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Identification of a mutation in the *para* sodium channel gene of the cattle tick *Rhipicephalus (Boophilus) microplus* associated with resistance to synthetic pyrethroid acaricides

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Abstract

Resistance against synthetic pyrethroid (SP) products for the control of cattle ticks in Australia was detected in the field in 1984, within a very short time of commercial introduction. We have identified a mutation in the domain II S4-5 linker of the para-sodium channel that is associated with resistance to SPs in the cattle tick *Rhipicephalus (Boophilus) microplus* from Australia. The cytosine to adenine mutation at position 190 in the *R. microplus* sequence AF134216, results in an amino acid substitution from leucine in the susceptible strain to isoleucine in the resistant strain. A similar mutation has been shown to confer SP resistance in the whitefly, *Bemisia tabaci*, but has not been described previously in ticks. A diagnostic quantitative PCR assay has been developed using allele-specific Taqman® minor groove-binding (MGB) probes. Using the assay to screen field and laboratory populations of ticks showed that homozygote allelic frequencies correlated highly with the survival percentage at the discriminating concentration of cypermethrin.

Keywords: *Rhipicephalus microplus*; Cattle tick; Synthetic pyrethroid; Acaricide resistance; quantitative PCR

1. Introduction

Even before the commercial release of synthetic pyrethroid (SP) products for the control of cattle ticks in Australia in the early 1980s, Jim Nolan and associates (Nolan et al., 1977) had demonstrated that an organochlorine-resistant strain of cattle ticks (*Rhipicephalus* (*Boophilus*) *microplus*) showed cross-resistance to experimental SPs. These ticks had resistance ratios (ratio of the concentration required to kill a given proportion of a sample of resistant ticks compared with the concentration required to kill the same proportion of a sample of susceptible ticks, e.g. LC_{50}(resistant)/LC_{50}(susceptible)) of up to 10 in larvae (Nolan et al., 1977). The mechanism was subsequently determined to be increased esterase activity (Schnitzerling et al., 1983). Resistance was indeed detected against SPs in the field in 1984, within a very short time of their commercial introduction (Nolan et al., 1989). In one strain (Marmor) the resistance ratios were 3, 9.5 and 6 for flumethrin, cyhalothrin and cypermethrin, respectively, and were due to increased detoxification. However, some cases of field resistance were associated with much higher resistance ratios and there was no evidence of increased detoxification. For example, the Parkhurst strain was associated with resistance ratios of 114 against cypermethrin, 130 against cyhalothrin, 152 against deltamethrin and 446 against flumethrin. A second mechanism conferring SP resistance, independent of increased detoxification, seemed likely (Nolan et al., 1989).

In the USA, Miller and co-workers demonstrated a similarly diverse pattern of resistance to SP products in Mexican ticks (Miller et al., 1999). They showed that resistance ratios were consistently greater than 1,000 for the Corrales and San Felipe strains, in which there was no evidence of increased detoxification mechanisms. In contrast, detoxification was evident in the Coatzacoalcos strain, which had a

resistance ratio of 166, with synergist ratios with permethrin of 19.7 and 13.0 for triphenylphosphate and piperonyl butoxide, respectively (Miller et al., 1999). The synergist ratio is the ratio of the concentration of the active compound required to kill a given proportion of ticks in the absence of the synergist to the concentration of the active compound required to kill the same proportion of ticks in the presence of the synergist. Pyrethroid insecticides primarily target the voltage-gated sodium channel and point mutations in the gene have been linked to SP resistance in numerous insect species including flies, cockroaches, moths, aphids, mosquitoes, beetles, thrips and fleas (reviewed by Dong, 2007). The gene consists of four domains (I-IV) each containing six segments (S1-6). A resistance-linked mutation in the domain III S6 *para*- sodium channel that caused an amino acid substitution from phenylalanine (F) to isoleucine (I) was discovered in the Corrales and San Felipe *R. microplus* strains (He et al., 1999), but has not been reported in ticks in Australia.

Most mutations that confer SP resistance in arthropods are found in the domain II S6 or in the linker between domain II S4-5 (Williamson et al., 1996; Guerrero et al., 1997; Morin et al., 2002). In houseflies, a leucine (L) to phenylalanine (F) replacement in the domain II S6 alone confers knockdown resistance (*kdr)*, and a methionine (M) to threonine (T) replacement in the domain II S4-5 linker in addition to the *kdr* mutation confers *super-kdr* resistance (Williamson et al., 1996). In some cases, such as the whitefly *Bemisia tabaci*, a mutation in the domain II S4-5 linker alone has been associated with resistance to SPs (Morin et al., 2002).

Various bioassay techniques have been developed for detecting acaricide resistance in ticks, however the most definitive method for many acaricides has been the larval packet technique (LPT) (Stone and Haydock, 1962). This method has been adopted by the Food and Agriculture Organization of the United Nations (FAO) as the

standard for acaricide resistance detection and measurement (Jonsson et al., 2007). In the LPT, live larvae are exposed to filter paper packets impregnated with acaricide, incubated for acaricide-specific time periods and then larval mortality is assessed. The LPT can be used to detect resistance to organochlorines, organophosphates, synthetic pyrethroids, amidines and macrocyclic lactones, and can be used for single and multi-host ticks. It is a repeatable test that performs better than the widely used adult immersion test (Jonsson et al., 2007). Although the LPT provides repeatable indications of the overall level of resistance to acaricides of a given population, it does not provide an indication of the proportion of a population that carries resistance-conferring genes and the requirement for larvae means that it takes at least 6 weeks to provide a result.

Allele-specific PCR assays and probe-based quantitative PCR assays have been developed to detect drug resistance in a variety of organisms (Wada et al., 2004; Moreno et al., 2008;Yoshida et al., 2008). Such assays offer sensitive and rapid alternatives to bioassays required to determine levels of drug resistance for the subsequent application of appropriate treatments. Further advantages of molecular-based assays are that specimens do not need to be maintained alive; ticks can be screened at any life-stage, reducing the time and cost associated with completing life cycles in the laboratory; and there is comparatively little exposure of laboratory technicians to the toxic compounds used in the bioassays. The obvious disadvantage is that in contrast to the LPT, more advanced equipment is required to conduct the tests. Another, perhaps more important disadvantage is that PCR-based assays can only detect the known mutations and will not detect new mutations in the same gene, mutations in other genes or enhanced detoxification through over-expression of

esterases, mixed function oxidases or GSTs. For this reason, PCR-based assays should not be viewed as a complete replacement for conventional bioassays.

In this paper we describe the identification of a mutation in the domain II S4-5 linker that is associated with resistance to SPs in the cattle tick *Rhipicephalus microplus*. The C-A mutation at nucleotide position 190 in Genbank *R. microplus* sequence **AF134216** results in an L to I substitution, the same as that described by Morin et al. (2002) in the whitefly. A molecular assay was developed to confirm the detection of this mutation in resistant tick strains.

2. Materials and methods

2.1. Laboratory tick strains for assay development

A strain of ticks resistant to SPs (Parkhurst; Nolan et al., 1989) and a strain susceptible to all acaricides (NRFS, or N; Stewart et al., 1982), are maintained at the Queensland Department of Primary Industries and Fisheries (DPI&F) Animal Research Institute. Parkhurst ticks are resistant to all SPs, including flumethrin, deltamethrin, cyhalothrin and cypermethrin (Nolan et al., 1989). Homozygous resistant ticks were selected from the SP resistant (Parkhurst) strain by selecting larvae that survived exposure to the discriminating concentration of cypermethrin expected to kill all susceptible ticks but no resistant ticks (0.3% w/v). Homozygous susceptible (wild type) ticks were obtained from the NRFS (N) strain which has 100% mortality with cypermethrin treatment (Stewart et al., 1982).

2.2. Field isolates of ticks for validation of assay

The DPI&F provides a diagnostic acaricide resistance testing service to cattle producers throughout Australia, using the LPT (Stone and Haydock, 1962). Larvae remaining after diagnostic testing are stored frozen at -20º C to use for research on the development of molecular diagnostic tests and for population genetic studies. The database of results from diagnostic samples submitted to the DPI&F for acaricide resistance using the LPT were examined to identify field populations with a range in survivorship when exposed to cypermethrin. Diagnostic submissions were identified for which survivorship at the discriminating dose of cypermethrin (0.3% w/v) was 0%, 28%, 53% and 75% and from which sufficient frozen, stored larvae were available. Cypermethrin is used routinely in the LPT bioassay because all ticks resistant to cypermethrin are expected to be resistant to all SPs (Nolan et al., 1989). One hundred larvae from the Parkhurst strain as well as 100 larvae from each of the four additional field isolates were screened with the new quantitative PCR diagnostic assay to evaluate its performance.

2.3. DNA extraction, amplification and sequencing

Single tick larvae were crushed with forceps in a 200 µl microfuge tube. Fifty microlitres of lysis buffer (PCR buffer containing 67 mM Tris–HCl pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 0.2 mg/ml Gelatin, 2.5 mM Mg, 0.25 µl Tween 20) were then added to the crushed ticks followed by 0.125 µl of 20 mg/ml Proteinase K. The ticks were incubated overnight at 56^{o}C then heat inactivated at 95^{o}C for 45 min.

PCR genomic DNA primers were designed to amplify the exon region between domain II, S4 loop and domain II, S5 because it is an area where resistance-conferring mutations have been described previously. Intron position was determined from an alignment of partial sodium channel *R. microplus* RNA (Mexican strain

GenBank accession number **AF134216**) against *Ixodes scapularis* sodium channel DNA sequences obtained searches using the BLASTn algorithm for matches against *Ixodes* contigs (*Ixodes* genome: http://iscapularis.vectorbase.org). Genomic DNA amplification was between forward primer BmNaF5 5' TACGTGTGTTCAAGCTAGC (position 103 in *R. microplus* GenBank accession number **AF134216**) and reverse primer BmNaR5 5' ACTTTCTTCGTAGTTCTTGC (position 260 in *R. microplus* GenBank accession number **AF134216**) producing a 167 bp product. PCR reactions contained 0.5 µM of each primer, combined with 10-100 ng of template DNA, 10 x Taq buffer, 0.8 mM dNTP, 3.75 mM magnesium and 0.05 units/µl of Taq polymerase (Geneworks BTQ-1). This mix was amplified in an Applied Biosystems 2720 thermocycler for 30 cycles. Cycle 1 was 95ºC for 60 s, 50ºC for 45 s and 72ºC for 90 s. This was followed by 29 shorter cycles, 95ºC for 30 s, 50ºC for 30 s and 72ºC for 90 s. The mix was held at 72ºC for 7 min to complete extension then dropped to 4ºC. Products were viewed on an ethidium bromide stained 1.5% agarose and tris(hydroxymethyl)aminomethane, acetic acid, EDTA (TAE) gel.

PCR products were concentrated and desalted prior to sequencing using Exosap-it® (USB Corporation distributed by GE Healthcare Bio-Sciences, Rydalmere NSW, Australia). PCR products were sequenced using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit Version 3.1 (PE Applied Biosystems, California, USA) and the products were run on an ABI 3130xl automated sequencer. Forward and reverse sequences were aligned and edited using ChromasPro (Technelysium Pty Ltd, Tewantin, Australia).

2.4. Dual probe quantitative PCR diagnostic assay

Generic primers and two diagnostic Taqman® MGB probes (Table 1) were designed around the target mutation site using Primer Express (Version 2.0 Applied Biosystems, California, USA). Quantitative PCR assays were run on both Rotor-Gene 3000 and Rotor-Gene 6000 (Corbett Research, Mortlake, NSW, Australia) thermocyclers. Reactions of 20 µl total volume containing 8 µl RealMasterMix Probe (Eppendorf), PCR primers at a concentration of 300 nM, the two MGB Taqman® probes at 200 nM each, 4% DMSO and 5 µl of undiluted extracted DNA. Amplification conditions were 2 min at 95°C followed by 45 cycles of 15 s at 95°C, 20 s at 58°C and 20 s at 68°C, acquiring the differently coloured FAM™ and VIC™ fluorescence (Applied Biosystems) at the end of the extension step. At the completion of the run the dynamic tube was turned on and the data was slope corrected. After preliminary testing the threshold line was set to 0.01 for all assays. Ticks were scored using the allelic discrimination function. Known homozygous and manually mixed heterozygous samples were run alongside all unknown ticks as standards and a negative PCR control, substituting water for DNA, was also included in every run.

2.5. Allele-specific PCR assay

Allele-specific primers were designed using the Web-based Allele Specific Primer designing tool (WASP) Bioinformatics Laboratory, BIOTEC 2006-2007 (http://bioinfo.biotec.or.th/WASP) (Table 1). A mis-match of a C to a T was incorporated at the penultimate base of the forward allele-specific primers to reduce the possibility of mis-primed amplification, thus increasing the primers' specificity. Amplification of genomic DNA was carried out in two separate PCR reactions (forward susceptible + reverse common), (forward resistant + reverse common), each producing a 102 bp product. PCR reactions contained 0.5 µM of each primer,

combined with 10-100 ng of template DNA, 10 × Taq buffer, 0.3 mM dNTP, 3.8 mM magnesium and 0.05 units/µl of Taq polymerase (Geneworks BTQ-1). This mix was thermocycled in an Applied Biosystems 2720 thermocycler for 35 cycles of 94ºC for 10 s, 52ºC for 15 s and 72ºC for 20 s. The mix was held at 72ºC for 7 min to complete extension then dropped to 4ºC. Products were viewed on an ethidium bromide stained 2.0% agarose and TAE gel.

2.6. Analyses

Field populations were tested to determine whether allele frequencies conformed to the Hardy–Weinberg equilibrium (HWE) using GENEPOP 3.4 (Raymond and Rousset, 1995). Those that did not conform were further tested to determine whether distortion from HWE resulted from deficient or excessive heterozygosity (Raymond and Rousset, 1995).

3. Results

A single point mutation at position 190 in GenBank *R. microplus* sequence **AF134216** substituting a C for an A was identified in the resistant Parkhurst *R. microplus* strain. The mutation is non-synonymous, causing an amino acid change from L in the susceptible strain to I in the resistant strain. Table 2 shows the amino acid sequence alignment of this area of the gene for several species.

On the basis of this mutation, a dual probe quantitative PCR assay was developed using FAM and VIC labelled probes (Table 1). The assay is sensitive enough to detect the alleles in DNA extracted from single tick larvae. Homozygous susceptible ticks (from the NFRS population) produced strong fluorescence in the

FAM channel alone. Homozygous resistant ticks (Parkhurst) produced strong fluorescence in the VIC channel and occasionally produced a weak false signal in the FAM (susceptible) channel. This false signal was partly overcome by the introduction of DMSO (4%) into the reaction mix and by adopting three-step temperature assay conditions (adding an annealing step of 58°C). During screening, any heterozygotes identified with weak amplification of the susceptible allele (FAM channel) were confirmed using an allele-specific conventional PCR assay.

The distribution of susceptible and resistant alleles in Parkhurst and field collected populations of ticks with varying pyrethroid resistance (based on LPT) are shown in Table 3. Both alleles were detected in all populations with the frequency of the resistant allele (R) ranging from 0.03 to 0.97. The presence of the resistant allele was strongly correlated with the reduced mortality observed in the bioassays with cypermethrin. Only the fully susceptible population (0%) was found to be in HWE; the remaining field populations displayed either heterozygote excess or deficiency (Table 3). The relationship between allele frequency and mortality in the bioassay is illustrated in Fig. 1. There was a strong correlation between the percentage of resistant homozygote ticks and the proportion of survivors in the LPT bioassay (r^2 = 0.98) but only a weak relationship between the proportion of heterozygotes and survival (r^2 = 0.071), suggesting that the allele conferring resistance to SPs might be recessively inherited.

4. Discussion

We believe this to be the first report of a single point mutation in the domain II S4-5 linker of the sodium channel gene in *R. microplus* that is associated with

cypermethrin resistance. The mutation causes an amino acid change from L in susceptible ticks to I in resistant ticks. A similar mutation (L925I) has been discovered in whitefly, *B. tabaci* , in which it confers resistance to SP insecticides (Morin et al., 2002). In houseflies, mutations in the domain II S 6 have been associated with knockdown resistance (*kdr*) and an additional mutation in domain II S4-5 linker confers a highly resistant phenotype (*super kdr*) (Williamson et al., 1996). Given the absence of a *kdr* mutation in cattle ticks to date, the use of the terminology *kdr* and *super kdr* does not seem to be appropriate.

The C-A mutation at position 190 was identified first in ticks of the cultured Parkhurst strain of *R. microplus* with high resistance to SPs. Ninety-seven percent of the ticks genotyped from that population were homozygous for the mutation. In contrast, 94% of the ticks from the susceptible field population were homozygous for the wild-type allele, with 6% heterozygotes. The hypothesis that the mutation is associated with resistance was supported by the close correlation (R^2 = 98%) between homozygote frequency and survival in the bioassay, using three field populations with intermediate acaricide resistance status. The results suggest that the mutation is a major mechanism for pyrethroid resistance in these field populations. Similarly, the inconsistent association between heterozygotes and survival suggests that the trait is recessive. Controlled mating studies would be required to substantiate this possibility, however.

The observed shift from an excess of heterozygotes in populations displaying low level resistance (28-53%) to synthetic pyrethroids, to a deficit of heterozygotes in populations displaying a high level of resistance (75-100%), suggests that allele frequencies in tick populations exposed to SPs are strongly driven by selection.

A diagnostic quantitative PCR assay has been developed using allele-specific Taqman® MGB probes. By amplifying the DNA with generic primers and distinct probe fluorophores (FAM or VIC), single larvae can be screened for both alleles in one multiplexed reaction. Together with assays for detoxification mechanisms that likely exist in the field, the quantitative PCR assay will enable researchers to confirm the role of the mutation in the expression of resistance in the field. The assay will also enable the rapid confirmation of suspected resistance to synthetic pyrethroid acaricides in samples of ticks collected from the field. Because of the potential role of detoxification mechanisms and other mutations, the molecular assay should be used in conjunction with, rather than as a replacement for, the existing LPT bioassay.

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367

Figure legend

Fig. 1. Correlation between population genotype and survivorship in the larval packet bioassay at a discriminating concentration of cypermethrin. Black diamonds represent the resistant allele as an homozygous genotype only while grey squares represent the resistant allele as an homozygous or an heterozygous genotype.

375 Figure 1
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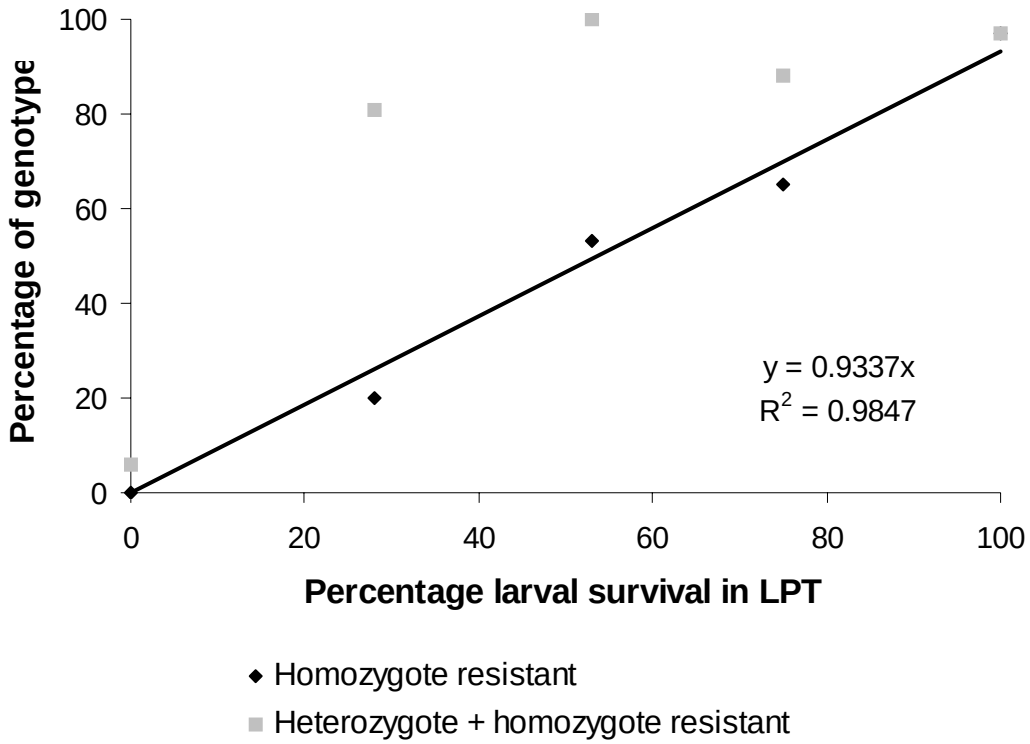

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Table 1. Primers and probes for dual-probe quantitative PCR assay and allele-specific conventional PCR assay targeting a single nucleotide mutation (underlined) in the sodium channel that confers synthetic pyrethroid (SP) resistance in Australian *Rhipicephalus (Boophilus) microplus*. The mismatched penultimate base in the conventional PCR forward primers is italicised.

Primer/Probe	Position in AF134216	Sequence 5' -
Quantitative PCR assay		
Forward primer (common)	132	CAAATCGTGGCCTACCCTTA
Reverse primer (common)	198	TTCCCAGGACAAAGGTCAAG
Susceptible MGB probe (C) FAM™ fluorophore	181	ATCGGTGCCCTCG
Resistant MGB probe (A) VIC™ fluorophore	180	CATCGGTGCCATC
Conventional PCR assay		
Forward primer (susceptible)	173	GGAAAACCATCGGTGC*T*C
Forward primer (resistant)	173	GGAAAACCATCGGTGC*T*A
Reverse primer (common)	255	CTTCGTAGTTCTTGCCAAAG

MGB, minor groove-binding.

Table 2. Amino acid sequence alignment for the domain II S4-5 linker region from several species of diverse taxa, showing the leucine to isoleucine mutation in cattle tick and the whitefly, highlighted in black. A second mutation in the whitefly is underlined. Highly conserved residues across species are shaded.

Rhipicephalus microplus	
NRFS[a] (susceptible)	RVFKLAKSWPTLNLLISIMGKTIGALGNLTFVLGIIIFIFAVMGMQLFGKNYEES
Parkhurst (resistant)	RVFKLAKSWPTLNLLISIMGKTIGA**I**GNLTFVLGIIIFIFAVMGMQLFGKNYEES
B. tabaci (susceptible)	AKSWPTLNLLISI**M**GRTVGALGNLTFVLCIIIFIFAVMGMQLFGKNYTDN
B. tabaci (resistant)	AKSWPTLNLLISI**V**GRTVGA**I**GNLTFVLCIIIFIFAVMGMQLFGKNYTDN
D. melanogaster	RVFKLAKSWPTLNLLISIMGRTMGALGNLTFVLCIIIFIFAVMGMQLFGKNYHDH
M. domestica	RVFKLAKSWPTLNLLISIMGRTMGALGNLTFVLCIIIFIFAVMGMQLFGKNYIDH
B. germanica	RVFKLAKSWPTLNLLISIMGRTVGALGNLTFVLCIIIFIFAVMGMQLFGKNYYDN
L. opalescens	RVFKLAKSWPTLNMLISIVAGTMGALGNLTLVLGIIVFIFAVMGQQLFGANYEKP
G. gallus	RVFKLAKSWPTLNMLIKIIGNSVGALGNLTLVLAIIVFIFAVVGMQLFGKNYKEC
M. musculus	RVFKLAKSWPTLNMLIKIIGNSVGALGNLTLVLAIIVFIFAVVGMQLFGKSYKEC
H. sapiens	RVFKLAKSWPTLNMLIKIIGNSVGALGNLTLVLAIIVFIFAVVGMQLFGKSYKEC

[a]Non-resistant field strain.

Accession numbers in GenBank are as follows: *Bemisia tabaci* (Whitefly) **CAD29437**, *Drosophila melanogaster* (Fruit Fly) **P35500**, *Musca domestica* (House Fly) **U38814**, *Blattella germanica* (German Cockroach) **U71083**, *Loligo opalescens* (Squid) **L19979**, *Gallus gallus* (Chicken) **XP_424477**, *Mus musculus* (Mouse) **CAM23795**, *Homo sapiens* (Human) **P35499**.

Table 3. Distribution of observed and expected genotype frequencies and results of tests for Hardy-Weinberg (H-W) equilibrium for sodium channel alleles in different populations of ticks with varying synthetic pyrethroid resistance.

Isolate (LPT resistance)	Genotype frequencies observed (expected) SS	RS	RR	Total	*P* value H-W exact test	Heterozygote deficit or excess
Field 0%	94 (94)	6 (6)	0 (0)	100	1.000	-
Field 28%	19 (25)	61 (50)	20 (26)	100	0.045 [a]	excess
Field 53%	0 (6)	47 (36)	53 (59)	100	0.001 [a]	excess
Field 75%	12 (6)	23 (36)	65 (59)	100	<0.0001 [a]	deficit
Parkhurst 100%	3 (0)	0 (6)	97 (94)	100	<0.0001 [a]	deficit

[a] significant at $\alpha = 0.05$ level,

S = allele with C at position 190 linked to susceptible phenotype, R = allele with A at position 190 linked to resistant phenotype, hence SS = putative susceptible homozygote, RS = heterozygote, RR = putative resistant homozygote

LPT, larval packet technique.