Studies on Control of Acaricide Resistant Ticks in Uganda

（ウガンダ共和国における薬剤耐性マダニの対策法に関する研究）

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Abbreviations

A. vari: Amblyomma variegatum

AfDB: African Development Bank

AIT: Adult immersion test

B. decol: Rhipicephalus (*Boophilus*) *decoloratus*

BHC: β-hexachlorocyclohexane

CDL: Central Diagnostic Laboratory

COF: Co-formulation

COVAB: College of Veterinary Medicine, Animal Resources and Biosecurity

CPD: Continued Professional Development

CXE: Carboxylesterase

DD: Discriminating Dose

DDT: Dichlorodiphenyltrichloroethane

DNA: Deoxy nucleotide adenosine triphosphate

DVO: District Veterinary Officer

EBATIC: Evidence Based Acaricide (chemical) Tick Control

ECF: East Coast fever

FAO: Food and Agriculture Organization

GABA: gamma-amino butyric acid

H. leach: *Haemaphysalis leachi*

HEST: Higher Education, Science and Technology support

IT-IE: Identify, Test, Intervene and Eradicate

JICA: Japan International Cooperation Agency

JSPS: Japanese Society for Promotion of Science

Kdr: knock down resistance

LPT: Larval packet test

LTT: Larval tarsal test

MAAIF: Ministry of Agriculture, Animal Industry and Fisheries

NDA: National Drug Authority

NT: not tested due to few larvae

OP: Organophosphate

PCR: Polymerase chain reaction

Pop: Tick population

R. app: *Rhipicephalus appendiculatus*

RdNaDII: *Rhipicephalus* (*Boophilus*) *decoloratus* sodium channel domain II

RFLP: Restriction fragment length polymorphism

RTC: Research Center for Ticks and Tick-borne diseases Control

SFG: Spotted fever group

SP: Synthetic pyrethroid

SNP: Single nucleotide polymorphism

spp: species

SPPS: Statistical Package for Social Scientists

super-kdr: Super knock down resistance

TBD: Tick-borne diseases

VSSC: Voltage sensitive sodium channel

WHO: World Health Organization

Unit abbreviations

bp: base pair

^{o}C: degree celcius

mg/ml: milligram per milliliter

ml: milliliter

ng: nanogram

µl: microliter

%: percentage

General Introduction

Ticks are one of the ubiquitous ectoparasites that infest animals globally. Over 800 species of ticks are known, with 80% of them being ixodid ticks (hard ticks) (72). Despite animals being the dominant host, over 20% of the ixodid ticks also infests man (46). The economic cost of ticks and tick-borne diseases (TBD) is very high in the tropics and subtropics due to their aboundance and diversity (72). The annual losses associated with ticks and TBD has been estimated to be 364 million USD in Tanzania (88) and 5.6 million USD due to cowdriosis alone in Zimbabwe (118). In Uganda, a more conservative annual loss of 0.3 million USD was reported in 2009 (123). The above estimated losses due to TBD in Africa grossly under-represent the true economic lossess due to ticks and TBD (72). This is due to the fact that, an earlier study in Australia estimated losses between 75 USD and 115 million USD despite the country only having *R.* (*B.*) *decoloratus* as the most economically important tick for livestock. In the tropics and subtropics, given that more than one ecomomically important tick species infest and vector TBD in livestock, the economic losses should be higher than that reported in Australia (72). What remians undisputed, at least in the Ugandan context, is that control of ticks and TBD constitutes upto 85.6% of the total farm diseases control cost (123). It was found that the bulk of the cost incured on TBD control is attributed to costs on control of ticks (123). The most economically important ixodid ticks that parasitize domestic animals in Africa belong to the genera *Amblyomma*, *Rhipicephalus* and *Hyalomma* (72, 168).

The genus *Amblyomma* has diverse species, with 129 that are known (72). Of the most important *Amblyomma* species in Africa, *A. variegatum* is widely distributed across all the

regions, followed by, *A. lepidum* (eastern Africa), *A. gemma* (parts of eastern Africa) and *A. pomposum* (southcentral Africa) (168). *Amblyomma* ticks vector pathogens that infect animals and humans. *Anaplasma* spp. (143) and *Ehrlichia* spp. (101) vectored by *Amblyomma* ticks cause anaplasmosis and cowdriosis in livestock, respectively. They also vector spotted fever group (SFG) rickettsia, which are zoonotic intracellular bacteria (44). Among the SFG, *Rickettsia africae*, vectored by *A. variegatum* is wide spread across Africa (86, 95, 102, 172), including Uganda (119). The prevalence of *Anaplasma* spp. in cattle in Uganda was reported to be high (>70%) in Karamoja region where *Amblyomma* tick population is equally high (21, 22). On the other hand, bovine *Ehrlichia* infection appears to be low in Uganda, with a prevalence of 1.7% (115).

The subgenus *Boophilus* is among the economically important livestock ticks in Africa. Two species, *R.* (*B.*) *decoloratus* and *R.* (*B.*) *microplus* are widely distributed in tropical areas of Africa (168). The *R.* (*B.*) *microplus* is native to Asia, and invasion in Africa has been attributed to cattle trade (72, 157). The *R.* (*B.*) *microplus* ticks have now colonized several countries in the west, east and southern Africa (16, 96, 99, 100). Given its high reproductive efficiency, *R.* (*B.*) *microplus* is displacing *R.* (*B.*) *decoloratus* in African countries like Benin (33), Ivory coast (100), South Africa (122, 159), Zimbabwe (155) and Tanzania (99). While *R.* (*B.*) *decoloratus* is distributed in Uganda (22), *R.* (*B.*) *microplus* is not yet reported despite the fears that it may be present due to its distribution in neighboring countries like Tanzania (99) and Kenya (54). The *Boophilus* ticks are vectors for *Babesia* spp. and *Anaplasma* spp. that cause babesiosis and anaplasmosis respectively, in animals (71, 72, 94, 139). The prevalence of bovine babesiosis reported in Uganda ranged from 0.6% to 6.7% (9, 84, 116).

Another genus *Rhipicephalus*, presents the highest threat to the livestock production in Africa. *Rhipicephalus appendiculatus* (brown ear ticks) and *Rhipicephalus evertsi* (red-legged

tick) are the two species that are distributed in east, central and southern Africa (135, 168). The brown ear tick is a three-host tick that vectors *Theileria parva*, which causes a highly fatal disease of cattle called East coast fever (ECF) (90, 135). *R. evertsi* is further distributed in western Africa, and the tick vectors *Babesia caballi* and *T. equi* in horses and *A. maginale* in cattle (66, 168). Other species of *Rhipicephalus* ticks found in Africa includes *R. muhsamae, R. simus, R. pravus* and *R. pulchellus* (22, 168). The economic impact of ECF in Uganda has been widely reported (112, 123, 126). The prevalence of *T. parva* documented in earlier studies ranged from 5.3% to 47.4% (22, 84, 117).

The ticks in the genus *Hyalomma* (*Hy*) are widely distributed in the dryland regions of Africa. *Hy. anatolicum* is a vector for *T. annulata* (tropical theileriosis), *T. lestoquardi* (malignant ovine theileriosis), *T. equi* (equine theileriosis), *Ba. caballi* (causes equine babesiosis) and *Trypanosoma theileri* (benign bovine trypanosomiasis). The tick also vectors the zoonotic Crimean-Congo haemorrhagic fever virus (CCHFV) that causes a fatal hemorrhagic disease in humans. *Hy. marginatum rufipes*, is one of the most widely distributed *Hyalomma* ticks in Africa (168), including Uganda (82). It is the most important vector for CCHFV in humans, in addition to transmitting *A. marginale*, *Rickettsia conorii* that causes typhus in humans, *Ba. occultans* to cattle (168). *Hy. truncatum* is another widely distributed tick in Africa, although rarely found in Uganda (22). It causes severe physical trauma on domestic animals, toxicosis and also vectors *Ba. caballi* and *Rickettsia conorii* (168). *Hy. impeltatum* also transmits *T. annulata* and CCHFV (168). Other species includes *Hy. scupense* that vectors *T. annulata* and *T. equi*, and *Hy. lusitanicum* that transmits *T. annulata* and the zoonotic bacterium *Coxiella burnetii*. *Hy. marginatum* vectors *Ba. caballi* in horses and CCHFV in humans (168).

The control of ticks on livestock is an important component of animal husbandry. The

demand for control of ticks on livestock is also reflected in the overall cost incurred on the control of ticks to prevent TBD. For example, in Uganda, up to 87.9% of the total cost incurred on TBD accrued from purchase of acaricides (123). The history of use of acaricides for controlling ticks has been traced back to arsenic compounds in 1893 (8) and organochlorines in 1930's (8, 52, 85). Chemical tick control in Uganda was first reported in 1935 but actively implemented with the introduction of organochlorines in 1950s (131). Organochlorine compounds were reported to have been tested in Uganda included β-hexachlorocyclohexane (BHC), Dichlorodiphenyltrichloroethane (DDT) and Toxaphene, with the latter being used extensively between 1950s and early 1960s (131) until resistance was reported in 1970 (87). Organochlorines are sodium channel modulators that were effective against ticks but concerns over their persistence in the environment and endocrine disruption (146, 167), led to the ban on their use internationally under the United Nation Stockholm convention (27, 50).

The current generation of acaricides used for tick control includes organosphophates (OP), synthetic pyrethroids (SP), amidine, macrocyclic lactones, phenylpyrazole, benzoyl phenyl urea and spinosyns (52, 56). The organophosphate compounds were introduced in 1960s (55) and used to control organochlorine resistant ticks in Uganda (131). The examples of OP used in Uganda include dioxathion, chlorfenvinphos and oxinothiophos. They were first used in 1960s for implementation of the compulsory tick control program implemented under the Uganda National Tick program (131). The OP act by inhibiting tick cholinesterase enzymes leading to paralysis (1, 52). SP pyrethroids on the other hand were introduced in 1970s (56) and tested in Uganda in late 1980s (131). The third generation pyrethroids have been considered very effective against both ticks and insects, making them popular in African countries where both fly and tick control are a necessity on livestock farms. Examples of

commonly used SP include cypermethrin, flumethrin, delatamethrin, cyhalothrin, fenvalerate and cyfluthrin (52, 141). Generally, pyrethroids act by activating the voltage gated sodium channel, leading to paralysis of the tick (7, 52). Amidines on the other hand were first introduced in 1970s (56) and tested in South Africa in 1980s (52). In Uganda, amitraz-based formulations were introduced in 1991 and 1994 (131) but they were initially shelved and reserved for future use, under the national acaricide rotation system practiced in 1980s. Since its introduction, amitraz has been used widely in other African countries (81, 104, 111). Amitraz acts on octopamine receptors and its unique mode of action has been exploited in controlling OP and pyrethroid resistant ticks (51, 52, 56).

Other acaricides used include the macrocyclic lactones such as ivermectin, milbemycin, abamectin and eprinomectin (6, 47). They act by modulating glutamate-gated chloride channel leading to hyperpolarization and tick paralysis (93, 129). Phenylpyrazole such as fipronil is also used as acaricide and it act as gamma-amino butyric acid (GABA) receptor blocker, thus blocking chloride conductance (25, 28). Benzoyl phenyl urea such as diflubenzuron and fluazuron are a growth regulator that inhibits chitin biosynthesis thus affecting molting of insects and ticks (36, 136). Fluazuron has been used for control of *R.* (*B*.) *microplus* in Latin American countries (31, 47, 138). Spinosyn such as spinosad is used as agricultural pesticide (67, 149) and acaricide for tick control (77, 141). The macrocylic lactones, fipronil, benzoyl phenyl urea and spinosads have been used in various countries as adjunct chemical for management of resistance against the conventional acaricides such as OP, SP and amitraz (1, 52, 77, 141).

Acaricide resistance is a natural phenomenon in which previously susceptible ticks to the discriminating dose of a given chemical survives against the same dose (1, 141). Generally, acaricide resistance may occur through mutations at the target site or by increased metabolic

breakdown of the chemical by the tick (1, 52, 141). For example, resistance against SP has been attributed to mutations in the voltage sensitive sodium channel (VSSC) domains II and III (1, 58, 97, 152). Such mutations usually lead to alteration in amino acid residues at the target site, thus affecting the binding affinity of the receptor for its ligand, leading to a knock-down (*kdr*) or super knock-down (*super-kdr*) resistance (38, 165). Target site resistance against amitraz due to mutation in octopamine receptor has also been reported (13, 30). Metabolic resistance on the other hand is mediated by three major pathways: esterases, glutathione transferases and mixed function oxidases (1). Organophosphate resistance has been attributed to increased hydrolytic activity of esterase enzymes (157, 91). Hydrolytic breakdown of SP by oxidases, carboxylesterase (CXE) and glutathione transferases have also been reported (1, 43, 70).

Diagnosis of acaricide resistance is based on *in vitro* assays and genetic techniques (52). The common *in vitro* tests include adult immersion test (AIT), larval immersion test (LIT), larval tarsal test (LTT) and larval packet test (LPT) (49, 89). Other *in vitro* tests include enzyme kinetic studies such as esterases and glutathione transferases biochemical activity profiling (55, 92). The genetic techniques involve PCR assays specific for detection of mutations (56, 64, 97, 152) or restriction fragment length polymorphism (RFLP) (13, 60, 64). Since there is limited information on the current status of acaricide resistance in Uganda, despite wide spread complaint about acaricide failure, this study evaluated effectiveness acaricides currently used for tick control. The study also developed strategies for diagnosis and intervention approach against acaricide resistant ticks in Uganda. The specific objectives for the study included;

I. To assess chemical tick control practices in Uganda.

II. To determine the effectiveness of the different classes of acaricides against ticks.

III. To determine the genetic basis of super synthetic pyrethroid-resistant ticks and developing a rapid genetic diagnostic method.

IV. To develop an evidence-based intervention strategy for management of tick acaricide resistance in Uganda.

Chapter 1

Chemical tick control practices in southwestern and northwestern Uganda

1. Introduction

Ticks and TBD have become the leading concern to cattle production in African countries including Uganda. *R. appendiculatus*, *R.* (*B.*) *decoloratus* and *A. variegatum* are among the most important tick species in Uganda (22). Beside the physical damage which ticks cause on cattle, they also vector diseases that are associated with severe economic losses (18, 72). The climate in Uganda (82) favors tick survival throughout the year. Thus, cattle farmers continuously have to use acaricides throughout the year to be able to reduce production losses associated with TBD (72). However, prolonged use of acaricides usually predispose to emergence of acaricides resistance (1, 58). Acaricide resistance is a natural response to selection pressure (140) although inappropriate farm tick control practices may induce its rate of progression. Acaricide application practices are the most important factor that influences the pace at which resistance occurs (1). Frequent use of the same molecule on a farm is amongst the leading drivers of selection for resistance (76). In view of wide spread complaint on acaricide failure especially in western Uganda, this study assessed the practices involved in acaricide usage and determined the common types of ticks in four selected districts in southwestern and northwestern Uganda.

2. Materials and Methods

2.1. Study area

The study was conducted in four districts, namely Adjumani, Mbarara, Mitooma and Rukungiri between July and September 2015 (Fig. 1). Three of the districts in Uganda (Mbarara, Mitooma and Rukungiri) lie within the high acaricide pressure zone in Uganda's dairy shed areas (11), where complaint of acaricide failure by farmers were wide spread. Adjumani district on the other hand is located in the northwestern part of Uganda where acaricide use (pressure on ticks) is generally lower. Adjumani is at an altitude of 900-1,500 meters above sea level and receives an average rainfall of 1,125 mm per annum (5). Mixed farming (crop-livestock) is the major economic activity in with Zebu cattle as the dominant breed reared. Mbarara, Mitooma and Rukungiri are located in southwestern Uganda and livestock production is one of the core economic activities (11).

2.2. Study design

This was a cross sectional study that involved use of semi-structured questionnaire to assess the knowledge, attitude and practice of farmers regarding tick control. Ticks were also collected from cattle and identified to determine species distribution in the four districts. In southwestern Uganda, farms with and without complaints of acaricide failure at the time of the study were identified by the local district veterinary office or drug shop outlet operators in the community. A total of 85 farms were purposively selected from the four districts. In southwestern Uganda, 33 farms with complaints of acaricide failure as evidenced by over 10 ticks collected on cattle following acaricide application were sampled. Other 30 farms that had less than 10 ticks collected (no acaricide failure) at the time of this study were also

included. In Adjumani district, 22 farms identified by the district veterinary office were used for obtaining data on chemical tick control practices in northwestern Uganda.

2.3. Baseline survey to identify gaps in tick control

A semi-structured questionnaire was used to capture on-farm tick control practices in southwestern and northwestern Uganda. The key variables assessed included characteristics of the farms, equipment and facilities used for tick control, acaricide dilution and application practices, strategies for coping (overcoming) acaricide failure, brands of acaricides used currently, intermediate and previously, acaricide toxicity to animals and farm workers. Data on acaricide used at the time of the study and previously was used to determine the acaricide brands and classes used and correctness of rotation. Acaricide rotation was considered wrong if the change of acaricide was effected within the same class of acaricide, change from co-formulated acaricide to respective mono-formulations, not being sure of the brand name of the acaricide used before (intermediate) changing to the current acaricide in use. The volume of acaricide mixed with 20 liters or per liter of water for application on animals was used to determine whether manufacturer's recommendation for dilution of the acaricide in use was followed. Dilution was deemed wrong if the acaricide strength was higher, lower than manufacturers' recommendation, estimated or the respondent was not sure.

2.4. Collection and identification of ticks

At each farm, at least half of the cattle were randomly driven to the holding yard or kraal (Fig. 2A). The cattle were restrained using either crush or ropes and visible ticks were hand-picked from their various attachment sites. The ticks were transferred into aerated sample bottles, sorted and identified to species level using tick identification guide (168).

2.5. Data analysis

The data captured were coded and entered in MS excel and analyzed in SPPS version 21 (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.). Tick population data were further analyzed to determine the distribution of the different types of tick species per district.

2.6. Ethical considerations

The study was approved by the College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University (Approval number: VAB/REC/15/104). Questionnaires were administered to only participants who consented to the study. The identity of the respondents was kept confidential.

3. Results

3.1. Characteristics of the farms

A total of 85 farms participated in this survey, 63 of which were from southwestern region districts of Mbarara (25/63), Mitooma (19/63) and Rukungiri (19/63) (Table 1). In northwestern region, 22 farms from Adjumani participated in this study. Of the 85 farms, 47.1% (40/85) were owned by people whose occupation was farming as a full time job while the rest had the farm as a secondary enterprise. Majority (78.8%, 67/85) of the respondents for the survey were the owners of the farm while the rest were farm managers (21.2%, 18/85). All the farms in Adjumani district reared local cattle for multiple purposes (beef, milk and draught power). In contrast, 95.2% (60/63) of the farms in southwestern Uganda reared mainly exotic cattle crosses for dairy production. Most of the farms had small (<20 head) (29/85) or medium (21-100 heads) (45/85) cattle herd size. These were kept along with small ruminants in 52.9% (45/85) of the farms. Interaction between livestock and wildlife was reported in 38.8% (33/85) of the farms, 27 of which occurred in farms in southwestern Uganda. Paddocking was practiced by 85.7% (54/63) of the farms in southwestern Uganda, while communal grazing was the main system of cattle production in Adjumani district (81.8%, 18/22).

3.2. Method of tick control and associated facilities

All the respondents reported that chemical (acaricide) application was the only means of tick control strategy employed on their farms (Table 2). Overall, spraying accounted for 90.6% (77/85) of the methods of acaricide application. Up to 81.2% (69/85) of the farms used crush (Figs. 2B and 2C) for restraining cattle during acaricide application with the spray

method. Dipping and scrubbing with cloth was encountered in 6 and 2 farms, respectively. Of concern was that 38.8% (33/85) of the farms used wrong equipments such as knapsack sprayer (17/85), hand sprayer (14/85) and scrubbing cloth (2/85) for acaricide application. Hand-held 1 or 2 liter spray pumps were predominantly used in Adjumani district (12/22). Bucket pump (recommended spray equipment) was used by 68.3% (43/63) of the farms in southwestern Uganda. The most common equipments used for measurement of acaricide were calibrated bottle tops (57.6%, 49/85) and syringes (30.6%, 26/85). However, ungraduated acaricide bottle tops were also used by 4 farms. The common source of water for mixing acaricides was tap water (14.1%, 12/85) and water harvested directly from natural reservoirs (85.9%, 73/85) obtained from river, stream, valley dam, and swamp. The quality of swamp water from 13 farms was characterized as dirty and not suitable for diluting acaricides. Of the 33 farms in southwestern with complaint of acaricide failure, 97% (32/33) were applying acaricides using the spray method.

3.3. Sources of acaricide and advice, acaricide dilution and application interval

Majority (71.8%, 61/85) of the respondents reported that veterinarians were the main source of advice on tick control (Table 3). However, 28.2% (24/85) of the farmers either relied on their personal judgement or sought advice on tick control from Veterinary drug shop attendants and fellow farmers. Veterinary drug shops were the main source of acaricides (69.4%, 59/85) although some farmers obtained acaricides from veterinary pharmacies/distributors (14.1%, 12/85) and illicit open markets (9.4%, 8/85). Mixing of acaricides were mainly done by farm workers such as herdsmen (39/85), farm managers (17/85) and children (3/85). Up to 81.2% (69/85) of the farms diluted acaricide wrongly while only 18.8% (16/85) diluted correctly following the manufacturers' recommendation (Table 3).

In northwestern region, only 27.2% (6/22) of the farms followed the manufactures' dilution rate, the rest used estimate (22.7%, 5/22), low acaricide strength (13.6%, 3/22), higher strength (9.1%, 2/22) and 22.7% (5/22) were not sure of the dilution rate. Similarly, in southwestern region, only 19.0% (12/63) of the farms followed the manufacturers' recommended dilution rate, the rest practiced dilution malpractices such as use of higher concentration (50.8%, 32/63), low concentration (11.1%, 7/63), estimate (1.6%, 1/63) and 17.5% (11/63) were not sure about the dilution rate. Apparently, half of the farms in southwestern region with acaricide failure (48.5%, 16/33) and those without acaricide failure (53.3%, 16/30) were using higher acaricide strength.

Acaricide application was carried out frequently by herdsmen (56.5%, 48/85) while supervision of mixing and application was done by the farm owner (45.9%, 39/85), farm manager (28.2%, 24/85) and fellow herdsmen (23.5%, 20/85). The recommended weekly acaricide application during rainy season was practiced by 76.5% (65/85) of the farms. However, 69.4% of the farms also applied acaricide weekly during dry seasons, contrary to the recommended interval of 2 weeks. Worryingly, 23.8% (15/63) and 17.5% (11/63) of the farmers from southwestern Uganda applied acaricides on cattle at least twice a week during rainy and dry seasons, respectively. One-month acaricide application interval especially during dry season (12/22) was practiced in Adjumani district. In both regions, no farmer applied the FAO recommended 10 liters of mixed acaricide solution per cattle. Only 17.6% (15/85) of the farms used 20 liters of mixed acaricide solution to spray utmost 7 heads of cattle, while the rest of the farmers applied the same quantity of mixed acaricide solution on 8 to over 40 heads of cattle. The average number of cattle sprayed with 20 liters of mixed acaricide solution was 38 and 12 in northwestern and southwestern regions, respectively. Farmers in northwestern region applied on average 0.98 liters of acaricide solution per cattle,

compared to 2.15 liters in southwestern region ($p = 0.0001$).

3.4. Type of acaricides used and strategies for overcoming acaricide failure

Of the 20 brands of acaricides reported, 11 were currently used (Fig. 3A). Six of the seven brands of amidine, were currently used, with amitix (24.4%, 21/86) and milbitraz (11.6%, 10/86) being the most frequent. Eight brands of pyrethroids were mentioned, only two (alfapor and vectocid) were currently used. Of the 3 brands of co-formulated acaricides, two (duodip and protaid) were currently used. Only one brand of mono-formulated organophosphate (supona extra) was encountered. The proportion of uncertainty (not sure) in brands used currently, intermediate and previously was at 1.2% (1/86), 29.5% (26/88) and 60.0% (51/85), respectively.

Comparison of the classes of acaricides used currently, intermediate and previously, showed a general increase in the current usage of amidine and co-formulated acaricides but a decline for SP (Fig. 3B). Amidines (48.2%, 41/85) and co-formulation (37.6%, 32/85) accounted for 85.9% (73/85) of the currently used classes of acaricide (Fig.2). In southwestern region, 47.6% (30/63) of the farms used co-formulation, 53.3% (16/30) of which was from Mbarara district. Of the 41.3% (26/63) of the farms in southwestern region that used amidine, Rukungiri had the highest (46.2%, 12/26) while Mbarara and Mitooma had each 26.9% (7/26) farms. In northwestern region, amidine and pyrethroid were currently used by 68.2% (15/22) and 22.7% (5/22) of the farms, respectively. Generally, mono-formulated pyrethroids (9.4%, 8/85) and organophosphate (3.5%, 3/85) acaricides were the least used in the 85 farms.

Acaricide failure was encountered in 33 out of 63 farms in western Uganda (Table 4). The major coping strategies against acaricide failure in southwestern included change from

one brand of acaricide to another (56%, 35/63), using higher (double to quadruple) acaricide concentration (19%, 12/63) and increasing frequency of acaricide application (3%, 2/63). The frequency of change of acaricides varied from less than 5 months (12.9%, 11/85) to over 2 years (20%, 17/85). Of major concern was 41.2% (35/85) of the respondents were not sure about how frequently acaricides were changed. Among the 49 farms that adopted strategies for overcoming acaricide failure, 51% (25/49) reported that the strategies were not effective. Mixing of two or more acaricides together was also reported as a strategy for overcoming acaricide failure by 3 respondents, however 29.4% (25/85) of the respondents think such approach is not effective against acaricide failure. Overall, only 48.2% (41/85) of the farms rotated acaricides from one class to another correctly, the rest (51.8%) either rotated wrongly (21.2%, 18/85) or could not remember the previous acaricide used (30.6%, 26/85) (Table 4). No farm had written records on acaricides used for tick control in the last 2 years.

3.5. Interaction of animals in neighborhood and quarantine of newly introduced animals

Majority (68.2%) of the 85 farms were in close proximity with neighboring farms but separated by a fence (Table 5). Interaction of animals at neighboring farms was reported for (57.6% (49/85) of the farms. Moreover, 44.7% (38/85) of the farms reported that their animals interacted with those of neighbors' daily or weekly. Interestingly, 51.8% (44/85) of the respondents were not aware about acaricide used in their neighboring farms and 45.9% (39/85) of the respondents were also not aware when their neighbors applied acaricides. None of the neighboring farms attempted to synchronize either the days or the class of acaricide used for tick control. It was also found that 36.5% (31/85) of the farmers had introduced new animals on their farm from sources such as cattle market, neighboring farms and districts. Moreover, 17.6% (15/85) of the farms that brought in new animals neither quarantined nor

sprayed the cattle with acaricides prior to introduction in their farms.

3.6. Acaricide safety concerns in animals and workers

Only 23.5% (20/85) of the farms kept acaricides in a designated storage facility (store), while 55.3% (47/85) kept acaricides in their residential houses (Table 6). Of concern was that 31.8% (27/85) of the farms reported occupational toxicity of farm workers during acaricide application. The most frequent signs of toxicity associated with exposure of workers to acaricides included dizziness, itching of the skin and the eye and coughing. Relatedly, 12.9% (11/85) of the respondents reported toxicity to cattle associated with acaricide application as evidenced by signs such as damaged skin, blindness and death of cattle.

3.7. Tick species identified from the farms in southwestern and northwestern regions

A total of 2,520 ticks were collected and identified as *R. appendiculatus* (54.3%), *R.* (*B.*) *decoloratus* (22.2%), *A. variegatem* (18.2%), *R. evertsi* (2.7%) and *Hyalomma* spp. (2.7%) (Table 7). Of the 1,023 ticks collected from Adjumani, *A. variegatum* (44.8%) were the prevalent followed by *R. appendiculatus* (28.4%), *R.* (*B.*) *decoloratus* (13.7%) and *Hyalomma* spp. (6.5%). In contrast, *R. appendiculatus* and *R.* (*B.*) *decoloratus* were the only tick species found on farms from southwestern Uganda. *R. appendiculatus* population was consistently higher than *R.* (*B.*) *decoloratus* in all the three districts from southwestern Uganda.

4. Discussion

This study presents six fundamental findings that characterize the current tick control practices in southwestern and northwestern Uganda. I) In southwestern Uganda, farmers rely exclusively on chemicals (acaricides) for tick control as opposed to integrated tick control approaches. II) The exotic cattle (crossbreeds) keepers in southwestern Uganda have adopted high acaricide application pressure (high acaricide strength over recommended concentration) as a possible compensatory strategy against acaricide ineffectiveness. Thus, absence of ticks on cattle in southwestern may not necessarily reflect acaricide effectiveness but also excessive use of acaricide to eliminate "stubborn" ticks. III) The agro-pastoral community in northwestern Uganda lacked knowledge on appropriate tick control but their local cattle are tolerant and may survive high tick burdens with limited need for acaricides. IV) Less than optimal acaricide application practices were wide spread and does not only present a real threat to emergence and spread of acaricide resistance but also pose a serious public health threat. V) Even where animals interacted on neighboring farms, there was lack of evidence on synchronization of type of acaricides used and acaricide application practices due to lack of strategic tick control policy to enforce rational acaricide rotation. VI) Chemical tick control records were not kept, making it difficult for farmers to implement appropriate acaricide rotation. The implications of these fundamental findings are further discussed below.

The study found that spraying was the most common method (Table 2) of acaricide application since it is widely perceived as convenient and cheaper for small holder farms (112, 113). However, almost all the farms in southwestern that complained of acaricide failure were using the spray method, suggesting that this method may predispose to tick acaricide resistance. A previous study in South Africa also reported that the spray method is one of the

factors that promotes resistance against acaricides (151). Associated with the spray method are other factors such as inappropriate restraint facilities (poor crush and use of *boma*) (Fig. 1), hardship of spraying large herd size, lack of adequate supervision, frequent acaricide dilution and rotation malpractices which makes the spray method inefficient and vulnerable to acaricide failure (Tables 2-4). The above irrational acaricide application practices during spraying were also reported previously as a potential precursor for acaricide resistance in Uganda (112, 113). While dipping is considered the most effective technique for acaricide application, the initial cost of investment is prohibitive for medium size farms (35). The spray-race method of acaricide application is recommended as an alternative to dip plunge on medium-scale farms for appropriate acaricide application and tick control outcome (76).

The study also found a general decline in the use of pyrethroids but an increase in the use of amidine and co-formulations (organophosphate and pyrethroid) in southwestern Uganda (Fig. 3B). The decline in use of pyrethroids from earlier report (112) may be attributed to wide spread resistance by *R.* (*B*.) *decoloratus* and *R. appendiculatus* ticks in the southwestern region. The use of amitraz as an alternative acaricide in rotational control of pyrethroid resistant ticks has been widely reported (7, 52, 56). Increase in the use of co-formulation further re-enforces the premise regarding possible emergence of resistance against mono-formulated pyrethroid acaricides. It is also speculated that increased marketing of co-formulated acaricides by the suppliers and Veterinary drug shops might have influenced the choice of the farmers, as reported in other studies (114, 151). The short acaricide application interval (twice a week) and use of higher concentrations of amidine and co-formulation (Tables 3 and 4) are also early warning signs that resistance may be developing against the acaricides in southwestern Uganda. Continuous improper acaricide rotation and application practices can be expected to trigger and sustain acaricide resistance.

In the northwestern region where farmers reared *Bos indicus*, amidine was widely used, possibly due to the low cost of amitraz as opposed to only its effectiveness. This is consistent with the findings that farmers in Adjumani purchased small volume of amitraz regardless of the herd size as previously reported (112, 113). Despite the poor acaricide application practices in northwestern region, factors such as limited chemical use and extensive grazing system helps to prevent development of acaricide resistance. Nevertheless, farmers in Adjumani district needs to be trained in appropriate use of acaricides to prevent emergence of resistance in the future.

It was also found that animals on neighboring farms mixed frequently (Table 5). This presents a threat especially in southwestern Uganda since acaricide resistant ticks can be easily exchanged across farms (73). In the absence of tick control policy in Uganda, there is no regulation in the type of acaricides to be used in a particular area. As such, farmers use different classes of acaricides, even within the same area. It is therefore postulated that lack of synchronized rotation and free movement of animals across farms may accelerate the emergence and spread of multi-acaricide resistant ticks. Such risks can be avoided by identifying the class of acaricide molecules that are effective against ticks in the different regions of the country and enforcing compulsory rotation. The state-led acaricide rotation is important in delaying acaricide resistance (158), ensuring adherence to a specific molecule (12, 112) and creating acaricide reservoir molecule for future use.

The current study also found poor acaricide application practices in almost equal proportion among farms with and those without acaricide failure. Notably, farmers in the southwestern region that had no tick challenge were likely using double or even quadruple acaricide concentration above the manufacturers' recommendations. Increasing the concentration of acaricides alone would only cause a temporary relief against acaricide failure,

but with the possibility of selecting for more stable resistance. With some farmers opting to source advice from fellow farmers and drug shop attendants (who may be unqualified), the threat of a complex acaricide crisis should be expected. Additional factors that may exacerbate acaricide crisis will be absence of centralized acaricide rotation, and inadequate technical farmer advisory services.

Poor acaricide handling (Table 6) appeared to be a neglected public health concern. The short acaricide application interval (twice a week) was practiced and increasing the concentration 2 or 3 folds above manufacturers' recommendation is not only unsafe to the animal but also the farm workers who handle acaricides. A farmer in Mitooma district quadrupled the concentration of chlorfenvinfos leading to the death of cattle. Moreover, with 31.8% level of human occupational toxicity (Table 6), there is needed to educate the livestock keeping communities on safe acaricide handling practices. Interventions on safe acaricide use should be a joint initiative between veterinarians and health officials in a one health approach. The effectiveness of one health intervention approach in chemical tick control was earlier reported in Zambia and Burkina Faso (35). Such joint intervention is expected to avert the adverse effect of acaricides (32) on animal welfare, food safety and public health.

This study also identified *R. appendiculatus* and *R.* (*B.*) *decoloratus* as the common species of ticks infesting cattle in southwestern Uganda (Table 7). However, Adjumani had more diverse tick species that also includes *A. variegatum* and *Hyalomma* spp. The difference in species dynamics between farms from southwest and Adjumani district could be attributed to high acaricide pressure in the southwest which wipes off susceptible ticks on cattle. With the high population of susceptible exotic breeds, farmers in southwestern Uganda have to cleanse their cattle with acaricides weekly thereby reducing the population of susceptible ticks. This is consistent with previous report (76) that related frequent application of acaricides to

selection pressure and resistance in *R.* (*B.*) *microplus* ticks.

The major limitation of the study accrues from the small sample size and the subjective criteria for selection of farms with or without acaricide failure. Such criteria may not be able to give prediction of true acaricide failure since other factors such as use of high acaricide strength and increased frequency of acaricide application may temporarily reduce resistant tick population, thus giving false impression on absence of acaricide failure. In the absence of written records on tick control, response bias due to selective memorization by the study participant could have introduced bias in their response to the research questions on sequence of acaricides used. Nevertheless, the malpractices in chemical tick control in the two regions reflect the level of concern that requires urgent intervention to prevent a complex acaricide resistance crisis from emerging in Uganda.

The short-to-medium term interventions proposed against tick control includes extensive education of farmers on appropriate acaricide use, increased farmer access to veterinary advisory services and building human and laboratory capacity for prompt acaricide resistance diagnosis. The threat posed by uncoordinated tick control in neighboring farms, district and region requires harmonization by Ministry of Agriculture, Animal Industry and Fisheries. At farm level, strengthening biosecurity (48) through proper fencing, proper inspection, quarantine and spraying of new animals brought to the farm is recommended. One of the widely agreed recommendation for fighting acaricide failure is adoption of integrated tick control strategies for farm management that minimizes tick challenge, promotion of vaccination against ECF (112) and maximizing the benefits of indigenous breeds (1, 32, 73, 109). However, a national tick control policy for Uganda is needed to enforce a programmed acaricide rotation and use of other emerging technologies as integral part of integrated tick and TBD diseases control.

5. Conclusion

This study identified over dependence on chemicals (acaricides) as the only method for tick control and widespread inappropriate acaricide application practices that may predispose to acaricide failure and emergence of resistance, especially in southwestern Uganda. The key recommendations include adoption of integrated tick control to reduce use of acaricides, educating farmers on acaricide stewardship and implementation of area-wide rotation policy by relevant government authorities to preserve the efficacy of acaricides. Overall, the findings from this study are expected to provide a basis for developing intervention strategies for enhancing prudent chemical tick control and safe acaricide handling practices that will ensure animal welfare, food safety and public health.

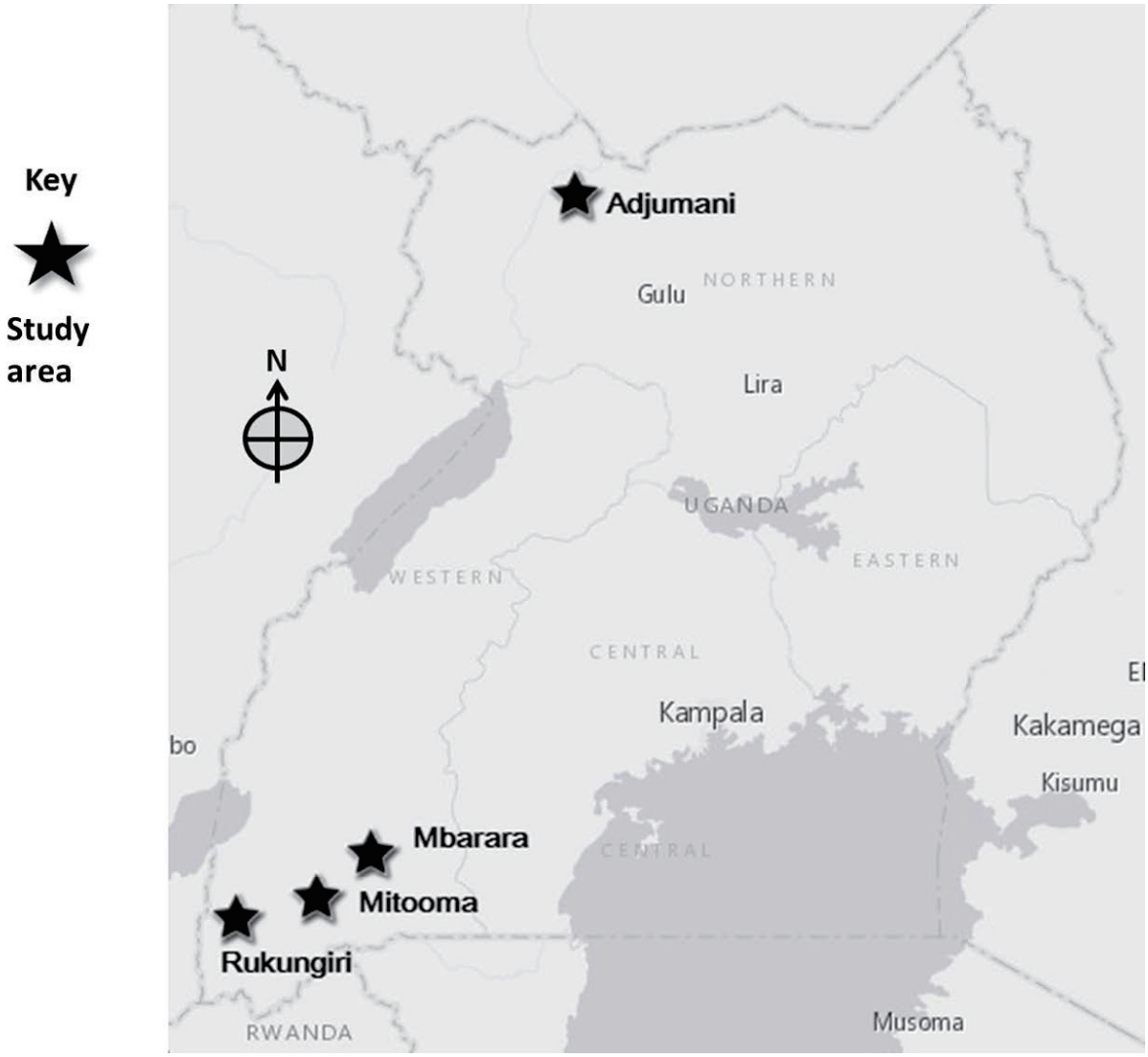

Fig. 1. Map of Uganda showing the study areas.

The black stars represent the districts in southwestern (Mbarara, Mitooma and Rukungiri) and northwestern (Adjumani) Uganda.

Fig. 2. Tick control facilities used for restraint of cattle during spraying in southwest and northwest Uganda.

(A) A kraal with a spraying area known as *boma* indicated in yellow arrow. The red arrow shows a crush that was abandoned due to poor design (too wide) and while cattle are in the crush it was difficult the access tick predilection sites during acaricide application. **(B)** A crush that is poorly cited (close to natural fence) and constructed with weak materials.**(C)** A crush in that is poorly constructed; too wide that animals form 3 rows in the crush, making it difficult to effectively spray.

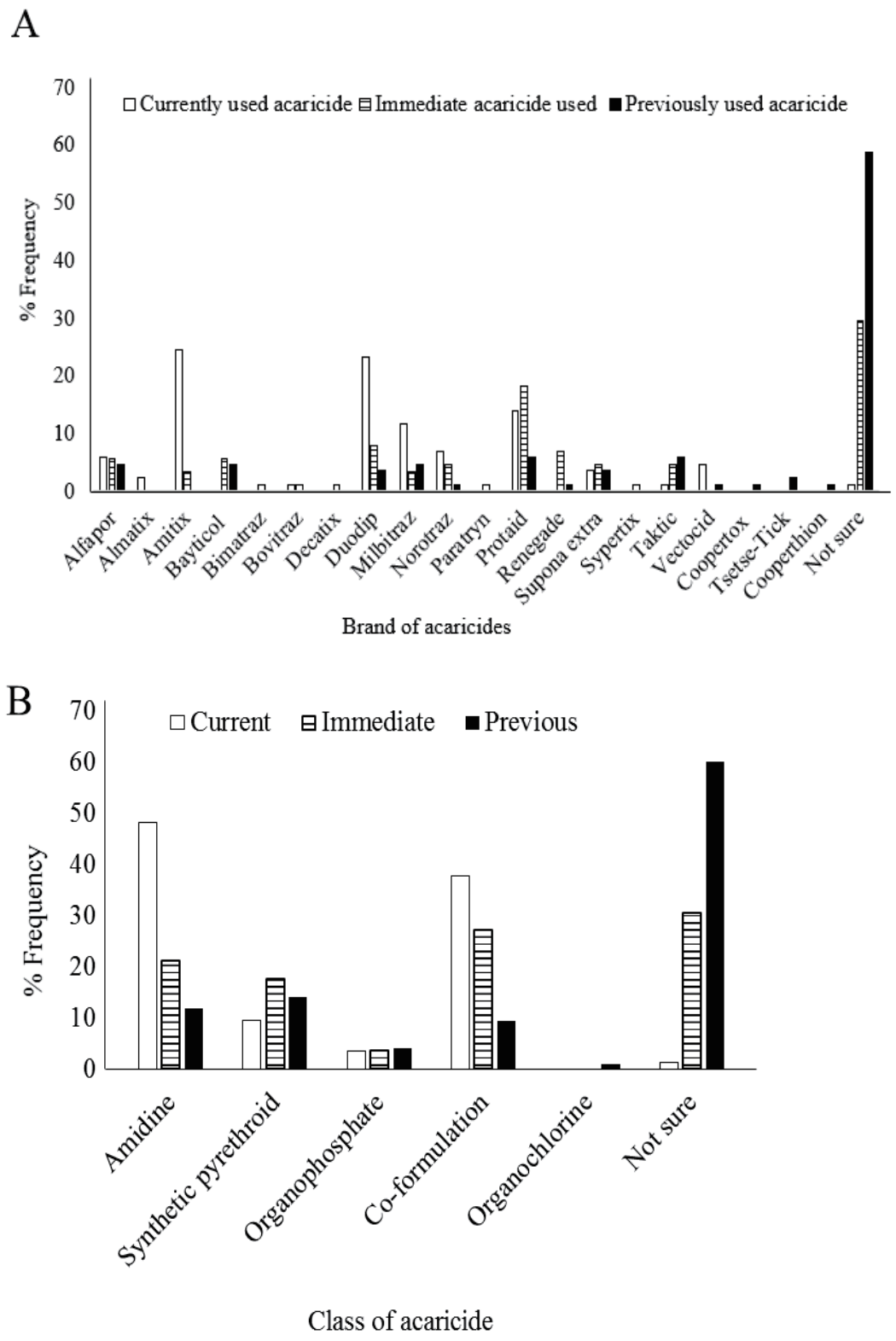

Fig. 3. Brands and classes of acaricides used for tick control in southwestern and northwestern Uganda.

(A) Brands of acaricides used currently, intermediate and previously for tick control. **(B)** Classes of acaricides used currently, intermediate and previously for tick control.

Table 1. General characteristics of the study farms in southwestern and northwestern Uganda.

Query	Response	Region		Total	%
		Southwestern	Northwestern		
Location	Adjumani	-	22	22	25.8
	Mbarara	25	-	25	29.4
	Mitooma	19	-	19	22.4
	Rukungiri	19	-	19	22.4
Occupation	Business	9	1	10	11.8
	Civil servant	7	17	24	28.2
	Engineer	2	1	3	3.5
	Farmer	38	2	40	47.1
	Livestock technician	1	0	1	1.2
	Politician	3	1	4	4.7
	Retired	3	0	3	3.5
Position at the farm?	Manager	11	7	18	21.2
	Owner	52	15	67	78.8
Breed of cattle reared?	Crosses	60	0	60	70.6
	Local	3	22	25	29.4
Purpose for keeping cattle?	Dairy	44	0	44	51.8
	Multipurpose	19	22	41	48.2
Herd size?	Small (<20)	25	4	29	34.1
	Medium	34	11	45	52.9
	Large (>100)	4	7	11	12.9
Presence of small Ruminants?	Yes	41	4	45	52.9
	No	22	18	40	47.1
Wildlife-Livestock Interaction?	Yes	18	15	33	38.8
	No	45	7	52	61.2
Livestock management system?	Communal	6	18	24	28.2
	Paddocks	54	0	54	63.5
	Ranching	1	1	2	2.4
	Tethering	0	3	3	3.5
	Zero grazing	2	0	2	2.4
Total		63	22	85	100.0

Table 2. Method of tick control on farms in southwestern and northwestern Uganda.

Query	Response	Region		Total	%
		Southwestern	Northwestern		
Method of tick control?	Acaricides	63	22	85	100.0
Method of restraint (during spraying)?	*Boma**	1	3	4	4.7
	Cattle crush	62	7	69	81.2
	Ropes	0	12	12	14.1
Method of acaricide application used?	Dipping	5	1	6	7.1
	Scrubbing	2	0	2	2.4
	Spraying	56	21	77	90.6
Equipment used for acaricide application?	Bucket pump	43	3	46	54.1
	Dip	5	1	6	7.1
	Hand sprayer	0	12	14	16.5
	Knapsack sprayer	11	6	17	20.0
	Scrubbing cloth	2	0	2	2.4
Equipment used for acaricide Measurement?	Calibrated bottle top	38	11	49	57.6
	Acaricide bottle for	5	1	6	7.1
	Non-calibrated bottle	4	0	4	4.7
	Syringe	16	10	26	30.6
Water used for mixing acaricides?	Tap water	8	4	12	14.1
	Clean natural	50	10	60	70.6
	Dirty natural	5	8	13	15.3

* A holding yard in a cattle kraal where cattle are restrained during spray.

Table 3. Methods of acaricide application, sources of acaricides and sources of advice in farms in southwestern and northwest Uganda.

Query	Response	Region		Total	%
		Southwestern	Northwestern		
Source of advice on tick control?	Drug shop attendant	5	0	5	5.9
	Drug shop attendant and fellow farmer	1	0	1	1.2
	Drug shop attendant and veterinarian	2	0	2	2.4
	Fellow farmer	1	3	4	4.7
	Fellow farmer and Veterinarian	1	0	1	1.2
	Veterinarian	43	18	61	71.8
	None	10	1	11	12.9
Source of acaricide?	Local drug shop	46	13	59	69.4
	Open market	5	3	8	9.4
	Pharmacy	7	5	12	14.1
	Veterinarian	5	1	6	7.1
Who mixes the acaricide?	Children	3	0	3	3.5
	Farm manager	15	2	17	20.0
	Herdsman	25	14	39	45.9
	Owner	19	6	25	29.4
	Veterinary pharmacy	1	0	1	1.2
Strength (concentration) of diluted acaricide	High	32	2	34	40.0
	Recommended	12	6	18	21.2
	Not sure	11	6	17	20.0
	Lower	7	3	10	11.8
	Estimates	1	5	6	7.1
Acaricide dilution verdict	Wrong	51	18	69	81.2
	Correct	12	4	16	18.8
Who applies acaricides?	Children	3	0	3	3.5
	Farm manager	8	1	9	10.6
	Herdsman	33	15	48	56.5
	Owner	19	6	25	29.4
Number of cattle sprayed with 20 liters of mixed acaricide wash?	2 (FAO recommended)	0	0	0	0
	At most 7	15	0	15	17.6
	8-20	40	6	48	56.5
	21-40	3	7	10	11.8
	>40	0	6	6	7.1
	Not applicable (dip)	5	1	6	7.1

Table 3. Methods of acaricide application, sources of acaricides and sources of advice in farms in southwestern and northwest Uganda (continued).

Query	Response	Region		Total	%
		Southwestern	Northwestern		
	Children	2	0	2	2.4
	Farm manager	18	6	24	28.2
	Owner	33	6	39	45.9
	Herdsmen	10	10	20	23.5
Acaricide application interval in rain season?	Thrice a week	1	0	1	1.2
	Twice a week	10	4	14	16.4
	Weekly	51	14	65	76.5
	Fortnight	0	1	1	1.2
	Monthly	1	3	4	4.7
Acaricide application interval in dry season?	Twice a week	7	4	11	12.9
	Weekly	53	6	59	69.4
	Fortnight	1	1	2	2.4
	Monthly	2	11	13	15.3
Total		63	22	85	100.0

Table 4. Strategies for coping (overcoming) tick acaricide failure by farmers.

Query	Response	Region		Total	%
		Southwestern	Northwestern		
History of acaricide failure	Yes	33	0	33	38.8
	No	30	22	52	61.2
Strategies used for overcoming acaricide failure?	Change acaricide	35	0	35	41.2
	Double concentration and change of acaricide	6	0	6	7.1
	Double concertation	3	0	3	3.5
	Increase the frequency of spraying	2	0	2	2.4
	Triple acaricide concentration	3	0	3	3.5
	Not applicable	14	22	36	42.4
Are the coping strategies effective?	Yes	19	0	19	22.4
	Somehow	5	0	5	5.9
	No	25	0	25	29.4
	Not applicable	14	22	36	42.4
Time spent with previous acaricide before changing to current	Less than 5 months	11	0	11	12.9
	6 – 9 months	6	1	7	8.2
	1 – 2 years	15	0	15	17.6
	Above 2 years	14	3	17	20.0
	Not sure	17	18	35	41.2
Knowledge about people mixing two or more	Yes	3	0	3	3.5
	No	60	22	82	96.5
Source of advice on mixing two or more acaricides together	Fellow farmer	2	0	2	2.4
	Trial and error	1	0	1	1.2
	Not applicable	60	22	82	96.5
Does mixing two or more acaricide together solve acaricide failure?	Yes	3	0	3	3.5
	No	25	0	25	29.4
	Not sure	35	22	57	67.1
Correctness of acaricide rotation	Correct	34	7	41	48.2
	Not sure	12	14	26	30.6
	Wrong	17	1	18	21.2
Total		63	22	85	100.0

Table 5. Proximity to neighbors, knowledge on tick control in neighborhood and quarantine of newly control of ticks on newly introduced animals in southwest and northwestern Uganda.

Query	Response	Region		Total	%
		South-western	North-western		
Distance with neighboring farm (kilometers)?	0 (separated by fence)	57	1	58	68.2
	> 1	2	16	18	21.2
	1-3	3	3	6	7.1
	>4	1	2	3	3.5
Presence of fence?	No	5	19	24	28.2
	Yes	58	3	61	71.8
Interaction of your animals with that of neighbors?	Daily	14	15	29	34.1
	Weekly	5	4	9	10.6
	Monthly	8	3	11	12.9
	Never	35	0	35	41.2
	Not sure	1	0	1	1.2
Knowledge of acaricide use by neighbors?	Yes	24	17	41	48.2
	No	39	5	44	51.8
Knowledge on the days of the week when neighbor sprays?	Yes	29	17	46	54.1
	No	34	5	39	45.9
New animals (cattle) introduced in the farm?	Yes	29	2	31	36.5
	No	34	20	54	63.5
Origin of new animals (cattle) introduced in the farm?	Cattle market	10	0	10	11.8
	Neighboring district	15	1	16	18.8
	Neighboring farm	4	1	5	5.9
	None	34	20	54	63.5
Did you quarantine animals (new) and spray before mixing with other animals?	Yes	16	0	16	18.8
	No	13	2	15	17.6
	Not applicable	34	20	54	63.5
Total		63	22	85	100.0

Table 6. Acaricide safety concerns in animals and humans in southwestern and northwestern Uganda.

Query	Response	Region		Total	%
		Southwestern	Northwestern		
Location where acaricide is stored?	At dip tank	5	1	6	7.1
	Drug box	2	5	7	8.2
	In the bush at the kraal	0	1	1	1.2
	Designated store	20	0	20	23.5
	Residential house	35	12	47	55.3
	No response	1	3	4	4.7
Have you seen or heard of animals with damaged skin damage & due to acaricide (poisoning)?	Yes	9	2	11	12.9
	No	48	8	56	65.9
	Not sure	6	12	18	21.2
Has any person applying acaricide on this farm suffered from adverse (bad) effects of acaricides?	No	36	22	58	68.2
	Yes	27	0	27	31.8
If yes, which effects?	Blindness	1	0	1	1.2
	Coughing	2	0	2	2.4
	Dizziness	8	0	8	9.4
	Eye and skin Itching	1	0	1	1.2
	Eye itching	8	0	8	9.4
	Skin itching	6	0	6	7.1
	Skin itching and Coughing	1	0	1	1.2
	None	36	22	58	68.2
Total		63	22	85	100.0

Table 7. Distribution of the tick species collected from farms in southwestern and northwestern Uganda.

Region	District	Tick species identified											
		R. appendiculatus		*R.* (*Boophilus*) *decoloratus*		*R. evertsi*		*A. variegatum*		*Hyalomma* spp.			
		Number	%	Number	%	Number	%	Number	%	Number	%	Total	%
Southwest	Mbarara	396	62.9	234	37.1	0	0.0	0	0.0	0	0.0	630	25.0
	Mitooma	198	71.5	79	28.5	0	0.0	0	0.0	0	0.0	277	11.0
	Rukungiri	484	82.0	106	18.0	0	0.0	0	0.0	0	0.0	590	23.4
Northwest	Adjumani	291	28.4	140	13.7	67	6.5	458	44.8	67	6.5	1023	40.6
	Total	1369	54.3	559	22.2	67	2.7	458	18.2	67	2.7	2520	100.0

%; percentage.

Chapter 2

Emergence of multi-acaricide resistant *Rhipicephalus* ticks and its implication on chemical tick control in Uganda

1. Introduction

Ticks are among the leading vectors that cause serious economic loss to Africa's livestock industry (88, 118). Ticks also vector TBD such as ECF, babesiosis and anaplasmosis that affect the productivity of especially exotic cattle and their crosses in Africa (72). In Uganda, over 30% of calf crop is lost to TBD and the cost of controlling ticks and TBD accounts for nearly 90% of total diseases control costs and over 60% of total farm inputs (123). One of the ways through which TBD can be prevented is through control of ticks using chemicals (acaricides). The high burden of ticks in African countries like Uganda necessitates monthly or weekly application of acaricides on cattle (56). This has created a huge demand and market for acaricides in Uganda. The liberalization of veterinary drug industry in the country has enabled supply of various classes of acaricides to meet the demand by farmers (126). Because of limited control of acaricide supply and usage, cases of irrational use of acaricides by farmers have been widely reported (112, Chapter 1). Wrong dilution, application methods and increased acaricide pressure are among the factors that accelerate development of acaricide resistance (1, 76). Acaricide resistance was first reported in Uganda in 1970 against organochlorine toxaphene by *R.* (*B.*) *decoloratus* and *R. evertsi* (89). Lack of tick acaricide resistance monitoring system since early 1990's to date implies that the performance

of various molecules on the Ugandan market are unknown. However, the increased cases of farmers' complaint on acaricide failure especially in western and central Uganda raises serious suspicion of possible emergence of acaricide resistant ticks in the country as reported in Chapter 1. In the rest of the world, tick resistance to various classes of acaricides has been extensively reported (1, 52, 56). The current study therefore, determined the acaricide resistance profile of ticks collected from farms complaining of acaricide failure using LPT.

2. Materials and Methods

2.1. Study area

The primary study area for this research was cattle farms in western and central Uganda that were experiencing acaricide failure between December 2013 and January 2015. Western and central Uganda has the highest population of exotic cattle (especially dairy breeds) and their crosses (11). Due to the susceptibility of the improved cattle breeds against ticks and TBD, farmers have to rely on extensive use of acaricides for tick control and prevention of TBD. A total of 14 districts from central and western Uganda were included in this study (Fig, 4). The districts were identified during an earlier investigation of complaints of acaricide failure by the National Drug Authority (NDA) of Uganda and the researcher. The farms from the 14 districts (central 16 and western 34 farms) were sampled based on history of acaricide failure reported to the respective district veterinary office and animal health workers. However, 4 additional samples were obtained from 1 district in the north (Gulu) and 2 districts in the eastern (Serere and Mbale) parts of Uganda. The sample from Gulu was collected from cattle in the abattoir to establish possible spread of resistant ticks through cattle trade. The tick samples from Mbale were collected electively for purposes of finding a susceptible reference tick. Overall, ticks were collected from 54 study sites designated as farms in this study (Fig. 4).

2.2. Tick collection

Ticks were collected from 6-20 randomly sampled cattle per farm. Dogs were also included for tick collection in farms that had dogs. In addition, ticks were collected on goats and sheep from one farm in Kampala, central Uganda. Both engorged and semi-engorged

ticks from each farm were carefully picked and put in perforated sample bottles and transported to the Central Diagnostic laboratory (CDL) at College of Veterinary Medicine, Animal Resources and Biosecurity (COVAB), Makerere University.

2.3. Taxonomic identification of tick samples

Ticks were identified to species level based on morphological features described by Walker et al (168). For each farm, identified ticks were categorized based on their species to determine the dominant species associated with acaricide failure at farm and district levels. The engorged female ticks were immediately transferred into individual tubes and incubated at 27 ± 1^{o}C and 80% relative humidity for oviposition. After hatching, the larvae were kept in the incubator until they were 18 days old and used for acaricide efficacy assays.

2.4. Acaricides used for tick resistance assay

Commercial acaricide formulations that represented all the classes of acaricide on Ugandan market were purchased from the local importers and used for LPT. They were coded as; A4 (12.5% amitraz, Kenya), SP3 (10% α-cypermethrin, Kenya), SP10 (5% deltamethrin, Tunisia), OP (100% chlorfenvinphos, Italy), COF1 (co-formulation, 30 % chlorfenvinphos and 3% α-cypermethrin, Italy). The commercial (brand) names of the acaricides used were coded for anonymity to avoid any misinterpretation as promotion or demotion of such products based on their efficacy result.

2.5. Tick bioassays for acaricide efficacy

A total of 31 tick populations from 30 farms were tested for acaricide susceptibility. For logistical reasons, the method proposed for insecticide resistance testing by World Health

Organization (WHO) was adopted (169). The manufacturers recommended concentration was considered as the diagnostic/discriminating dose (DD) for all the chemicals. However, one additional dose level, which was twice the above dose (2×DD), was also applied. The diluent used for all the acaricides was trichloroethylene and olive oil mixed in a ratio of 2:1 (26). For amitraz, the method by Miller *et al* (107) was used. Briefly, 0.25 mg/ml (DD) and 0.5 mg/ml (2×DD) amitraz were prepared using the diluent. For cypermethrin and deltamethrin, 0.05 mg/ml (DD) and 0.1 mg/ml (2×DD) were prepared. For OP-chlorfenvinphos, 0.5 mg/ml (DD) and 1 mg/ml (2×DD) were prepared for the bioassays. The concentration of the COF_1 prepared was 0.3:0.03 mg/ml (DD) and 0.6:0.06 mg/ml (2×DD).

The choice of substrate used for impregnation of the chemicals was based on Food and Agriculture Organization recommendation (FAO) (49). Filter paper (Whatman No.1, Whatman, Madstone, United Kingdom) was used as a substrate for cypermethrin, deltamethrin, chlorfenvinphos and co-formulated acaricide. Nylon fabric was used for amitraz. The substrates were labelled with pencil and impregnated with 0.7 ml of the corresponding acaricide solution prepared. After impregnation, trichloroethylene was evaporated in a fume hood for 2 hours. Each filter paper or nylon fabric was folded into a packet and loaded with, on average, 60 larvae from the same farm and same species. The packets were then secured with alligator clips and incubated at 29 ± 1^{o}C and 80% relative humidity for 24 hours. Each experiment was carried out in duplicates. In all the assays, contamination was avoided by starting every experiment with the negative control followed by the lower concentration and changing gloves between different acaricide molecules. In the absence of laboratory reference susceptible *Rhipicephalus* ticks in the country, *Haemaphysalis leachi* and *A. variegatum* larvae that were 100% susceptible to all the acaricides were taken as reference ticks for

acaricide resistance assay. The reliability of this approach was later verified using 6 populations of susceptible *R. appendiculatus* and *R.* (*B.*) *decoloratus* reference ticks collected from low acaricide pressure farms in Adjumani district of north western Uganda during the study in Chapter 1.

After 24 hours, the packets were removed from the incubator. Three independent enumerators (who were previously trained on identifying dead and live ticks using magnifying lens and stereo-microscope) counted the number of ticks that died and those that were alive for each set of experiments. Mortalities were expressed as percentage of the total number of larvae exposed to the acaricide. There were no mortalities recorded in the control groups that were exposed to only the diluent.

2.6. Data on acaricide application practices

A semi-structured interview with farmer and/or farm workers was carried out from 52 of the 54 farms since data could not be retrieved from the two farms. The data captured included breeds of cattle reared, sequence of acaricide brands used in the last two years, method of acaricide application, dilution of acaricide(s) used, application interval at the time of the study and mixing of two or more acaricide formulations at one time. The data on sequence and brands of acaricides were used to determine the correctness of rotation from one molecule to another. Rotation was considered wrong if a farmer changed acaricide brand within the same molecule following acaricide failure. However, a change from SP to COF and OP molecule following acaricide failure to SP was also considered a wrong rotation due to the possible cross resistance between SP and OP (1, 169). The farm data on acaricide usage was also used to establish the brand preference for the different acaricide molecules on the market. The registration status of various brands of acaricides stated by the farmers was either

established from NDA or verified using the NDA's Veterinary Register (http://www.nda.or.ug/vet_list.php).

2.7. Data analysis

The mortality data for the 31 tick populations tested were recorded in MS Excel, and mean mortality and standard error were determined. The WHO percentage mortality cut-off values for susceptibility and resistance against insecticides determined using DD was used to categorize the mortality data (169). Ticks that showed at least 80% mortality against a given chemical were considered susceptible while those that showed less than 80% mortality were considered resistant. The above data together with the qualitative data on acaricide use was analyzed using SPPS version 21 (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.). Pearson chi square analysis was done with MedCalc for Windows, version 12.5 (MedCalc Software, Ostend, Belgium) to determine the factors associated with multiple acaricide resistance at 95% confidence and p value <0.05 was considered statistically significant.

2.8. Ethical considerations

The study was approved by the institutional review board (No. VAB/REC/15/104) of the College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University. To ensure biosecurity of ticks, all experiments were carried out under strict in-house procedure for avoiding escape of larvae. All materials used were either autoclaved or soaked in hot water at 99^{o}C. Larvae that were kept for further molecular studies were preserved in 70% ethanol. The commercial (brand) names of all the acaricides were coded to ensure confidentiality.

3. Results

3.1. Farm characteristics and tick species identified

Of the 54 cattle farms, 83.3% (45/54) of the farms kept crosses of exotic cattle and 16.7% (9/54) of them had only local cattle. Up to 90.4% (47/52) of the farms used hand-sprayer for acaricide application while only 3.8% (2/52) used plunge dip and another 1.9% (1/52) of them used spray-race. Complaint of acaricide failure was reported in 94.4% (51/54) of the farms. A total of 1,357 ticks were identified from the 54 farms. *Rhipicephalus* ticks accounted for 95.6% (1,297/1,357) of the tick populations although *A. variegatum* and *H. leachi* constituted 3.5% (48/1,357) and 0.9 % (12/1,357), respectively (Table 8). Amongst the *Rhipicephalus,* 55.1% (715/1,297) was the one-host tick *R.* (*B.*) *decoloratus* and 44.9% (582/1,257) was the three-host tick *R. appendiculatus.* On the other hand, 70.8% (34/48) of the *A. variegatum* ticks were from eastern Uganda. Only one out of the 12 *H. leachi* was collected on cattle, the rest were from dogs. No *Rhipicephalus* tick was found on dogs. For the 51 farms that had complaint of acaricide failure, 98.0% (1,257/1,283) of the ticks belonged to the genus *Rhipicephalus.* *R.* (*B.*) *decoloratus* tick was 55.7% (714/1257) and 42.3% (543/1257) was *R. appendiculatus. A. variegatum* formed only 1.1% (14/1,257) of the ticks from the 51 farms.

3.2. Acaricide molecules and brand preferences by farmers

The veterinary drug register showed that a total of 25 commercial brands of acaricides had been marketed in Uganda. Synthetic pyrethroids (SP1-SP15) constituted 60.0% (15/25) of the total commercial brands marketed, followed by amitraz (28.0% A1-A7), co-formulation (8.0% COF1-COF2) and only one brand of mono-formulated OP (4.0%). Up to 68.0% (17/25)

of the commercial brands of acaricide registered were found to have been used in the study farms. Overall, amitraz accounted for 36.9% (48/130) of the total acaricide formulations used on the farm followed by COF (30.0%, 39/130), SP (27.7%, 36/130) and mono-formulated OP (5.4%, 7/130) (Table 9). Within the same molecule, clear brand preferences were recorded. For example, two brands of amitraz (A3 and A4), four brands of SP (SP1, SP2, SP3 and SP13) and 1 brand of COF (COF1) were preferred by 75.0% (36/48), 69.4% (25/36) and 71.8% (28/39) of the farmers, respectively. Majority of the farmers (81.3%, n = 48) used at least two classes of acaricides within the last 2 years. Acaricide registration pattern showed that the rapid influx of different acaricide brands began in 1997 and its climax attained in 2007. Between 1997 and 1998, all the three classes of acaricides (amidine, SP and OP) were on the Ugandan market, suggesting that they have been in use for over 16 years in Uganda.

3.3. Strength variation of SP acaricides sold on the Ugandan market

As shown in Table 9, all amitraz brands available on the market had concentration of 12.5% (wt/vol.). However, the brands of SP had concentration ranging from 2% to 15%. The 38.5% (5/13) of SP brands were 5% (wt/vol.) followed 10 % wt.vol (4/13), 2% wt/vol. (2/13), 7% wt/vol (1/13) and 15% wt/vol (1/15). Moreover, aside from one molecule, the rest were prescribed in a dilution ratio of acaricide to water of 1 ml: 1 liter, giving wide concentration range for chemical tick control in Uganda. Similarly, the two co-formulations on the market had wide concentration range despite the same dilution ratio of acaricide to water of 1:2.

3.4. Susceptibility of tick larvae against the various molecules used

The percentage mortalities of larvae against the different acaricides used in the bioassay at DD and 2×DD are shown in Table 10. 93.5% (29/31) of the tick populations tested

had resistance to at least one class of acaricide molecule. Acaricide resistance was detected in *R. appendiculatus* and *R.* (*B.*) *decoloratus ticks* only.

3.4.1. Resistance to synthetic pyrethroids

At DD, 90.0% (27/30) of the ticks tested were resistant to both cypermethrin and deltamethrin. Doubling the concentration (2×DD) of both chemicals did not cause any significant increase in mortality of the above ticks since 86.7% (26/30) remained resistant (Table 10, Fig. 5). Moreover at 2×DD, 60.0% (18/30) and 63.3% (19/30) were super resistant (0% mortality) against deltamethrin and cypermethrin, respectively. Of major concern was the fact that the *R. appendiculatus* collected from cattle in Gulu abattoir (northern region) was among the super resistant ticks. Information obtained from the abattoir indicated that cattle from which the *R. appendiculatus* ticks were collected had originated from central Uganda. On the other hand, both *A.variegatum* from Gulu and *H. leachi* from Kiruhura districts were 100% susceptible at DD for cypermethrin and deltamethrin.

3.4.2. Resistance to organophosphate

Mono-formulated OP (chlorfenvinphos) at DD was efficacious in 86.7% (26/30) of tick populations screened (Table 10). However, 13.3% (4/30) of the one host tick *R.* (*B.*) *decoloratus* were resistant to DD of chlorfenvinphos (Fig. 5). The four tick populations that were resistant were collected from Wakiso, Mbarara and Kiruhura districts.

3.4.3. Resistance to co-formulation

At DD of co-formulation, resistance was detected in 43.3% (13/30) of the tick populations tested (Table 10). Interestingly, even at 2×DD, the co-formulated acaricide

could not provide the level of effectiveness that was shown by mono-formulated chlorfenvinphos at DD since 23% (7/30) tick populations tested remained resistant (Fig. 5). Of the 13 *Rhipicephalus* tick populations that were resistant to co-formulation, 76.9 % (10/13) were *R.* (*B.*) *decoloratus.*

3.4.4. Resistance to amitraz

At the DD, only 12.9% (4/31) of the tick populations tested had amitraz-resistant *Rhipicephalus* ticks with mortalities ranging from 15.4% to 68.1% (Table 10). However, increasing the dose of amitraz to 2×DD did not result into commensurate level of mortality. Three of the amitraz-resistant tick populations were *R.* (*B.*) *decoloratus* from the greater Bushenyi area (Bushenyi and Mitooma district) (Fig. 4). One amitraz-resistant *R. appendiculatus* tick population was from a farm in Rukungiri district. In the current study, amitraz resistance was only recorded in western part of Uganda.

3.4.5. Multi-acaricide resistance by *Rhipicephalus* ticks

The presence of single or multiple acaricide resistance in the study area is shown in Fig. 6A-C. Resistance to single and multi-acaricide molecules was detected in 48.2% (13/29) and 55.2% (16/29) of tick populations from farms with acaricide resistance, respectively (Fig. 6A). Of the multi-acaricide-resistant *Rhipicephalus* ticks, 75% (12/16) were *R.* (*B.*) *decoloratus* and the rest were *R. appendiculatus* ($p < 0.05$) (Fig. 6A). All the farms that used either SP and co-formulation or SP, OP and COF within the last 2 years had 100% (14/14) multi-acaricide resistant ticks. There was significant association between use of both SP and COF with resistance to two classes ($p < 0.001$) (Fig. 6B). Kiruhura district had 100% (4/4) multi-acaricide resistant tick populations, followed by Mbarara (75%; 3/4) in the western

Uganda. Ticks from the two farms in Wakiso district (central Uganda) were also multi-acaricide resistant.

3.4.6. Farm practices aimed at mitigating acaricide failure

To overcome acaricide failure, various coping strategies have been adopted by farmers although they were considered to potentially worsen the existing tick challenge. Buying different brand(s) of acaricide with little or no regard to similarity in active molecules with previous brand(s) used on the same farm was encountered. In two years, 64.5% (20/27) of the farms whose tick acaricide resistance status was determined used two to three acaricide molecules, and 55.6% (15/27) of them rotated the molecules wrongly. Rotation within the same molecule through purchase of different brands was recorded in 40.7% (11/27) of the farms. In addition, 25.9% (7/27) of the farmers increased the concentration of acaricide at least twice over the recommended dosage. Also, 14.8% (4/27) of the farmers shortened acaricide application interval to twice a week (every three days). Mixing of two different acaricide formulations was encountered in 7% (2/27) of the farms and one of the farms mixed co-formulation and amitraz, thus exposing ticks to all the three molecules at the same time. In a farm that mixed two acaricides and sprayed twice every week, damage to the skin of cattle due to frequent spraying with higher acaricide strength was encountered. As a result, the ticks were easily picked with the damaged skin (Fig.7).

4. Discussion

This is the first report that has comprehensively investigated tick acaricide resistance since the introduction of SP, co-formulations and amitraz in Uganda. *Rhipicephalus* ticks are widespread in the country (130), posing serious threat to especially improved cattle. Thus, TBD especially ECF is ranked by farmers as the most important constraint to cattle production in Uganda (79, 116). Acaricides are, therefore, perceived as the most efficient way of controlling ticks and preventing the above diseases. However, with over 25 brands of all the major classes of acaricides circulating on the market (Table 9), farmers are "spoiled for choice". SP and amitraz accounted for 88% of the total acaricide brands marketed although amitraz was the most preferred by farmers during the study. This finding is consistent with what was previously reported in north eastern Uganda (12). Of concern was the variation in strength of the different SP whose dilutions are similar, thus giving different concentrations. It may be possible that amongst cypermethrin, variation in strength may reflect the proprietary difference in composition of the active components (*cis* and *trans* isomers). However, there is need for regulatory harmonization of strength of SP formulations with similar active ingredients, notwithstanding inappropriate application practices by farmers. A noticeable example of inappropriate acaricide use was wrong rotation of acaricides between molecules and rotation of acaricides within the same molecule under different brand names. It was also widely believed by farmers that acaricide failure could only be caused by "fake" chemicals. This clearly indicates that farmers lacked knowledge on possibility of ticks becoming resistant to chemicals due irrational acaricide use.

In this study, 93.5% (29/31) of the larval population tested had resistance to at least one class of acaricide molecule; all of them belonging to the genus *Rhipicephalus* (*R.*

appendiculatus and *R*. (*B*.) *decoloratus*). In Uganda, acaricide resistance was first diagnosed in *Rhipicephalus* ticks against organochlorine, toxaphen in 1970s (87). This occurred mainly due to increased acaricide pressure considering a compulsory tick control committee enforced weekly dipping of cattle across the country. However, subsequent zoning of acaricides and restricting circulation to the district veterinary office were reported as efficient strategies in delaying acaricide resistance. Unfortunately, the political strife in early 1970s (20, 127) and further liberalization of the veterinary drug sector (30) ended both zoning and control in supply of acaricides, leading to widespread inappropriate acaricide use. Of major concern now is the high level of resistance to SP (90%) and emergence of super resistant *R. appendiculatus* and *R.* (*B*.) *decoloratus* ticks in at least 60% of the tick populations investigated in this study (Table 10). Since their introduction, SP formulations have been preferred to other molecules such as amitraz and OP due to their dual effect against both ticks and flies (12). However, its irrational use for over 16 years especially by farmers who use spray method, could have selected for stable resistance. Studies carried out in related tick, *R.* (*B*.) *microplus* have attributed such level of resistance to multiple mutations in SP target site, VSSC domains II and III (58, 74, 110, 152). Similar level of resistance was first observed in insects and attributed to knock-down resistance (*kdr*) in the sodium channel (39, 40, 137, 148, 156). It should be noted that the prevalence of SP resistance by *Rhipicephalus* ticks reported in this study (Table 10) is amongst the highest compared to those previously known in South America (37, 106, 166), India (145) and the rest of Africa (3, 98, 105, 121). Possible evidence of cross resistance between SP and OP was also observed in 30% of the tick populations from farms that used co-formulated acaricides (Table 10). Previous studies showed that ticks that were resistant to SP and OP had elevated esterase activity (64, 142). The apparent lack of synergism between SP and OP observed in this study possibly emanates from the fact that the most dominant

co-formulation used in Uganda (COF1) is prescribed at 1.7 times lower concentration than their corresponding mono-formulations. While the pharmacological basis for such formula is justifiable under ideal conditions, its efficacy is bound to be low in a situation where resistance has emerged against one of the chemicals. This eventually could act as a recipe for emergence of resistance against what otherwise would be the effective molecule (OP) in the co-formulation due to sub-optimal exposure dose. This possibly explains the low efficacy recorded against OP in farms with SP-resistant ticks that were also previously exposed to co-formulated acaricides. The mono-formulated OP chlorfenvinphos showed promising efficacy, partly because it is not widely used. The low farm use may be attributed to factors such as shorter application interval recommended for its use and low margin of safety compared to other classes of acaricides. However, emergence of resistance against co-formulation containing OP is an early indication that resistance to this group of acaricide is progressively building amidst fear of possible cross-resistance with SP.

Amitraz resistance was the least detected (12.9%) in the current study (Table 10). This finding is consistent with previous studies (41, 75). Although amitraz formulations have been the dominantly mentioned acaricides (36.9%, Table 9), their routine use has remained low due to their narrow spectrum of benefit compared to SP, as far as fly repellence is concerned. This explains why some farmers irrationally mixed amitraz and SP formulations. On the other hand, the increase in amitraz use may be an indicator that farmers were getting better tick control result with amitraz following negative experience while using SP and COF. However, the resistance observed against amitraz in 12.9% of the tick populations may be mediated by mutation in amitraz target, octopamine receptor (15, 26, 30). Nevertheless, the high level of multi-acaricide resistance (55.2%, Table 10) and emergence of isolated amitraz resistance ticks further emphasize the need for accelerated intervention to combat their spread across the

country. The super SP-resistant *R. appendiculatus* collected in Gulu abattoir from cattle bought from central Uganda should be an example of how such ticks can be easily spread through cattle trade and/or movement. Therefore, creation of farm awareness, vigilance amongst veterinarians and cattle traders, and promoting use of amidines in farming communities with ticks that are resistant to SP and coformulation could potentially lead to containment of resistant tick populations. However, the use of amitraz should factor into account the balance between need for tick and tsetse fly control, especially in areas that are known to be tsetse fly infested as previously reported (12). In absence of technical intervention, the strategies employed by farmers for overcoming acaricide failure are likely to worsen the existing challenge. This includes exponential rise in irrational admixing of various acaricide formulations into cocktail and short application interval that will cause collateral damage to cattle (Fig. 7), food safety and public health. Although alternative technologies such as vaccination of cattle with Muguga cocktail ECF vaccine is being promoted and said to be effective against ECF (120), the emergence of acaricide resistant *R.* (*B.*) *decoloratus* undermines such efforts. Without controlling the above ticks, babesiosis and anaplasmosis will certainly cause economic losses despite immunization against ECF. Therefore, there is need for various actors in the animal industry to jointly identify strategies for mitigation of acaricide resistance in Uganda. However, this requires close collaboration between the various stakeholders in the acaricide supply chain and research animal health institutions in the country (55).

5. Conclusion

This research is the first in Uganda to report emergence of super SP-resistant and multi-acaricide resistant *R. appendiculatus* and *R.* (*B.*) *decoloratus* ticks. These results further shows that farmer-led strategies for overcoming acaricide failure may potentially worsen acaricide resistance and limit future chemical tick control options. While understanding the molecular basis of such resistance and countrywide epidemiological studies are necessary, a multi-faceted approach directed towards containment and eradication of acaricide resistant ticks is urgently needed in Uganda.

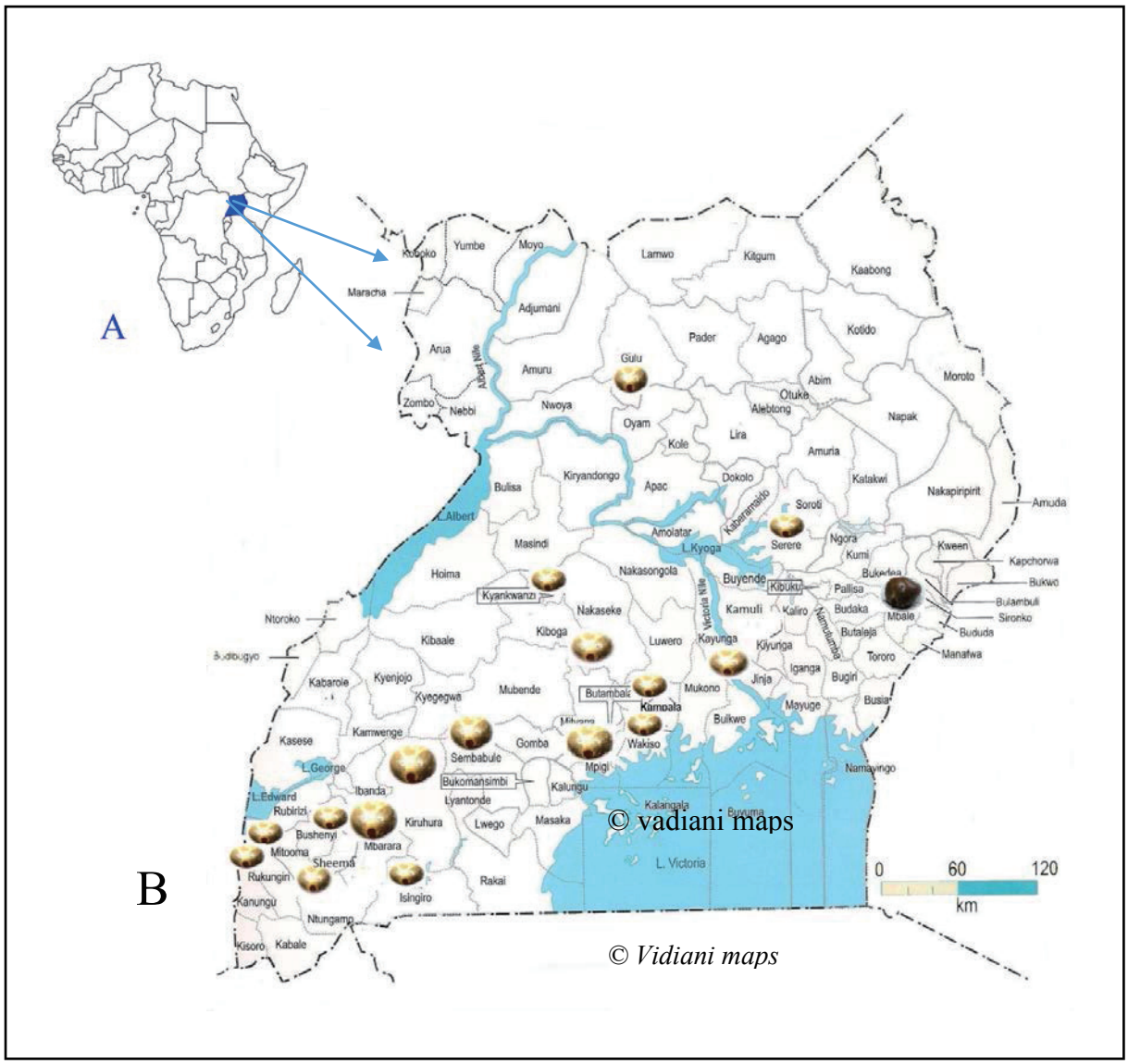

Fig. 4. Map of Uganda showing the various districts from which tick samples were collected.

(A) Map of Africa showing Uganda. **(B)** Map of Uganda showing the areas from which ticks were collected (depicted by ticks).

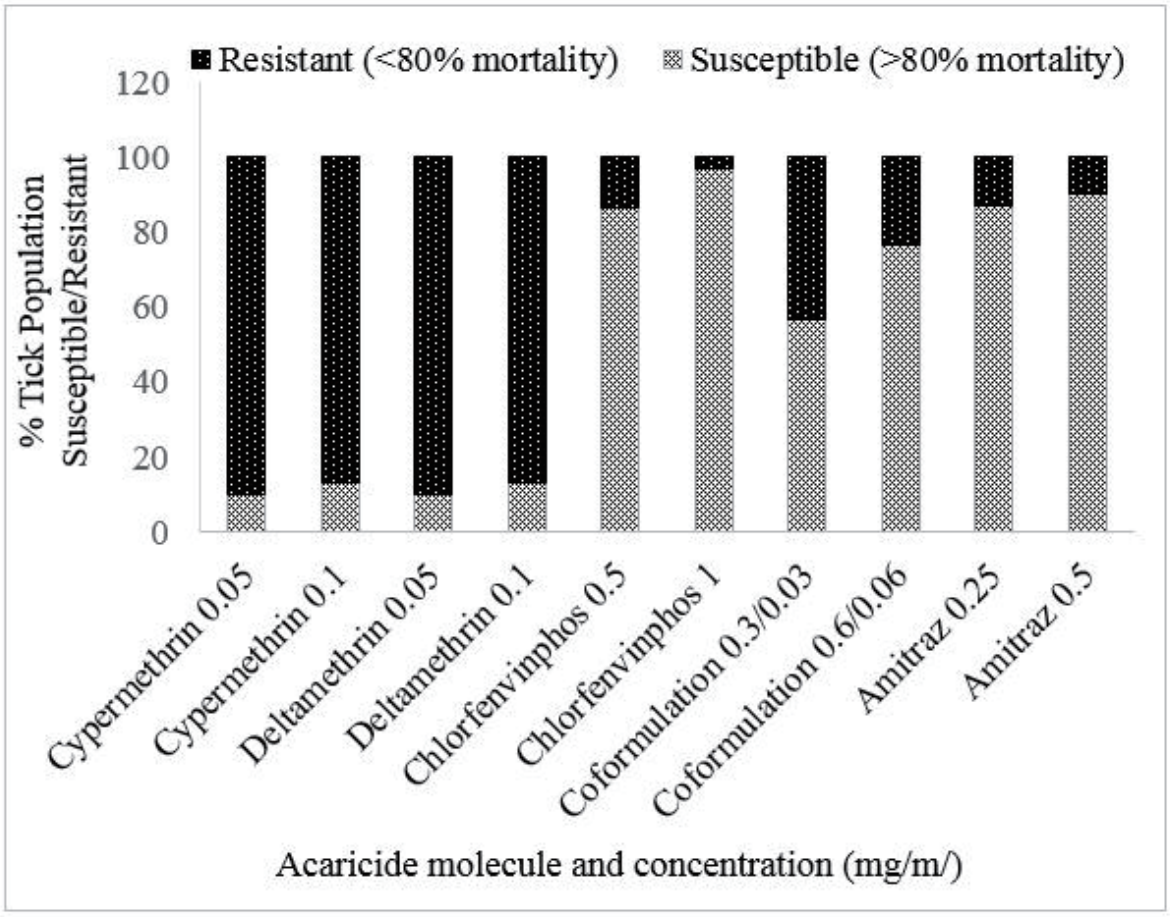

Fig. 5. Tick resistance status against various classes of acaricides.

Thirty-one tick populations from 30 farms were tested for determining amitraz resistance. Tick resistance to SP, OP and COF were determined using 30 tick populations from 30 farms.

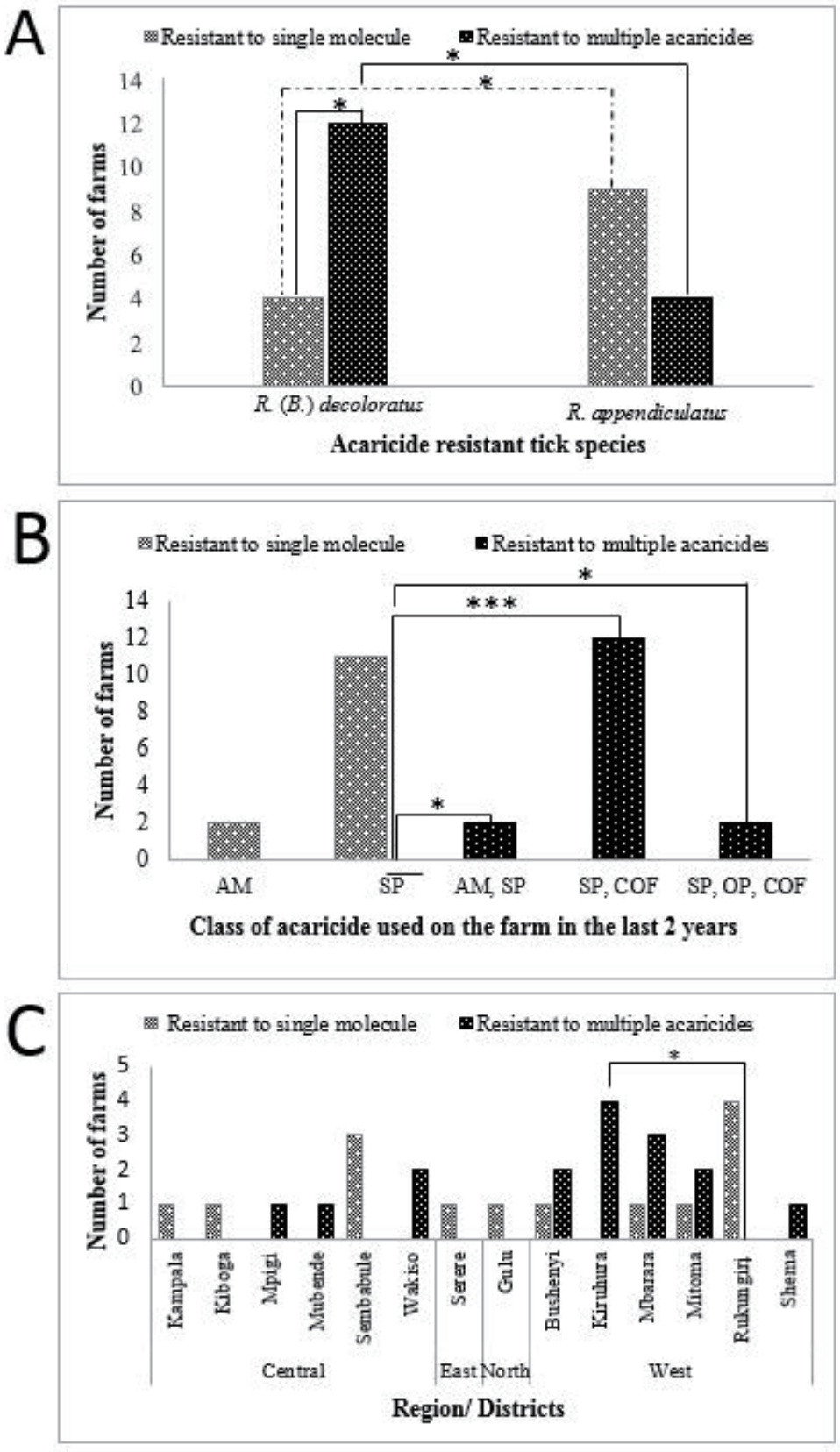

Fig. 6. Factors associated with occurrence of multi-acaricide resistance.

Fig. 6. Factors associated with occurrence of multi-acaricide resistance (continued).

(A) Tick species associated with multiple acaricide resistance. Comparison of proportion of ticks with single and multiple resistance within each species showed that *R.* (*B.*) *decoloratus* were significantly associated with multiple acaricide resistance (p = 0.0133; 95% CI = 11.3% to 75.1%, χ^2 = 6.125). Comparison of multiple acaricide resistance between the two tick species showed that *R.* (*B.*) *decoloratus* was significantly associated with multiple resistance (p = 0.0461, 95%CI = 2.9% to 72.1%, χ^2 = 4.020) compared to *R. appendiculatus.* However, *R. appendiculatus* was significantly associated with single resistance when compared to population of *R.* (*B.*) *decoloratus* resistant to single acaricide molecule (p = 0.0461, 95% CI = 2.9% to 72.1%, χ^2 = 3.978). **(B)** Acaricide molecule resisted by ticks in the farms. Comparison of proportion of farms that used only one molecule (SP) to those that used two to three molecules showed that multiple resistance was associated with use of at least two classes of acaricides; SP, COF (p < 0.0001, 95% CI = 61.1% to 100%, χ^2 = 19.167); AM (amitraz), SP (p = 0.0111, 95% CI = 11.1% to 100%, χ^2 = 6.453); SP,OP,COF (p = 0.0111, 95% CI = 11.1% to 100%, χ^2 = 6.453). **(C)** Source (district) of origin of the ticks. Ticks from Kiruhura district were significantly multi-acaricide resistant when compared to those from Rukungiri district (p = 0.0339, 95% CI = 14.8%-100%). However, there was no statistical difference in the occurrence of multiple acaricide resistance between the central and western region of Uganda. * = p < 0.05; *** = p < 0.001.

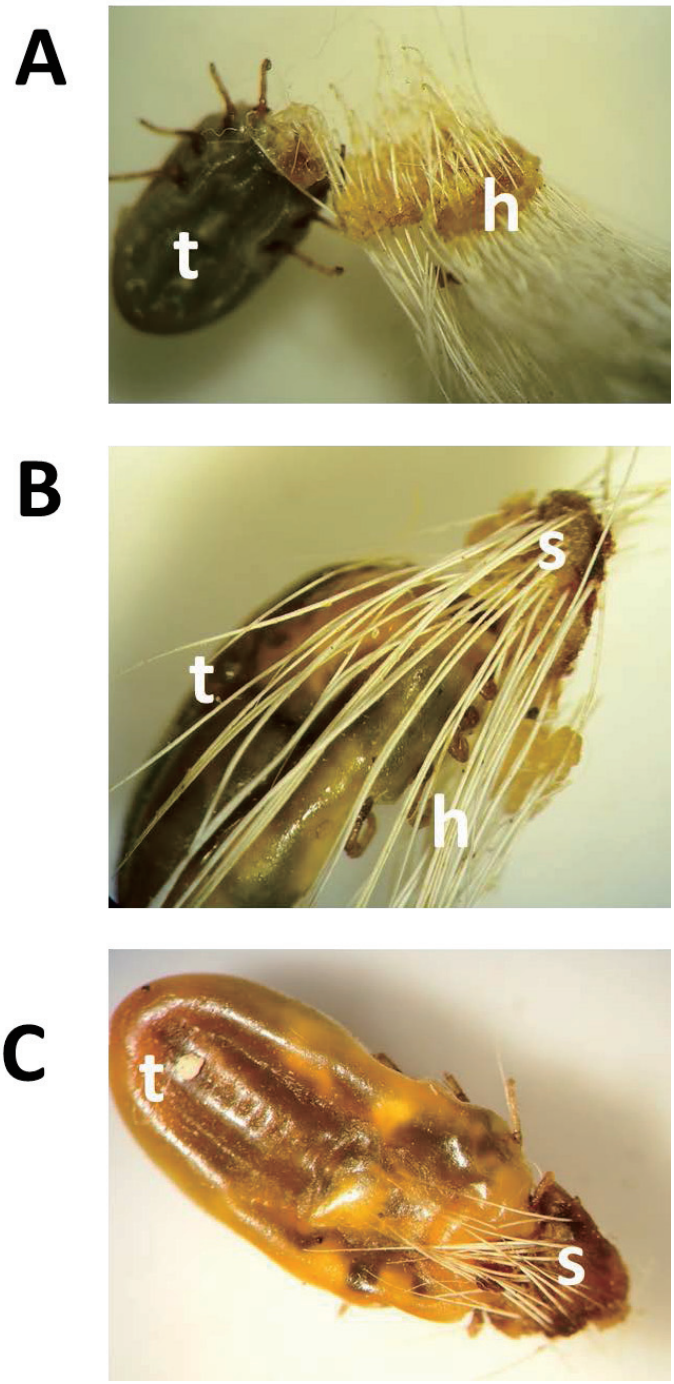

Fig. 7. *R.* (*B.*) *decoloratus* picked from cattle with acaricide induced skin damage.

(A) Hair bundle (h) that detached from the skin of cattle as the tick (t) was picked. **(B)** Damaged cattle skin (**s**) that was easily detached with the tick (t). **(C)** The piece of damaged skin (**s**) firmly attached to the mouth part thus altering the gross morphological appearance of the cephalus region of tick (t). Farmer considered these "new" species of ticks.

Table 8. Species of ticks identified from 17 districts in Uganda.

Region	District	No. farms	Number and frequency (%) per district								Total
			R. appendiculatus		*R. (B.) decoloratus*		*A. variegatum*		*H. leachi*		
			No.	%	No.	%	No.	%	No.	%	
Central	Kampala	1	17	100.0	0	0.0	0	0.0	0	0.0	17
	Kiboga	1	6	60.0	4	40.0	0	0.0	0	0.0	10
	Kyankwanzi	1	1	9.1	4	36.4	2	18.2	4	36.4	11
	Mpigi	1	30	100.0	0	0.0	0	0.0	0	0.0	30
	Mubende	1	0	0.0	35	100.0	0	0.0	0	0.0	35
	Nakasongola	1	15	48.4	9	29.0	7	22.6	0	0.0	31
	Sembabule	7	79	73.1	29	26.9	0	0.0	0	0.0	108
	Wakiso	3	31	52.5	28	47.5	0	0.0	0	0.0	59
East	Mbale	2	1	2.9	1	2.9	33	94.3	0	0.0	35
	Serere	1	0	0.0	13	92.9	1	7.1	0	0.0	14
North	Gulu	1	38	97.4	0	0.0	1	2.6	0	0.0	39
West	Bushenyi	4	3	0.9	347	99.1	0	0.0	0	0.0	350
	Kiruhura	12	28	17.8	121	77.1	0	0.0	8	5.1	157
	Mbarara	6	96	61.5	56	35.9	4	2.6	0	0.0	156
	Mitooma	3	20	29.4	48	70.6	0	0.0	0	0.0	68
	Rukungiri	8	217	95.6	10	4.4	0	0.0	0	0.0	227
	Sheema	1	0	0.0	10	100.0	0	0.0	0	0.0	10
Total	17	54	582	42.9	715	52.7	48	3.5	12	0.9	1357

No., Number.

Table 9. Acaricide molecules registered in Uganda and report of their use by the farmers.

Classification	Generic name	Brand names, total number (%)	Dilution Acaricide (milliliter): Water (liter)	Concent-ration (%)	Frequency of usage by farmers in study area	% within class	Overall (%)	Year licensed by NDA
Amidine	Amitraz	A1	2:1	12.5	1	2.1	0.8	2000
		A2	2:1	12.5	8	16.7	6.2	2001
		A3	2:1	12.5	25	52.1	19.2	1998
		A4	2:1	12.5	11	22.9	8.5	1997
		A5	2:1	12.5	3	6.3	2.3	1997
		A6	2:1	12.5	0	0.0	0.0	1998
		A7	2:1	12.5	0	0.0	0.0	2007
Sub-total		7 (28.0)			48	100.0	36.9	
Synthetic Pyrethroid	α-Cypermethri	SP1	1:1	5.0	8	22.2	6.2	2002
		SP2	1:1	5.0	5	13.9	3.8	1998
		SP3	1:2	10.0	7	19.4	5.4	2009
		SP4	1:1	7.0	3	8.3	2.3	2011
	Cypermethrin	SP5	1:1	10.0	1	2.8	0.8	1998
		SP6	1:1	10.0	0	0.0	0.0	1998
		SP7	1:1	15.0	0	0.0	0.0	2007
		SP8	1:1	10.0	0	0.0	0.0	2005
	Deltamethrin	SP9*	1:1	5.0	2	5.6	1.5	-
		SP10	1:1	5.0	4	11.1	3.1	2007
		SP11	1:1	5.0	0	0.0	0.0	-
	Flumethrin	SP12[q]	-	-	0	0.0	0.0	1997

Table 9. Acaricide molecules registered in Uganda and report of their use by the farmers (continued).

Classification	Generic name	Brand names, total number (%)	Dilution Acaricide (milliliter): Water (liter)	Concent-ration (%)	Frequency of usage by farmers in study area	% within class	Overall (%)	Year licensed by NDA
	Flumethrin	SP13	1:1	2.0	5	13.9	3.8	1997
		SP14	1:1	2.0	0	0.0	0.0	2010
	Cyhalothrin	SP15	1:1	5.0	1	2.8	0.8	2013
Sub-total		15 (60.0)			36	100.0	27.7	
Organophosphate	Chlorfenvinphos	OP (1(4))	1:2	100	7	100.0	5.4	1997
Co-formulation	Chlorfenvinphos + α-cypermethrin	COF1	1:2	30:3	28	71.8	21.5	2004
	Chlorpyriphos + Cypermethrin	COF2	1:2	50:5	11	28.2	8.5	2013
Sub-total		2 (8.0)			39	100.0	30.0	
Total		25 (100)			130		100	

(*) deregistered, (^{q}) pour-on, NDA National Drug Authority

Table 10.Mortality of larvae against various classes of acaricides determined with LPT.

District	Farm ID	Tick species	% Mortality (Mean ± SEM)									
			Amitraz (mg/ml)		Cypermethrin:SP (mg/ml)		Deltamethrin:SP (mg/ml)		Chlorfenvinphos:OP (mg/ml)		Chlorfenvinphos/ cypermethrin (COF) (mg/ml)	
			0.25	0.5	0.05	0.1	0.05	0.1	0.5	1.0	0.3/0.03	0.6/0.06
Kampala	C1	*R. app.*	100 ± 0.0	100 ± 0.0	0	0	0	0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
Wakiso	C2	*R. app.*	100 ± 0.0	100 ± 0.0	0	0	0	0	100 ± 0.0	100 ± 0.0	14.7 ± 0.4	100 ± 0.0
	C3	*R. decol.*	100 ± 0.0	100 ± 0.0	0	0	0	0	67.5 ± 0.5	94.5 ± 0.5	21.0 ± 5.0	39.0 ± 3.0
Mubende	C4	*R. decol.*	100 ± 0.0	100 ± 0.0	0	0	0	0	100 ± 0.0	100 ± 0.0	70.4 ± 0.7	93.7 ± 0.8
Mpigi	C5	*R. app.*	100 ± 0.0	100 ± 0.0	11.0 ± 0.0	11.5 ± 0.5	0	12.5 ± 2.5	82.5 ± 5.5	100 ± 0.0	79.0 ± 1.0	87.5 ± 2.5
Kiboga	C6	*R. app.*	100 ± 0.0	100 ± 0.0	0	0	0	0	100 ± 0.0	100 ± 0.0	98.9 ± 1.2	100 ± 0.0
Gulu	N1	*R. app.*	100 ± 0.0	100 ± 0.0	0	0	0	0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
	N2	*A. vari.*	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
Mbarara	W1	*R. app.*	100 ± 0.0	100 ± 0.0	0	0	0	0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
	W2	*R. decol.*	100 ± 0.0	100 ± 0.0	0	0	0	0	50.6 ± 2.7	92.5 ± 0.6	0	19.6 ± 0.4
	W3	*R. app.*	100 ± 0.0	100 ± 0.0	0	0	0	0	100 ± 0.0	100 ± 0.0	71.0 ± 0.0	92.0 ± 0.5
	W4	*R. app.*	100 ± 0.0	100 ± 0.0	6.8 ± 1.1	36.6 ± 7.0	0	46.5 ± 11.5	100 ± 0.0	100 ± 0.0	94.0 ± 0.9	100 ± 0.0
Kiruhura	W5	*R. decol.*	100 ± 0.0	100 ± 0.0	0	0	0	0	68.7 ± 3.8	76.5 ± 9.8	25.1 ± 0.6	57.1 ± 11.3
	W6	*R. decol.*	100 ± 0.0	100 ± 0.0	0	0	0	19.7 ± 10.3	74.45 ± 3.9	91.2 ± 4.0	62.7 ± 5.7	93.3 ± 2.9
	W7	*H. leach.*	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
	W8	*R. decol.*	100 ± 0.0	100 ± 0.0	0	0	0	0	100 ± 0.0	100 ± 0.0	66.5 ± 2.2	76.3 ± 0.7
	W9	*R. decol.*	100 ± 0.0	100 ± 0.0	0	0	0	0	100 ± 0.0	100 ± 0.0	56.0 ± 1.0	65.0 ± 2.5
Bushenyi	W10	*R. decol.*	68.1 ± 1.9	74.5 ± 1.5	0	10.8 ± 1.5	0	16.3 ± 2.5	92 ± 0.5	96.7 ± 0.9	96.2 ± 1.6	98.8 ± 0.0
	W11	*R. decol.*	100 ± 0.0	100 ± 0.0	0	0	0	0	80.2 ± 0.2	100 ± 0.0	49.3 ± 2.7	60.0 ± 1.6

Table 10. Mortality of larvae against various classes of acaricides determined with LPT (continued).

District	Farm ID	Tick species	% Mortality (Mean ± SEM)									
			Amitraz (mg/ml)		Cypermethrin:SP (mg/ml)		Deltamethrin:SP (mg/ml)		Chlorfenvinphos:OP (mg/ml)		Chlorfenvinphos/ cypermethrin (COF) (mg/ml)	
			0.25	0.5	0.05	0.1	0.05	0.1	0.5	1.0	0.3/0.03	0.6/0.06
Mitoma	W12	*R. decol.*	100 ± 0.0	100 ± 0.0	5.0 ± 0.0	13.5 ± 1.5	8.0 ± 3.0	15.5 ± 0.5	100 ± 0.0	100 ± 0.0	53.5 ± 0.5	100 ± 0.0
	W13	*R. decol.*	41.5 ± 0.5	62.5 ± 1.5	65.5 ± 1.5	72.0 ± 2.0	73.5 ± 1.5	91.5 ± 2.5	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
	W14	*R. decol.*	45.0 ± 1.0	NT	NT	NT	NT	NT	NT	NT	NT	NT
Shema	W15	*R. decol.*	100 ± 0.0	100 ± 0.0	0	0	0	0	100 ± 0.0	100 ± 0.0	56.7 ± 0.9	62.7 ± 2.4
Rukungiri	W16	*R. decol.*	100 ± 0.0	100 ± 0.0	0	0	0	0	100 ± 0.0	100 ± 0.0	95.8 ± 0.3	100 ± 0.0
	W17	*R. app.*	100 ± 0.0	100 ± 0.0	0	0	0	0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
	W18	*R. app.*	15.4 ± 0.1	16.7 ± 1.3	97.7 ± 0.5	100 ± 0.0	98.3 ± 0.1	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
	W19	*R. app.*	100 ± 0.0	100 ± 0.0	10.7 ± 0.4	23.2 ± 1.9	12.0 ± 1.3	27.9 ± 0.7	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
Sembabule	W20	*R. decol.*	100 ± 0.0	100 ± 0.0	15.0 ± 1.0	26.0 ± 3.0	15.5 ± 0.5	24.0 ± 2.0	100 ± 0.0	100 ± 0.0	88.5 ± 0.5	95.0 ± 0.0
	W21	*R. app.*	100 ± 0.0	100 ± 0.0	0	0	0	0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
	W22	*R. app.*	100 ± 0.0	100 ± 0.0	0	0	0	0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
Serere	E1	*R. decol.*	100 ± 0.0	100 ± 0.0	79.2 ± 4.7	91.7 ± 1.1	78.7 ± 6.1	95.0 ± 1.9	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0

N1 and N2 are two tick population collected from abattoir (designated as "one farm" for purpose of this study). *A. vari.*, *A. variegatum*; *R. decol.*, *R.* (*B.*) *decoloratus*; COF, coformulation; *H.leach.*, *H. leachi*; NT, not tested due to few larvae; *R. app.*, *R. appendiculatus*.

Chapter 3

Genetic mutations in sodium channel domain II and carboxylesterase genes associated with phenotypic resistance against synthetic pyrethroids by *Rhipicephalus* (*Boophilus*) *decoloratus* ticks in Uganda

1. Introduction

R. (*B.*) *decoloratus* is widely distributed in Africa and transmits economically important livestock diseases, such as anaplasmosis and babesiosis, in sub-Saharan Africa (83, 133). SP and their co-formulation withOP are used globally for tick control on livestock (55). The SP was introduced in late 1970's and has been extensively used for controlling both ticks and flies on livestock (52). In Uganda, SP has been the mainstay of tick and fly control since their introduction in the late 1990's as shown in Chapters 1-2. However, wide spread acaricide resistance against SP by *R.* (*B.*) *decoloratus* ticks in central and western Uganda has made SP ineffective as shown in Chapter 2. Generally, pyrethroids act by modulating arthropod sodium channel and have been considered highly effective at even low concentrations (1, 56). However, resistance mediated by mutations in VSSC against SP has been reported especially in *R.* (*B.*) *microplus* (74, 110, 152). The common type of mutations in sodium channel includes knock-down (*kdr*) and *super-kdr* (38, 152). Both forms of *kdr* have been widely reported in insects compared to ticks (147). For example, *super-kdr* mutation in housefly sodium channel segment IIS6 and linker II (S4–S5) confers up to 500-fold resistance against SP deltamethrin (163). In ticks, *kdr* mutations have been reported mainly in *R.* (*B.*) *microplus* from India (92), South Africa (165) and USA (152). In Africa, only two studies in South

Africa have established a shared *kdr* mutation (C190A) in *R.* (*B.*) *decoloratus* and *R.* (*B.*) *microplus* among SP-resistant ticks (97, 165). *In silco* modeling revealed that *kdr* mutations alter amino acid residues in sodium channel thereby, reducing the binding affinity of SP to its target site and affecting sensitivity of the sodium channel to SP (38, 165).

Apart from target site mutations, metabolic hydrolysis by tick enzymes may also confer resistance against SP (58, 42). CXE enzyme is one of the metabolizing enzymes that have been associated with resistance against SP (10, 61, 64, 69). It was found that SP-resistant *R.* (*B.*) *microplus* had high transcript of CXE compared to susceptible ticks, suggestive of its role in metabolic-mediated resistance (63). Interestingly, the same tick population with high CXE transcript had mutation at nucleotide 1,120 from guanine to adenine (G1120A), which introduced *Eco* RI restriction site making it a diagnostic mutation for detection of CXE-mediated SP resistance. The resultant amino acid substitution from the above mutation (D374N) was hypothesized to lead to SP resistance by increasing the affinity of CXE for SP or by enhancing its ability to hydrolyze SP (63).

As shown in Chapter 2, *R.* (*B.*) *decoloratus* tick populations from central and western Uganda were super resistant against SP, with majority having 100% survival at discriminating dose of either cypermethrin or deltamethrin. Thus, it was hypothesized that the stable resistance established against SP could be mediated by both target site alterations and metabolic pathways. The aim of this study was to determine the genetic basis of super resistance against SP in *R.* (*B.*) *decoloratus* ticks by investigating mutations in VSSC domain II and CXE genes. Furthermore, novel restriction sites in CXE gene was explored for possible development of rapid diagnostic technique using RFLP.

2. Materials and methods

2.1. Tick populations used in the study

A total of 20 tick populations collected from 20 farms in 10 districts were used for this study (Table 11 and Fig. 8). The susceptibility of 11 tick populations (TSR, WKB, 2SBL, 2KRH, 2MTM, ISHM, 4MBR, 10KRH, 1BUS, 2BUS and 3BUS) to SP, OP and COF were determined in Chapter 2. Additional 9 tick populations (AM08, A16, AJ17, M22, KD01, KMD, KKK, KKN and KMH) were collected and identified to species level using morphological features (168), and their susceptibility was determined by LPT. Tick population AM08 collected from Adjumani in northwestern Uganda district was used as reference susceptible population. The percentage mortality of larvae were determined at discriminating concentration of deltamethrin (0.05 mg/ml), chlorfenvinphos (0.5 mg/ml) and co-formulation containing chlorfenvinphos and cypermethrin (0.3/0.03 mg/ml) or chlorpyrifos and cypermethrin (0.5/0.05 mg/ml). A total of 12 tick populations were used for investigating mutations, while 18 tick populations were used for validation of RFLP assay.

2.2. Extraction of genomic DNA

Tick genomic DNA was extracted using NucleoSpin Tissue® DNA extraction kit (Macherey-Nagel, German), according to the manufacturer's instruction. Approximately 30 pooled ethanol preserved larvae were washed with 1×phosphate-buffered saline and crushed in a Bio-masher II tubes with Bio-masher motor (Nippi, Japan). The concentration of the eluted DNA was determined by Nano Drop 2000 (Thermo Fisher Scientific Inc, USA), and the samples were stored at -30 ^{o}C until they were used.

2.3. Amplification of *R.* (*B.*) *decoloratus* voltage sensitive sodium channel domain II S4-5 linker

A total of 10 *R.* (*B.*) *decoloratus* (1 susceptible and 9 SP-resistant) tick populations were used to determine mutations associated with SP resistance. Amplification of sodium channel domain II was carried out using previously described primers (152). The primers, RmNaDIIF1 (5'-TACGTGTGTTCAAGCTAGCCAA-3') and RmNaDIIR1 (5'-CTTTCTTCGTAGTTCTTGCCAA-3'), were designed from *R.* (*B.*) *microplus* sodium channel gene. The PCR reaction was carried out in 50 µl volume containing 0.4 mM dNTP, 1×KOD FX Neo buffer, 2 ng of DNA template, 0.5 µM of each primer and 0.014 U of KOD FX Neo (Toyobo, Japan) with Veriti Thermal Cycler (Applied Biosystems, USA). The gradient thermal cycling condition included initial denaturation at 95^{o}C for 5 min and 10 cycles of 65^{o}C for 1 min, 72^{o}C for 1 min while decreasing annealing temperature by 1 degree. This was followed by 30 cycles of denaturation at 95^{o}C for 1 min, annealing 55^{o}C for 1 min, extension at 72^{o}C for 1 min and final extension at 72^{o}C for 7 min. An aliquot of 10 µl of the PCR product was electrophoresed in 1.5% agarose gels (Nacalai Tesque, Inc., Japan), stained with ethidium bromide (Nacalai Tesque, Inc., Japan) and visualized with Atta Type-FX-II UV Transilluminator (Atta Corporation, Japan).

2.4. Amplification of *R.* (*B.*) *decoloratus* carboxylesterase gene

The partial carboxylesterase (CXE) gene from 10 (1 susceptible and 9 SP-resistant) *R.* (*B.*) *decoloratus* tick populations were amplified with Veriti Thermal Cycler. The primers designed previously (63) (GS138B: 5′-GCATCGACCTCTCGTCCAAC-3′ and GS139R: 5′-GTCGGCATACTTGTCTTCGATG-3′) were used for DNA amplification (63). The PCR was performed in 40 µl of mixture that contained 0.1 µM of each primer, 0.2 µM of dNTP, 1× PCR buffer for Blend Taq plus, 1 ng of DNA template and 1 U of Blend Tag® -Plus (Toyobo,

Japan). The thermal cycling condition included initial denaturation at 95^{o}C for 2 min and 33 cycles of denaturation at 94^{o}C for 30 sec, annealing at 64^{o}C for 30 and extension at 68^{o}C for 1 min. The elongation was further extended for 5 min at 68^{o}C. An aliquot of 10 µl of the PCR product was electrophoresed in 1.5% agarose gels (Nacalai Tesque, Inc., Japan), stained with ethidium bromide and visualized with Atta Type-FX-II UV Transilluminator (Atta Corporation, Japan).

2.5. Cloning and sequencing of carboxylesterase and sodium channel domain II genes

The amplicons with the expected band size for CXE and sodium channel domain II genes were purified with Wizard® SV Gel and PCR Clean-up System (Promega, USA), according to the manufacturer's instruction. Since KOD FX polymerase has a proof reading activity, the resultant PCR products (sodium channel gene) were further treated with 10×Attachment mix (Toyobo, Japan) to introduce adenosine overhang at 3' end, according to the manufacturer's instruction. The genes were cloned using the pGEM-T easy ligation kit (Promega, USA) and transformed into ECOS™ competent *Escherichia coli* DH5α (Nippon gene, Japan). Four to five positive colonies that were confirmed to have the desired insert were multiplied in Luria-Bertaini broth (with 100 µg/ml ampicillin) and purified using NucleoSpin® Plasmid Easy Pure kit (Marcherey-Nagel, Germany), following manufacturer's instruction. The CXE and sodium channel domain II gene inserts were sequenced with SP6 (reverse) and T7 (forward) promotor primers, using BigDye v3.1 Terminator Cycle Sequencing Kit and the 3730×l DNA Analyzer (Applied Biosystem, USA).

2.6. Diagnostic RFLP protocol for detection of SP resistance in *R.* (*B.*) *decoloratus* ticks

The presence of novel single nucleotide polymorphism (SNP) loci in CXE genes from SP-resistant ticks was explored using restriction mapper. Two novel restriction sites; *Eco* RI

and *Eco* RII were identified in SP-resistant *R.* (*B*) *decoloratus* ticks. This was confirmed by virtual digestion of CXE genes *in silco*. The validation assay for *Eco* RII restriction was carried out with purified CXE PCR products from reference susceptible and resistant tick populations. The RFLP was performed in 15 µl reaction volume containing 0.3 µg of DNA template, 1×M buffer and 0.33 U/µl *Eco* RII enzyme (Nippon gene). The restriction mixture was incubated at 37^{o}C for 4 hours. The RFLP validation assay for *Eco* RI was done in 20 µl reaction volume that contained 0.8 U/µl *Eco* RI (Nippon gene), 0.4 µg of purified esterase PCR product and 1×H buffer and the mixture incubated at 37^{o}C for 1 hour. PCR products for 18 tick populations of known susceptibility against SP and formulations of SP and OP were assayed. The resultant digestion products were electrophoresed in 2% agarose gels, stained with ethidium bromide and visualized under UV lamp.

2.7. Data analyses

The nucleotide sequences were edited with DNASTAR (Ver. 7.1.0, DNAstar Inc., Madison, WI). The similarities of CXE and sodium channel domain II genes obtained in the current study were compared with those of *R.* (*B.*) *microplus* in the GenBank using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide sequences were translated to their corresponding amino acid sequences with EMBOSS Transeq online tool (http://www.ebi.ac.uk/Tools/st/emboss_transeq/). Mutations in the coding region of both genes were determined by multiple sequence alignment using BioEdit version 7.2.5 (Tom Hall Ibis Biosciences, CA). The percentage identity of *R.* (*B.*) *decoloratus* CXE with that of *R.* (*B.*) *microplus* was determined by basic local alignment tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Novel restriction sites associated with phenotypic resistance against SP and COF were determined using online restriction mapper tool (RestrictionMapper; http://www.restrictionmapper.org/).

3. Results

3.1. Nucleotide sequence accession number

Nucleotide sequence data reported in this chapter have been deposited in the DDBJ/EMBL/GenBank databases. The GenBank accession numbers for *R.* (*B.*) *decoloratus* sodium channel genes are KY659478- KY659487, while for *R.* (*B.*) *decoloratus* CXE gene are KY659488- KY659497 (Table 12).

3.2. Mutation in *R.* (*B.*) *decoloratus* sodium channel domain II gene associated with stable resistance against synthetic pyrethroids

The 167-bp sodium channel domain II gene for the reference susceptible tick population AM08 was 100% homologous to that of SP susceptible *R.* (*B.*) *microplus* Accession # AF134216 (Fig. 9). The gene for one tick population from Serere district (TSR), which was previously identified as SP-resistant, was also 100% identical to that of the reference tick (AM08). However, 7 tick populations with history of SP resistance from central and western Uganda (high acaricide pressure areas) had the T58C mutation in the partial sodium channel gene. The T58C mutation corresponded to T170C *super-kdr* mutation in the sodium channel gene based on previous nomenclature (62). This mutation leads to amino acid change from methionine to threonine (M19T) as shown in Fig. 10. One SP-resistant tick population (KD01) had two unique non-synonymous mutations C91T and C92T, which led to single amino acid substitution from threonine to isoleucine (T30I) (Fig. 10). A synonymous mutation G128A was observed only in highly SP-resistant ticks (>80% SP survival) from central and western Uganda.

3.3. Mutations *in R.* (*B.*) *decoloratus* CXE gene associated with resistance against synthetic pyrethroids

The *R.* (*B.*) *decoloratus* CXE gene for the susceptible reference tick (AM08) was 99% identical to that of *R.* (*B.*) *microplus* Deutsch strain (Accession # HM193855) (57) (Fig. 11). In contrast, the highest CXE identity score for *R.* (*B.*) *decoloratus* ticks from high acaricide pressure area was 95% compared to that of *R.* (*B.*) *microplus* (Accession # KC710047) (78) (Fig. 11). Multiple single nucleotide polymorphisms (SNPs) were found in 36 loci (Table 13 and Fig. 11), 15 of which were non-synonymous and 21 were synonymous. Interestingly, 94.4% (34/36) of the total SNPs were only unique to ticks from central and western Uganda where acaricide pressure was high. Only 2 SNPs A180G and G300A were shared between SP-resistant ticks from Serere district (TSR) and the rest of the ticks from central and western Uganda. The above mutations correspond to A1000G and G1120A according to nomenclature by Guerrero and Nene (59) based on complete CXE gene sequence (Accession # DQ533868). For the purpose of this study, both nomenclature have been used and depicted as A180G/A1000G and G300A/G1120A. Furthermore, 13 additional non-synonymous SNPs were found in *R.* (*B.*) *decoloratus* ticks that were resistant to SP (Table 13 and Fig. 12). The same mutations occurred in ticks with various levels of resistance against co-formulation containing SP and OP. One non-synonymous mutation A342G/A1162G with its corresponding amino acid substitution I114V/I388V was only unique to tick population MS22 that was resistant to SP, OP and COF. Three additional non-synonymous mutations (G114A/ G934A, A283G/ A1103G and T295C/ T1115C) were unique to tick population 4MBR that had 100% survival rate against SP and COF in addition to being resistant against OP (Table 13).

3.4. Validation of RFLP based on *Eco* RI and *Eco* RII

The restriction mapper tool identified two SNPs, G195C/G1015C and

G300A/G1120A, that led to introduction of *Eco* RII restriction and *Eco* RI restriction sites in CXE for SP-resistant ticks, respectively. Aside from tick population 2MTM, *Eco* RII sites was found in all the SP-resistant ticks from central and western Uganda, while *Eco* RI sites was found in 7 out of 9 SP-resistant ticks. However, validation assay showed that *Eco* RII was specific for identification of highly SP-resistant (>80% survival against deltamethrin) *R.* (*B.*) *decoloratus* ticks (Table 15). The *Eco* RII digestion product had two distinct bands. One band was at approximately 371-bp while the other band contains the two fragments of 193-bp and 178-bp produced after restriction of the heterozygous mutant genotypes. The size of homozygous wildtype DNA fragment (susceptible) was 371-bp. Due to the small difference between the two fragments, the band appeared as single in 2% gel (Fig. 13). However, validation of *Eco* RI restriction showed some of the CXE for both SP-susceptible and resistant ticks were digested without clear selectivity based on resistance level.

4. Discussion

The current study found 100% homology between the 167-bp *R.* (*B.*) *decoloratus* sodium channel domain II gene for the susceptible reference population (Accession# KY659478) and that of SP-susceptible *R.* (*B.*) *microplus* (Accession # AF134216). The T58C mutation in the partial sodium channel gene (Fig. 9) that corresponds to T170C *super-kdr* mutation reported previously (62) led to amino acid change from methionine to threonine (M19T). The ticks that had the above mutation showed extremely low to zero mortality against discriminating dose of SP (deltamethrin). The *super-kdr* (T170C) was previously reported in SP-resistant insects (68, 132, 154) and *R.* (*B.*) *microplus* ticks (152). Other studies also showed that the *super-kdr* mutation usually occurs together with *kdr* mutation (L to F mutation) in highly resistant insects (38, 144) contrary to findings in this study. It is postulated that the weekly acaricide exposure of the one-host tick, *R.* (*B.*) *decoloratus*, creates high selection pressure that necessitates a strong survival fitness likely conferred by *super-kdr* mutation. On the other hand, the C91T and C92T which led to a single amino acid substitution from threonine to lysine-T30I in tick population KD01 is reported in ticks for the first time by this study (Table 13, Fig. 10). Since the tick population, KD01 exhibited zero mortality against discriminating dose of deltamethrin, it may be deduced that T30I mutation possibly offers the same survival fitness like *super-kdr* mutation. This is consistent with previous reports in insects that revealed that the T to I mutation at position 929 alone desensitized the sodium channel and was considered as a *super-kdr* mutation (38, 163). It has been reported that M to T, T to I and L to F mutations in the binding pockets of IIS4-S5 linker and IIS5 domains maintained the close-state of the sodium channel, hence reducing opening of the channel and subsequently reducing the binding affinity of SP to its target site (162, 163, 165). This helps to withstand the toxic effect of pyrethroids especially where the chemicals were applied in shorter interval and at higher concentration as reported in

Chapters 1-2. Other researchers have also highlighted the effect of frequent acaricide application as a factor influencing the rate of development of stable resistance (1, 48, 65). The current study further noted that the use of SP containing co-formulated acaricides helps to sustain selection pressure (30) and lack of tick immigration further increases the dominance of resistance gene pool in the population (65). Another interesting finding was the 100% homology of the sodium channel domain II for the susceptible reference (Accession# KY659478) and the SP-resistant *R.* (*B.*) *decoloratus* ticks from Serere district (TSR) (Fig. 9), which suggests that SP-resistance in TSR may be mediated by alternative pathways. These may include pyrethroid metabolizing esterase, mixed function oxidases and glutathione transferase (42, 58).

To-date, all the available reports on SP resistance attributed to pyrethroid metabolizing CXE are based on *R.* (*B.*) *microplus* ticks (60, 63). Hence, there is gross lack of information on the role of CXE in SP resistance in *R.* (*B.*) *decoloratus* ticks in Africa. This study found out high nucleotide similarity score for CXE gene from susceptible *R.* (*B.*) *decoloratus* ticks (AM08) and *R. microplus* Deutsch strain (Accession # HM193855) (57). This indicates that partial 371-bp CXE gene is highly conserved amongst the two tick species. Tick populations that had higher resistance to pyrethroids tended to have more mutations in CXE gene. Of the 15 non-synonymous (Table 13), one *Eco* RII site conferring mutations in CXE gene G195C/G1015C that lead to amino acid substitution from valine to leucine (V65L/V339L) was associated with highly SP-resistant ticks from central and western Uganda. The exact role of the V65L transversion mutation in SP resistance is not known and warrants further investigation. This study also identified the *Eco* RI site conferring mutation G300A/G1120A but further validation studies showed that the mutation occurred in both susceptible and resistant *R.* (*B.*) *decoloratus* ticks (Tables 13 and 15). Previous studies on SP metabolizing CXE have attributed the G300A/G1120A mutation with SP resistance in *R.* (*B.*) *microplus*

ticks (10, 60, 64). Hernandez *et al* (64) hypothesized that the amino acid substitution D374N increases the affinity of CXE for SP thus enhancing its ability to hydrolyze SP. Whether or not the V65L/V339L mutation also increases SP-hydrolytic efficiency requires further investigation.

Although the contribution of the G195C/G1015C mutation to the overall survival fitness remains unclear, its co-occurrence with *super-kdr* mutation in the sodium channel domain II was associated with very high survival rates (85-100%) against SP. Lovis *et al* (97) suggested that CXE confers less resistance fitness compared to target specific mutations in tick sodium channel gene but may be more frequent metabolic resistance pathway. A recent study by Eiden *et al* (42) found out that increased esterase activity conferred stronger pyrethroid (permethrin) resistance compared to cytochrome P450-dependent metabolic detoxification in *Rhipicephalus sanguineus* ticks. Other studies have also suggested that the role of CXE in SP resistance in arthropods emanates from its upregulation and increased hydrolytic efficiency associated with increased production (2, 43, 173). This eventually leads to alteration of the concentration of SP molecules required to cause toxicological effect in the tick (10). Overall, the current findings suggest that CXE may provide additional resistance pathway to target specific mutations in VSSC leading to stronger survival fitness where SP selection pressure is high.

This study further explored the RFLP diagnostic potential of the G195C/G1015C and G300A/G1120A mutations that conferred *Eco* RII and *Eco* RI restriction sites in CXE, respectively. Mutations that lead to introduction of restriction sites have been previously explored in development of RFLP for rapid detection of resistant ticks (13, 64). The RFLP validation assay revealed that *Eco* RII restriction enzyme had sensitivity threshold at least 80% survival against SP (Table 15). The two tick population with low survival (mild resistance) did not show an *Eco* RII restriction site. This may suggest that SP resistance in the

two tick populations (TSR and 2MTM) may be mediated by other alternative metabolic pathways such as cytochrome P450 and glutathione transferase (42, 58). However, the efficiency of *Eco* RII-based RFLP (Fig. 13) in detecting highly SP-resistant ticks made it a novel diagnostic technique for diagnosis of SP-resistant *R.* (*B.*) *decoloratus* ticks in Uganda. On the other hand, *Eco* RI RFLP reported previously for detection of SP resistance in *R. microplus* ticks (60, 64) was not specific and could not be used for detection of SP resistance in *R.* (*B.*) *decoloratus* from Uganda. The new diagnostic method using *Eco* RII RFLP will help to reduce the time taken to diagnose SP resistance from 4 weeks with LPT to 2 days.

Taken together, this study indicates that stable SP resistance by *R.* (*B.*) *decoloratus* may be additively mediated by both *super-kdr* in sodium channel gene and mutations in CXE genes. The underlying managemental factors contributing to the above mutations may be attributed to prolonged irrational use of SP-based acaricide formulations in Uganda, as reported in Chapters 1-2. The existence of dual acaricide resistance mechanism against SP suggests that reversal of such resistance is expected to take long. Moreover, the continued use of either mono or co-formulated SP-based acaricide formulations against resistant ticks will contribute towards maintaining selection pressure and fitness of the resistant ticks. In absence of appropriate rotation to alternative acaricides, incidence of acaricide failure associated with SP-based formulations will increase.

5. Conclusion

This study reported *super-kdr* mutation in sodium channel domain II and multiple mutations in CXE genes in SP-resistant *R.* (*B.*) *decoloratus* ticks from Uganda. These mutations possibly act simultaneously to mediate stable resistance against SP in *R.* (*B.*) *decoloratus* ticks. One mutation G195C/G1015C that conferred *Eco* RII site in CXE gene was novel for RFLP diagnosis of highly SP-resistant *R.* (*B.*) *decoloratus* ticks. This study, therefore, confirmed wide spread SP resistance in *R.* (*B.*) *decoloratus* ticks in Uganda and provides valuable information for developing strategies for control of ticks in Uganda. It is essential that farmers and extension workers are trained on appropriate acaricide rotation, use of alternative molecules to remove selection pressure against SP and building technical capacity for containment of acaricide resistant *R.* (*B.*) *decoloratus* ticks in Uganda.

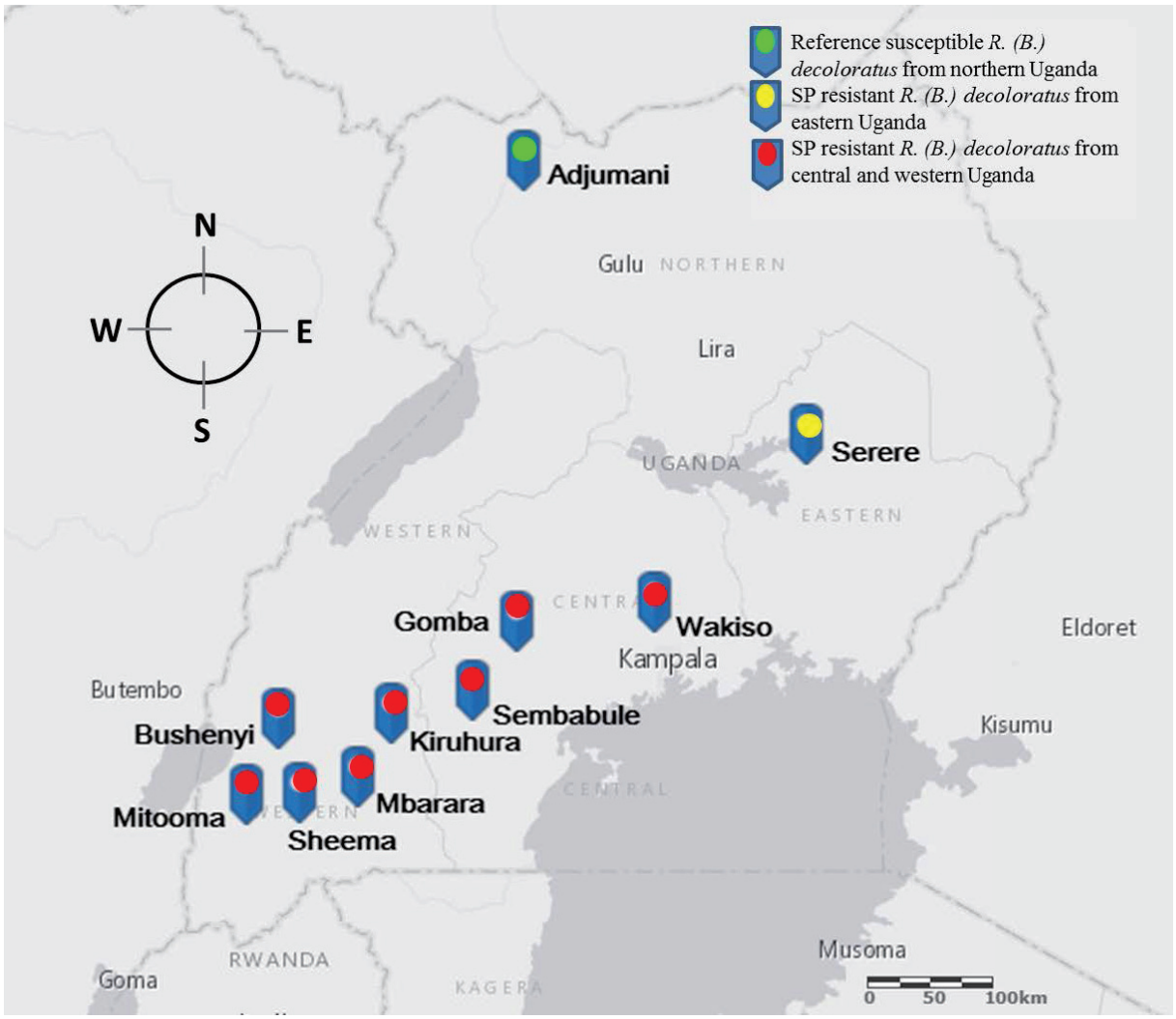

Fig. 8. Map of Uganda showing the districts from which ticks were collected.

Susceptible ticks were collected from northwestern district of Adjumani (green) were farmers keep local zebu cattle and rarely use acaricides for tick control. SP resistant ticks (red) were collected from central, east and western Uganda. Both central and western Uganda lies in the high acaricide pressure zone due to exotic cattle production that predispose to extensive acaricide application to prevent the susceptible cattle from TBD infection.

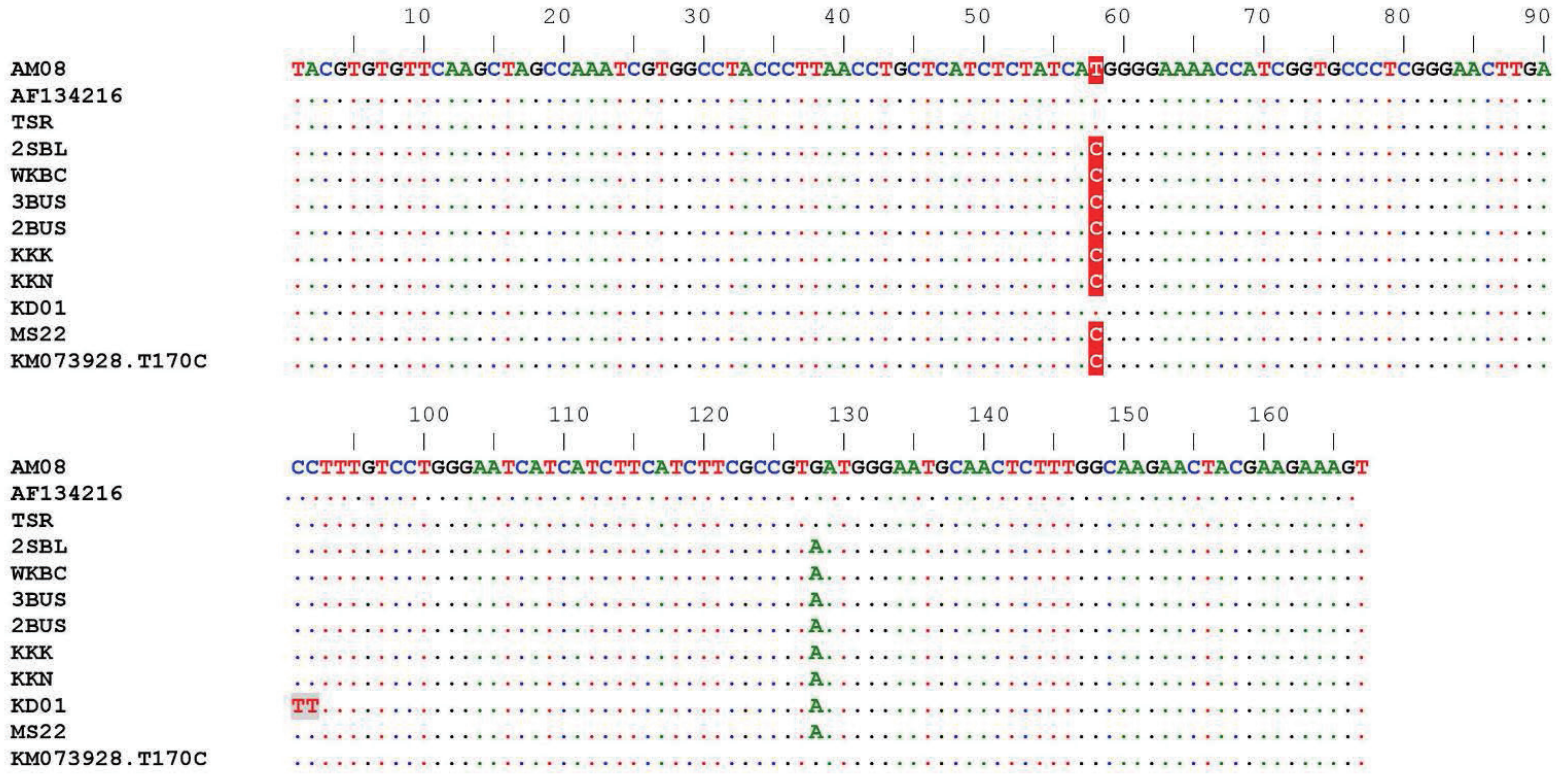

Fig. 9. *R.* (*B.*) *decoloratus* voltage sensitive sodium channel domain II nucleotide mutations that confer resistance against synthetic pyrethroids.

AF134216 (Accession # for *R.* (*B.*) *microplus* sodium channel gene for reference susceptible strain; KM073928.T170C (Accession # for *R. (B.) microplus* sodium channel gene for *super-kdr* mutation denoted as T170C). Red highlight shows the T58C *super-kdr* loci. Grey highlight shows novel C91T and C92T.

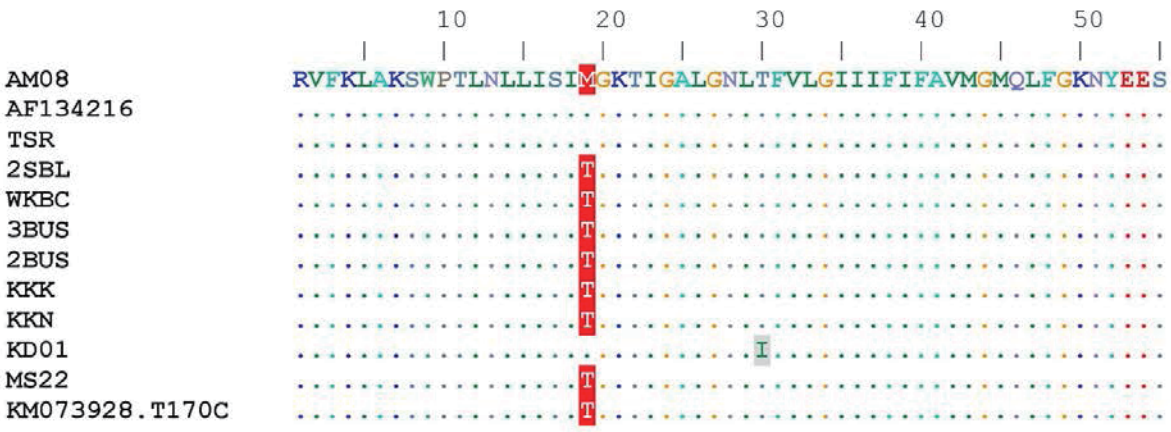

Fig. 10. *R.* (*B.*) *decoloratus* voltage sensitive sodium channel domain II amino acid substitutions that confer resistance against synthetic pyrethroids.

AF134216 (Accession # for *R.* (*B.*) *microplus* sodium channel gene for susceptible reference strain; KM073928.T170C (Accession # for *R. (B.) microplus* sodium channel gene for *super-kdr* mutation denoted as T170C). The red highlight shows *super-kdr* amino acid substitution M19T in highly resistant ticks. The grey highlight indicates a novel threonine to isoleucine amino acid mutation that was reported to confer *kdr* resistance in insects.

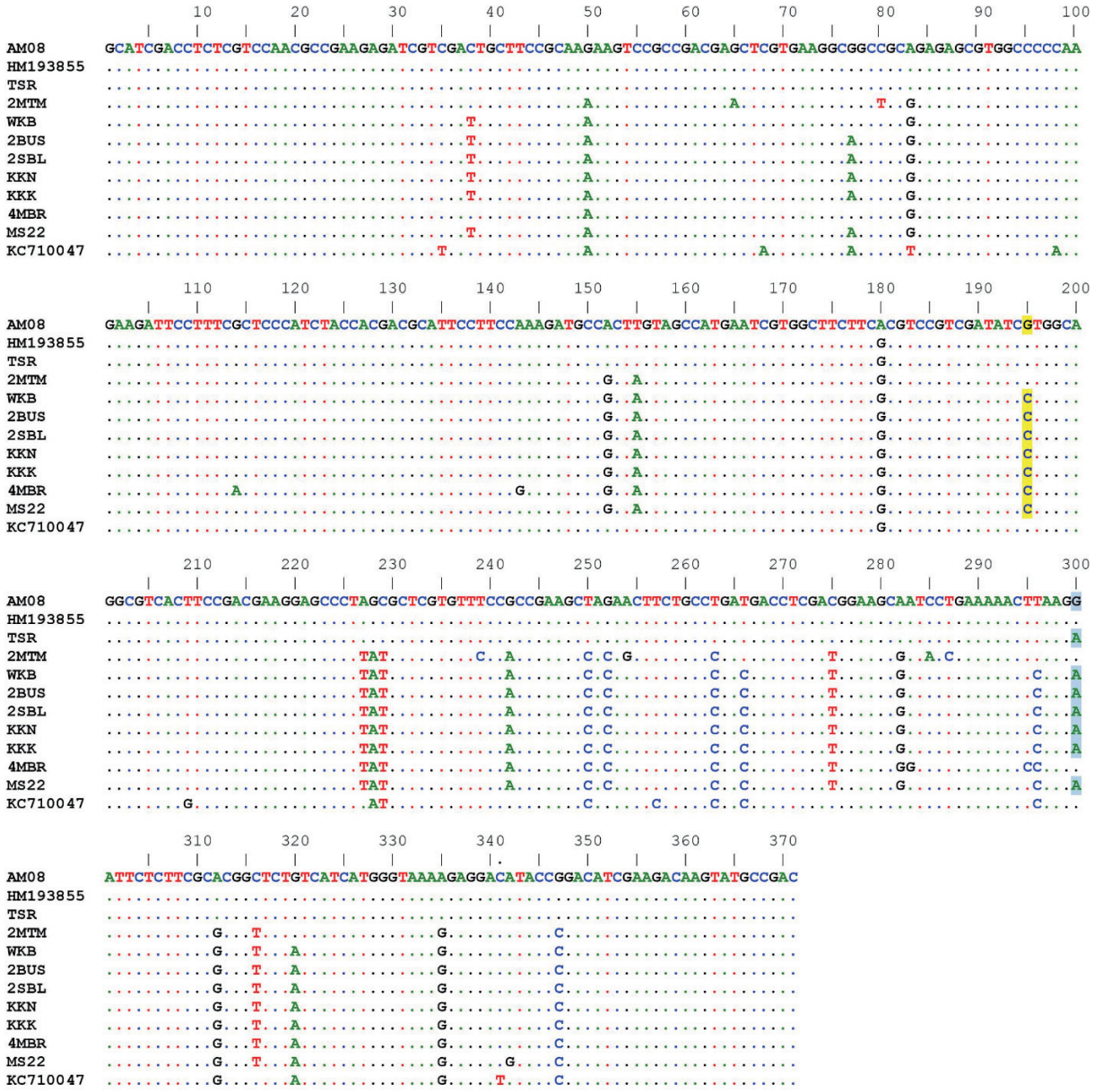

Fig. 11. Multiple mutations in *R.* (*B.*) *decoloratus* carboxylesterase gene that confer resistance against synthetic pyrethroids.

A total of 36 mutations were observed; The G195C (yellow highlight) and G300A (blue highlight) conferred *Eco* RII and *Eco* RI restriction sites, respectively: The G195C mutation was associated with stable resistance (85-100% survival) against discriminating dose of deltamethrin or cypermethrin.

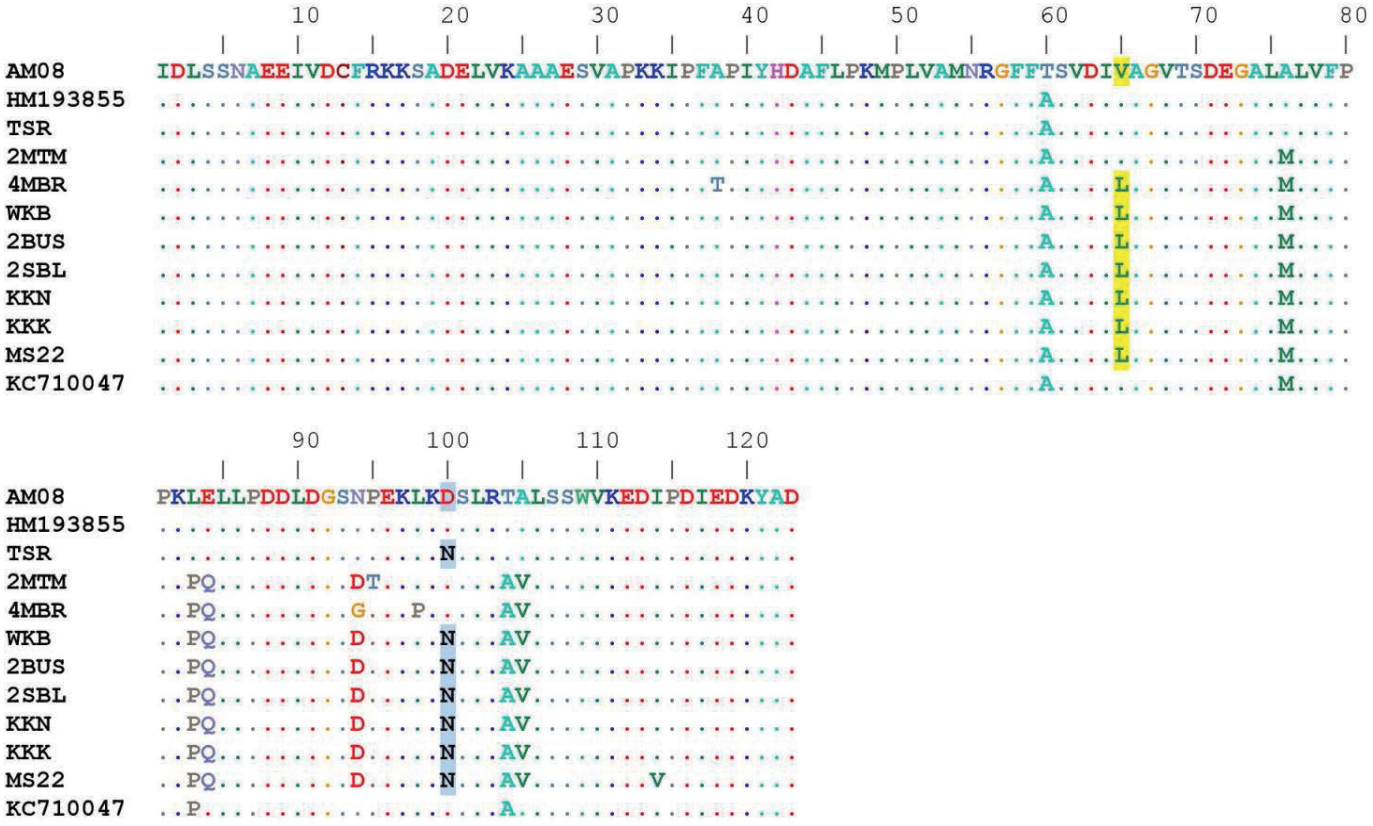

Fig. 12. Amino acid substitutions in *R.* (*B.*) *decoloratus* carboxylesterase gene associated with resistance against synthetic pyrethroids. A total of 15 non-synonymous mutations that led to amino acid change was found. The V65L (yellow highlight) amino acid substitution was associated with stable resistance against SP, making it a novel genetic marker for detection of SP-resistance. The D100N mutation (blue highlight) was not only specific to SP-resistant ticks.

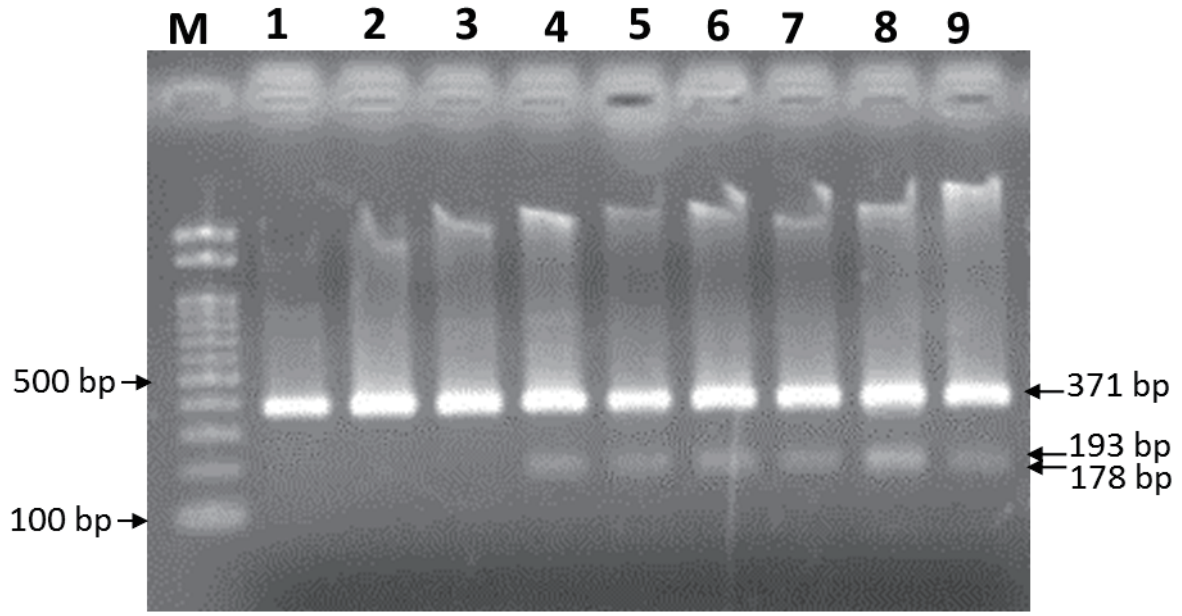

Fig. 13. RFLP showing bands after *Eco* RII digestion of carboxylesterase gene for 8 hours.

Lanes 1-9: CXE *Eco* RII digestion assay for different tick populations with known survival rate against discriminating dose of synthetic pyrethroid (SP) deltamethrin. Lane 1: AM08 (reference susceptible tick); lane 2: TSR (mild SP-resistance with 21.3% survival rate); lane 3: 2MTM gene (mild SP resistance with 26.5% survival rate); lane 4: KKK (highly SP-resistant with 99% survival); lane 5: KMH (highly SP-resistant with 99.3% survival rate); lane 6: WKB (highly SP-resistant with 100% survival); lane 7: 1BUS (highly SP-resistant with 100% survival); lane 8: 10KRH (highly SP-resistant with 100% survival); lane 9: 4MBR(highly SP-resistant with 100% survival). M: 100-bp DNA marker.

Table 11. Origin and characteristics of *R. (B.) decoloratus* tick populations used in the study.

Category	Tick Population ID	Region	District	History of exposure to OP or SP based acaricides in 2 years	SP-resistant status	OP-resistant status	COF-resistant status	Target gene(s)
Reference susceptible	AM08	North	Adjumani	None	S	S	S	RdNaDII and Esterase
Susceptible	A16	North	Adjumani	None	S	S	S	RD
	AJ17	North	Adjumani	None	S	S	S	RD
Resistant	TSR*	East	Serere	SP	R	S	S	RdNaDII and Esterase
	WKB*	Central	Wakiso	COF[a]	R	R	R	RdNaDII and Esterase
	2SBL*	Central	Sembabule	OP, COF[a]	R	I	R	RdNaDII and Esterase
	M22	Central	Gomba	SP, OP, COF[.b]	R	R	R	RdNaDII and Esterase
	2MTM*	West	Mitooma	None[NR]	R	S	S	Esterase
	ISHM*	West	Sheema	COF[.b]	R	S	R	RD
	4MBR*	West	Mbarara	SP	R	R	R	Esterase
	1BUS*	West	Bushenyi	SP, OP	R	I	I	RD
	2BUS*	West	Bushenyi	SP	R	I	I	RdNaDII and Esterase
	3BUS[q]	West	Bushenyi	SP, COF[a]	NT	NT	NT	RdNaDII
	2KRH*	West	Kiruhura	None	R	R	R	RD
	10KRH*	West	Kiruhura	SP, COF[a], COF[b]	R	S	R	RD
	KD01	West	Kiruhura	COF[a,b]	R	R	R	RdNaDII
	KMH	West	Kiruhura	COF[b], OP	R	R	R	RD
	KMD	West	Kiruhura	COF[a], COF[b]	R	R	R	RD
	KKN	West	Kiruhura	COF[a,b]	R	R	R	RdNaDII and Esterase
	KKK	West	Kiruhura	SP, OP, COF[.b]	R	R	R	RdNaDII and Esterase

I: Intermediate (81-98%); R: Resistant (0-80% mortality; S: Susceptible (99-100%). [q]: Resistance status unknown due to low larval population for LPT but farmer complained of acaricide failure against SP and COF; NT: Not tested by LPT; SP: Synthetic pyrethroids; OP: Organophosphate-Chlorfenvinphos; COF[a]: SP-OP coformulation containing Chlorfenvinphos and α-cypermethrin; COF[b]: Co-formulation containing Chlorpyriphos and cypermethrin; COF[a,b]: Farm that used both COF[a] and COF[b] ; NR, No record of previous acaricide but amitraz was used on the farm at the time of this research. RdNaDII, *R.* (*B.*) *decoloratus* sodium channel domain II. RD, additional ticks used for RFLP validation assay.*Tick population whose susceptibility was determined in chapter 2 (TSR/E1, 2MTM/W13, 2SBL/W20, ISHM/W15, 2KRH/W5, 4MBR/W2, WKB/C3, 1BUS/W10, 2BUS/W11, 10KRH/W9).

Table 12. GenBank accession numbers for *Rhipicephalus* (*B.*) *decoloratus* sodium channel domain II and carboxylesterase genes.

SN	Tick population	GenBank accession number	Gene
1	AM08 (Reference susceptible)	KY659478	Sodium Channel
2	TSR	KY659479	Sodium Channel
3	2SBL	KY659480	Sodium Channel
4	WKB	KY659481	Sodium Channel
5	3BUS	KY659482	Sodium Channel
6	2BUS	KY659483	Sodium Channel
7	KKK	KY659484	Sodium Channel
8	KKN	KY659485	Sodium Channel
9	MS22	KY659486	Sodium Channel
10	KD01	KY659487	Sodium Channel
11	AM08 (Reference susceptible)	KY659488	Carboxylesterase
12	TSR	KY659489	Carboxylesterase
13	2MTM	KY659490	Carboxylesterase
14	WKB	KY659491	Carboxylesterase
15	2BUS2	KY659492	Carboxylesterase
16	2SBL	KY659493	Carboxylesterase
17	KKN	KY659494	Carboxylesterase
18	KKK	KY659495	Carboxylesterase
19	4MBR	KY659496	Carboxylesterase
20	MS22	KY659497	Carboxylesterase

SN, serial number

Table 13. Single nucleotide polymorphism in *R.* (*B.*) *decoloratus* CXE gene and phenotypic characteristics of various ticks.

#	SNP loci in partial CXE nucleotide sequence	SNP loci in complete CXE nucleotide sequence	Amino acid substitution loci in partial CXE gene	Amino acid substitution loci in complete gene	Identity of ticks with the mutations	Resistance Phenotype		
						SP	OP	COF
1	C 38T	C858T	None	None	WKB, 2BUS, 2SBL, KKN, KKK, MS22	R	R/I	R/I
2	G 50A	G870A	None	None	WKB, 2BUS, 2SBL, KKN, KKK, MS22,4MBR	R	R/I	R/I
3	G65A	G885A	None	None	2MTM	R	S	S
4	G 77A	G897A	None	None	WKB, 2BUS, 2SBL, KKN, KKK, MS22	R	R/I	R/I
5	C80T	G900A	None	None	2MTM	R	S	S
6	A83G	A903G	None	None	WKB, 2BUS, 2SBL, KKN, KKK, MS22, 4MBR	R	R/I	R/I
7	**G114A**	**G934A**	**A38T**	**A312T**	**4MBR**	**R**	**R**	**R**
8	A143G	A963G	None	None	4MBR	R	R	R
9	A152G	A972G	None	None	WKB, 2BUS, 2SBL, KKN, KKK, MS22,4MBR	R	R/I	R/I
10	T155A	T975A	None	None	WKB, 2BUS, 2SBL, KKN, KKK, MS22,4MBR	R	R/I	R/I
11	**A180G**	**A1000G**	**T60A**	**T334A**	**2MTM, 4MBR, TSR, WKB, 2BUS, 2SBL, KKN, KKK, MS22**	**R**	**R/I/S**	**R/I/S**
12	**G195C**	**G1015C**	**V65L**	**V339L**	**2MTM, 4MBR,WKB, 2BUS, 2SBL, KKN, KKK, MS22**	**R**	**R/I**	**R/I**
13	A227T	A1047T	None	None	4MBR, WKB, 2BUS, 2SBL, KKN, KKK, MS22	R	R/I	R/I
14	**G228A**	**G1048A**	**A76M**	**A350M**	**2MTM,4MBR, WKB, 2BUS, 2SBL, KKN, KKK, MS22**	**R**	**R/I**	**R/I**
15	**C229T**	**C1049T**	**A76M**	**A350M**	**2MTM, 4MBR, WKB, 2BUS, 2SBL, KKN, KKK, S22**	**R**	**R/I**	**R/I**
16	T239C	T1059C	None	None	2MTM	R	S	S
17	G242A	G1062A	None	None	4MBR, WKB, 2BUS, 2SBL, KKN, KKK, MS22	R	R/I	R/I
18	**T250C**	**T1070C**	**L83P**	**L357P**	**2MTM, 4MBR, WKB, 2BUS, 2SBL, KKN, KKK, S22**	**R**	**R/I**	**R/I**
19	A254G	A1074G	None	None	2MTM	R	S	S
20	**G252C**	**G1072C**	**E84Q**	**E358Q**	**2MTM, 4MBR, WKB, 2BUS, 2SBL, KKN, KKK, MS22**	**R**	**R/I**	**R/I**
21	T263C	T1083C	None	None	**4MBR,** WKB, 2BUS, 2SBL, KKN, KKK, MS22	R	R/I	R/I
22	T266C	T1086C	None	None	**4MBR,** WKB, 2BUS, 2SBL, KKN, KKK, MS22	R	R/I	R/I

Table 13. Single nucleotide polymorphism in *R.* (*B.*) *decoloratus* CXE gene and phenotypic characteristics of various ticks (continued).

#	SNP loci in partial CXE nucleotide sequence	SNP loci in complete CXE nucleotide sequence	Amino acid substitution loci in partial CXE gene	Amino acid substitution loci in complete gene	Identity of ticks with the mutations	Resistance Phenotype		
						SP	OP	COF
23	C275T	C1095T	None	None	**4MBR,** WKB, 2BUS, 2SBL, KKN, KKK, MS22	R	R/I	R/I
24	**A282G**	**A1102G**	**N94D/G***	**N368D/G***	**2MTM, 4MBR, WKB, 2BUS, 2SBL, KKN, KKK, MS22**	**R**	**R/I**	**R/I**
25	**A283G**	**A1103G**	**N94G**	**N368G**	**4MBR**	**R**	**R**	**R**
26	**C285A**	**A1105G**	**P95T**	**P369T**	**2MTM**	**R**	**S**	**S**
27	T287C	T1107C	None	None	2MTM	R	S	S
28	**T295C**	**T1115C**	**L98P**	**L372P**	**4MBR**	**R**	**R**	**R**
29	T296C	T1116C	None	None	WKB, 2BUS, 2SBL, KKN, KKK, MS22	R	R/I	R/I
30	**G300A**	**G1120A**	**D100N**	**D374N**	**TSR, WKB, 2BUS, 2SBL, KKN, KKK, MS22**	**R**	**R/I/S**	**R/I/S**
31	**A312G**	**A1132G**	**T104A**	**T378A**	**2MTM, 4MBR, WKB, 2BUS, 2SBL, KKN, KKK, MS22**	**R**	**R/I**	**R/I**
32	**C316T**	**C1136T**	**A105V**	**A379V**	**2MTM, 4MBR, WKB, 2BUS, 2SBL, KKN, KKK, MS22**	**R**	**R/I**	**R/I**
33	G320A	G1140A	None	None	4MBR, WKB, 2BUS, 2SBL, KKN, KKK, MS22	R	R/I	R/I
34	A335G	A1155G	None	None	2MTM,4MBR, WKB, 2BUS, 2SBL, KKN, KKK, MS22	R	R/I	R/I
35	**A342G**	**A1162G**	**I114V**	**I388V**	**MS22**	**R**	**R/I**	**R/I**
36	G347C	G1167C	None	None	2MTM,4MBR, WKB, 2BUS, 2SBL, KKN, KKK, MS22	R	R/I	R/I

Non-synonymous mutation is indicated by boldface; CXE; Carboxyl esterase; Phe, phenotypic characteristics against acaricides; I: Intermediate (81-98% mortality); R: Resistant (0-80% mortality; S: Susceptible (99-100% mortality). * A282G mutation leads to amino acid substitution N94G/ N368G in 4MBR. Bold font indicates non-synonymous mutation; susceptibility of tick populations TSR (E1), WKB (C3), 2SBL (W20), 2KRH (W5), 2MTM (W13), 4MBR (W2), 2BUS (W11) determined in chapter 2.

Table 14. Number of mutations in CXE gene and survival against discriminating concentration of SP, OP and COF acaricides.

Tick ID	No. Mutations in CXE	% Survival against SP	% Survival against OP	% Survival against COF
AM08	0	0[a]	0	0
TSR*	2	21[a]	0	0
2MTM*	24	27[b]	0	0
4MBR*	26	100[a]	49	100
WKB*	24	100[a]	33	79
2SBL*	25	85[b]	0	12
2BUS*	25	100[a]	20	51
KKM	25	100[a]	94	30
KKK	25	99[a]	92	61
MS22	26	100[a]	34	16

CXE (carboxylesterase); SP [a] deltamethrin 0.05 mg/ml, [b] cypermethrin 0.05 mg/ml); OP (chlorfenvinphos 0.5 mg/ml); COF containing chlorfenvinphos + cypermrthrin 0.3/ 0.03 mg/ml for tick populations AM08, TSR, 2MTM, 4MBR, WKB, 2SBL and 2BUS; COF containing chlorpyrifos + cypermethrin, 0.5/ 0.05 mg/ml for tick populations KKM, KKK and MS22. *Tick susceptibility determined in chapter 2 (TSR/E1, 2MTM/W13, 2SBL/W20, 4MBR/W2, WKB/C3, 2BUS/W11).

Table 15. Comparison of diagnostic efficiency of *Eco* RI and *Eco* RII against synthetic pyrethroid susceptible and resistant *R.* (*B.*) *decoloratus* ticks.

Tick ID	PCR-RFLP restriction		% survival against 0.05 mg/ml (DD) deltamethrin	Resistance status based on % survival at DD
	Eco RI	*Eco* RII		
AM8	+	-	0	S
A16	-	-	0	S
AJ17	+	-	0	S
TSR*	+	-	21.3	R
2MTM*	+	-	26.5	R
2SBL*	-	+	85.0[b]	R
KMG	+	+	95.4	R
KKK	+	+	99.0	R
KMH	-	+	99.3	R
ISHM*	-	+	100	R
2KRH*	-	+	100	R
4MBR*	-	+	100	R
MS22	+	+	100	R
WKB*	+	+	100	R
KKN	-	+	100	R
KMD	+	+	100	R
1BUS*	-	+	100	R
10KRH*	-	+	100	R

(+) positive digestion; (-) no digestion; S (susceptible); R (resistant); DD discriminating dose; [b] DD of cypermethrin 0.05 mg/ml and corresponding survival for deltamethrin was 84.5%;

* Tick population whose susceptibility was determined in chapter 2 (TSR/E1, 2MTM/W13, 2SBL/W20, ISHM/W15, 2KRH/W5, 4MBR/W2, WKB/C3, 1BUS/W10, 10KRH/W9).

Chapter 4

Evidence-based tick acaricide resistance intervention strategy in Uganda: concept and feedback of farmers and stakeholders

1. Introduction

The use of pesticides in crop and animal production has greatly increased agricultural production through suppression of pest populations below the economic threshold (150). Ticks vector pathogens and cause physical damage to animals, hence the need for routine control using acaricides (55). The most economically important tick species that parasitize domestic animals in Africa include *Rhipicephalus* spp., *Rhipicephalus* (*Boophilus*) spp., and *Amblyomma* spp. These ticks vector parasites that cause fatal diseases such as theileriosis, babesiosis, anaplasmosis and cowdriosis (32). Several classes of acaricides have evolved and marketed globally to combat ticks (1). In Africa, the history of chemical tick control has been traced back to arsenic and organochlorines (85) before the introduction of OP, SP and amidine. However, persistent use of chemicals for control of ticks often leads to the selection of resistant strains (1, 58, Chapters 1-3).

In Uganda, acaricide failure due to tick resistance against organochlorine was first reported in 1970 (87). In the 1960's, Uganda had a streamlined mechanism for control of acaricide supply chain through zonation, implemented by the Ministry of Animal Industry. However, the structural adjustment programs in 1990's led to a merger of the Ministry of Animal Industry with Ministry of Agriculture, leading to the collateral loss of some of the structures and functions that supported effective tick control (124). Subsequently, lack of

national policy on ticks and tick-borne diseases control and widespread irrational acaricide use has led to the emergence of multiple acaricide resistance in especially western and central Uganda as described in Chapters 2 and 3.

The future of chemical tick control is under serious threat due to reports of emergence of multiple acaricide resistance (106, Chapter 2). Recent findings that revealed the emergence of tick resistance against ivermectin, fipronil (24, 106) and fluazuron (138) suggest that care must be taken to preserve the efficacy of the existing chemicals, lest there would be no options. Whenever acaricides fail, there is an exponential increase in tick population leading to tick worries, increase in the incidence and costs associated with treatment of tick-borne diseases (48).

Tick acaricide resistance management strategies are therefore an essential component of chemical tick control. However, lack of tick acaricide resistance surveillance data in Uganda for the last one decade meant that the country lacked the relevant information to inform strategy. This may be partly attributed to lack of a specialized laboratory for diagnosis of acaricide resistance and pragmatic monitoring of the efficacy of licensed acaricide molecules in the country. Since farmers are likely not to have knowledge on acaricide resistance, whenever chemicals fail, they are tempted to think that the acaricide in use is fake and weak. Thus, cases of increasing concentration of acaricide beyond the manufacturers' recommendation and shortening acaricide application interval from 1 week to 3 days have been practiced as a means of overcoming acaricide failure due to the perceived "fake and weak" acaricides. As shown in Chapter 2, such practices would potentially worsen acaricide resistance with possible adverse effect on public. This study, therefore, sought to develop a simple and sustainable intervention approach that can be adopted for prudent chemical tick control following emergence of tick acaricide resistance in Uganda. The study further reports the perception of farmers, extension workers and selected stakeholders in Uganda's animal

industry on whether the proposed evidence-based acaricide tick control (EBATIC) intervention approach will improve rational chemical tick control and management of acaricide failure and resistance in Uganda.

2. Materials and methods

2.1. The study area

This study was carried out in Adjumani, Mbarara, Mitooma and Rukungiri districts where chemical tick control practices were investigated in Chapter 1 (Fig. 1). The same districts were used to extend an intervention approach aimed at creating awareness on acaricide resistance, enhancing farmers' knowledge on prudent chemical use and building a technical support system for diagnosis of acaricide resistance towards prompt intervention. The three districts in southwest included Mbarara, Mitooma, and Rukungiri while Adjumani district is located in northwestern Uganda. Southwestern Uganda is the backbone of the country's dairy industry and contributes up to 25% of the total milk production (11). The population of cattle in Mbarara and Rukungiri district were estimated as 149,992 and 60,061, respectively (108). In Mitooma district, it was estimated that 19.5% of the household own cattle as part of mixed (crop-livestock) farming (160). Adjumani district, on the other hand was reported to have 105,229 heads of cattle in the 2008 livestock census (108). The three districts in southwestern Uganda have been confirmed to have acaricide resistant ticks in Chapter 2 and threats of possible spread were feared. Livestock production is considered as an integral part of the household food and income security in the study areas and any surge in ticks and tick-borne diseases, especially in the southwest would not only cause worries but affect livelihoods.

2.2. Study design

This was a community action research in which an intervention approach against tick acaricide failure was developed and transferred to the community. The intervention approach included conceptualization of evidence-based tick control approach, development of

knowledge enhancement tools for farmers and animal health workers, conducting training seminars on rational chemical tick control, stakeholder workshop for creating awareness on acaricide resistance and the establishment of a specialized laboratory for tick susceptibility testing to enhance rational acaricide prescription. Knowledge transfer training seminars were implemented at community level in the respective districts and the perception of 199 participants was assessed using semi-structured questionnaires. The category of respondents who participated in the training included farmers, district extension staff (veterinary and agricultural service providers) and district administrators. A stakeholders' workshop was organized to foster dialogue on tick acaricide resistance, towards identifying actor specific solutions. The workshop was attended by participants from the four study districts, National Drug Authority (NDA), the Ministry of Agriculture, Animal Industry and Fisheries (MAAIF), National Livestock Resources Research Institute (NaLIRRI) and academic institutions. The perception of the stakeholders and key informants from the four districts on EBATIC approach were also assessed using questionnaires. The development of the intervention approach is detailed below.

2.3. Designing the intervention approach

2.3.1 Conceptualizing the approach

A conceptual framework for intervention was developed based on critical gaps in chemical tick control identified in the baseline survey in Chapter 1. The approaches were categorized as; i) development of knowledge enhancement kit for farmers and extension workers; ii) establishment of technical capacity for acaricide susceptibility and resistance diagnosis; iii) Community and stakeholders' engagement. The knowledge enhancement kit consisted mainly of posters, guide/manual on appropriate chemical tick control, farm assessment report that identified gaps in tick control and laboratory findings and

recommendations. Technical capacity building involved the establishment of the Research Center for Ticks and Tick-borne disease Control (RTC), training of laboratory personnel on tick taxonomy, rearing, bionomics and *in vitro* tick-acaricide resistance assays. Blending technical capacity and knowledge enhancement was used to deliver a unified intervention approach referred to as EBATIC, which aimed at taking the laboratory findings to the community for improved tick control outcomes.

2.3.2. Intervention

The farmers were mobilized by the veterinary departments in the four districts. Each farmer whose farm was profiled in our earlier baseline study on chemical tick control practices received their farm report and recommendations on appropriate tick control. The training workshop was organized to train participating farmers on appropriate acaricide use practices to minimize acaricide failure and prolong the effectiveness of acaricides. The major areas of training included ticks and their importance, farm structures for appropriate acaricide application, understanding instructions on acaricide bottles, proper acaricide dilution and application, acaricide safety tips, detection of acaricide failure or resistance, and procedures for collection of ticks and submission for testing. After the training, the farmers whose farms were baselined were each given a manual on appropriate chemical tick control.

2.3.3. District extension (Veterinary) staff

Technical staff under the district production (Veterinary department) participated in a separate training seminar on appropriate tick control. The Agriculture staff and district administrators from southwestern region requested to participate in the training because they also owned cattle and were concerned about the widespread tick acaricide failure in their community. Like the farmers, each of training participants received a guide/manual on

appropriate chemical tick control for strengthening their technical capacity.

2.4. Assessing perception of the participants on EBATIC approach in solving tick acaricide failure and resistance

A semi-structured questionnaire was used to assess the perception of 199 training participants (farmers, extension workers, and district administrators) who attended the training on the effectiveness of EBATIC approach in solving tick acaricide resistance. The key variables assessed included challenges with acaricide failure or presence of ticks on cattle, the usefulness of the training session in enhancing their knowledge on appropriate chemical tick control and whether they would recommend EBATIC intervention to other farmers. The participants also reflected on their own irrational acaricide application practices in relation to the knowledge acquired during the training and proposed areas they will improve. In addition, 12 district technical staff was randomly selected after the training to rank their level of satisfaction with the performance of EBATIC intervention approach and whether they would integrate it into the district extension system.

2.5. Engagement of stakeholders in the animal industry and their perception

A one-day feedback workshop on EBATIC intervention was organized and actor specific solutions were proposed through group discussions. Separate groups included; i) Local government (district) veterinarians and farmers' representatives; ii) Veterinary pharmaceutical drug suppliers; iii) Regulatory bodies (NDA and MAAIF); iv) National research and training institutions (NaLIRRI and Makerere University). Each category of groups was tasked to discuss and propose short-to-medium (2-3 years) and long-term (over 4 years) intervention strategies against tick acaricide resistance. In addition, a semi-structured questionnaire was used to assess the perception of 47 participants on the effectiveness of the

EBATIC approach in addressing acaricide resistance in Uganda.

2.6. Data analysis

The responses from the questionnaire data were coded, entered in Microsoft excel and analyzed in SPSS version 21 (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.). The resultant statistical outputs were presented as frequency tables. Data generated in the focused group discussion were synthesized, categorized and presented as stakeholder specific recommendations in tabular format.

2.7. Ethical considerations

The study was approved by the College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University (Approval number: VAB/REC/15/104). Ticks were handled under strict internal procedure involving restriction of access to tick incubation room, autoclaving all materials used for larval packet test (LPT) or immersing them in hot water at 99^{o}C for 30 minutes. Questionnaires were administered to only those participants who consented to the study and the identities of the respondents were kept confidential. Each farmer whose farm was used for baseline data collection received a report and recommendations for improvement of tick control practices.

3. Results

3.1. Description of the EBATIC approach

The framework and mechanism through which tick control service providers, regulators and researchers can effectively work together to detect and intervene against acaricide resistance in the EBATIC approach is shown in Fig. 14. The approach involved integrated activities aimed at generating evidence to inform appropriate farm intervention against tick acaricide resistance and also foster multi stakeholder dialogue for generating ideas and solutions against tick acaricide failure and resistance in Uganda. The approach recognized the multiplicity of actors who played key role in ticks and TBD control such as district veterinarians, drug shop owners, non-governmental organizations, the drug regulator and the ministry in-charge of the animal industry and interest groups in the livestock industry. A specialized laboratory such as RTC was not only central in generating evidence (farm tick control gap appraisal and tick testing) for informing on-farm intervention but also generates the relevant scientific data for guiding regulatory actions aimed at eradication of acaricide resistance.

3.2. Feedback from participants after training on EBATIC intervention approach

The sensitization and training seminar attracted more farmers and district extension and administrative staff as shown in Table 16. A total of 199 participants attended the training, 77.9% (155/199) and 22.1% (44/199) were from the southwestern region and northwestern district of Adjumani, respectively. At the time of the seminar, 89.0% (138/155) of the participants from southwestern Uganda reported that they had acaricide failure in the last 6 months. On the other hand, 37 out of 44 farmers in Adjumani district also had ticks on their cattle. After the training, 80.9% (161/199) of the farmers reported that they gained valuable knowledge and their expectations were met. Only 6.5% (13/199) of the participants were not

satisfied with the seminar. Furthermore, 98.1% (152/155) of the participants in the southwest noted that EBATIC training impacted knowledge that is useful in fighting tick acaricide failure or resistance in their areas, 95.5% (42/44) of the participants from Adjumani also agreed that their knowledge on controlling ticks was improved. As such, 95.5% (190/199) of participants from both southwest and Adjumani district reported that they would recommend the training to other farmers. Also, 91.6% (142/155) of the respondents in southwest acknowledged that the EBATIC approach will reduce tick acaricide failure or resistance in their farms. Similarly, 88.6% (39/44) of the participants in Adjumani district noted that the knowledge acquired will help them reduce tick burden on their animals. Overall, 92.5% (184/199) of the training participants noted that they would like to see the EBATIC intervention approach sustained.

3.3. Reflection of the participants on why acaricides failed and what they will change on their farms after the training

Of the 199 participants who were asked to give their opinion about the factors that might have led to acaricide failure in their area (southwest) or presence of ticks on cattle (northwest), 119 (59.8%) participants responded while 80 (40.2%) participants declined to respond (Table 17). Of the 97 participants from southwestern Uganda who disclosed the reasons for acaricide failure in their area, lack of knowledge due to poor extension (24/97), wrong acaricide mixing due to inappropriate measuring equipments (24/97) and using double or triple concentrations (15/97) were mentioned as the main drivers of acaricide failure. Moreover, 67.1% (104/155) of the participants from the southwest pledged to take immediate action to improve gaps in tick control they have identified after the training. These actions included proper acaricide rotation and seeking advice from veterinarians (26/155), adherence to manufactures' instruction (14/155), improving fence and crush (13/155) and synchronizing

tick control with the neighbor (10/155). Overall, 35.7% (71/199) of the participants proposed that more sensitization of farmers and improved extension and teamwork were important considerations towards finding lasting solutions against tick acaricide failure and resistance.

3.4. Feedback of key informants from district veterinary department

The feedback of 12 key informants (district veterinary and agriculture staff) who participated in the EBATIC activities such as farm appraisal and training is shown in Table 18. All the key informants (100%) reported that the tick control manual, farm reports and training seminar were the key benefit of the EBATIC intervention approach. The key informants were either satisfied (5/12) or highly satisfied (7/12) regarding the content and relevance of the EBATIC manual. Similarly, 7/12 of them were satisfied and another 5/12 was highly satisfied with the relevance of the EBATIC farmers report and recommendations. Overall, the performance and importance of the EBTAIC activities in the four districts were ranked as excellent (5/12), very good (5/12) and good (2/12). As such, the idea of integration of EBATIC in their extension practice was rated mainly as very good (5/12) and excellent (4/12). They stakeholders further considered the EBATIC approach relevant (100%) in solving tick acaricide resistance and recommended that it should be rolled out (100%) to other districts.

3.5. Stakeholders feedback

The stakeholders' feedback on EBATIC approach and proposed short-to-medium and long-term intervention strategies against tick acaricide resistance is shown in Tables 19 and 20, respectively. The main short-to-medium-term solutions proposed by all the stakeholders (Table 20A) included increased farmer access to extension services, sensitization and training on appropriate chemical tick control, increased access to acaricide strength (concentration) and tick susceptibility testing services at regional level, promotion of integrated tick control

(Fig. 15), instituting local by-laws to enforce proper tick control practices, strengthening veterinary drug regulation, supporting research on ticks and TBD and increased financial allocation for ticks and TBD control. The major long-term strategies by the proposed stakeholders (Table 20B) included enacting a law to govern ticks and TBD control, introduction of new acaricide molecules and vaccines against ticks and TBD, close partnership between local researchers and veterinary drug manufactures in identifying and trial of novel products against ticks and TBD.

The feedback of the 47 stakeholders who participated in the workshop is shown in Table 19. The EBATIC intervention approach was rated by 83.0% (39/47) of the stakeholders as either very good (24/47) or excellent (15/47) in solving tick acaricide resistance challenge in Uganda. The stakeholders were mostly satisfied with the useful research findings on factors that predispose to acaricide failure or resistance and the EBATIC intervention approach (36.2%, 17/47); as well as fostering inclusive dialogue on actor specific discussions (46.8%, 22/47) aimed at finding short-to-medium and long-term solutions against tick acaricide resistance in Uganda. All the 47 stakeholders reported that the EBATIC approach will help in solving tick acaricide resistance. However, strengthening stakeholder alliance and upscaling the EBATIC approach to more farmers across the country was recommended by 66.0% (31/47) of the stakeholders.

4. Discussion

Tick acaricide resistance management is an integral part of chemical tick control. The widespread acaricide failure in Uganda is an indication that there has been lapse in strategic use of chemicals for tick control in the past one decade. One of the strategies employed in acaricide resistance management in 1960's was zonal distribution of acaricide by the central government. This essentially meant that there was regional zonation and rotation of acaricide that allowed some molecules to be reserved for the future. Thus, the initial episode of tick acaricide resistance reported in 1970's by Kitaka et al (87) was swiftly managed by switching from the organochlorine –Toxaphene to organophosphate Supona® (chlorfenvinphos) and Steladone® (chlorfenvinphos) in 1980 (131). However, the central control of acaricide supply by the Ministry of Animal Industry was lost following structural reforms and liberalization of the economy in 1990's. Divesting tick control to the local governments in 1997 and designation of ticks and TBD as private good further created a vacuum in centralized institutional arrangements for effective monitoring of acaricide resistance. The consequence of the above vacuum has now manifested as unprecedented level of acaricide resistance in western and central Uganda, amidst lack of national acaricide resistance management strategy. Given the challenges stated above, the EBATIC intervention approach (Fig. 14) was developed to enhance the knowledge of farmers and extension workers on prudent acaricide use. The core components of the EBATIC approach are discussed below;

Identify: Both government (district extension staff) and private service providers (animal health workers and drug suppliers) were recognized as pivotal in identifying acaricide failure hotspots in livestock farms or communities. Regulatory bodies (ministry responsible for animals and drug regulatory authority) also received complaint from farms on acaricide infectiveness and referred the farmers to the RTC laboratory. It was therefore evident that the

above entities formed a key intermediary between farmers and tick testing laboratory. The EBATIC approach therefore emphasizes the importance of fostering relationship between RTC laboratory and farm service providers to guarantee a sustainable information loop, tick submission and referral. However, the laboratory also initiated community outreach to identify farms with tick acaricide failure based either on the request by a concerned farmer or farmer groups.

Test: Central to the EBATIC intervention approach was the establishment of RTC as a dedicated tick acaricide resistance testing service center in Uganda. Upon sample reception, ticks were identified to species level based on morphology. The engorged ticks were incubated so that first generation larvae were produced for carrying out various panels of acaricide tests by larval packet test to identify chemicals which were effective. Comprehensive farm reports containing farm specific recommendations were compiled based on both laboratory evidence and farm tick control gaps identified. Where few engorged ticks have been collected, farmers were given reports containing recommendations based on farm tick control gaps identified during farm appraisal. The information generated from continuous testing of ticks can also be used as surveillance tool for monitoring performance of existing and newly introduced molecules, as well as informing future acaricide rotation and zonation. The importance of laboratory testing in efficacy of tick control outcomes has also been highlighted by Moyo and Masika (111). Since its establishment, RTC has received exponential number of tick submissions by both farmers and veterinarians. This has helped farmers to know the status of acaricide performance in their farms and to institute evidence-based acaricide rotation. The veterinarians who submitted samples also used the RTC results for evidence-based acaricide prescription, where possible. The benefit of evidence-based acaricide application includes increased success rate of tick control outcome and reducing losses resulting from purchase of non-effective classes of acaricides. The

establishment of RTC laboratory was a very important step towards sustainability of the EBATIC approach and tick acaricide resistance surveillance in Uganda (Figs. 14 and 15). Prescription of effective acaricide may lead to reduction in the incidence of TBD infection and losses associated with treatment of the clinical disease.

Intervene: Evidence of inappropriate farm tick control practices and where possible laboratory tick tests were key in the intervention. Three approaches were used;

Farm level: During the EBATIC pilot study, a feedback sensitization and training seminar was organized at the time of delivery of results. The intervention farmers were also given the knowledge enhancement kit which mainly included the RTC guide on appropriate chemical tick control, EBATIC brochure and poster. Based on the feedback post training (Tables 16 and 17), it was clear that the participants (mainly farmers) lacked enough information on appropriate use of chemicals for tick control. Sharing practical evidence of wrong tick control practices identified in their area enabled the farmers to reflect, realize and commit to making positive changes in both facilities and tick control practices as shown in Table 17. It was expected that the knowledge enhancement tools like the guide on appropriate tick control and brochures will positively re-enforce the commitment of farmers towards use of recommended practices for chemical tick control.

Government and private extension service providers: Since acaricide resistance was a relatively new phenomenon to some extension service providers, it was prudent that the knowledge of service providers was enhanced. A separate training seminar was conducted for technical and administrative staff on tick acaricide resistance, causes and predisposing factors for its occurrence and management strategy. They were also trained on appraisal of tick control gaps, tick collection and submission to RTC and interpretation of RTC reports. Like the farmers, each extension staff and drug shop owners were given the knowledge

enhancement kit. The importance of information in promoting rational chemical tick control was also reported by George *et al* (52).

Stakeholders' engagement and collective dialogue: The EBATIC intervention approach and its findings were shared with the stakeholders in the animal industry such as the drug regulatory authority, Directorate of Animal Resources, Veterinary pharmaceutical distributors, District Veterinarians, researchers and farmers' representatives. This helped to create awareness on acaricide resistance, EBATIC intervention approach and collective dialogue on what each actor can do to contribute towards preventing and solving tick acaricide resistance in the country.

Based on the feedback of the respondents (Tables 16 and 17), farmers' knowledge on proper use of acaricides can be achieved through mass sensitization, training and demonstration of appropriate techniques for tick control. This has to be simultaneously carried out with continuous professional development (CPD) for animal health service providers so that they are equipped with the knowledge on acaricide resistance management. It is worth noting that the deficiencies in veterinary extension services have been widely reported as one of the major constraints to animal production in Uganda (19, 80, 103, 161). A survey by International Food Policy Research Institute (IFPRI) on the state of public service delivery in Uganda reported glaring gap in both the level of access and quality of livestock extension services (80). The previous study found that a mere 11.0% of the rural households received one visit by livestock professional within a year and only 8.0% claimed to have received knowledge and expertise from visiting an extension officer (80). The researchers at the Economic Policy Research Center (EPRC) in Uganda further argued that the country's agricultural extension human resource level has reached a crisis level following the ban in the recruitment of public extension officers at district level (14). This ban has created an estimated 86.0% extension human resource deficit at sub-county level (14). Therefore,

employing more veterinarians at lower administrative units such as sub-counties will help to bridge the current gap in animal health extension service delivery in areas experiencing acaricide resistance crisis. Increased routine farm visits by area veterinarians is critical in early identification of farms with acaricide failure, inspection of animals on transit, submission of tick samples for testing and using the result to intervene early before the resistant ticks spread to neighboring areas

Eradicate: The implementation of EBATIC recommendations at farm level such as improvement of tick control practices, evidence-based acaricide rotation or both were crucial to successful management of acaricide failure. Further submission of samples for testing in the laboratory allows active and passive monitoring of performance of chemicals recommended for intervention. However, the EBATIC intervention approach may be futile for farms that had ticks that were resistant to all the acaricides on the market (multiple acaricide resistance). This implied that regulatory oversight and restricted release of acaricide molecules at a time to create reserve is essential in sustainable acaricide rotation and long-term acaricide resistance eradication program.

Strengthening regulation of veterinary drugs and acaricides to ensure professionalism in drug dispensing and promotions, pharmacovigilance and ensuring only effective molecules are in circulation was suggested as an action for the drug regulator and MAAIF (Tables 20A and 20B). However, sustainable regulation of veterinary drugs under the current unified veterinary drug regulation requires close collaboration between the drug regulator and MAAIF. Furthermore, farmers in Uganda use drug shops as an alternative extension service point, thus the technical capacity of human resources at the shop determines the quality of advice farmers get from the drug outlets. Regular inspection of veterinary drug outlets to weed out unqualified personnel is crucial. However, the most viable option for on-farm management of acaricide failure is to promote integrated tick control to reduce over

dependence on acaricides (Fig. 15). The proposed integrated approaches include rotational pasture grazing and spelling for farms with paddocked pasture, intensification of dairy cattle management with alternative feeding technologies such as silage and hay to reduce contact between ticks and cattle, immunization against theileriosis (124, 133), and rearing tick and TBD resistant breeds of cattle (82). Integrated tick control will substantially reduce the over dependence on acaricides and lessen selection pressure by ticks, thus preserving the efficacy of chemicals and reducing incidence of acaricide resistance (71, 109, 171).

The long-term strategy for control of ticks and TBD will ultimately depend on harnessing technologies like vaccines against both the ticks and TBD as suggested by stakeholders (Table 20B). Already, anti-tick vaccines have been reported to be effective in controlling acaricide resistant *R. microplus* ticks in Cuba and Venezuela (153, 164). Such existing anti-tick vaccines developed against *R. microplus* could be tested against *R.* (*B.*) *decoloratus* for possible adoption in Uganda. However, due to limited cross-protection (34), the long-term strategy should focus on establishing collaborative research between the local scientists and leading anti-tick research and development companies for identifying novel antigens from other economically important tick species such as *R. appendiculatous* and *A. variegatum* to produce a broad spectrum anti-tick vaccine. Furthermore, there is need to invest resources in research and development of vaccines against TBD, especially babesiosis and anaplasmosis based on the local strains. For example, Australia is among the countries that have used babesia and anaplasma cocktail vaccine successfully in cattle (17). For Uganda to fast-track development of such vaccine, there are needs for a deliberate policy and financial resources to support research and technology development for control of the above diseases as part of an integrated and evidence-based ticks and TBD control initiative.

5. Conclusion

This study proposed evidence-based approach (EBATIC) as a short-to-medium-term intervention pathway for management of tick acaricide resistance in Uganda. Building national laboratory and technical human capacity is pivotal in prompt detection of acaricide resistant ticks, evidence-based acaricide rotation and monitoring the efficacy of acaricide resistance eradication interventions. Moreover, both laboratory and farm-based evidence can be used to support development of community sensitization and training packages for behavioral change and adoption of appropriate tick control practices. Such efforts should be complemented with a broader stakeholder dialogue aimed at identifying actor specific solutions that will constitute a foundation for national acaricide resistance management strategy.

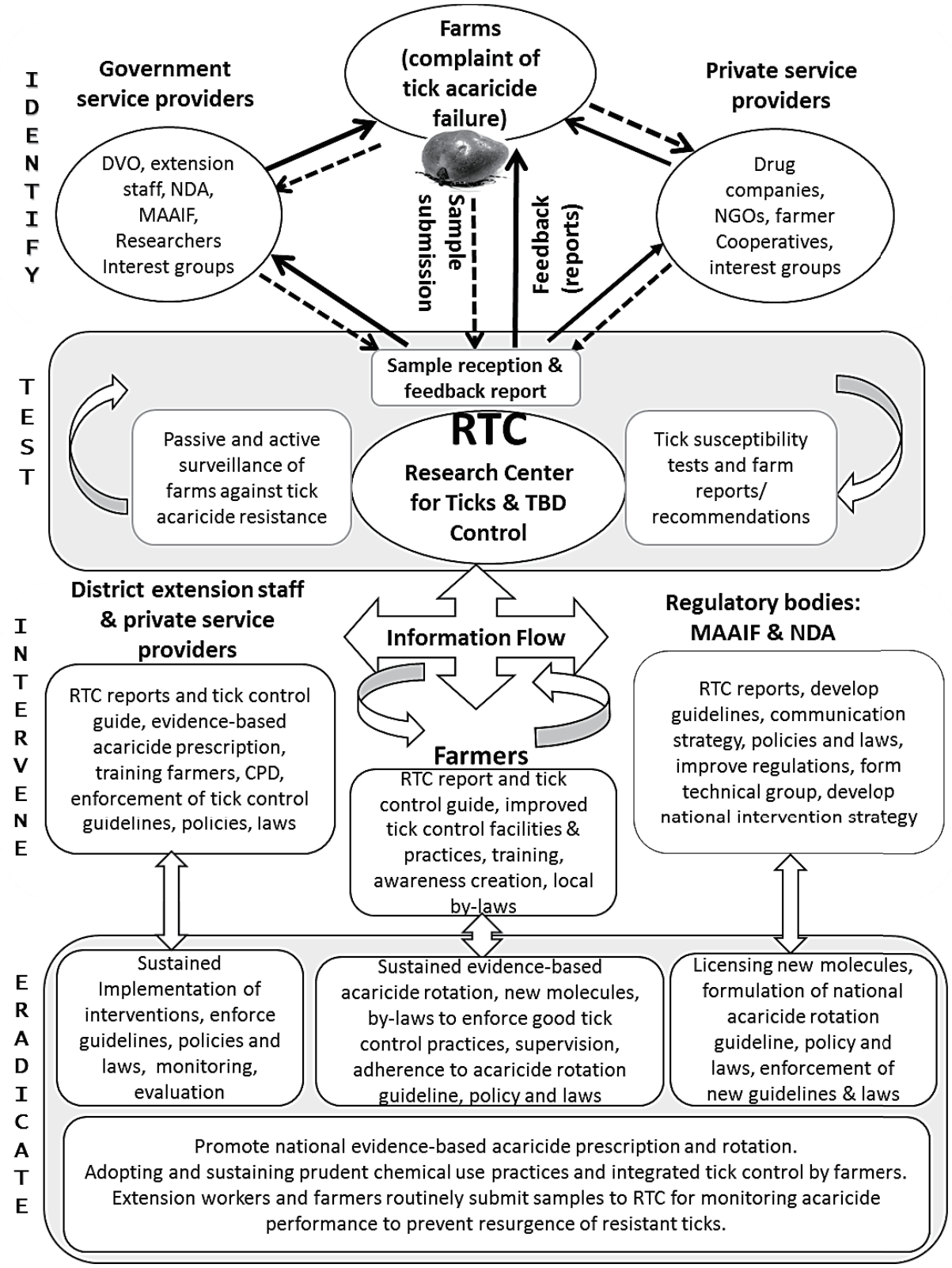

Fig. 14. Evidence-based acaricide tick control (EBATIC) intervention approach against tick acaricide resistance in Uganda.

EBATIC involves identifying farms with acaricide failure and gaps in tick control, collecting tick samples and testing in the laboratory and using the test and farm tick control gaps identified to intervene and eradicate acaricide resistance. CPD, Continued Professional Development; DVO, District Veterinary Officer; NDA, National Drug Authority; MAAIF, Ministry of Agriculture, Animal Industry and Fisheries; RTC, Research Center for Ticks and Tick-borne diseases control; TBD, Tick-borne diseases.

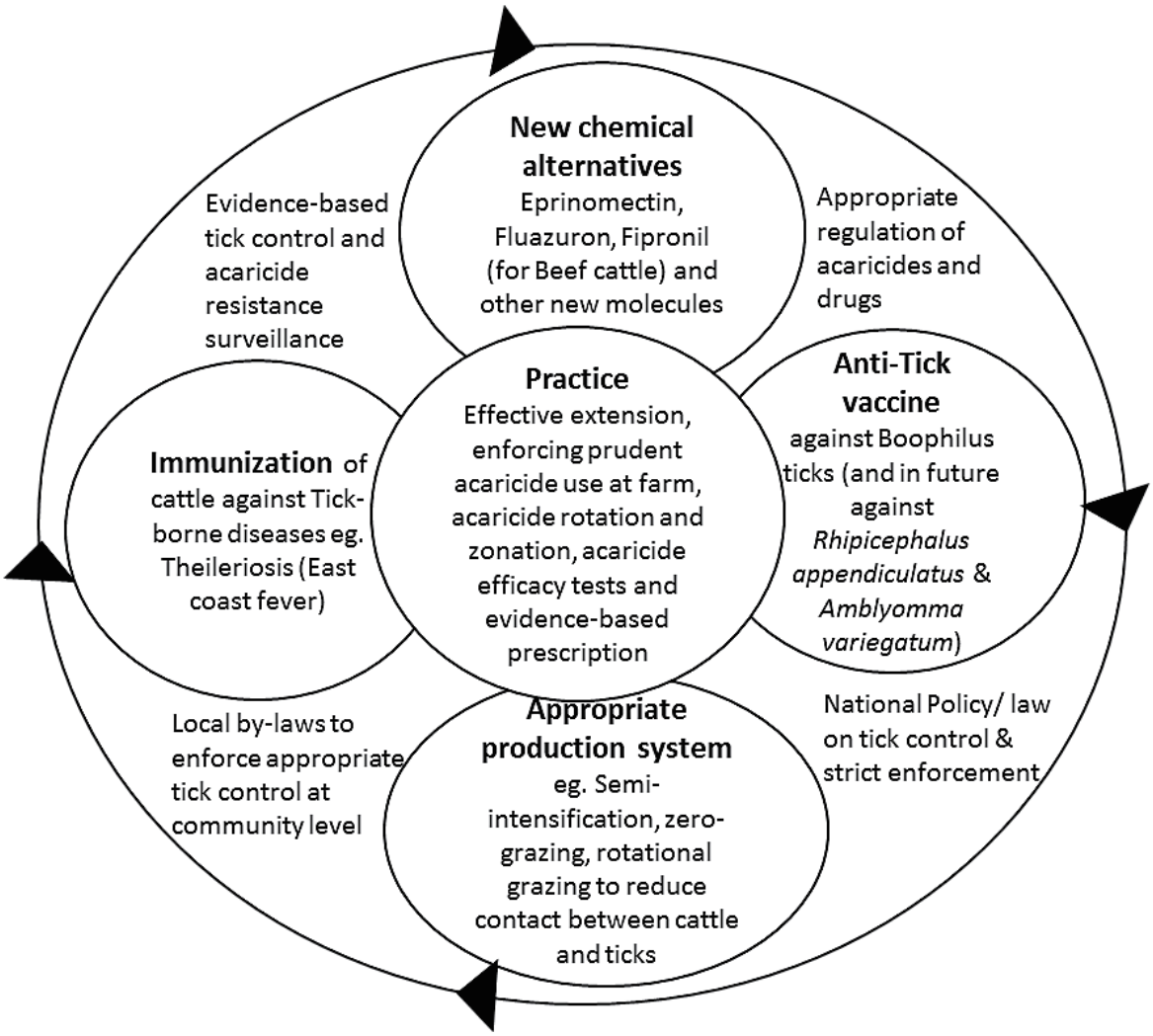

Fig. 15. Roadmap for integrated tick control approach for management of tick acaricide resistance in Uganda.

Roadmap generated during EBATIC stakeholders workshop as a pathway for management of tick acaricide resistance in Uganda.

Table 16. Feedback from the EBATIC sensitization and training seminar on acaricide resistance and appropriate chemical tick control for farmers and district extension staff.

Characteristics	Variables	Region		Total	%
		Southwestern	Northwestern		
Intervention district	Mitooma	88	0	88	44.2
	Adjumani	0	44	44	22.1
	Rukungiri	38	0	38	19.1
	Mbarara	29	0	29	14.6
Gender of participant	Male	123	40	163	81.9
	Female	32	4	36	18.1
Category of participants in the sensitization and training seminar	Farmer	100	23	123	61.8
	Animal husbandry officer	23	5	28	14.0
	No response	12	12	24	12.1
	District veterinarian	7	1	8	4.0
	District administrative officer	4	1	5	2.5
	Drug shop attendant	4	0	4	2.0
	Agriculturalist	5	2	7	3.5
Do you have challenge of acaricide failure on your farm or tick burden?	Yes	138	37*	175	87.9
	No	15	6	21	10.6
	No response	2	1	3	1.5
Were your expectations met in the sensitization/ training seminar	Yes	130	31	161	80.9
	No	7	6	13	6.5
	No response	18	7	25	12.6
Do you think this kind of seminar is useful for fighting tick in your area?	Yes	152	42	194	97.5
	No	2	1	3	1.5
	No response	1	1	2	1.0
Would you recommend other farmers for the same training?	Yes	149	41	190	95.5
	No	2	2	4	2.0
	No response	4	1	5	2.5
Do you think the EBATIC approach will reduce the tick acaricide resistance challenge in your area?	Yes	142	39	181	91.0
	No	5	3	8	4.0
	No response	8	2	10	5.0
Would you like to see the EBATIC approach sustained?	Yes	145	39	184	92.5
	No	1	3	4	0.6
	No response	9	2	11	5.5
Any suggestion regarding EBATIC approach?	Regular sensitization seminars at grassroot for farmers and extension officers	34	14	48	24.1
	Extend EBATIC model and research to other farmers and districts in Uganda	23	9	32	16.1
	Government should enact strict laws to govern acaricides use and tick control	7	0	7	3.5
	Providing extension material like the EBATIC manual to all farmers	3	2	5	2.5
	No response	88	19	107	53.8
Total		155	44	199	100.0

* No acaricide failure but presence of ticks due to irregular acaricide application

Table 17. Reflection of participants on wrong practices on the farms that might have led to acaricide failure or tick challenge and what they will do to improve tick control.

Question	Response	Region		Total	%
		Southwestern	Northwestern		
In your opinion, what are the factors that led to acaricide failure in your farm or area?	No response	58	22	80	40.2
	Wrong acaricide mixing and inappropriate measuring equipment	24	11	35	17.6
	Farmers lack of knowledge and poor extension services	24	5	29	14.6
	Irrational acaricide use (doubling or tripling concentration)	15	1	16	8.0
	Low pressure pumps use and not wetting the animal properly	8	0	8	4.0
	Improper farm structures and poor farm management	3	3	6	3.0
	Irregular spraying of animals	5	1	6	3.0
	Farmers failure to adhere to professional advice	5	0	5	2.5
	Poor acaricide regulation (all classes are on the market)	4	0	4	2.0
	Acaricide overuse for long time	4	0	4	2.0
	lack of consensus with neighbors on the type of acaricides to use	1	1	2	1.0
	Getting advice from wrong (unqualified) people	1	0	1	0.5
	Inadequate supervision by farm owners during spraying	1	0	1	0.5
	Other animals like goats and dogs are not sprayed	1	0	1	0.5
	Acaricide is washed off quickly in rainy season	1	0	1	0.5
After today's seminar, which aspect of tick control will you improve on your farm?	No response	51	21	72	36.2
	Proper rotation of acaricide and seeking veterinary advice	26	0	26	13.1
	Proper spraying to wet the animal with acaricide and reach all tick attachment sites	17	4	21	10.6
	Use right amount of acaricide and water for mixing as instructions and instructed by manufacturer	14	3	17	8.5
	Proper dilutions and change from hand spray to bucket/foot pump	7	8	15	7.5
	Fencing and improving crush and other farm structures	13	2	15	7.5
	Synchronizing day of spraying with neighbor	10	1	11	5.5
	Proper dip management and charging dip tank	7	2	9	4.5
	Proper record keeping for acaricides used	4	1	5	2.5
	paddocking and improves farm management	1	2	3	1.5
	Training my workers and those of neighboring farm on proper tick control	3	0	3	1.5
	Improve supervision of spraying on my farm	2	0	2	1.0

Table 17. Reflection of participants on wrong practices on the farms that might have led to acaricide failure or tick challenge and what they will do to improve tick control (continued).

Question	Response	Region		Total	%
		Southwestern	Northwestern		
In your opinion, how best do you think the current problem tick acaricide failure can be solved?	Sensitization of farmers and improved extension service delivery	56	15	71	35.7
	No response	49	17	66	33.2
	Team work among stakeholders and the government	17	3	20	10.1
	Follow instructions from manufacturers and veterinary professionals regarding acaricide use	12	2	14	7.0
	Proper acaricide mixing, measurement and application	8	3	11	5.5
	New policies and regulations for acaricides and tick control	5	2	7	3.5
	Proper rotation within different classes of acaricides	7	0	7	3.5
	Regular spraying of animals	1	2	3	1.5
Total		155	44	199	100.0

Table 18. Feedback of key informants on performance of EBATIC approach in appropriate control of ticks and management of acaricide failure and resistance.

Characteristics	Variable	Region		Total	%
		South-western	North-western		
Characteristics of key informants at the intervention district	Veterinary officer	2	2	4	33.3
	AHO	3	1	4	33.3
	Agriculturalist	2	0	2	16.7
	Entomologist	1	0	1	8.3
	Lab technologist	1	0	1	8.3
How did you/ your district benefit from the project	EBATIC manual, sanitization/training seminar & Farm reports	9	3	12	100.0
Rate your level of satisfaction with the content and relevance of EBATIC Tick control manual	Highly satisfied	6	1	7	58.3
	Satisfied	3	2	5	41.7
Rate your level of satisfaction with relevance of EBATIC Farm reports and recommendations	Satisfied	5	2	7	58.3
	Highly satisfied	4	1	5	41.7
Rate your level of satisfaction on EBATIC farmers sensitization seminar on tick control and acaricide resistance	Highly satisfied	5	2	7	58.3
	Satisfied	3	1	4	33.3
	Moderately satisfied	1	0	1	8.3
Rate the performance and importance of EBATIC project activities in your district	Excellent	5	0	5	41.7
	Very good	4	1	5	41.7
	Good	0	2	2	16.7
Integration of EBATIC in extension	Very good	2	3	5	41.7
	Excellent	4	0	4	33.3
	Fair	2	0	2	16.7
	Good	1	0	1	8.3
Rate relevance of EBATIC in solving acaricide resistance	Highly relevant	7	0	7	58.3
	Relevant	2	3	5	41.7
Should EBATIC be rolled to other districts with tick challenge?	Yes	9	3	12	100.0

AHO, Assistant Animal Husbandry Officer; EBATIC, Evidence based tick acaricide control; Lab, Laboratory

Table 19. Perception of stakeholders' on the relevance of EBATIC intervention approach in solving tick acaricide resistance in Uganda.

Characteristics	Variables	Frequency	%
Gender of respondents	Male	40	85.1
	Female	7	14.9
Category of stakeholders in the EBATIC dialogue workshop	Extension service provider	8	17.0
	Academia	7	14.9
	Regulatory body (NDA & MAAIF)	5	10.6
	Research institution	3	6.4
	Farmer representative	3	6.4
	Farmers' cooperative union	1	2.1
	Non-governmental organization	1	2.1
	Pharmaceutical representative	1	2.1
	Others	18	38.3
Rate relevance of EBATIC initiative solving the current tick acaricide resistance challenge in the country	Very good	24	51.1
	Excellent	15	31.9
	Good	7	14.9
	Fair	1	2.1
Which part of EBATIC approach satisfied you most	Stakeholders' focused group discussions and sharing	22	46.8
	EBATIC model , its research findings and suggested way forward	17	36.2
	Farmer representative presentation	6	12.8
	Understanding acaricide classes and rotation	1	2.1
	Collaboration with partners	1	2.1
Do you think EBATIC approach will help in solving acaricide resistance in Uganda?	Yes	47	100.0
If yes, how can it be fully operationalized and sustained?	Stakeholders alliance and synergy to solve tick resistance and TBDs	18	38.3
	Upscaling EBATIC approach to more farmers and other districts	13	27.7
	Regulatory bodies should be strict and enact the policies for tick control	8	17.0
	Multiple farmer sensitization seminars across districts affected by tick resistance	7	14.9
	No response	1	2.1
Total		47	100.0

NDA-National Drug Authority; MAAIF- Ministry of Agriculture, Animal Industry and Fisheries

Table 20A. Short-to-medium-term intervention strategies against tick acaricide resistance proposed by stakeholders during EBATIC workshop.

Stakeholders	Short-to-medium-term strategies for acaricide resistance management (2-3 years)
Local government and farmers' representatives	- Mobilization and sensitization of cattle farmers and leaders on acaricide resistance management. - Adequate staffing of extension staff at district and sub county level. - Supervision and inspection of veterinary drug shops by DVOs and NDA. - Regular feedback meeting with stakeholders on acaricide resistance interventions. - Put in place Bi-laws to ensure proper tick control at community level. - Clear channel of information sharing and dissemination on acaricide resistance management strategies. - Renovation and supervision of communal cattle dipping where possible. - Intensification of zero grazing practices where applicable.
Pharmaceutical actors	- Continue availing quality products to the market. - Sensitize farmers on proper application of the acaricides and the recommended equipment and structures required for tick control. - Submission of tick to and acaricide samples to relevant stakeholders (RTC, NaLIRRI, MAAIF, and NDA) to enhance the EBATIC program. - Recommend the right acaricide to the farmers based on the proper analysis of the history of acaricide use on the farm and test results from the laboratory. - Discourage mixing of different molecules while spraying animals. - Uphold professionalism in promotion of acaricides and other pharmaceutical products. - Cost effective products in form of a range of volumes that are friendly to all farmers. - Provide calibrated measuring cups attached to each acaricide bottle.
The Regulators (Ministry of Agriculture, Animal Industry and Fisheries (MAAIF) and National Drug Authority (NDA))	- MAAIF and NDA should promote integrated tick control. - MAAIF should secure financial resources both locally (Ministry of Finance) and internationally (donors) for intervention program. - MAAIF to co-ordinate different stakeholders in tick and tick borne disease management to build on the EBATIC approach. - Conduct massive community sensitization and training together with all the actors. - Continued professional development to the extension workers on tick acaricide resistance management and EBATIC approach. - Strengthening the extension and regulatory services through increased recruitment of veterinarians in both MAAIF and NDA. - Re-instituting the Uganda Veterinary Board to regulate standard of personnel in Veterinary drug outlets so as to weed out quarks who misadvise farmers.
National Research and training institutions	- Collaborative mapping of tick acaricide resistance to identify acaricide resistance hotspots and the classes of chemicals resisted. - Assessing the economic losses associated with acaricide failure and resistance, as well as tick-borne diseases in the affected areas. - Sharing information and experience among researchers on ticks and tick-borne disease control research to avoid duplication of efforts. - Further building of the capacity of both NaLIRRI, RTC and regional laboratories to be able to offer acaricide susceptibility services at large scale towards sustaining the EBATIC approach. - Sensitization of key stakeholders in the country based on available findings. - Formation of acaricide resistance working group to advance research and information needed by stakeholders and inform policy.

DVO, District Veterinary Officer; EBATIC, Evidence-Based Acaricide Tick Control; MAAIF, Ministry of Agriculture, Animal Industry and Fisheries; NaLIRRI, National Livestock Resources Research Institute; RTC, Research Center for Ticks and Tick-borne Diseases Control; NDA, National Drug Authority.

Table 20B. Long-term intervention strategies against tick acaricide resistance proposed by stakeholders during EBATIC workshop.

Stakeholders	Long-term strategies for acaricide resistance management (> 4 years)
Local government and farmers' representatives	- Decentralization of acaricide strength testing facilities at regional laboratories. - Establishment of demonstration farms (field schools) for training farmers on appropriate technologies for tick and tick-borne diseases control. - Sustaining integrated tick control.
Pharmaceutical actors	- Introduction of new molecules on the market with different mode of action from the ones available in Uganda. - Link experts in the manufacturing industry to the researchers in Uganda to enhance synergy in testing novel products.
The Regulators (Ministry of Agriculture, Animal Industry and Fisheries (MAAIF) and National Drug Authority (NDA))	- Advocating for tick and TBD control policy. - Strengthening regulations at importation, distribution and use of the acaricides. - Review of the Veterinary Surgeons Act 1958, to strengthen professional ethics in the practice. - Reviewing the Animal Disease Act, should consider issues of tick acaricide resistance management. - Lobbying for resources by the Ministry of Agriculture, Animal industry and fisheries to carryout mass tick acaricide resistance intervention program.
Research and training institutions	- Collaboration with international research groups with experience in tick acaricide resistance research. - Vaccine research against ticks and tick-borne diseases as part of integrated tick control. - Search on alternative chemicals and natural products against ticks. - Collaboration with Pharmaceutical industry to try new novel products against ticks and tick-borne diseases.

General discussion and conclusion

The importance of ticks and TBD as a constraint to livestock production in Africa is well documented (72, 88, 118, 123). Despite their importance, ticks and TBD receive the lowest resources for their control by governments in Africa (134). Moreover, there are reports that suggests that the economic distress caused by ticks and TBD will continue to rise due to climate change that favors tick population dynamics (45, 128). This means that countries in Africa need to increase financial allocation for controlling ticks and TBD. In Uganda, ticks and TBD such as ECF, anaplasmosis and babesiosis are recognized among the leading causes of cattle mortalities and farm losses (23, 112, 114, 123, 126). Contrary to their importance, ticks and TBD control receives limited support from the central government in Uganda. Thus, the responsibility of controlling ticks and TBD lies in the hands of individual farmers. This includes costs of construction of farm structures (spray-race, dip and crush) and purchase of inputs for tick control such as equipments, acaricides and drugs for treatment against TBD. The government's role is to register and quality assures drugs and acaricides as well as employing veterinarians at local governments to provide extension services on disease control and treatment. But with declining level of extension services across the country, farmers have been largely left on their own to battle ticks and TBD as reported in this study (Chapters 1, 2 and 4) and other reports (14, 80). This is contrary to the tick and TBD control strategies employed in 1960-1970s, where the responsibility of tick control was actively affected by the government (131). The old arrangement of the 1970s ensured that government established communal dips and functional structures for mandatory tick control and centralized supply of acaricide supply (112, 124, 131). The controlled acaricide supplies enable zonation and rotational use of acaricides (124, 131).

Under the current private sector-led drug and acaricide supply system, veterinary drug

outlets and farmers have more role in veterinary drug and acaricide supply, dispensing and use. This consequently led to flooding of the Ugandan market with all the classes of acaricides and numerous generic brands, without any restriction and creating future reserve as reported in Chapters 1 and 2. The irrational use of SP, OP and amitraz at the same time in the country may lead emergence of complex multiple acaricide resistance crisis that will take very long time to be solved.

This study found that acaricide resistance by *R. appendiculatus* and *R. (B.) decoloratus* has emerged in southwestern and central Uganda (Chapters 2 and 3). Stable resistance against SP were wide spread across cattle farms in the two regions. Moreover, detection of resistance against co-formulations containing SP and OP, amitraz and OP (in Chapter 2) is an indication that multi-acaricide resistant ticks have emerged in Uganda. The current study predicts that the rate of development and spread of multi-acaricide resistant ticks will increase if the wrong acaricide licensing and application practices reported in Chapters 1, 2 and 4 continue unabated. Other factors that will accelerate the rate of spread of resistant tick will include poor regulation of animal movement and unstreamlined acaricide use within neighboring farms where tick exchange occurs. Therefore, whenever acaricide resistance emerges, the responsibility of its control should be taken over by the government so as the curb the practices that promotes further emergence and spread of the resistant ticks. The most feasible solution against multiple acaricide resistance lies in tactical regulatory withdrawal of some of the failed molecules and co-formulations, promoting prudent acaricide use and adoption of integrated tick control approaches.

Withdrawal of already resisted acaricides is one of the ways through which selection pressure can be reduced or even abolished. In Chapter 3, this study has demonstrated that *R.* (*B.*) *decoloratus* ticks in southwestern and central Uganda have acquired super knock-down resistance (*super-kdr*) mutation in the SP target-sodium channel. The same highly SP-resistant

ticks also exhibited high levels of non-synonymous mutations in the partial gene of the pyrethroid metabolizing enzyme CXE. The implication of the above mutations on the time required for SP resistance to recede in the absence of further selection pressure is not known. Further research is therefore recommended to investigate the acaricide rotation strategy and SP withdrawal period that can alleviate the *super-kdr* in the sodium channel and multiple mutations in the CXE gene. A study done by Jonnson *et al* (77) revealed that rotation of amitraz with spinosad led to loss of resistance within a period of only two months. This suggests that rotational acaricide application is very important in managing amitraz resistance. Therefore, similar studies for amitraz, SP and OP-resistant tick populations are recommended in Uganda.

This study also attempted to develop the most feasible short-to-medium term intervention strategy against tick acaricide resistance based on the challenges identified in Chapters 1 and 2, in Uganda. The EBATIC: Identify, Test, Intervene and Eradicate (IT-IE) was conceptualized, developed and operationalized at community level. The established RTC Laboratory for testing ticks against acaricides helped to resolve the challenge of irrational acaricide prescription. Since its establishment, RTC has continued to receive samples from farms and the test results helps to guide decisions on the choice of acaricide rotation and interventions against acaricide failure. Continuous engagement of farmers, extension workers, regulators and ministry in-charge of animal sector, towards promoting the EBATIC approach is crucial for its sustainability. Already, the national drug authority is promoting the EBATIC approach and regularly recommends farmers and veterinarians to submit tick samples for testing.

(http://www.nda.or.ug/files/downloads/BRIEF%20ON%20CURRENT%20ACARICIDE%20RESISTANCE.pdf). The Directorate of Animal Resources (DAR) in MAAIF, in its press release on tick acaricide resistance, also recommended farmers to submit ticks for testing at

RTC Laboratory so as to identify the effective acaricides to control the resistant ticks. With increased adoption of the EBATIC approach, training of farmers on prudent use of acaricides and adoption of integrated tick control strategies, there is more hope that acaricide resistance can be overcome. However, further research is recommended on the most appropriate intervention strategy against ticks that are resistant to all the three conventional classes of acaricides: SP, OP and amidine. Clinical trial of anti-tick vaccine (125) and other alternative chemicals such as zero-withdrawal period macrocyclic lactone against the multi-acaricide resistant ticks are needed. The long-term solution against tick acaricide resistance in Uganda will include reforms in the regulation of drugs/acaricides that creates reserve molecule, increasing farmers' access to quality veterinary extension, introduction of policy on ticks and TBD control in which the government plays active central role, professionalism in supply and dispensing of acaricides and adoption of integrated tick control as described in the EBATIC approach.

Overall, the finding in this study also provides useful insight into the growing challenge of acaricide resistance in Africa (4, 13, 165, 170). The level of resistance reported in Uganda should be taken as the tip of ice-berg in the east African region and the tropical areas of Africa. With increasing livestock trade between neighboring countries and across Africa, there is need for regional bodies such as Interafrican Bureau for Animal Resources (AU-IBAR), regional economic blocks in Africa, World Animal Health (OIE) and Food and Agriculture Organization (FAO) to recognize the growing threat of tick acaricide resistance to the livestock industry and public health in Africa. This should lead to formation of *inter alia* regional consortium and technical working group against tick acaricide resistance in Africa. This will ensure that the threat of complex resistance scenario that presents as crisis to the livestock industry is either prevented or controlled through prompt intervention.

In conclusion, this study provided useful baseline information on both farm-based and

regulatory errors that could have additively favored the emergence and spread of acaricide resistant ticks in Uganda. The short-to-medium-term solutions involved enhancing the knowledge of farmers and veterinarians on on-farm acaricide resistance management strategies and upscaling the EBATIC: IT-IE intervention strategy for rational acaricide rotation. However, the long-term solutions against tick acaricide resistance in Uganda depends on the pace of implementing reforms in acaricide regulation to restrict the spectrum of acaricide molecules licensed, as well as development and implementation of a national acaricide resistance management strategy to prevent further spread of the resistant ticks across the country.

General summary

Ticks and TBD are among the leading constraints to livestock production in Africa. TBD such as theileriosis/ECF, babesiosis and anaplasmosis present a major threat to the productivity of especially exotic dairy and beef cattle. In Uganda, over 30% of the calf crop is lost to TBD. Ticks and TBD control also accounts for 85.6% of the total disease control costs and over 60% of the total farm inputs. Given the year round abundance of ticks due to the favorable climatic condition, cattle farmers rely extensively on acaricides to control ticks and prevent TBD. Historically, chemical tick control in Uganda has evolved from the use of arsenic and organochlorines to the newer molecules such as OP, SP and amidine. In 1960-1970s, acaricide supply and use was highly controlled by the central government through zoning and rotation. However, the structural adjustment programs in early 1990s led to the liberalization of veterinary drugs, making the supply of acaricides private sector-led, through pharmacies and drug shops. The government only licenses, assures and monitors the quality of acaricide through the National Drug Authority (NDA). Over the last 2 decades, the increase in the population exotic cattle that are susceptible to TBD has raised the demand for acaricides. This has led to massive influx of various brands of the three classes of acaricides on the market. With apparently no policy to control the flow of acaricides coupled with poor extension services, irrational use of acaricides became wide spread at farm level. The inappropriate acaricide application practices have led to unprecedented level of acaricide failure and surge in TBD in central and western Uganda. In the absence of a national acaricide resistance management strategy, the country's dairy and beef industry is threatened by the increased burden of ticks and TBD. Therefore, this study examined the chemical tick control practices, determined the tick susceptibility to acaricides and genetic basis of stable SP-resistance by *R.* (*B.*) *decoloratus* ticks. The study further developed a rapid diagnostic

method for detection of pyrethroid resistance and established a stakeholder-centered conceptual framework for intervention against tick acaricide resistance in Uganda.

In Chapter 1, the chemical tick control practices by cattle farmers in southwestern (Mbarara, Mitooma and Rukungiri districts) and northwestern (Adjumani district) Uganda was assessed to identify wrong practices that predispose to acaricide failure. A total of 85 farms were assessed and ticks were collected to determine species distribution. It was found that all the farms used chemical method for tick control. However, farmers in southwestern districts (exotic cattle keepers) used acaricides extensively compared to those in northwestern district whose indigenous cattle were naturally resistant to TBD. The low acaricide pressure in northwestern district was associated with diverse tick population unlike in southwestern districts where only *R. appendiculatus* and *R.* (*B.*) *decoloratus* ticks dominated, possibly due to high acaricide pressure and selection of resistant phenotypes. Inappropriate chemical tick control practices were widely encountered in southwestern districts among farms with or without complaint of acaricide failure. It was concluded that wrong acaricide application practices were widespread in both southwestern and northwestern Uganda although acaricide failure in southwestern districts was possibly due to suspected acaricide resistance.

In Chapter 2, acaricide failure was investigated in 54 farms from 17 districts in Uganda. The acaricide resistance status of 31 tick populations collected from 30 farms was determined using larval packet test (LPT). The LPT test was carried out at discriminating dose (DD) and 2 × DD of five panels of commercial acaricide molecules belonging to amidine, SP, OP and OP-SP co-formulations. Overall, 94.4% (51/54) of the farms had history of acaricide failure and resistance was detected in 93.5% (29/31) of tick populations (*R. appendiculatus* and *R.* (*B.*) *decoloratus*) tested. 90.0% (27/30) of the tick populations tested were resistant to SP. Worryingly, 63.0% (19/30) of the above ticks were super resistant (0% mortality) against 2 × DD cypermethrin. Acaricide resistance to at least 2 molecules (multi-acaricide) was detected

in 55.2% (16/29) of the resistant *Rhipicephalus* ticks. Multi-acaricide resistance was significantly ($p < 0.05$) associated with *R.* (*B.*) *decoloratus* ticks, use of both SP and COF in the last 2 years, and Kiruhura district. It was concluded that super SP-resistant and multi-acaricide resistant *Rhipicephalus* ticks have emerged in Uganda and amitraz was the efficacious acaricide against SP and COF-resistant ticks. However, LPT was time consuming, thus genetic studies for understanding basis of SP resistance and developing a quick diagnostic method was necessary.

In Chapter 3, the genetic basis of stable SP resistance by *R.* (*B.*) *decoloratus* was investigated and novel diagnostic mutations for rapid detection of SP resistance was developed and validated. Genomic DNA was extracted from 20 larval populations (19 of known SP susceptibility and 1 unknown susceptibility). The voltage sensitive sodium channel (VSSC) domain II (SP target) and partial carboxylesterase (CXE) (SP metabolizing enzyme) genes were amplified by PCR, cloned and sequenced. A *super knock-down resistance* (*kdr*) mutation T58C in *R.* (*B.*) *decoloratus* VSSC was associated with stable SP resistance. Furthermore, multiple non-synonymous mutations in CXE of SP-resistant ticks were identified. One of the mutations conferred a novel *Eco* RII (G195C) restriction site for restriction fragment length polymorphism (RFLP) detection of SP resistance. It was deduced that the *super-kdr* mutation in the VSSC and multiple mutations in CXE genes may concurrently mediate stable resistance against synthetic pyrethroids in *R.* (*B.*) *decoloratus* ticks from Uganda. The *Eco* RII based RFLP seemed to be a useful diagnostic tool for rapid detection of stable SP-resistant *R.* (*B.*) *decoloratus* ticks. However, devising intervention strategies against acaricide resistant ticks would be essential.

In Chapter 4, a short-to-medium-term intervention approach called EBATIC: Identify, Test, Intervene and Eradicate (IT-IE) was developed. The approach involved establishment of a specialized tick laboratory for identifying and testing (IT) ticks for prompt intervention and

acaricide resistance surveillance. Intervention and acaricide resistance eradication (IE) were centered on using the laboratory test results and farm tick control gaps identified in guiding acaricide resistance management strategies such as evidence-based acaricide rotation, development and dissemination of extension materials, training of farmers and extension workers, and stakeholders' dialogue. Feedback on the EBATIC approach revealed that all the 47 stakeholders and 91.0% (181/199) of the farmers and extension workers reported that EBATIC will help in solving the tick acaricide resistance crisis in Uganda. Overall, the positive feedback from farmers, district veterinarians and stakeholders in the animal industry suggested that the EBATIC approach was a useful proof-of-concept on scalable intervention pathway against tick acaricide resistance in Uganda with possibility of adoption in other African countries.

In conclusion, these findings suggest that uncontrolled acaricide supply, wide spread inappropriate acaricide use and inadequate knowledge of farmers were the possible operational factors that precipitated acaricide failure. The emergence of multiple acaricide resistant *Rhipicephalus* ticks complicates effectiveness of farm tick control efforts. However, laboratory testing of ticks to support evidence-based acaricide rotation and enhancing the knowledge of farmers on appropriate acaricide use was proposed as the short-to-medium-term intervention strategy against tick acaricide resistance in the country. The proposed long-term strategies include promoting integrated tick control, establishing a national tick control policy and acaricide resistance management strategy for sustainable control of ticks and TBD in Uganda.

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