. infestans* from the Gran Chaco studied are shown in Table 2. We focused on the regions where resistance was reported: Tarija-Salta (Argentinean-Bolivian border) and the Argentinean Chaco Province (Fig. 1). The three populations that were tested for resistance to deltamethrin during the present study ranged from incipient resistance levels (RR = 2.08 for PG and RR = 5.51 for LG) to extremely high resistance level (RR > 2000 for EJ). Moreover, all populations were susceptible to fenitrothion, with the exception of EM that showed an incipient resistance level (RR = 2.75).

3.2. Geographic distribution of resistance and kdr alleles in populations of infestans from the gran chaco

Regarding resistance SNPs, four possible alleles of the gene could exist: L925 + L1014 (wild type), L925 + F1014, I925 + L1014 and I925 + F1014. The sequencing results revealed the existence of L925 + F1014 allele in every high resistant population from the Tarija-Salta border, whereas I925 + L1014 allele was present in every high resistant population analyzed from the Argentinean Chaco Province. Pampa Grande and La Gerónima (classified as insipient resistance) presented the "sensitive" wild-type L925 + L1014 allele (Table 2). The potential double *kdr* I925 + F1014 allele was not detected in any of the samples analyzed.

3.3. PASA and REA validation and frequency estimations

To propose PASA and REA assays for their reliable use in the whole endemic area, they should be validated for different populations. We performed the assays using the genomic DNA isolated for all the populations under study. We observed similar results to those obtained for sequencing (Fig. 2 and Fig. 3), indicating that these methods can reliably be used in a wide range of populations.

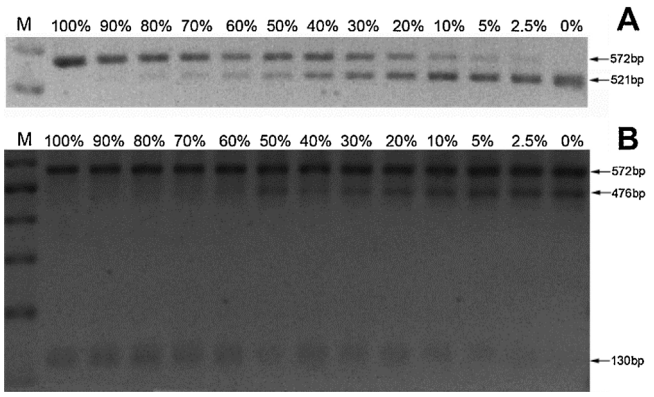

Fig. 4. Sensitivity determination for REA (A) and PASA (B) assays. M indicates the position of the standard size marker. The upper numbers indicate the percentage of resistance allele in each sample.

Once the molecular assays were validated, we used them to estimate whether the *kdr* SNPs are in high frequency in the populations, or if more than one mutation is present at the time. Individuals from the different populations were genotyped. We found that *kdr* SNP is present in all the individuals analyzed from M (L1014F), VC (L1014F) and LE (L925I), and is present at high frequencies in EJ and EM (L925I) (Table 2).

3.4. PASA and REA sensitivity determination

We studied the lower limit of detection of our molecular assays. A set of standard plasmidic DNA template mixtures of known composition were adjusted to contain an increasing frequency of one of both resistant SNPs. In the conditions assayed, the lower detection limit for the L925I SNP with REA is 5% (Fig. 4A). Likewise, the lower detection limit of PASA is also 5% (Fig. 4B). Therefore, insects could be pooled and analyzed with REA and/or PASA in single assays, and the presence of one heterozygous individual will still be detected.

4. Discussion

Present results demonstrate that mutations conferring high pyrethroid resistance are widespread in Bolivian and Argentinean Gran Chaco, suggesting that insecticide resistance could be an important reason for the failures in the elimination of *T. infestans* in the Gran Chaco ecoregion. Two villages in Argentinean Chaco exhibited the highest levels of resistance: EJ (RR>2000) and EM (RR=1031), even several Bolivian populations were also highly resistant. There are also two populations with incipient resistance in the Argentinean Chaco, considered not to be an immediate threat for the spraying campaigns with pyrethroids: LG (RR:5.51) and PG (RR: 2.08).

In this study, we detected that Bolivian-Argentinean border and Argentinean Chaco resistance foci are characterized by the presence of different *kdr* SNPs, L1014F in the former and L925I in the latter. Even though the first detected Bolivian-Argentinean border resistant populations were collected in the field almost 10 years ago, observations with populations collected in 2013 in this region confirm that pyrethroid resistance due to L1014F *kdr* mutation is still occurring in the zone (Gonzalo Roca-Acevedo, personal communication).

The results presented here show that *kdr* SNPs are present in *T. infestans* populations whenever high pyrethroid resistance is detected. The former suggests that the principal cause for pyrethroid resistance in *T. infestans* is *kdr* mutations; however, different levels of resistance observed in populations carrying the same mutation could suggest that other insecticide resistance mechanisms collaborate. Indeed, enhanced metabolism and reduced penetrance, were reported for resistant *T. infestans* (Santo-Orihuela et al., 2008; Pedrini et al., 2009). Hence, present and former results point to several resistant mechanisms contributing to the high resistance levels observed in *T. infestans*, even though mutations in the target site seem to be a major cause.

Even though the frequency results obtained here should be interpreted cautiously, as in many cases the number of individuals that could be analyzed is low and some populations were maintained in the laboratory for many generations, they suggest that the *kdr* frequencies in resistant field populations are fixed or very high. They also show that the differences observed in the levels of resistance are not directly related to differences in the frequencies of *kdr* mutations. For example, in Argentinean Chaco, L925I SNP is present in all the samples from LE (RR: 233.42), but not in all the samples from the more resistant EJ (RR>2000) and EM (RR: 1031). Likewise, the most resistant population from the Bolivian-Argentinean border focus analyzed here is TN (RR: 541.6), where the L1014F mutation is present at lower frequency than in the less resistant M (RR: 35.7).

To overcome the resistance problem, monitoring strategies should be promptly implemented. Hence, an important contribution of the present work is the validation of the molecular tests for the detection of resistance-associated SNPs in different populations, and the determination of their sensitivity. Efficient monitoring of resistance SNPs in *T. infestans* populations should involve the processing of hundreds of insects per region. Hence, the use of a pool of insects in a single assay would increase the throughput and reduce the costs of the detection. The methods presented require basic equipment and simple technical skills. They are proposed for the monitoring of *kdr* SNP spreading in *T. infestans*. REA and PASA can be used for a wide range of field populations, allowing the use of pooled samples. Besides, qPCR based techniques such as TaqMan could be harnessed to even improve sensitivity, even though it would require more expensive equipment and supplies.

Regarding present and previous results (Germano et al., 2014), organophosphates could be suggested as an alternative insecticide when *kdr* SNPs are detected. In this way, resistance spreading could be treated, anticipating control failures. Integrated control strategies including housing improvements, community-based surveillance and environmental management measures should be considered in the long term to reduce the dependence on insecticides.

5. Conclusions

The results presented here are relevant in the context of the implementation of resistance management strategies for the control of Chagas vectors in the Gran Chaco. We show that *kdr* mutations are always present in populations with pyrethroid resistance, in two differentiated foci: L925I in the Argentinean Chaco and L1014F in the Bolivian-Argentinean border. The molecular assays validated here could be useful tools for the early detection of pyrethroid resistance in the field. As an alternative, the use of fenitrothion could be implemented as an alternative to pyrethroids whenever *kdr* mutations are detected. The rational alternation of insecticides will help to prevent or delay the fixation of resistance.

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