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Identification of a mutation in the *para*-sodium channel gene of the cattle tick *Rhipicephalus microplus* associated with resistance to flumethrin but not to cypermethrin

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ABSTRACT

A mutation in the domain II S4–5 linker region of the *para*-sodium channel gene has been associated previously with synthetic pyrethroid (SP) resistance in the cattle tick (*Rhipicephalus microplus*) in Australia. This is a C → A mutation at nucleotide position 190, which results in a leucine to isoleucine amino acid substitution (*L64I*). In a survey of 15 cattle tick populations with known SP resistance status, sourced from Queensland and New South Wales in Australia, there was a strong relationship ($r = 0.98$) between the proportion of ticks carrying the *L64I* homozygous resistant genotype and the survival percentage after exposure to a discriminating concentration of cypermethrin in the bioassay, as expected. However, among populations resistant only to flumethrin, the *L64I* homozygous genotype was not found. The sequence obtained for a 167 bp region including domain II S4–5 linker in flumethrin-resistant ticks identified a G → T non-synonymous mutation at nucleotide position 214 that results in a glycine to valine substitution (*G72V*). The frequency of the *G72V* homozygous genotype in each population was found to be moderately related to the survival percentage at the discriminating concentration of flumethrin in the larval packet test ($r = 0.74$). However, a much stronger relationship between genotype and resistance to flumethrin was observed when the heterozygotes of *L64I* and *G72V* were added to the *G72V* homozygotes ($r = 0.93$). These results suggest that there is an interaction between the two mutations in the same gene, such that flumethrin resistance might be conferred by either two copies of the *G72V* mutation or by being a *L64I* and *G72V* heterozygote.

1. Introduction

Synthetic pyrethroid (SP) resistance is common among cattle ticks (*Rhipicephalus microplus*) around the world. In Australia, the prevalence of SP resistance was estimated to exceed 50% (Jonsson et al., 2000). More recent data from an unpublished survey undertaken in 2006 by the Department of Employment Economic Development and Innovation (DEEDI, formerly Queensland Department of Primary Industries and Fisheries (DPI&F)) indicate that the prevalence of resistance to SP is 54% (72 of 133 samples). The effective use of SP products is therefore limited to fewer than 50% of farms in Queensland and if SP products are to be used efficiently there is a need for rapid and accurate diagnosis of the resistance status on farms.

Among Australian *R. microplus*, it has been shown that some populations are resistant to flumethrin but not to cypermethrin whereas any population with resistance to cypermethrin is also expected to be resistant to flumethrin (Nolan et al., 1989). The corresponding reference isolates that were maintained by Biosecurity Science Laboratories (BSL) of the DEEDI as cultured strains are known as the Lamington strain, which is resistant only to flumethrin, and the Parkhurst strain, which is resistant to flumethrin, cyhalothrin, permethrin and cypermethrin. The resistance ratios (RR) of these two strains were shown to differ; Lamington-strain ticks had RR of 29 for flumethrin at the concentration that caused mortality of 50% of larvae (LC_{50}), compared with 446 in the Parkhurst strain (Nolan et al., 1989). The Lamington strain was also shown to have very low levels of resistance to cypermethrin, cyhalothrin and deltamethrin (RR of 3.2, 3.0 and 3.6, respectively). The Parkhurst strain was maintained in culture until 2007, when it was replaced by a new field strain (Louise Jackson, DEEDI, personal communication) but the Lamington strain has not been in culture for many years. The practical significance of the Lamington strain as distinct from Parkhurst is that cattle producers can select SP products based on flumethrin if there is no SP resistance at all (i.e. no Lamington and no Parkhurst), or they can continue to use

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cypermethrin or deltamethrin products if there is flumethrin resistance (Lamington) only.

We have recently identified a mutation in the *para*-sodium channel gene that is associated with resistance to cypermethrin in Australian *R. microplus* (Morgan et al., 2009). This cytosine to adenine mutation at position 190 in the *R. microplus* sequence AF134216 corresponds to the domain II S4–5 linker region and results in a leucine to isoleucine substitution (*L64I*). Another mutation in the domain III S6 region of this gene had previously been shown to confer high levels of resistance to SP in Mexican *R. microplus* (*F1550I*; He et al., 1999a). Early work on SP resistance in Australian ticks suggested that the major mechanism of resistance was most likely to be target site insensitivity rather than metabolic detoxification (Schnitzerling et al., 1983; Nolan et al., 1989). However, SP resistance in surveys of Mexican ticks has not been uniformly explained by the presence of the mutation in domain III S6 (Jamroz et al., 2000; Rosario-Cruz et al., 2009).

The purpose of the present study was to sequence the domain II S4–5 linker region of the *para*-sodium channel gene in samples of *R. microplus* ticks with diverse SP resistance phenotypes and from geographically distant locations in Queensland and New South Wales, Australia, to determine whether the *L64I* or other mutations in this region of the gene are associated with all cases of SP resistance.

2. Materials and methods

2.1. Tick samples

Tick samples for this study were frozen larvae drawn from two sources. Samples R9102-Q, R9113-S, R9399-Z, R9128-I, R9180-V-J, R9206-X, TP30-E, R9177-G, R9109-L, R9166-CB, R9172-CD and R9166-CE were extracted from the archive of diagnostic samples held by the BSL of DEEDI at Yeerongpilly in Brisbane, Queensland. These larvae were the progeny of females that were submitted between August 2003 and June 2009 for diagnostic acaricide resistance testing for flumethrin and cypermethrin using the larval packet test. Larvae surplus to requirements for the bioassay were retained frozen at −20 °C.

In addition, samples from acaricide-resistant reference strains were provided by the BSL in December 2003. These samples were the non-resistant field strain (NRFS), a multiple resistant strain known as Ultimo (resistant to amitraz and cypermethrin) and a cypermethrin resistant Parkhurst strain. The isolation and establishment of the NRFS was first described in 1982 (Stewart et al., 1982). The Parkhurst strain was isolated from the field in 1987 (Nolan et al., 1989) and maintained as a distinct strain until 2007, when it was replaced by a new field isolate (L. Jackson, DEEDI, personal communication). The Ultimo strain was initially isolated from Central Queensland near Rockhampton in 1992 and demonstrated resistance against all synthetic pyrethroids and amitraz (Kunz and Kemp, 1994). The Ultimo and Parkhurst strains were not selected within a year prior to their initial collection in 2003.

Samples were chosen for this study to provide a range of values of resistance to cypermethrin and flumethrin, a variety of ratios of flumethrin to cypermethrin resistance, and to ensure wide geographic representation. The resistance values for each of the populations and strains are shown in Table 1. They range from 0% to 100% in the case of cypermethrin and from 0% to 60% for flumethrin.

2.2. Bioassays

Bioassays were conducted by BSL staff at the time of sample submission. The bioassay used was the larval packet test (Stone and Haydock, 1962) and the percentage of resistance in a population was considered to be the percentage of survivors among ticks exposed to a discriminating concentration of each acaricide (0.3% w/v for cypermethrin; 0.02% w/v for flumethrin). The mean percentage of mortality of two replicate samples, each of approximately 100 larvae, is recorded after exposure to flumethrin and cypermethrin in separate bioassays. The percentage of survival after exposure to 0.3% w/v cypermethrin is called the percentage of Parkhurst resistance (R_P), and the percentage Lamington resistance (R_L) is considered to be the difference between the percentage of mortality after exposure to 0.03% cypermethrin (M_c) and the percentage of mortality to 0.02% flumethrin (M_f).

$$R_L = M_c - M_f$$

Because it has been observed that all ticks with resistance to cypermethrin are also resistant to flumethrin (Nolan et al., 1989), it is expected that $M_c \geqslant M_f$ and hence, $R_L \geqslant 0$ in all cases. For example, a sample might have a mean mortality of 80% to cypermethrin (M_c) and 50% to flumethrin (M_f). This equates to 20% Parkhurst resistance ($1 - M_c$) and 30% Lamington resistance ($M_c - M_f$). Occasional samples are submitted for which $M_c \leqslant M_f$. In these cases the laboratory practice has been to record the result as Parkhurst resistance without evidence of any contribution from Lamington-strain ticks and to attribute the difference to natural variation among samples in their response to the bioassay.

2.3. DNA extraction, amplification and sequencing

These procedures are described in detail elsewhere (Morgan et al., 2009) and are briefly recorded here for ease of reference.

Single tick larvae were crushed with forceps in a 200 μl microfuge tube to which 50 μl 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) was added, followed by 2.5 μg Proteinase K. The larvae were incubated overnight at 56 °C and then heat inactivated at 95 °C for 45 min.

PCR primers were designed from an alignment of partial sodium channel gene sequences of *R. microplus* sequences (Mexican strain GenBank Accession No. AF134216) and *Ixodes scapularis* (*Ixodes* genome: http://iscapularis.vectorbase.org) to amplify the exon region between domain II, S4 loop and domain II, S5. The primers, which amplified a 167 bp product, were: BmNaF5 TACGTGTGTTCAAGCTAGC (position 103 in *R. microplus* GenBank Accession No. AF134216) and BmNaR5 ACTTTCTTCGTAGTTCTTGC (position 260 in *R. microplus* GenBank Accession No. AF134216). PCRs contained 0.5 μM of each primer, combined with 10–100 ng of template DNA, 10× Taq buffer, 0.8 mM dNTP, 3.75 mM magnesium and 0.05 units/μl of Taq polymerase (Geneworks BTQ-1, Australia). PCR conditions were one cycle of 95 °C for 60 s, 50 °C for 45 s and 72 °C for 90 s, followed by 29 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 90 s and a final extension at 72 °C for 7 min. PCR products were cleaned using Exosap-it® (USB Corporation distributed by GE Healthcare Bio-Sciences, Rydalmere NSW, Australia), sequenced using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit Version 3.1 (PE Applied Biosystems, Foster City, California, USA) and run on an ABI 3130xl automated sequencer. Forward and reverse sequences were aligned and edited using ChromasPro (Technelysium Pty Ltd., Australia).

2.4. Data analyses

The association between the percentage of resistance in a population to the specified acaricide and the proportion of each genotype in each population was tested using Pearson's product moment correlation in Minitab v13 (Minitab Inc., 2000. Minitab