Ticks and Tick-borne Diseases xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

An integrative approach to understanding pyrethroid resistance in *Rhipicephalus microplus* and *R. decoloratus* ticks

Roelof DJ van Wyk[a,1], Samantha Baron[b,1], Christine Maritz-Olivier[b,*]

[a] Department of Biochemistry, University of Pretoria, Pretoria, South Africa
[b] Department of Genetics, University of Pretoria, Pretoria, South Africa

ARTICLE INFO

Article history:
Received 8 September 2015
Received in revised form 8 January 2016
Accepted 15 January 2016
Available online xxx

Keywords:
Rhipicephalus microplus
Rhipicephalus decoloratus
pyrethroids
acaricide resistance
SNPs
protein modelling
drug docking

ABSTRACT

Rhipicephalus microplus and *Rhipicephalus decoloratus* species occur in regions with savannah and temperate climates, typically in grassland and wooded areas used as cattle pasture. Both species are associated with the transmission of *Anaplasma* and *Babesia* spp., impacting livestock health and quality of livestock-associated products. In Africa, tick control is predominantly mediated with the use of acaricides, such as synthetic pyrethroids. After several years on the market, reports of resistance to synthetic pyrethroids escalated but limited field data and validation studies have been conducted to determine the extent of acaricide resistance in Africa. Without this data, knowledge-based tick control will remain problematic and selection pressure will remain high increasing the rate of resistance acquisition.

To date, several pyrethroid resistance associated single nucleotide polymorphisms (SNPs) have been reported for arthropods within the voltage-gated sodium channel. Three SNPs have been identified within this channel in pyrethroid resistant *R. microplus* ticks, but none has been reported for *R. decoloratus*. This study is the first to report the presence of a shared SNP within the voltage-gated sodium channel in both *R. microplus* and *R. decoloratus*, which is directly linked to pyrethroid resistance in *R. microplus*. As the mode of action by which these SNPs mediate pyrethroid resistance remains unknown, this study aims to set hypotheses by means of predictive structural modelling. This not only paves the way forward to elucidating the underlying biological mechanisms involved in pyrethroid resistance, but also improvement of existing acaricides and ultimately sustainable tick control management.

Introduction

Rhipicephalus microplus and *Rhipicephalus decoloratus* ticks are widely distributed in suitable habitats throughout Africa, south of the Sahara. These species often occur together with *R. annulatus* and *R. geigyi* and are associated with the spread of babesiosis and anaplasmosis to cattle (Walker et al., 2003). *R. microplus* is well known for the detrimental socio-economic impact it inflicts on the cattle industry in terms of beef and dairy production (Jonsson, 2006; Jonsson et al., 2001).

The foremost tick control strategy implemented by farmers in South Africa, and most of southern Africa, is the use of chemical acaricides. The main concern at this point is the declining efficacy of current acaricides due to the development of resistance. Resistance to organophosphates (OPs) first came about in the 1980's (Aguirre et al., 1986) after which synthetic pyrethroids and amitraz were introduced to circumvent organophosphate-resistant ticks (Aguirre et al., 1986; Vargas et al., 2002). This led to the extensive use of synthetic pyrethroids and consequently the prevalent eruption of pyrethroid resistance in the 1990's (He et al., 1999; Miller et al., 1999).

Metabolic resistance towards pyrethroids has been documented in the Coatzacoalcos Mexican *R. microplus* tick strain based on synergistic assays with piperonyl butoxide (PBO) and triphenyl phosphate (TPP) (Miller et al., 1999). Within this particular strain, there was also an up-regulation in the expression of an esterase (CzEst9) predicted to be involved in the rapid hydrolysis of permethrin (Pruett et al., 2002). Subsequent studies confirmed that this carboxylesterase enzyme played a key role in pyrethroid resistance in the Mato Grosso Brazilian strain (Baffi et al., 2007).

Three single nucleotide polymorphisms (SNPs) in the voltage-gated sodium channel have been reported to be associated with pyrethroid resistance in ticks, evidently indicating that target site resistance is the most prevalent resistance mechanism against

* Corresponding author. 8th Floor, Agricultural Sciences Building, University of Pretoria, Hatfield, Pretoria, 0083, South Africa. Tel.: +27 (012) 420 3945. fax: +27 (012) 362 5327.
E-mail address: Christine.maritz@up.ac.za (C. Maritz-Olivier).
[1] Authors contributed equally

http://dx.doi.org/10.1016/j.ttbdis.2016.01.007
1877-959X/

pyrethroids in *R. microplus* ticks. The first SNP was discovered in domain III of the sodium channel in pyrethroid resistant tick populations from Mexico, and was also reported to be confined to North America (Guerrero et al., 2012; He et al., 1999). Target site mutations resulting in pyrethroid resistance were predominantly found to occur in domain II of the voltage-gated sodium channel in a number of other arthropods that were investigated (Soderlund and Knipple, 2003). This subsequently led to the discovery of two SNPs in domain II segments 4-5 (DIIS4-S5) of the linker region of the sodium channel in *R. microplus* (Jonsson et al., 2010; Morgan et al., 2009). Of the three reported mutations it has been shown that the one described by Morgan et al. (2009) occurs in South African *R. microplus* ticks (Guerrero et al., 2012). No pyrethroid resistance associated mutations have been reported in the sodium channel for *R. decoloratus*.

A step towards understanding the impact of these SNPs on the structure and biological function of the voltage-gated sodium channel is through *in silico* predictive modelling. To date only 58 crystal structures are available (according to RCSB PDB database) for voltage-gated sodium channels, with most of the structures only partially resolved.

Previous attempts to construct a model for the *R. microplus* voltage-gated sodium channel utilised an existing housefly model, constructed from several voltage-gated potassium channel template structures with only a few corresponding amino acid substitutions (O'Reilly et al., 2014). Research by O'Reilly et al. (2014) illustrated a pyrethroid resistance-associated SNP (position F1519I) that occurred in the S6 transmembrane helix of domain III. This does not correspond to the most prevalent SNP marker (position L64I) that occurs in domain II, which has been documented in both Mexico and Brazil as a viable resistance marker (Guerrero et al., 2012). To date, only the domain II S4-S5 linker region marker has been observed globally for pyrethroid resistant *R. microplus* ticks.

The model proposed in this study focuses on the L64I marker and therefore contributes insight to resistance mediated by domain II. The study by O'Reilly et al. (2014) and colleagues was used as criteria for determining relevant docked poses for cypermethrin, however the amino acid sequence identity for the housefly model with the corresponding crystal templates was below 30%. Therefore, we set out to construct a higher quality model with higher sequence coverage in amino acid sequence alignments.

Insight into the status of pyrethroid resistance within South Africa will aid in the development of improved tick control strategies in the hope of prolonging the use of current acaricides. With increased selection pressure promoting the development of acaricide resistance, the progression towards novel control strategies is more imperative than ever before.

Methods and materials

Tick Collection, identification and DNA extraction

Ticks were collected by representatives of Zoetis South Africa (Pty) Ltd. from108 farms across South Africa, including Swaziland with written consent from each farmer. Collection points are shown in Figure S1. Ticks were sorted into genera according to published guidelines (Madder and Horak, 2010; Walker et al., 2003). Distinction between *Rhipicephalus* species was done using microscopy to evaluate hypostome dentition and adanal spur features (Madder and Horak, 2010; Walker et al., 2003). Morphology results were validated through molecular identification using restriction fragment length polymorphism (RFLP) analysis (Lempereur et al., 2010). A salt based extraction protocol was used for genomic DNA extraction from individual adult ticks (Baron et al., 2015). A full repository of all ticks used in this study is shown in Table S1 and S2.

PCR amplification of R. microplus *voltage-gated sodium channel gene fragment*

Published primers were used to amplify a fragment of domain II segment 4-5 region of the sodium channel (Morgan et al., 2009). Each reaction contained 200 ng of gDNA template, 10 pmol of each primer and KAPA2G^{TM} Robust HotStart ReadyMix (200 μM dNTPs, 2.0 mM $MgCl_2$) in a final 25 μl reaction. The cycling parameters were as follows; 94 °C for 4 min, followed by 40 cycles of 94 °C for 30 s, 51 °C for 30 s and 72 °C for 1 min with a final extension at 72 °C for 7 min.

Allele-specific PCR for the voltage-gated sodium channel of R. decoloratus

Allele-specific PCR of *R. decoloratus* voltage-gated sodium channel was conducted using allele-specific primers (Guerrero et al., 2001). This was done in order to reduce the cost of sequencing and to increase overall productivity. Several samples were sequenced for validation of the amplified PCR products. For each sample two reactions were set up. One reaction containing the susceptible forward primer (5'-GGAAAACCATCGGTGCTC-3': L64IS) and the diagnostic/reverse primer (5'-GAACTTGTGTTTACTTTCTTCGTAGT-3'). The second reaction contained the resistant forward primer (5'-GGAAAACCATCGGTGCTA-3': L64IR) as well as the diagnostic reverse primer. PCR conditions for both susceptible (L64IS) and resistant (L64IR) reactions were as follows: a 25 μl reaction made up using water (SABAX®, ADCOCK-INGRAM), 100 ng gDNA, 10 pmol forward and reverse primer and Econo*Taq*® PLUS GREEN 2X Master Mix. The cycling parameters were 94 °C for 4 min, followed by 40 cycles of 94 °C for 30 s, 51 °C for 30 s and 72 °C for 1 min with a final extension at 72 °C for 7 min.

Sequencing and analysis of amplified PCR products

PCR products were sequenced by Macrogen Inc. (Netherlands). Individual chromatograms were manually curated using BioEdit sequence alignment editor version 7.2.0 (Hall, 1999). Multiple alignments were constructed using the online MAFFT program version 6 (http://mafft.cbrc.jp/alignment/software/) (Katoh and Standley, 2013) to identify resistance associated mutations. To test for neutrality within DNA sequences, the Tajima D value (Tajima, 1989) was calculated using MEGA5 (Tamura et al., 2011). This test for neutrality was conducted on the multiple sequence alignment to determine if directional or balancing selection was acting on domain II S4-5 linker region of the voltage-gated sodium channel.

Template selection and model construction

Protein BLAST (BLASTp algorithm) was performed for all of the target protein sequences from literature of *R. microplus* against the protein data bank (PDB). Maximum coverage (above 80%) and sequence identities (above 30%) were used as cut off values. Protein crystal structures were obtained from PDB (http://www.rcsb.org/). Pfam seed alignments were included for target protein sequences (http://pfam.sanger.ac.uk/). Multiple-sequence alignments were done for the template and seed alignments using MAFFT version 6. All alignments were performed using the G-INS-i parameter global alignment algorithm (Needleman-Wunsch algorithm) and the BLOSUM62 matrix. The sequence for the voltage-gated sodium channel of *R. microplus* was used for model building (AF134216.2). Homology models were generated using Modeller 9v1 within the Discovery studio 4.0® suite (Accelrys software Inc, USA). From

Please cite this article in press as: Wyk, R.D.v., et al., An integrative approach to understanding pyrethroid resistance in *Rhipicephalus microplus* and *R. decoloratus* ticks. Ticks Tick-borne Dis. (2016), http://dx.doi.org/10.1016/j.ttbdis.2016.01.007