Rapid detection of pyrethroid and amitraz acaricide status of R. microplus and R. decoloratus ticks

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EXECUTIVE SUMMARY

Introduction

Rhipicephalus microplus is a major ectoparasite of cattle that causes large economic losses. This is due to the diseases it transmits resulting in high mortality and morbidity in bovines, as well as indirect losses such as a reduced milk and meat yield, damage to hides and secondary infections which increase the need for use of antibiotics. Synthetic pyrethroids, formamidines, carbamates and organophosphates are commonly used in Africa to control the R. microplus population, as these acaricides are the most cost-effective ways to combat high tick loads.

However, R. microplus has developed resistance to the above mentioned chemical acaricides. In order to overcome this problem an effective tick control strategy with rapid access to the resistance status of a population is required to enable knowledge-based selection of a suitable acaricide. This study provides an update on the resistance status of R. microplus to synthetic pyrethroids and amitraz in the farming community of Mnisi, Mpumalanga, South Africa using conventional single nucleotide polymorphism (SNP) analyses. A high-throughput method of identifying the resistance status of R. microplus cattle ticks to synthetic pyrethroids and amitraz by making use of TaqMan genotyping technology has been tested in this study. These TaqMan assays were designed for the rapid detection of SNPs located in the voltage-gated sodium channel and Octopamine/tyramine receptor genes that have been linked to resistance. Briefly, field samples were collected and a cost-effect genomic DNA extraction methods was developed for cost-effective rapid screening of tick's samples. Individual ticks were analysed to confirm their species via ITS2 sequencing and for their resistance status using PCR, DNA sequencing. For testing of the TaqMan assays, we successfully developed all positive controls required for a diagnostic assay and tested these alongside the validated field

samples to evaluate the accuracy of the TaqMan assays. From the conventional SNP analyses, it is evident that there is a high level of resistance in R. microplus to synthetic pyrethroids and growing levels of amitraz resistance in the Mnisi communal area. The TaqMan assays as a high-throughput detection for pyrethroids were successful and is now being further validated for commercialisation.

Objective Statements

AIM 1: To establish a rapid, high-throughput platform for the detection of pyrethroid and amitraz resistance. Ticks will be collected from cattle farms and analysis using an Open Array TaqMan probe platform.
AIM 2: Evaluation of novel compounds as second generation acaricides to overcome amitraz resistance. A subset of amitraz resistant larvae will be subjected to inhibitors of the pregnenolone pathway (endoxifen) and 3 additional proprietary compounds, RNA sequenced and mode of action described.

Project Aims

- To establish a rapid, high throughput platform for the detection of pyrethroid and amitraz resistance of R. microplus and R. decoloratus ticks.
- To evaluate novel compounds as second generation acaricides to overcome amitraz resistance in R. decoloratus or R. microplus.

Results

Pyrethroid resistance assay: The accepted level of accuracy for an assay to be considered suitable as a high throughput detection tool is generally 95% and above (McGuigan and Ralston, 2002). Both DII_F2R2_v2 and DIII_F3R3_v2 were able to correctly genotype all samples (100% call rate). However, a false weak signal was recorded in the FAM™ channel of DIII_F3R3_v2 when genotyping homozygous susceptible (VIC./VIC.) samples. This issue needs to be addressed before this assay can be upscaled to commercial use. Both DII_F2R2_v2 and DIII_F3R3_v2 showed species specificity to R. microplus which was illustrated by the absence of fluorescent signal when using bovine gDNA as template as well as confirmed by the BLAST analysis which produced no significant hits. This result is important because ticks can contain bovine DNA contaminants which they acquire from feeding.

Cross reactivity between the TaqMan. genotyping assays and bovine gDNA would severely limit the application of these assays to be used as a high-throughput detection tool as samples acquired for this purpose will be field samples which would have likely been feeding on cattle. If cross-reactivity were present the reliability and accuracy of the assays would be compromised. The TaqMan genotyping assays were able to produce accurate genotype calling using crude gDNA extracts. No prior PCR or purification steps were necessary following gDNA extraction, saving both time and money.

Amitraz resistance detection: The Oct 2 TaqMan SNP genotyping assay may prove to be a valuable tool for diagnosing amitraz susceptible and amitraz resistant R. microplus ticks, although further optimization is required for the identification of heterozygous field samples. The Oct 1 TaqMan SNP genotyping assay is not able to distinguish between homozygous susceptible and heterozygous genotypes but it can still potentially be used to screen for homozygous resistant ticks. To improve the diagnostic capabilities of the Oct 1 assay, it could be redesigned in future studies. The ITS2 TaqMan SNP genotyping assay for species identification was only able to correctly identify all R. decoloratus samples but only 40% of

the R. microplus samples. However, the concept of the species identification may still hold potential and an additional ITS2 species assay could be designed and tested in future studies either as a new TaqMan SNP genotyping assay or as a TaqMan qPCR assay.

Analyses of rural field samples for amitraz resistance in Mpumalanga: The results show that the frequencies of susceptible (AA/TT), heterozygous (AC/TC) and resistant (CC/CC) genotypes were 0.07, 0.87 and 0.06, respectively. The frequency of the homozygous susceptible genotype has largely decreased over the past 6 years (from 0.45 to 0.07) whilst the heterozygous genotype has largely increased (from 0.52 to 0.87). Only a slight increase was observed for the homozygous resistant genotype (from 0.03 to 0.06). The observed shift to a heterozygous population over the past years is of concern and as such monitoring of these amitraz-associated SNPs are essential as long as amitraz is being used at the dip stations.

Analyses of rural field samples for pyrethriod resistance in Mpumalanga: The data collected in this study is representative of the resistance status of the adult female R. microplus ticks collected from eight different dip stations in the Mnisi communal area, Mpumalanga, South Africa for the year 2019. A similar study was conducted by our research group in 2016 with samples acquired from March 2012 to May 2013 (Robbertse et al., 2016). In the last seven years since the original study, there has been no change in the allele frequencies of homozygous resistant DII L64I mutants at the dip stations for which we had samples in both 2012/2013 and 2019. For dip stations we obtained samples for the first time in 2019 (Clare B, Utah B, Allandale A, and Dumfries C) we showed that all of the ticks analysed are homozygous resistant. This strengthens the hypothesis that indeed the entire Mnisi area is already homozygous resistant towards SPs, and that the use of SPs should be terminated and replaced with a new class of acaricide. The deficiency in heterozygotes indicates that the DII L64I mutation is favourable and strongly driven by selection and as a result has become fixed in the population. this finding will be communicated to the relevant government divisions and state veterinarians to alter the dipping strategy of the region.

With regards to the testing of endoxifen as a possible new acaricide for control amitraz resistance, the work was delayed due to the COVID pandemic and ability to collect field samples and analyse them using Larval packet tests. We did however manage to do this in 2021 in collaboration with AfriVet and will be treating the resistant larvae in Feb 2021.

Conclusion

Aim 1: This aim was completed and a TaqMan assay for the rapid detection of pyrethoid resistance in R. microplus was developed. In addition to the 96 individual ticks sequenced from Mpumalanga, more field samples (from KwaZulu Natal) will now be analysed to obtain statistical proof supporting the validation of this assay. A TaqMan assay to discriminate between R. microplus (causative agent for Babesia bovis) and R. decoloratus was also developed. This assay was able to correctly identify R. decoloratus ticks and further studies will be done to optimise the assay for detection of R. microplus. TaqMan SNP genotyping assays to detect the 2 SNPs in the octopamine/tyramine receptor gene linked to resistance has been tested and partially optimised. Future studies will now screen additional parameters and probes to improve upon the amitraz assay.

Aim 2: Ticks samples from KwaZulu Natal have been collected and amitraz resistant larvae identified using standard larval packet test (done in Jan 2021, postponed due to COVID pandemic). These larvae will now be exposed to endoxifen, the viability calculated and then subjected to RNA sequencing to gain insight into the ode of action of these possible second

generation acaricides. The sequencing of these treated larvae is planned for Feb 2021, RNA sequencing and data analysis for March-April 2021.

Popular Article

Title for Popular Article

The era of knowledge-based selection of acaricides for livestock

1 January 2021

Livestock production is a fundamental source of revenue, with cattle farming constituting a major agricultural industry with some 2 million people depending on it for their livelihood. With 80% of the global cattle population reported as exposed to ticks and at risk to tickborne diseases (TBDs), these ectoparasites and their associated diseases remain an important contributor to malnutrition, poverty and food insecurity.

Acaricide resistance against all major classes of acaricides has been reported in tick populations around the world. In South Africa, amitraz and pyrethroids are the most commonly used acaricides, with resistance already reported for a number of livestock specific tick species. With regards to “blue ticks” affecting cattle, such as Rhipicephalus microplus (the vector for Babesia bovis, the causative agent for Asiatic redwater) and R. decoloratus (the vector for Babesia bigemina causing African redwater) evidence is fast growing and becoming of great concern.

Conventional methods to detect acaricide resistance by the collection of female ticks and testing their offspring for resistance to a specific class of acaricide is time-consuming, with results only available after a month. It also does not provide information on the possible development of resistance in a tick population which limits the development of future tick control strategies, selection of the correct acaricides and early intervention to prevent resistant tick populations to become established. Due to these limitations, new rapid diagnostic approaches are needed to streamline resistance screening protocols to ensure knowledge-based intervention.

During our research endeavours over the past years, we have moved into genetic testing for acaricide resistance in ticks. As such, we identified genes in South African populations associated with being susceptible or resistant towards pyrethroids and amitraz, respectively. With this information at hand, we were able to develop genetic tests for the detection of resistance in cattle Rhipicephalus species that can be completed within 3-4 days. To further improve the turnover time, we started working on cost-effective, high throughput platforms that can enable the screening of thousands of ticks within 48 hours. To date, we have successfully developed such as system for the rapid detection of pyrethroid resistance in R. microplus ticks which can provide information on ticks being susceptible (homozygous susceptible, which means that bot of their DNA copies are normal), resistant (homozygous resistant, which means that both of their DNA copies contain mutations that render the tick resistant to the acaricide) or heterozygous (their DNA already contain one mutated copy and resistance is starting to develop) (see figure). Work on optimising such a platform for the rapid detection of amitraz resistance detection is ongoing and promising. Within 2021, we hope to offer a service to livestock farmers to rapidly detect both pyrethroid resistance via our high-throughput system and amitraz resistance screening via our conventional genetic tests.

To gain insight into the situation in South Africa, we started with evaluation of resistance in R. microplus ticks in Mpumalanga. In the Mnisi area, where farmers make use of communal grazing and are facing serious challenges associated with high tick loads, we found that 87% of the population was heterozygous resistant for amitraz and 100% resistant to pyrethroids. This stresses the need for rethinking their acaricide program to prevent the development of complete amitraz resistance, and also to conduct cost-effective control strategies by not using inappropriate pyrethroids. With our ongoing studies of tick samples from KwaZulu Natal and the Eastern Cape we aim to expand our database on the current status of acaricide resistance in South Africa in blue ticks from cattle, and use this information in collaboration with famers, government and industry partners to improve acaricide mediated tick control strategies.

In parallel with the development of acaricide resistance the need for additional and/or next generation of acaricides to control tick loads is evident. In this regard, we have used a number of technologies to provide the 1st mechanism by which amitraz resistant R. decoloratus ticks are able to thrive. This provided us with additional druggable targets for testing novel compounds to control amitraz resistant ticks. Work on evaluating these compounds and understanding their mode of action is ongoing.

Combined, this research will benefit the agricultural industry as well as the South African economy by reducing capital losses to farmers as they will be able to purchase the correct acaricides and use them in a more effective way. This will also impact veterinary health disciplines in South Africa by decreasing the tick burden on cattle, which will hinder the spread of acaricide resistant ticks and per association lessen the transmission of tick-borne disease.

NOTE: A more comprehensive article with additional schematic presentations to explain the text will be provided for publication.

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