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Distribution patterns of three sodium channel mutations associated with pyrethroid resistance in *Rhipicephalus* (*Boophilus*) *microplus* populations from North and South America, South Africa and Australia

Leonore Lovis [a,b,*], Felix D. Guerrero [c], Robert J. Miller [d], Deanna M. Bodine [c], Bruno Betschart [a], Heinz Sager [b]

[a] University of Neuchâtel, Institute of Biology, Laboratory of Parasitology, Neuchâtel 2000, Switzerland
[b] Novartis Animal Health Research Center, St. Aubin (FR) 1566, Switzerland
[c] USDA-ARS Knipling-Bushland U.S. Livestock Insects Research Laboratory, Kerrville, TX 78028, USA
[d] USDA-ARS Cattle Fever Tick Research Laboratory, Moore Air Base, Edinburg, TX 78541, USA

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ABSTRACT

Resistance to synthetic pyrethroids (SP) in the cattle tick *Rhipicephalus* (*Boophilus*) *microplus* is widespread throughout its distribution area. Three single nucleotide substitutions identified in Domains II and III of the sodium channel gene of *R.* (*B.*) *microplus* are known to be associated with target site pyrethroid resistance. We developed a multiplex PCR using allele-specific primers to amplify wild type or mutated genotypes of the three mutations simultaneously. This assay was used to screen tick samples originating from Brazil, Argentina, Mexico, South Africa and Australia whose phenotype to flumethrin and cypermethrin had been determined by the use of the Larval Tarsal test (LTT) or the Larval Packet Test (LPT). These mutations were found to have distinct geographical distributions and result in different resistance phenotypes. The L64I Domain II mutation conferring resistance to several SP compounds was found in all the Brazilian, Argentinean and Australian populations and in one South African population, with frequencies between 38% and 100% in flumethrin and cypermethrin resistant populations. In contrast, this mutation was not found in samples from Mexico, while the Domain III mutation was found exclusively in this country. The G72V Domain II flumethrin-specific mutation was found in a single Australian population, with a very low resistant allele frequency (3%). The homozygous resistant RR genotype of the L64I Domain II mutation correlated significantly with the survival rates at the discriminating doses of flumethrin and cypermethrin. This survey shows the widespread distribution of the L64I Domain II mutation and provides evidence of its geographic separation from the Domain III mutation.

1. Introduction

Resistance to synthetic pyrethroids (SP) in the one-host cattle tick *Rhipicephalus* (*Boophilus*) *microplus* is widespread throughout its area of distribution (Kemp et al., 1998; FAO, 2004; Graf et al., 2004; Rosario-Cruz et al., 2009a). Pyrethroid insecticides target the voltage-gated sodium channel, an integral transmembrane protein consisting of four homologous domains (I–IV) each containing six membrane spanning segments (S1–6) (Dong, 2007). Single nucleotide substitutions in the gene have been shown to cause SP resistance in numerous insect species (reviewed by Dong, 2007). In Australian *R.* (*B.*) *microplus*, increased metabolic detoxification was the first identified mechanism of SP resistance (Schnitzerling et al., 1983; Nolan et al., 1989). However, increased detoxification was not observed in some SP resistant populations in their studies, suggesting that target site insensitivity was present. Later, patterns of SP resistance found in some Mexican populations of *R.* (*B.*) *microplus* led to the hypothesis that both increased metabolic enzyme activity (esterase and cytochrome P450) and target site insensitivity (*kdr* mutation) were involved in SP resistance in these populations (Miller et al., 1999). Among the two phenomena, target site insensitivity is likely the major mechanism of economically significant resistance to SP in most populations of *R.* (*B.*) *microplus* (Guerrero et al., 2012).

Three point mutations associated with resistance to SP have been reported in the sodium channel gene of *R.* (*B.*) *microplus* (Table 1) (He et al., 1999; Morgan et al., 2009; Jonsson et al., 2010). The first mutation was identified in two tick populations from Mexico (He et al., 1999) in which target site-based resistance had been reported (Miller et al., 1999). This mutation is located in the S6 segment of the Domain III of the sodium channel gene and results in a phenylalanine to isoleucine amino acid substitution. A PCR diagnostic assay was developed to allow the detection of this

* Corresponding author at: Novartis Animal Health Research Center, Chemin de la Petite Glâne, 1566 Saint-Aubin (FR), Switzerland. Tel.: +41 26 679 14 15; fax: +41 26 679 14 10.
E-mail address: leonore.lovis@unine.ch (L. Lovis).

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Table 1
Summary of the three point mutations in the sodium channel gene associated with resistance.

Domain	Segment	Nucleotide position	Nucleotide substitution	Amino acid substitution	Reference code	Identification	PCR assay
III	S6	2134	T → A	Phe → Ile	F1550I	He et al. (1999)	Guerrero et al. (2001)
II	S4–5 linker	190	C → A	Leu → Ile	L64I	Morgan et al. (2009)	Morgan et al. (2009)
II	S4–5 linker	215	G → T	Gly → Val	G72V	Jonsson et al. (2010)	None

Accession number in GenBank: AF134216.

mutation (Guerrero et al., 2001), and frequency of this mutation correlated with survival rates in permethrin dose-mortality bioassays. In addition, the allelic frequency of the mutation correlated with resistance ratios and survival rates at discriminating doses of SP assessed *in vitro* (Rosario-Cruz et al., 2005, 2009b; Li et al., 2007). Later, another mutation associated with SP resistance was identified in Australian populations of *R.* (*B.*) *microplus* (Morgan et al., 2009), and a PCR assay was developed to allow the detection of the mutation. More recently, an additional point mutation associated with resistance to flumethrin but not to cypermethrin was identified in Australian populations (Jonsson et al., 2010). These two mutations are located in the S4–5 linker of the Domain II of the sodium channel gene, leading to leucine to isoleucine (L64I) and glycine to valine (G72V) amino acid substitutions, respectively.

The mutation identified by He et al. (1999) has been found throughout Mexico (Rosario-Cruz et al., 2005, 2009b) and in an outbreak strain in the United States (Miller et al., 2007) while it has not been reported in Brazilian SP resistant populations (Andreotti et al., 2011; Domingues et al., 2012) nor in Australia (Chen et al., 2009). Although the two Domain II mutations were initially identified in Australia, recent work showed that the L64I mutation reported by Morgan et al. (2009) was found in ticks outside Australia. In contrast, the G72V mutation reported by Jonsson et al. (2010) appears to be isolated to Australia (Guerrero et al., 2012; Domingues et al., 2012).

Bioassays are complementary to molecular surveys and allow assessment of SP resistance without knowledge of the underlying mechanism. *In vitro* tests, such as the adult immersion test (Drummond et al., 1973), the larval packet test (LPT) (Stone and Haydock, 1962), both currently recommended by the FAO (FAO, 2004) and the recently developed larval tarsal test (LTT) (Lovis et al., 2011) can be used to identify resistant phenotypes. Ticks can be exposed to a range of doses of acaricides to establish a dose–response curve from which the doses inducing 50% or 90% mortality can be calculated and compared to a susceptible reference strain to determine the resistance ratios. Alternatively, ticks can be exposed to a single dose that has been pre-established based on data from bioassays conducted on a susceptible reference strain, and survival at this discriminating dose (DD) is considered as an indicator of resistance (FAO, 2004).

The objectives of this study were (1) to develop a multiplex diagnostic PCR assay to allow the simultaneous detection of the three known *R.* (*B.*) *microplus* sodium channel gene mutations that are associated with target site pyrethroid resistance, and (2) to investigate the presence of these three mutations in field and laboratory populations originating from Brazil, Argentina, South Africa, Mexico and Australia and, if present, to correlate their frequency to the phenotypic resistance to flumethrin and cypermethrin assessed *in vitro* with the LTT or LPT bioassays.

2. Materials and methods

2.1. Tick populations

Samples of field tick populations were collected between February 2009 and November 2010 from Brazil, Argentina, South Africa, and Australia. Details about the origin of these populations are listed in Table 2.

In addition, an Australian SP-resistant laboratory strain, Ultimo, was also included in the study. The Ultimo strain was originally collected in 1992 in central Queensland, Australia from SP and amitraz resistant ticks, maintained in colony at CSIRO and subsequently established at the Novartis Animal Health Research Centre (CRA), St-Aubin, Switzerland in 1999 where it was maintained without acaricide selection (Lovis et al., 2011). Ultimo ticks used for bioassays and molecular studies were from F31 to F33 generations.

Finally, three Mexican strains (San Felipe, Coatzacoalcos, and Rio Bravo) and an additional Brazilian strain (Santa Luiza), established as laboratory strains at the Cattle Fever Tick Research Laboratory (CFTRL), Edinburg, Texas were also included in this study. The three Mexican strains were collected from pyrethroid resistant populations. The San Felipe and Rio Bravo strains have predominant pyrethroid resistance due to target site insensitivity, while the Coatzacoalcos strain is resistant through esterase-based metabolism. The San Felipe and Coatzacoalcos strains were reared under SP selection pressure and their origin, rearing conditions and characterization were described by Miller et al. (1999). The Rio Bravo strain originated from the city of the same name in Tamaulipas, Mexico and was collected at the importation inspection vats in Reynosa, Tamaulipas at the border with Texas. This strain was selected for pyrethroid resistance. The origin of the Brazilian Santa Luiza strain, which has a pyrethroid resistant phenotype, has been described by Li et al. (2007). San Felipe and Coatzacoalcos larvae used for molecular studies were from F16 and F26 generations, respectively, while LPT data for these two strains were from F8 and F16 generations. Rio Bravo ticks used for bioassays and molecular studies were from F1 and F3 generations, respectively. Santa Luiza larvae used for molecular studies were from the first generation established at the CFTRL in 2000 while it was tested later *in vitro* with F13 ticks.

2.2. Bioassays

The LTT was used to assess the susceptibility of tick populations to technical grade flumethrin (Sigma–Aldrich, Switzerland) and cypermethrin (Novartis, Switzerland). The LTT was performed at the CRA, following the protocol described by Lovis et al. (2011) for the Argentinean, South African and Australian populations. For the Brazilian field populations the LTT was conducted at the Instituto Biologico (IB), São Paulo and at the Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF) with the modifications described by Lovis et al. (submitted for publication-a).

The LPT (Stone and Haydock, 1962) was carried out at the CFTRL as previously described (Miller et al., 1999; Li et al., 2007) to determine permethrin (FMC, Philadelphia, PA), cypermethrin (Hoechst-Roussel, Mexico) and flumethrin (Bayer, Mexico) toxicity to the San Felipe and Coatzacoalcos strains and permethrin toxicity to the Rio Bravo and Santa Luiza strains.

2.3. DNA extraction

Genomic DNA was isolated from individual tick larvae of the Rio Bravo, San Felipe, Coatzacoalcos and Santa Luiza strains as

Table 2
Origin of the field populations and laboratory strains and information about their previous characterisation.

Strain	Country	State/Province	Origin	Year of collection	Bioassay used for characterization
ST40	Brazil	São Paulo	Field pop	2011	LTT
ST41	Brazil	São Paulo	Field pop	2011	LTT
ST42	Brazil	São Paulo	Field pop	2011	LTT
ST44	Brazil	São Paulo	Field pop	2011	LTT
ST46	Brazil	Espirito Santo	Field pop	2011	LTT
ST47	Brazil	Rio Grande do Sul	Field pop	2011	LTT
ST48	Brazil	São Paulo	Field pop	2011	LTT
ST49	Brazil	Mato Grosso do Sul	Field pop	2011	LTT
ST50	Brazil	Mato Grosso do Sul	Field pop	2011	LTT
ST51	Brazil	Mato Grosso do Sul	Field pop	2011	LTT
ST52	Brazil	Paranà	Field pop	2011	LTT
ST55	Brazil	Rio Grande do Sul	Field pop	2011	LTT
ST57	Brazil	São Paulo	Field pop	2011	LTT
ST58	Brazil	Rio Grande do Sul	Field pop	2011	LTT
Santa Luiza	Brazil	na	Lab strain	2000[a]	LPT
ST21	Argentina	Corrientes	Field pop	2010	LTT
ST22	Argentina	Corrientes	Field pop	2010	LTT
ST24	Argentina	Corrientes	Field pop	2010	LTT
ST25	Argentina	Corrientes	Field pop	2010	LTT
ST26	Argentina	Corrientes	Field pop	2010	LTT
ST27	Argentina	Corrientes	Field pop	2010	LTT
ST29	Argentina	Corrientes	Field pop	2010	LTT
ST30	Argentina	Corrientes	Field pop	2010	LTT
San Felipe	Mexico	Tamaulipas	Lab strain	1996	LPT
Coatzacoalcos	Mexico	Veracruz	Lab strain	1994[a]	LPT
Rio Bravo	Mexico	Tamaulipas	Lab strain	1998	LPT
ST11	South Africa	Western Cape	Field pop	2010	LTT
ST12	South Africa	Western Cape	Field pop	2010	LTT
ST15	South Africa	Mpumalanga	Field pop	2010	LTT
Ultimo	Australia	Queensland	Lab strain	1992	LTT
Urah	Australia	Queensland	Field pop	2010	LTT

na: not available.
[a] Year of establishment in the CFTRL.

described by Guerrero et al. (2001). Briefly, pooled larvae were stored frozen in plastic vials until DNA purification. Larvae were placed on a Petri plate on dry ice, and individuals were transferred to pre-chilled 1.5 ml tubes (Kontes, Vineland, NJ). Liquid nitrogen-cooled disposable pellet pestles for 1.5 ml centrifuge tubes (Kontes, Vineland, NJ) were used to grind the larvae against the tube walls for 15 s. Twenty-five µl of GeneAmp 10X PCR buffer II (Applied Biosystems, Carlsbad, CA) were added, and larvae were ground an additional 15 s. The tubes were briefly centrifuged and boiled for 3 min. Samples were stored at −80 °C until PCR amplification.

Genomic DNA from the other tick populations was also extracted from individual larvae according to Guerrero et al. (2001) but with some modifications. Briefly, frozen larvae were transferred individually from plastic vials to pre-chilled 1.2 ml polypropylene tubes (Qiagen, Switzerland) kept on dry ice. Steel beads (5 mm diameter, Qiagen, Switzerland) were dispensed into the tubes using a 96-well dispenser (Qiagen, Switzerland) and 20 µl buffer (100 mM Tris, pH 8.3; 500 mM KCl) were added per tube. The tubes were grouped in a 96-well rack and placed in a mixer mill (Retsch, Haan, Switzerland, Type MM301) between two adaptors at an oscillation frequency of 25 Hertz for a total of 4 min (2 x 2 min) to grind the larvae. The tubes were centrifuged for 1 min at 1500*g* and 4 °C, boiled for 5 min and centrifuged again for 1 min at 1500*g* at room temperature. The DNA extracts were pipetted from the polypropylene tubes to 96-well PCR plates (Thermo Scientific, Switzerland). Plates were sealed with adhesive foil sheets (Thermo Scientific, Switzerland) and samples stored at −80 °C until PCR amplification.

2.4. PCR Conditions

PCR amplification was carried out in two separate reactions to detect both the pyrethroid susceptible and pyrethroid resistant alleles. Twenty µl reactions were optimized and performed in thin-walled 0.2 ml microcentrifuge tubes (Rainin, Oakland, CA). During optimization, we designed and tested several oligonucleotide primers and varied their concentrations (0.5–4.0 µM) and annealing temperature (54–64 °C). The concentration of $MgCl_2$ was varied from 1.0 to 2.5 mM, and the concentration of each dNTP was varied from 0.05 to 0.2 mM. Final optimized reaction conditions used 1 µl of DNA from a single tick larva, 1 µl of 10X PCR buffer II, 1.75 mM $MgCl_2$, 0.1 mM of each dNTP, and 0.2 µl of a 1:1 vol:vol mix of Ampli*Taq* (Applied Biosystems, Carlsbad, CA) and TaqStart antibody (Clontech, Mountain View, CA). To detect the susceptible alleles, reactions included 4 µM of primers FG-221 and FG-424, 1 µM of primers DB-011 and FG-447, and 0.5 µM of primers LL-001 and FG-446 (Table 3). To detect the resistant alleles, reactions included 4 µM of primers FG-222 and FG-424, 1 µM of primers DB-012 and FG-447, and 0.5 µM of primers LL-005 and FG-446 (Table 3). Amplification was carried out using a DNA Engine (MJ Research, Watertown, MA) programmed for 2 min at 96 °C, followed by 37 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. A final extension of 72 °C for 7 min was also included. PCR products were viewed on GelStar (Lonza, Rockland, ME) stained 4% NuSieve agarose and TBE gels (Lonza, Rockland, ME).

2.5. Data analyses

Statistical analysis of the LTT results was performed on the R software (version 2.12.0) using the drc package (version 2.0–1), specific for modelling dose–response curves (Ritz and Streibig, 2005). Concentrations inducing 50% (LC_{50}) and 90% (LC_{90}) mortality and their respective resistance ratios (RR50 and RR90) as well as the survival rates at potential discriminating doses (DD) were calculated as described previously (Lovis et al., 2011). Correlations

Table 3
Sequences of primers selected for the multiplex PCR assay.

Primer ID	Sequence	Description	Annealing Site in AF134216 (nt#)
Domain III mutation			
FG-221[a]	5′-TTATCTTCGGCTCCTTCT-3′	Wild type-specific sense	2117–2134
FG-222[a]	5′-TTATCTTCGGCTCCTTCA-3′	Resistant-specific sense	2117–2134
FG-424	5′-TCATTGAAATTGTCGATAATAACAC-3′	Downstream non-diagnostic	2156–2180
L64I Domain II mutation			
FG-447	5′-GAACTTGTGTTTACTTTCTTCGTAGT-3′	Downstream non-diagnostic	266–291
DB-011[b]	5′-GGAAAACCATCGGTGCTC-3′	Wild type-specific sense	173–190
DB-012[b]	5′-GGAAAACCATCGGTGCTA-3′	Resistant-specific sense	173–190
G72V Domain II mutation			
LL-001	5′-CTTGACCTTTGTCCTGGG-3′	Wild type-specific sense	198–215
LL-005	5′-CTTGACCTTTGTCCTGGT-3′	Resistant-specific sense	198–215
FG-446	5′-ACTTGTGTTTACTTTCTTCGTAGT-3′	Downstream non-diagnostic	266–289

[a] Primer sequences obtained from Guerrero et al. (2001).
[b] Primer sequences obtained from Morgan et al. (2009).

between the frequency of the RR genotype or resistant allele frequency and the survival rates at the DD were estimated using the Pearson's correlation coefficients on the R software for flumethrin and cypermethrin. Due to non-linear correlations, correlations with RR50 and RR90 were computed using the Kendall coefficient. These correlations were based on the populations which possessed PCR results for a minimum of 15 larvae. Statistical analysis of the LPT results was performed using Polo-PC (LeOra Software, 1987). Lethal doses inducing 50% and RR50 were calculated as described previously (Miller et al., 1999; Li et al., 2007). Tick population resistance phenotype was assigned based on three criteria: RR50, RR90 and survival at the DD. Populations were considered resistant when their RR50 or RR90 was greater than four or when the survival rate at the DD was over 10% (Lovis et al., submitted for publication-a,b).

3. Results

3.1. Multiplex PCR assay development

Guerrero et al. (2001) developed an allele-specific PCR assay to detect an T → A nucleotide substitution at the nucleotide #2134 of the tick sodium channel coding sequence (all numbering in this work is based upon the AF134216 GenBank nucleotide sequence fragment entry for *R.*(*B.*) *microplus*) using primers FG-221 and FG-222 as diagnostic primers and FG-227 as the downstream non-diagnostic primer. The two diagnostic primers from this assay were used for the new multiplex assay, but a new non-diagnostic primer (FG-424) was designed leading to an amplified product of 64 bp (Fig. 1). FG-424 performed better than FG-227, because self-hybridization of FG-227 at its 3′-end led to primer-dimer formation during the amplification. Primer concentrations in the multiplex were adjusted from the 1.0 μM concentration used in the Guerrero et al. (2001) protocol as needed to optimize the performance of the multiplex.

Morgan et al. (2009) designed primers for an allele-specific PCR targeting a C → A nucleotide substitution at the nucleotide #190 of the tick sodium channel coding region (L64I Domain II mutation). Their two diagnostic primers were used for the new multiplex assay and renamed DB-011 (wild type) and DB-012 (resistant), but a new non-diagnostic primer (FG-447) was designed to lengthen the amplified fragment to 119 bp and hence allow its differentiation from other amplified fragments (Fig. 1). We initially designed primers to detect the mutation on the sense and antisense strands and observed the presence of an intron in the diagnostic assay based on the antisense strand, preventing the use of this version for the multiplex. To enhance specificity of the multiplex, the mismatch of a C to a T incorporated at the penultimate base of the diagnostic primers by Morgan et al. (2009) was also incorporated into our protocol. Primers were used at a concentration of 1.0 μM.

Jonsson et al. (2010) identified a G → T mutation at the nucleotide #215 of the tick sodium channel coding region (G72V Domain II mutation) and we designed allele-specific primers LL-001 (wild type diagnostic), LL-005 (resistant diagnostic) and FG-446 (non-diagnostic) to allow detection of this mutation in the multiplex reaction, producing a 92 bp amplification product. As noted previously, detection on the antisense strand would be hampered by the presence of an intron upstream of the mutation reported by Morgan et al. (2009). An additional intron was observed on the coding strand between the nucleotides #353 and #434. FG-446 was therefore used as the non-diagnostic primer to avoid this intron. The concentration of these primers was optimized at 0.5 μM and typical results from the multiplex PCR assay obtained for different genotypes are shown in Fig. 2.

3.2. Bioassays

Resistance ratios to flumethrin and cypermethrin at 50% and 90% mortality, survival rates at the DD obtained with the LTT, as well as the RR50 estimated with the LPT are shown in Table 4. Around 86% (24/28) of the populations evaluated with the LTT were found to be resistant to cypermethrin based on our criteria noted previously. Resistance to cypermethrin based on the LTT was observed in 13 out of 14 Brazilian, 7 out of 8 Argentinean, both of the Australian and one of the three South African populations. The four populations found to be susceptible to cypermethrin were also susceptible to flumethrin (ST11, ST12, ST21, ST47). One additional Brazilian population (ST55) was susceptible to flumethrin, by all the bioassay criteria, but was cypermethrin resistant. Resistance statuses to flumethrin based on RR50, differed from those based on RR90 and DD for four Argentinean populations (ST22, ST25, ST26, ST27) which would have been considered susceptible based on RR50 but resistant based on RR90 and DD. Finally, the three Mexican strains, tested with the LPT, were resistant to flumethrin, cypermethrin and permethrin, and the Brazilian Santa Luiza population was resistant to permethrin based on LPT.

3.3. Genotype frequencies

The frequency of larvae with specific genotypes to the three mutations is listed in Table 4 and correlation between RR genotype frequency and survival rate at the DD of flumethrin and cypermethrin represented in Fig. 3. The Domain III mutation was found in two of the three Mexican populations, San Felipe and Rio Bravo

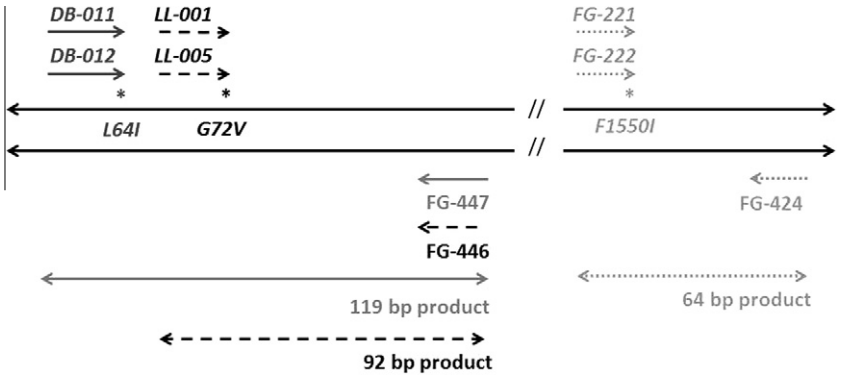

Fig. 1. Schematic representation of the locations of the primers selected for multiplex PCR assay. Asterisks indicate the location of the mutations. Italicised primers are the diagnostic primers.

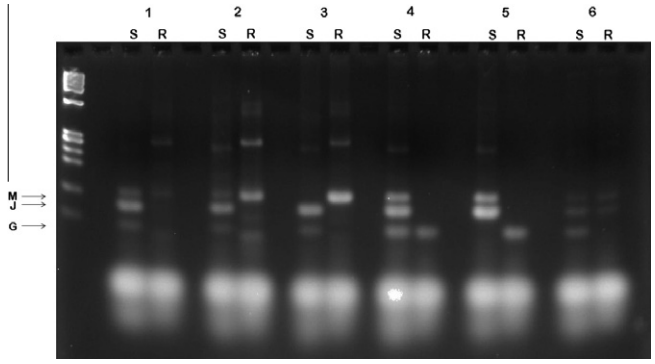

Fig. 2. Multiplex PCR assay of representative genotypes. Arrows point to the locations of the PCR amplification product that is diagnostic for each mutation: *G* = Domain III mutation, product size is 64 bp; *J* = G72V Domain II mutation, product size is 92 bp; *M* = L64I Domain II mutation, product size is 119 bp. Two separate reactions were carried out to detect the susceptible alleles (S) and the resistant alleles (R). (1) larva homozygous susceptible to all three mutations; (2) larva homozygous susceptible to *G* and *J*, heterozygote to *M*; (3) larva homozygous susceptible to *G* and *J*, homozygous resistant to M; (4) larva heterozygote to *G*, homozygous susceptible to *J* and *M*; (5) larva homozygous resistant to *G*, homozygous susceptible to *J* and *M*; (6) larva homozygous susceptible to *G*, heterozygote to *J* and *M*.

(Table 4). Both of these populations were phenotypically resistant to SP and had a resistant allele frequency of 94% and 100%, respectively. This Domain III mutation was not found in any samples from the other countries. The G72V Domain II mutation was found in only a single larva of an Australian population as a heterozygote. It was not found in any other country. The L64I Domain II mutation was found in all the Brazilian, Argentinean and Australian populations and in one of the three South African populations. In contrast, the L64I Domain II mutation was not found in the samples that originated in Mexico, and only the Domain III mutation was found in these populations. The frequency of the L64I resistant allele was greater than 38% in all the non-Mexican populations resistant to cypermethrin, with the exception of ST55 where it was 8% (Table 4). Three of the four populations which were susceptible to both flumethrin and cypermethrin (ST11, ST12, and ST21) did not possess any RR genotype individuals and none or few RS individuals (0–17%) while the fourth population (ST47) had a resistant allele frequency of 28%.

4. Discussion

Target site insensitivity has been shown to be the major resistance mechanism in most of the SP resistant populations of *R.* (*B.*) *microplus* in Mexico (Rosario-Cruz et al., 2005, 2009b) and Australia (Morgan et al., 2009; Jonsson et al., 2010). Metabolic detoxification, through increased esterase activity (Jamroz et al., 2000; Pruett et al., 2002) or mutation in an esterase gene (Hernandez et al., 2000, 2002), is a complementary mechanism to *kdr* resistance. The three currently known mutations in the sodium channel gene of *R.* (*B.*) *microplus* have distinct geographical distributions and result in different resistance phenotypes. The Domain III mutation, widespread throughout Mexico but apparently limited to this country, confers very high resistance to flumethrin, cypermethrin and permethrin. For example, the Corrales and San Felipe Mexican strains were reported by Miller et al. (1999) as possessing RR50 > 1000 to permethrin and RR50 > 2300 to cypermethrin following laboratory selection with SP. Guerrero et al. (2001) showed the Corrales and San Felipe strains had 99% and 86% resistant allele frequency, respectively. The two Domain II mutations seem to provide lower levels of resistance than the Domain III mutation as assayed in our study. The L64I Domain II mutation identified by Morgan et al. (2009) in Australian populations has also been found in Brazil by other researchers (Domingues et al., 2012) and provides resistance to the same spectrum of acaricides as the Domain III mutation, but with low to moderate resistance ratios. For example, the Parkhurst strain was reported by Nolan et al. (1989) to have RR50 to SP between 100 and 450 and Brazilian populations possessing very high frequency of this mutation had RR50 to cypermethrin between 16 and 25 (Domingues et al., 2012). The G72V Domain II mutation reported by Jonsson et al. (2010) confers low levels of resistance to flumethrin but offers no resistance to cypermethrin (Jonsson et al., 2010).

Allele specific PCR assays allow the identification of wild type or mutated alleles at the individual level. They offer several advantages in comparison to bioassays, requiring low numbers of larvae and allowing results to be obtained in less time than the larval *in vitro* tests, such as the LPT or LTT, which usually require 6 weeks for completion. In addition, PCR assays can detect resistance early in its emergence because resistant alleles can be detected at low frequency in populations that may still demonstrate a susceptible phenotype in bioassays (Rosario-Cruz et al., 2005). However, PCR assays require specific infrastructure, well-trained technicians and cold-chain sample maintenance to preserve nucleic acid quality. Furthermore, PCR assays can only identify the genotype of the mutation for which it was designed. Yet-to be discovered mutations or metabolic detoxification mechanisms cannot be detected. Hence, PCR assays should not substitute for bioassays but the two should be carried out in conjunction when possible. The use of the present multiplex PCR assay allowed the successful detection of the three currently known target site resistance-associated mutations in *R.* (*B.*) *microplus* in one assay, increasing significantly the efficiency of the detection of these mutations.

Table 4
Resistance ratios at 50% and 90% mortality to flumethrin and cypermethrin or permethrin and *kdr* genotypes to the three mutations, in populations of *R.* (*B.*) *microplus* ticks from Brazil, Argentina, Mexico, South Africa and Australia.

Strain	Country	Flumethrin			Cypermethrin			% of larvae larvae with specific *kdr* genotype												
								L64I Domain II					Domain III				G72V Domain II			
		RR50[a]	RR90[b]	Surv[c] (%)	RR50[a]	RR90[b]	Surv[c] (%)	SA[d]	RR[e]	SR[e]	SS[e]	R[f]	SA[d]	RR[e]	SR[e]	SS[e]	SA[d]	RR[e]	SR[e]	SS[e]
ST40	Brazil	57.9	29.5	99.9	104.8	71.3	97.1	7	86	14	0	93	8	0	0	100	9	0	0	100
ST41	Brazil	129.2	92.4	100	141	95.4	99	8	62	38	0	81	7	0	0	100	9	0	0	100
ST42	Brazil	88.7	64.8	99.8	201.1	407.5	92.6	8	100	0	0	100	9	0	0	100	9	0	0	100
ST44	Brazil	147.3	105.1	99.9	167.3	203.4	95.7	6	100	0	0	100	6	0	0	100	9	0	0	100
ST46	Brazil	92.2	78.1	99.2	109.9	78.7	85.5	9	89	11	0	94	0	No amplif.			9	0	0	100
ST47	Brazil	0.8	0.6	0.1	3.6	2.2	0.5	16	25	6	69	28	22	0	0	100	9	0	0	100
ST48	Brazil	63.3	55.5	100	203.9	120.4	95.4	20	100	0	0	100	29	0	0	100	9	0	0	100
ST49	Brazil	74.8	46.9	99.9	155.7	73.6	98.2	5	100	0	0	100	7	0	0	100	9	0	0	100
ST50	Brazil	40	37.5	85.4	71.3	85.3	68	5	40	60	0	70	6	0	0	100	7	0	0	100
ST51	Brazil	41.5	47.3	81	103.7	64.9	80.5	7	86	14	0	93	8	0	0	100	9	0	0	100
ST52	Brazil	116.1	91.1	100	221.5	339.4	95.7	8	50	12	38	56	16	0	0	100	15	0	0	100
ST55	Brazil	0.8	2.3	7.8	11	15.8	19.4	13	0	15	85	8	14	0	0	100	16	0	0	100
ST57	Brazil	83.5	65.6	100	309.3	735.5	96.2	10	80	10	10	85	17	0	0	100	17	0	0	100
ST58	Brazil	65.4	41.5	99.8	143.1	72.8	97.2	5	100	0	0	100	11	0	0	100	14	0	0	100
Santa Luiza	Brazil	na	na	na	na	na	90.7*	18	78	22	0	89	18	0	0	100	18	0	0	100
ST21	Argentina	<1.0	0.2	0	1.7	2.1	0	18	0	17	83	8	16	0	0	100	17	0	0	100
ST22	Argentina	1.2	36.5	34.5	6.8	21.7	20.5	17	24	29	47	38	11	0	0	100	18	0	0	100
ST24	Argentina	21.8	46.3	67.2	27.2	30.2	40.5	18	22	39	39	42	16	0	0	100	18	0	0	100
ST25	Argentina	2.5	31.3	31.9	6.6	16.4	16.7	15	47	47	7	70	11	0	0	100	17	0	0	100
ST26	Argentina	1.3	27.8	26.1	5.6	23.2	18.2	16	81	19	0	91	16	0	0	100	16	0	0	100
ST27	Argentina	0.9	32.2	33.4	4.2	19.4	16.1	16	19	75	6	56	18	0	0	100	13	0	0	100
ST29	Argentina	23	24	83.1	57	30.8	67.4	18	100	0	0	100	18	0	0	100	18	0	0	100
ST30	Argentina	na	na	na	21.5	116.2	41.2	17	88	12	0	94	17	0	0	100	17	0	0	100
San Felipe	Mexico	>24,300	na	na	>2300	na	>1000*	17	0	0	100	0	17	88	12	0	17	0	0	100
Coatzacoalcos	Mexico	15.7	na	na	57.2	na	166*	0	No amplification				17	0	0	100	17	0	0	100
Rio Bravo	Mexico	na	na	na	na	na	99.6**	17	0	0	100	0	17	100	0	0	17	0	0	100
ST11	South Africa	<4.0	<1.6	0	2.3	2.1	0.3	17	0	0	100	0	17	0	0	100	17	0	0	100
ST12	South Africa	<4.0	<1.6	0	1.7	1.4	0.1	18	0	0	100	0	18	0	0	100	18	0	0	100
ST15	South Africa	51.9	46.1	87.1	101.5	43.7	79.4	16	100	0	0	100	18	0	0	100	18	0	0	100
Ultimo	Australia	157.8	113.7	99.7	113.1	67.8	78.3	6	100	0	0	100	6	0	0	100	6	0	0	100
Urah	Australia	43.4	58.3	78	33.9	48.8	47.9	18	94	6	0	97	18	0	0	100	18	0	6	94

na: not available.
[a] Resistance ratios at 50% mortality.
[b] Resistance ratios at 90% mortality.
[c] Survival rates at DD.
[d] No. of larvae with successful amplification.
[e] RR = homozygous resistant genotype; SR = heterozygous genotype; SS = homozygous susceptible genotype.
[f] Percentage of resistant alleles: R = No. of resistant alleles divided by the total number of successful amplified alleles (= RR% + 0.5*RS%).
* Resistance ratios at 50% mortality of permethrin.
** Surviving rate at a DD of 0.2% AI of permethrin.

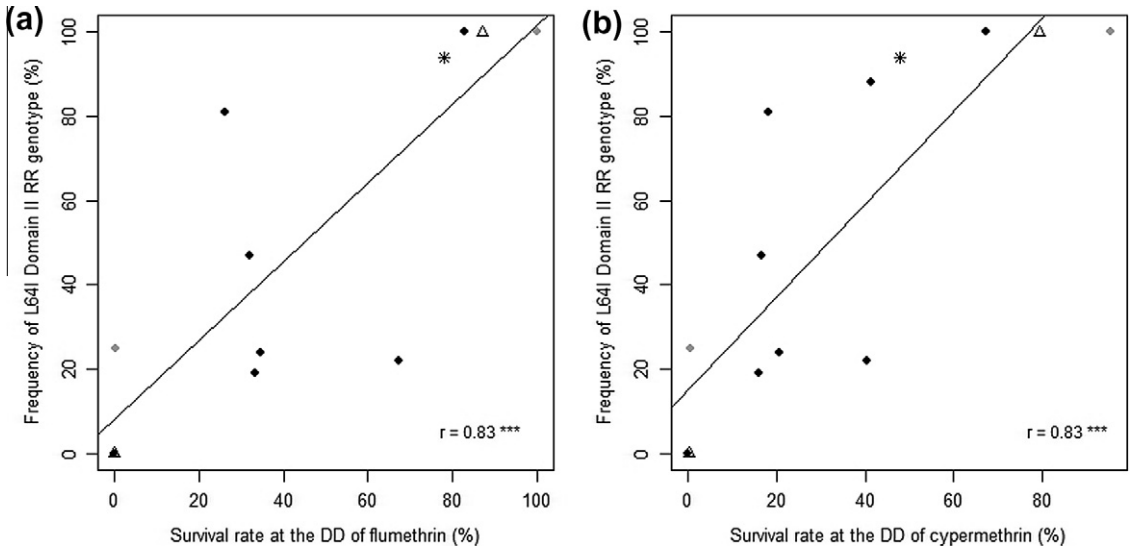

Fig. 3. Correlation between RR genotype frequency of the L64I Domain II mutation and survival rate at the DD of (a) flumethrin and (b) cypermethrin, measured with the LTT for the Brazilian (grey diamonds), Argentinean (black diamonds), South African (triangles) and Australian (stars) populations. The *r* value is the Pearson correlation coefficient; ***indicates that $p < 0.001$. Correlations were based on the populations which possessed PCR results for a minimum of 15 larvae.

In this paper, we report the widespread distribution of the L64I Domain II mutation outside Australia. This mutation was found in all the phenotypically SP resistant bioassayed populations of Brazil, Argentina, South Africa and Australia, with resistant allele frequency between 38% and 100% in all of them, except in one population from Brazil (ST55, 8%) (Table 4). Interestingly, the L64I Domain II mutation was not found in any of the three Mexican strains whereas the Domain III mutation was present in two of these strains, with resistant allele frequency over 95% (Table 4). Thus, the Domain III mutation was limited to Mexico, further supporting the absence of this mutation outside North America as previously reported (Chen et al., 2009; Andreotti et al., 2011; Domingues et al., 2012). In addition this study provides evidence that the L64I Domain II mutation, whose major importance has been demonstrated in Australia (Morgan et al., 2009; Jonsson et al., 2010) and very recently in Brazil (Domingues et al., 2012), is probably also a major mechanism conferring resistance to SP in Argentina, and is also present in South Africa. Our results suggest that this mutation may be the mechanism responsible for SP resistance in some of the phenotypically resistant Brazilian populations studied by Andreotti et al. (2011) that failed to show the Domain III mutation in this group's PCR assays. The low incidence of the G72V Domain II mutation, being limited to a single copy in an Australian population, is consistent with the phenotypes we observed and the one reported to be associated to the G72V Domain II mutation. Indeed, Jonsson et al. (2010) showed that this mutation provides resistance to flumethrin but not to cypermethrin while none of the populations included in the present study demonstrated a similar phenotype. The geographical boundary between the Domain III mutation found in Mexico and the Domain II mutation found in South America is not known yet. Further sampling in Central America and north and south of the Amazon would provide very interesting information on the region where the distribution areas of these two mutations meet. Most of the tick populations included in this study were collected in farms in which some lack of treatment efficacy had been reported by the owners. Therefore, our data do not allow extrapolating SP resistance prevalence. Furthermore, it would be interesting to test additional field tick populations from Mexico to confirm the absence of the L64I Domain II mutation since the three Mexican samples used in the present study are all laboratory strains which have been occasionally exposed to acaricidal pressure during their maintenance.

When excluding the Mexican populations, the frequency of the RR genotype of the L64I Domain II mutation was found to be linearly correlated with survival rates at DD of both flumethrin and cypermethrin (Fig. 3; $r = 0.83$ ($r^2 = 0.69$), $p < 0.001$, for both compounds). The correlation coefficients between the resistant allele frequency and the *in vitro* results were very close to those values. RR genotype and resistant allele frequencies were also found to be associated with RR50 and RR90, but with a nonlinear correlation whose coefficients were lower than those for DD (data not shown).

As we see from the correlation results (r^2 values), 69% of the variation in the survival at the flumethrin and cypermethrin DD can be explained by the variation in the frequency of the L64I Domain II mutation RR genotype. Hence there is still an important part of the variation in the phenotypic results which is not explained by the L64I Domain II mutation genotype. Thus, for example, a 50% frequency of the L64I Domain II mutation RR genotype was sufficient to lead to 100% survival at the flumethrin DD in ST52, whereas for ST29 a RR genotype frequency of 100% led to only 67.4% survival at the cypermethrin DD (Table 4). Variability was also observed when comparing the L64I Domain II mutation genotype frequency with RR50 or RR90. Hence, RR genotype frequency of the L64I Domain II mutation of 100% resulted in RR50 ranging from 23 for flumethrin up to >200 for cypermethrin. These differences may be due to the presence of additional mechanisms of resistance such as metabolic detoxification or a yet undiscovered mutation, which may increase the resistance conferred by the L64I Domain II mutation in some of the tick populations. Furthermore, increased metabolic activity is probably the major mechanism of SP resistance in the ST55 population. Indeed, this Brazilian field population was shown to be cypermethrin resistant and flumethrin susceptible while it possessed a resistant allele frequency of the L64I Domain II mutation of only 8%. Inversely, another Brazilian population (ST47) possessed a resistant allele frequency of 28% while it demonstrated a susceptible phenotype to both flumethrin and cypermethrin. Lack of efficacy of cypermethrin treatment was reported by the farmer who provided this strain, supporting the molecular diagnosis. Interestingly, the four Argentinean populations (ST22, ST25, ST26, ST27) which appeared susceptible to flumethrin based on RR50 but resistant based on RR90, indicating the emergence of SP resistance in these populations, possessed resistant allele frequency between 38% and 91% (Table 4), confirming their resistance status and the importance

of considering RR90 in addition to RR50 to allow the detection of resistance in development with bioassays.

The Brazilian Santa Luiza strain has previously been widely studied. In 2007, Li et al. reported a 100% susceptible genotype of the Domain III mutation in the Santa Luiza strain while its RR50 to permethrin was 90.7. Increased metabolic detoxification was first thought to be the cause of the resistance but later studies made detoxification less likely to be a dominant cause of SP resistance (Li et al., 2008). In fact, these authors noted the possibility that another mutation besides the Domain III mutation might exist in Santa Luiza (Li et al., 2008). Our report of the L64I Domain II mutation in the Santa Luiza strain therefore confirms their hypothesis. In addition, Li et al. (2008) presumed that the permethrin resistance in the Santa Luiza strain was inherited as an incomplete recessive trait, which is compatible with the hypothesis that the L64I Domain II mutation is a recessive trait (Morgan et al., 2009).

The three Mexican strains included in the present study have also been widely studied and our results corroborate the previous findings. The San Felipe strain is one of the two populations in which the Domain III mutation was originally discovered (He et al., 1999). The frequency of the resistant allele was shown to be between 80% and 86% (Guerrero et al., 2001; Li et al., 2007) while a lack of significant metabolic resistance had been shown (Miller et al., 1999). The resistant allelic frequency observed in the present study (94%, Table 4) is in line with the previous results. The Rio Bravo strain was selected in 1998–1999 for permethrin resistance for two generations (personal communication, Robert Miller) and the resistant allelic frequency we found (100%, Table 4) is therefore consistent. In contrast, the SP resistance in the Mexican Coatzacoalcos strain is known to be driven by increased metabolic activity. Indeed, metabolic resistance was first shown using synergists (Miller et al., 1999) and later confirmed with measures of esterase activity (Jamroz et al., 2000) while only very few individuals carrying the Domain III mutated allele (resistant allele frequency of 4%) were identified in this strain (Guerrero et al., 2001). In the present study, we did not find any of the three investigated mutations in Coatzacoalcos, supporting the view that metabolic resistance is likely a major mechanism of resistance in the Coatzacoalcos strain. The presence of an additional mutation not yet identified cannot be excluded.

The mutation found in the Mexican strains is located in the Domain III of the sodium channel gene of *R.* (*B.*) *microplus* and seems to provide higher resistance to SP than the two Domain II mutations. Single nucleotide substitutions in the sodium channel gene are either found as single mutations in resistant populations or in combination with another mutation, which can provide an additive or synergistic increase of resistance (Soderlund and Knipple, 2003; O'Reilly et al., 2006). Hence, very high resistance levels to SP are generally observed when there is the simultaneous presence of two mutations (Williamson et al., 1996; Guerrero et al., 1997; Liu et al., 2000). Mutations occurring in the Domain III are less common than those in the Domain II (O'Reilly et al., 2006), but have also been reported in insects (Pittendrigh et al., 1997). Pittendrigh et al. (1997) identified three mutations in the Domain III of *Drosophila melanogaster*, among which two occur at positions that are similar to the positions of mutations identified by Williamson et al. (1996) in Domain II in super-*kdr* house flies. Strains carrying both mutations possessed higher resistance levels to deltamethrin than the additive effects of the two single mutations. However, in the case of *R.* (*B.*) *microplus*, the Phenylalanine (Phe) to Isoleucine (Ile) Domain III S6 mutation provides very high levels of resistance (>1000) while it was not found to be combined with another mutation. Tan et al. (2005) demonstrated that the Domain III S6 mutation identified in *R.* (*B.*) *microplus* provides knockdown resistance in cockroaches, abolishing the sensitivity of the cockroach sodium channel expressed in *Xenopus laevis* oocytes to type I and type II pyrethroids, by reducing the pyrethroid binding to the sodium channel. The authors also observed that an aromatic residue at the position of the Phe to Ile Domain III S6 mutation is essential for the action of pyrethroids. O'Reilly et al. (2006) proposed a model of the housefly sodium channel where the pyrethroid binding site is located in a hydrophobic cavity delimited by the domain II S4-S5 linker and the Domain II S5 and III S6 helices. Their model suggests that the Phe to Ile Domain III S6 mutation could disrupt the interactions between the Domain III S6 helix and the Domain II S4-S5 linker and alter their relative positions. The crucial role of the aromatic amino acid at the pyrethroid binding site of the sodium channel may explain why this mutation confers such high levels of resistance to *R.* (*B.*) *microplus* and why the Leu to Ile and Gly to Val mutations of the Domain II S4-S5 linker confer such lower levels of resistance.

To conclude, this study provides evidence of the geographic separation of the three previously identified mutations. We provide data on the extent of the L64I Domain II mutation outside Australia, and show that the Domain III mutation is present only in North America. The L64I mutation is located in the Domain II of the sodium channel, the region that usually contains the *kdr* and super-*kdr* mutations in insects (Soderlund and Knipple, 2003). Perhaps this Domain is somehow more amenable to mutation compared to Domain III which may explain the widespread occurrence of the Domain II mutation throughout arthropods. In addition, the widespread distribution of the L64I Domain II mutation and its association to SP resistance is consistent with the hypothesis that only a few mutations in the sodium channel gene conferring SP-resistance may exist in *R.* (*B.*) *microplus* (Guerrero et al., 2001). Increased detoxification, such as hydrolytic esterase activity, is likely to complement the target site insensitivity observed in the present study. However, other metabolic resistance mechanisms in *R.* (*B.*) *microplus* such as detoxification through cytochrome P450 and glutathione S-transferase cannot be ruled out.

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