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Original article

Genotyping acaricide resistance profiles of *Rhipicephalus microplus* tick populations from communal land areas of Zimbabwe

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ABSTRACT

Acaricide resistance is one of the greatest threats towards the successful control of vector ticks worldwide. Communal farmers of Zimbabwe use amitraz as the most common acaricide with occasional usage of pyrethroids and organophosphates. As a strategy towards developing an effective acaricide resistance management system in Zimbabwe, screening was done by genotyping *Rhipicephalus microplus* tick populations using molecular markers associated with resistance to these chemicals. The frequency of the mutant allele for the octopamine/tyramine receptor marker associated with amitraz resistance was high (0.55) and a large proportion 78.5% (288/367) of heterozygote genotypes were observed indicating balancing selection. Of the communal dipping tanks where *R. microplus* occurred 37.8% (39/103) showed complete resistance genotypes for amitraz. The carboxylesterase marker that has been associated with resistance to organophosphate and pyrethroids indicated no selection pressure in these chemical groups with a low frequency (0.052) of the mutant allele and 89.6% (329/367) sampled ticks showing homozygous susceptibility genotypes. Heterozygous genotypes were present at 27.2% (28/103) of the dipping tanks. The L641 mutation in the voltage–gated sodium channel gene associated with pyrethroid resistance was not detected in Zimbabwean samples. This would suggest a different mechanism of resistance to pyrethroids in these tick populations. Sequence analysis of the octopamine/tyramine receptor gene revealed the presence of other mutations in this region, it will be important to investigate their association with amitraz resistance. These results present the first molecular genotyping of resistance profiles of *R. microplus* tick populations from Zimbabwe.

1. Introduction

The deployment of acaricides is the most widely used strategy for the control of ixodid ticks affecting livestock in tropical and sub-tropical countries of the world (Abbas et al., 2014). The greatest challenge facing its implementation success is the accumulation of resistance in ticks to these chemicals (Rosario-Cruz et al., 2009). Subsequently, effective tick management is premised on the ability to detect and periodically carry out surveillance programmes on acaricide resistance (Ghosh et al., 2015).

Bioassays such as the Larval Packet Test (LPT) (Stone and Haydock, 1962), Larval Immersion Test (LIT) (Shaw, 1966) and the Adult Immersion Test (AIT) (Drummond et al., 1973) have traditionally been used to detect and monitor the acaricide resistance status of tick populations while the Larval Tarsal Test was recently introduced (Lovis et al., 2013). The increased length of time by which results from a bioassay are obtained, the need for live tick specimens and the inability to detect the genotype status of resistant individuals, negatively influences the effectiveness of these techniques. The development of molecular tools heralded by the design of an allele-specific PCR for the diagnosis of pyrethroid resistance in *R. microplus* ticks (Guerrero et al., 2001) has greatly improved acaricide resistance management. Molecular tools offer the distinct advantage that they are quick, do not require live tick specimens and enable the genotyping of the resistance status of tick populations.

Mutations have been identified in the voltage gated sodium channel

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gene in *R. microplus* which result in insensitivity to pyrethroids (Miller et al., 1999; He et al., 1999; Jonsson et al., 2010a; Morgan et al., 2009). An esterase in a pyrethroid resistant Mexican *R. microplus* strain was isolated and a mutation in the encoding gene was found by Hernandez et al. (2000), the same mutation was found in organophosphate resistant strains by Baffi et al. (2007). Mutations in genes encoding carboxylesterases will increase esterase hydrolytic activity on an acaricide (Jamroz et al., 2000). Chen et al. (2007) identified two mutations in a putative octopamine receptor gene that will result in target site insensitivity to amitraz. These mutations have enabled the development of molecular markers (Guerrero et al., 2001; Morgan et al., 2009; Hernandez et al., 2002a,b; Baron et al., 2015) diagnostic for pyrethroid, organophosphate and amitraz resistance.

Ticks are an important constraint to livestock production in (sub)-tropical countries with annual losses estimated at USD$18.7 billion (De Clercq et al., 2012) and resource-poor communal farmers who own approximately 80% of the cattle are most affected (Rushton et al., 2002). These costs are related to their direct effects as blood sucking parasites which result in productivity losses and damage to hides affecting their quality and value on the market (Rajput et al., 2006). Indirect effects are foreseen in their being: vectors of pathogens which affect humans and animals, costs incurred for treating tick-borne diseases and controlling the vectors (de Castro, 1997). In Zimbabwe the most important tick-borne diseases are heartwater, babesiosis, anaplasmosis and theileriosis and these account for 60 percent of livestock mortalities annually (Sungirai et al., 2016).

In Zimbabwe communal farming systems, tick control is based on the use of a plunge dip, where acaricide chemicals are diluted in large volumes of water and cattle will be submerged in the dip wash. Farmers have to bring their cattle for dipping weekly and fortnightly during the rainy and dry season respectively. The central government supplies acaricides to these communal farmers who pay a nominal fee to the Department of Veterinary Services to have their cattle participate in these tick control programmes (Sungirai et al., 2016). It then becomes important for the governments to be made aware of the status of acaricide resistance so as to take remedial action. It has been observed that communal farming systems are characterised by: absence of acaricide rotation practises, poor surveillance of acaricide resistance, lack of training on the judicious use of acaricides and indiscriminate selling of these chemicals without the recommended active ingredients (Mendes et al., 2013). This may increase the selection pressure for acaricide resistance which may be difficult to reverse once it has been established.

Studies on acaricide resistance have been neglected in Africa although interest in the field is growing (Adakal et al., 2013; Adehan et al., 2016; Baron et al., 2015; Robbertse et al., 2016; Wyk et al., 2016). Much work on acaricide resistance has been reported from Central America, South East Asia, the Caribbean and Australia with the focus on *R. microplus* (*R. australis* in Australia) largely due to its undisputed global importance (Abbas et al., 2014). This tick species developed resistance to the major classes of acaricides in use in these areas, although the level of resistance differs between areas (Lovis et al., 2012).

The purpose of this study was to genotype the acaricide resistance profiles of *R. microplus* ticks collected from cattle in communal areas of Zimbabwe. The hypothesis was that due to the overreliance on the same type of acaricides by farmers and government, *R. microplus* populations in different parts of the country are undergoing selection pressure for resistance to amidines, pyrethroids and organophosphates. The results of this study will provide vital information to policy makers to come up with alternative strategies for improved tick control in light of current acaricide resistance profiles. This is the first report on the genotyping of acaricide resistance status in this tick species in Zimbabwe.

2. Materials and methods

2.1. Biological materials and DNA extraction

Following a nationwide tick survey conducted in Zimbabwe (Sungirai et al., 2017), 383 *Rhipicephalus microplus* ticks collected at 103/322 communal dipping tanks were used for this study. The identity of *R. microplus* ticks which could not be resolved through morphology was confirmed using the ITS2 PCR-RFLP test (Lempereur et al., 2010). Whole genomic DNA was extracted from *R. microplus* ticks using the QIAamp genomic DNA kit (Qiagen, Hilden, Germany).

2.2. PCR conditions for the octopamine/tyramine receptor gene, carboxylesterase genes and voltage-gated sodium channel genes

The PCR assay conditions for the amplification of the octopamine/tyramine receptor gene, carboxylesterase and voltage-gated sodium channel genes were carried out as described by Baron et al. (2015), Hernandez et al. (2002a,b) and Morgan et al. (2009) respectively. An additional PCR assay as described by Stone et al. (2014) was done to amplify a large fragment of the voltage-gated sodium channel gene for subsequent sequencing.

Each of the molecular markers were amplified for the 383 tick samples in a programmable thermocycler (Biometra©, Göttingen, Germany). For the carboxylesterase and octopamine/tyramine receptor genes, Restriction Fragment Length Polymorphism (RFLP) was done after amplification as described by Faza et al. (2013) and Baron et al. (2015) respectively. The PCR and restriction digest products were loaded with dye on a 3% (w/v) agarose gel together with a 100 bp DNA super ladder (Thermo Fisher Scientific©, Waltham, Massachusetts, USA) for sizing the fragments. The gel was stained in 0.001% ethidium bromide solution for 30 min and the fragments were visualised using the Gel Doc™ XR$^+$ gel documentation system (BioRAd©, Hercules, California, USA). Each reaction was run with a non-template control (nuclease free water) and positive controls of *R. microplus* which were resistant and susceptible to pyrethroids as well as amitraz. These were obtained from the Department of Genetics, University of Pretoria, South Africa.

2.3. PCR cloning and sequencing

Samples showing homozygous (susceptible and resistance) as well as heterozygous genotype profiles were selected for cloning and subsequent sequencing of the cloned products. Three samples for each of the profiles for the carboxylesterase gene, 4 for the voltage-gated sodium channel (all susceptible) and 10 for the octopamine/tyramine receptor (Fig. 2) were used. The QIAquick PCR purification kit (QIAGEN) was used to purify the PCR product according to the manufacturer's instructions. The TOPO® TA Cloning Kit (Thermo Fisher Scientific) was then used to clone the purified PCR products by chemical transformation. The positive clones were then sent to the VIB Genetic Service Facility at the University of Antwerp for forward and reverse sequencing.

2.4. Data analysis

Frequency counts were done for all the genotypes present for each marker and these were expressed as a proportion of the total number of individual ticks which had positively amplified. This enabled calculation of the overall proportion of the genotypes as well as the proportion at the dipping tank level. To test for evidence of selection pressure against the acaricides in use, Hardy-Weinberg Equilibrium (HWE) was examined for the markers where all the genotypes were recorded. This was done using the chi-square test by the software R and a package called "HardyWeinberg" (R Development Core Team, 2013). The forward and reverse sequences were edited using the BioEdit software