Short communication: Potential causes of Acaricide Resistance in *Rhipicephalus* and *Amblyomma* Ticks (Acari: Ixodidae) in Namwala District, Zambia

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Abstract

Acaricide resistance in ticks poses a great threat to livestock production in many parts of the world where ticks are a problem. The objectives of this study were to screen for acaricide resistance in *Rhipicephalus* and *Amblyomma* ticks using phenotypic and molecular assays, and to assess current tick control practices used by cattle farmers in the traditional sector of Namwala District. The larval packet test was used to screen for acaricide resistance in ticks covering concentrations up to twice the discriminatory dose for amitraz, diazinon and cypermethrin. Genetic mutations associated with resistance to amitraz (A22C-T8P and T65C-L22S), and organophosphates/ pyrethroids (G1120A) were screened using polymerase chain reaction and sequencing. Information on tick control practices at the household level was collected using a semi-structured questionnaire. Intermediate resistance (21-50%) to amitraz and cypermethrin was detected in both *Rhipicephalus* and *Amblyomma* ticks, with both tick genera showing susceptibility to diazinon (<10%). None of the ticks in this study had the reported acaricide resistance-conferring molecular markers that were screened for. The findings suggest that the resistance detected in the studied tick population may be due to other mechanisms that are yet to be identified. Tick control practices observed amongst the farmers, such as incorrect use of acaricide concentrations and rotations could be fuelling the development of acaricide resistance. It is critical to comprehensively unravel the factors that could be contributing to acaricide treatment failure as this would allow for application of appropriate remedial actions for effective tick control in Zambia.

1.0 Introduction

Ticks and tick-borne diseases (TBDs) impede the productivity of livestock, especially cattle, resulting in severe economic losses (Vudriko et al., 2016; Sungirai, 2017). Mixed tick infestations are a common occurrence in Zambia (Speybroeck et al., 2002), and the genera *Rhipicephalus* and *Amblyomma* are among ticks that transmit TBDs of economic importance, such as East Coast Fever (ECF)/theileriosis, anaplasmosis, ehrlichiosis, and babesiosis (Makala et al., 2003; Simuunza et al., 2011). In Zambia, ECF causes up to 10, 000 cattle deaths annually (Muleya et al., 2012) and high mortalities and production losses have continued to be reported, especially in poorly managed traditional cattle herds in Southern Province (Inambao, 2012; Laing et al., 2018). Namwala District is the largest cattle farming area in Southern Province and the country at large (Mumba et al., 2017). However, the economic viability of the traditional cattle sector is threatened by ticks and TBDs (Makala et al., 2003).

Globally, vector control using chemical acaricides is the main strategy implemented in the control of ticks and TBDs (Abbas et al., 2014). Unfortunately, this vector control method is now under threat from acaricide resistance (Rodriguez-Vivas et al., 2018), with widespread reports of phenotypic and genotypic resistance to major acaricide classes (organophosphates, pyrethroids, and formamidines) being documented across the world (Abbas et al., 2014; Klafke et al., 2017; Rodriguez-Vivas et al., 2018). Acaricide resistance has also been reported in various African countries (Vudriko et al., 2016; Yessinou et al., 2018; Arafa et al., 2020) including the Sub-Saharan region (Baron et al., 2015; Sungirai et al., 2018). In Zambia, phenotypic resistance to organophosphates and amitraz was previously documented in Namwala and Isoka Districts, respectively

(Luguru et al., 1984; Muyobela et al., 2015), but there is no data on potential molecular markers responsible for this resistance.

Recently, there have been complaints of acaricide treatment failure by farmers in different parts of Zambia, including Namwala District (LSCS, 2019), and this has deleterious effects on livestock productivity for especially resource-limited farmers (Sungirai, 2017). However, it is not clear whether the reported complaints of acaricide failure are due to drug resistance emergence or improper acaricide usage. Using both phenotypic and molecular assays, we investigated if the acaricide failure in *Rhipicephalus* and *Amblyomma* ticks was an indication of acaricide resistance. Furthermore, we sought to determine if the acaricide failure could be attributed to the farmer tick control practices.

2.0 Materials And Methods

2.1 Study site

The study was conducted in five (5) veterinary camps (Maala, Chitongo, Kabulamwanda, Nakamboma, and Namwala central) of Namwala District, in the Southern Province of Zambia (15.8222° S, 25.85 22° E) between May 2018 and October 2019 (Fig. 1).

2.2 Tick collection and identification

Adult ixodid ticks were collected from cattle in 12 households, following sampling techniques described by Steyn et al. (2010). The ticks were collected at crush pens by gently and carefully pulling them out from cattle without destroying the mouthparts and placed in perforated capped tubes each containing a fresh leaf for the purpose of providing moisture. Ticks were then transported to the laboratory where they were morphologically identified up to genus level using dichotomous keys (Walker et al., 2003).

2.3 Bioassay

Fully engorged adult female ticks of the genus *Rhipicephalus* (n = 58) and *Amblyomma* (n = 12) were kept in an incubator at 27°C ±1 and 85% ±1 relative humidity to allow for oviposition and larval emergence (FAO, 2004). 14 to 21-day old unfed larvae hatched from the engorged females were subjected to the larval packet test (LPT) as previously described (FAO, 2004). Commercial formulations of amitraz (12.5% *w/v*), diazinon (12.5% *w/v*) and alpha-cypermethrin (15% *w/v*) were used to represent synthetic amidines, organophosphates and pyrethroids, respectively. Working concentrations covering 0.0001–0.4% for each acaricide were made, in duplicates (Supplementary Table 1), the range of which included the manufacturer's recommended dose (MRD) (taken as the discriminatory dose (DD) expected to cause 99.99% mortality) and twice the discriminatory dose (2 X DD) (Vudriko et al.,2016; Rodriguez-Hidalgo et al., 2017).

2.4 Molecular assays

DNA was extracted from individual ticks (*Rhipicephalus* = 305 and *Amblyomma* = 61) using TRI Reagent® solution (Sigma Aldrich® Life Science, USA) as described by the manufacturer. To screen for amitraz resistance-conferring mutations, A22C-T8P and T65C-L22S, a 417 bp region of the *Octopamine/Tyramine*

(*OCT/Tyr*) receptor gene was amplified as previously described by Chen et al. (2007). In order to screen for the presence of G1120A mutation associated with resistance to pyrethroids and organophosphates, a 372 bp region of the *Carboxylesterase* (*CES*) was amplified as previously described (Hernandez et al., 2002; Faza et al., 2013).

The Wizard® SV Gel Clean-Up System (Promega, Madison, WI, USA) was used to purify amplified samples for each target gene (*OCT/Tyr* and *CES*). The purified DNA was then directly subjected to bidirectional sequencing using Brilliant Dye™ v3.1 Terminator Cycle sequencing kit (NimaGen BV, Nijmegen, Netherlands) on the ABI 3500 genetic analyser (Applied Biosystems, Foster City, CA, USA).

Sequence editing and assembly was done using GENETYX ATGC software version 7.5.1 (GENETYX Corporation, Tokyo, Japan). BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST) in NCBI GenBank was used to verify sequence identities of the amplified genes with a percentage similarity range of 90–100% to those in the public repository database considered as positive identity. The obtained partial sequences were then deposited in the DDBJ GenBank under the accession numbers LC658951-LC658955 and LC659236-LC659240 for the *CES* and *OCT/Tyr* gene, respectively. The *OCT/Tyr* nucleotide sequences were translated into predicted amino acid sequences using BioEdit software version 7.2.5 (https://bioedit.software.informer.com). Finally, in order to determine the presence of resistance-conferring mutations, ClustalW (https://www.genome.jp/tools-bin/clustalw) was used to align sequences obtained in this study against the respective reference sequences for the *OCT/Tyr* (Gonzalez amitraz susceptible strain, accession number EF490687.1 and Santa Luiza amitraz resistant strain, accession number EF490688.1) and *CES* (Gonzalez pyrethroid/organophosphate susceptible strain, accession number AF182283.1) genes downloaded from GenBank (Supplementary Fig. 1 and Fig. 2).

2.5 Questionnaire survey

A total of 357 traditional cattle farmers were interviewed at communal grazing sites and main trading places such as abattoirs, following a 20% sampling technique based on the farmer population that is in possession of cattle in Namwala District (Mpofu, 2020). The administered semi-structured questionnaire captured information on acaricides currently in use, mode of acaricide delivery on cattle and acaricide rotation (Supplementary Questionnaire). Correct acaricide rotation was considered to be the difference in chemical groups or mechanism of action between the rotated acaricides (Rodriguez-Vivas et al., 2018).

2.6 Data analysis

Probit tool (IBM SPSS v 21.0 for windows. Armonk, NY: IBM Corp) was used to generate lethal dose estimates (LD_{50} and LD_{90}) (FAO, 2004) (Supplementary Table 2 to 7). Resistance levels at doses equal to 2 X DD were categorized into four (4) groups i.e. high (>51%), intermediate (21-50%), low (11-20%) and susceptible (<10%) (Junte et al., 2009; Rodríguez-Hidalgo et al., 2017). Questionnaire data were entered in Microsoft Excel (2016) spreadsheets where it was sorted into frequencies and calculated into percentages based on the participant responses.

2.7 Ethical approval

Ethical approval to conduct the study was obtained from the University of Zambia Health Sciences Research Ethics Committee (UNZAHSREC) with protocol ID number 20190217068. Consent to acquire information on tick control methods and tick collection from cattle was obtained from the respondents in the selected study sites. Recommended tick collection methods as described by Steyn et al. (2010) were used in order to ensure the safety of animals from which ticks were collected.

3.0 Results And Discussion

Rhipicephalus and *Amblyomma* ticks showed intermediate resistance (21-50 %) at the 2 x DD to both amitraz and cypermethrin, with the lethal dose estimate for *Rhipicephalus* ticks being relatively higher than that of *Amblyomma* ticks. However, both tick genera showed susceptibility (<10 %) to diazinon (Table 1).

The level of resistance detected in the field ticks coupled with fitness cost associated with the amitraz resistance allele, and the aspect of refugia in the three-host ticks (Sungirari et al., 2018; Rodriguez-Vivas et al., 2018) suggests that ticks in the study area are undergoing high selection pressure towards resistance to amitraz and cypermethrin. Our study adds to the growing knowledge of amitraz resistance in the country, with previous reports indicating presence of resistance in the northern part of the Country (Muyobela et al., 2015). Amitraz resistance in ticks has also been documented across the world (Abbas et al., 2014; Rodríguez-Hidalgo et al., 2017; Rodriguez-Vivas et al., 2018; Makuvadze et al., 2020). Whilst resistance to synthetic pyrethroids is widespread in most parts of the world (Abbas et al. 2014: Klafke et al., 2017), this is the first time, resistance to cypermethrin is being reported in Zambia. The presence of multi-acaricide resistance in the studied tick population is a cause for concern as it undermines efforts to enhance livestock productivity, especially that the control of ticks and TBDs in the study area relies on chemical acaricides (Mumba et al., 2017). This finding also confirms the reports by farmers in Namwala District of reduced acaricide effectiveness (LSCS, 2019).

Despite the observed level of resistance to amitraz and cypermethrin, sequence analysis of the *OCT/Tyr* and *CES* receptor genes revealed none of the mutations commonly associated with resistance to amitraz (T8P and L22S) and cypermethrin (G1120A) (Supplementary Fig. 1 and Fig. 2). The difference between results from this study and those reported previously (Hernandez et al., 2002; Chen et al., 2007; Baron et al., 2015; Sungirai et al., 2018) could be that ticks in this study may be using different mechanisms of resistance, as other mechanisms have also been proposed to induce resistance against these classes of compounds (Stone et al., 2014; Jonsson et al., 2018). Future studies on ticks in this area should go further and screen for all published mutations, in order to ascertain the exact mechanisms conferring resistance in these tick species.

Contrary to previous reports of resistance to organophosphates in ticks from Southern Province and other areas of Zambia (Luguru et al 1984, 1987), the ticks in our study showed susceptibility to diazinon, a finding which was also supported by the absence of the G1120A mutation in the *CES* gene (Supplementary Fig 2) that is also linked to organophosphate resistance (Faza et al., 2013). Use of organophosphorus compounds was discouraged in the country due to reports of resistance as well as environmental toxicity (De Meneghi et al., 2016) and it is possible that their discontinued use in the area could have resulted in reduction in acaricidal pressure leading to total loss of the resistance allele and reversion into its wild genotype, especially

that the organophosphate resistance gene is semi-dominant (Stone et al., 2014). Essentially, this is indicative of the possible future consideration of diazinon for the control of ticks resistant to amitraz and cypermethrin in Namwala District.

Our questionnaire survey revealed that amitraz (84.62 %, 302/357) was the most widely used chemical acaricide, followed by cypermethrin (8.72 %, 32/357) and fluazuron (3.08 %, 11/357). This trend has been observed in previous studies in Zambia (Muyobela et al., 2015; Mumba et al., 2017) and neighbouring Zimbabwe (Sungirai et al., 2018). The popularity of amitraz amongst farmers could be due to its relatively cheaper cost (Mumba et al., 2017; Chitombo et al., 2021) and rapid knockdown effect compared to other acaricides (Jonsson et al., 2018). Frequency of acaricide application was based on tick abundance, with 43.4% (155/357) of farmers doing weekly applications and the rest applying acaricides bi-weekly (53.4%, 191/357) through knapsack spraying. However, dipping has been established as the most effective delivery method for acaricides because it offers full animal body coverage unlike knapsack spraying which may not successfully cover areas such as the perineum and inguinal regions, thus leaving a critical number of ticks unexposed to the acaricide (Sungirai, 2017). The shortfall of functional dip tanks in the area and the cost associated with their maintenance (Mpofu, 2020) could potentially explain the preference for the knapsack spray method by farmers in this study.

Amongst the acaricide use practices recorded in our study was incorrect acaricide mixing (50% of respondents), with the majority of farmers using doses higher than those recommended by manufacturers. Also observed was incorrect rotation of acaricide by farmers (32%, 114/357), with lack of knowledge on what entails proper rotation being noted as shown by the frequent change of trade names, despite compounds being in the same chemical class. The use of compounds with differences in active molecule and mechanism of action between the acaricide compounds been alternated is considered to be the correct approach (Rodriguez-Vivas et al., 2018). Improper mixing and rotation of acaricides are practices that have also been reported previously (Muyobela et al., 2015; Mekonnen, 2006; Vudriko et al., 2016) and these practices are implicated as drivers of acaricide resistance (Rodriguez-Vivas et al., 2018). Thus, taking these factors into account might possibly explain the resistance observed in our study as evidenced by tick survival at doses higher than the MRDs (Table 1 and Supplementary Table 1).

Whilst our questionnaire findings could possibly explain the reported inefficacy of acaricides in the area (LSCS, 2019), we did not go further to assess the quality of these acaricides. It is noteworthy that failure of commercial preparations has previously been reported (Vudriko et al., 2016; Rodríguez-Hidalgo et al., 2017), and use of sub-standard chemical acaricides also contributes to resistance (Wambua et al., 2019). With the liberalization of the pharmaceutical industry, there is a need to standardize discriminatory doses against MRDs and ascertain the quality of acaricide products on the Zambian market.

This is the first study in Zambia that has attempted to analyse the resistance of ticks to acaricides such as formamidine (amitraz) and organophosphorus/pyrethroid compounds using molecular methods. The major limitation of this study was the lack of a susceptible reference strain that is necessary to determine the degree of resistance in the studied tick population. The study findings, highlight the need to strengthen farmer training on the judicious use of chemical acaricides, the substitution of amitraz and cypermethrin with

formulations containing chemicals with different mechanisms of action, and the development of acaricide rotation strategies in order to prevent/delay development of resistance in ticks.

4.0 Author Disclosure Statement

The authors declare that there are no conflicts of interest.

4.1 Acknowledgements

4.2 Author contributions

Simbarashe Chitanga; Conceptualisation, Fund acquisition, Resource, Supervision, Methodology, and Writing-review and editing; Karen Sichibalo: Methodology, Investigation, Writing original script; Katendi Changula: Resource, Supervision and Writing-review and editing; Chisoni Mumba: Investigation and Writing-review and editing; Natasha Mwila: Investigation and Writing-review and editing; Kennedy Chibesa: Investigation and Writing-review and editing; Benjamin Mubemba: Writing-review and editing; King S. Nalubamba: Resource, and Writing-review and editing: Walter Muleya: Writing-review and editing; Edgar Simulundu: Data curation, Resource, Supervision, Writing-review and editing.

4.3 Data availability

Data is available upon request

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Tables

Table 1: Percentage resistance at 2 X DD and lethal dose estimates (LD_{50}, LD_{90}) obtained for each acaricide against the respective tick genera.

Genus	Acaricide	% Mortality at MRD	%Resistance at 2 x DD	Slope (SE)	HF	LD 50 (ml/L) (95% CL)	LD90 (ml/L) (95% CL)
Rhipicephalus							
	Amitraz	28.50	46.50	1.530 (±0.24)	1.49*	0.004 (0.003 – 0.005)	0.025 (0.019 – 0.035)
	Cypermethrin	41.00	40.50	0.630 (±0.13)	3.19	0.002 (0.000 – 0.004)	0.109 (0.037 – 0.690)
	Diazinon	74.00	5.50	0.660 (±0.18)	2.40*	0.001 (0.000 – 0.003)	0.044 (0.020–0.229)
Amblyomma							
	Amitraz	63.50	28.50	0.41 (±0.19)	1.24*	0.000 (0.000 -0.001)	0.261 (0.074 – 2.392)
	Cypermethrin	66.00	21.00	0.70 (±0.19)	1.44*	0.000 (0.000 – 0.001)	0.018 (0.011 – 0.031)
	Diazinon	71.00	0	0.79 (±0.18)	9.71	0.000 (0.000 – 0.001)	0.016 (0.003 – 1.226)

MRD: manufacturer's recommended dose, 2 x DD: 2 x discriminatory dose = 2 x MRD, SE: standard error, HF: heterogeneity factor, LD: Lethal dose, DD: discriminatory dose, 95% CL: 95% confidence limit. *: Data followed probit model ($p<0.05$).

Figures
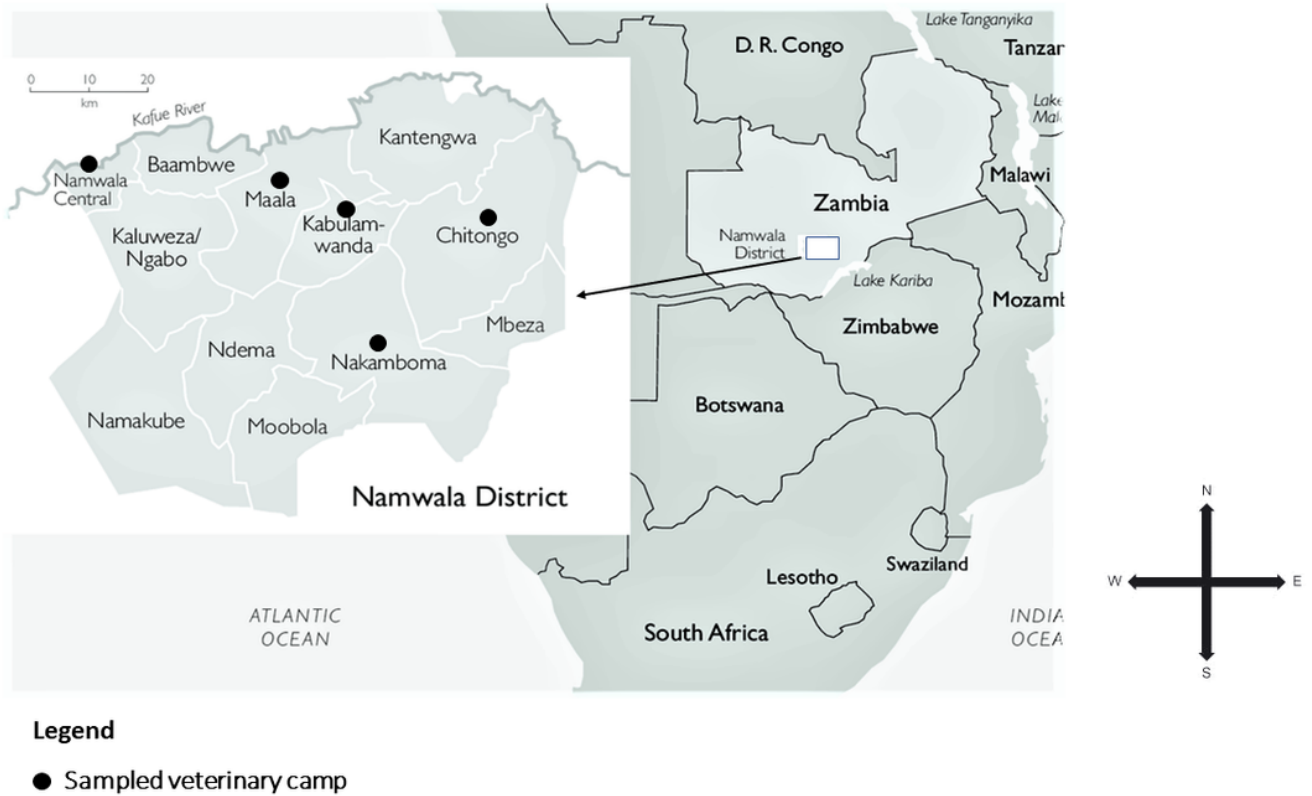
Figure 1

Map of Namwala District showing sampled veterinary camps (black dots). Map adapted from Mweemba et al. (2011) with slight modifications.

Supplementary Files

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