International Journal of Animal and Veterinary Advances 2(2): 51-58, 2010
ISSN: 2041-2908

Submitted Date: February 13, 2010 Accepted Date: March 05, 2010 Published Date: April 15, 2010

Prevalence and Characterization of *Theileria* and *Babesia* Species in Cattle under Different Husbandry Systems in Western Uganda

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Abstract: A total of 363 cattle taken from six sub counties of Kashaari county were tested for presence of *Theileria* and *Babesia* species using reverse line blot hybridization assay (RLB). The prevalences of *Theileria* and *Babesia* species were found to be 19.8% (CI = 95%, 15.7-23.9%) and 0.6% (CI = 95%, -0.2-1.4%) respectively with at least 68% (CI = 95%, 63.2-72.8) dually infected with more than one *Theileria* sp. *Theileria* sp. detected include; *T. parva*, *T. mutans, T. taurotragi, T. vilifera, T. buffeli, T.* spp. (*sable*), T. spp. (*buffalo*) and *T. bicornis* at 24% (CI = 95%, 19.6-28.4%), 18.4% (CI = 95%, 14.4-22.4%), 14% (CI = 95%, 10.4-17.6%), 13.7% (CI = 95%, 10.2-17.2%), 12.6% (CI = 95%, 9.2-16.0%), 10.4% (CI = 95%, 7.26-13.54%), 4.4% (CI = 95%, 2.3-6.5%) and 3.8% (CI = 95%, 1.8-5.8%) respectively. The prevalences of different *Theileria* and *Babesia* species among different cattle age groups, breeds, management systems and sub county of origin are presented and discussed. A 2.5 times risk of infection associated with cross bred cattle (OR = 2.5, 95% CI; 1.44-4.49) compared to that of local and exotic breeds was observed on logistic regression. Regardless of type of cattle breed; rate of acaracide application, restriction of calf movement, restricted grazing (paddocking) and zero grazing were the most important parameters that determined the risk of infection with TBs. RLB detected infections in animals which were negative by *Theileria* and *Babesia* Genera specific PCR. Such animals had low parasitemia that could not be detected by such non species-specific PCR. RLB is therefore a very sensitive and specific diagnostic tool that should be adopted in tick-borne hemoparasite epidemiological studies in Uganda.

Key words: Age, breed, Kashaari county (Uganda), management system, prevalence, reverse line blot hybridization, tick-borne diseases

INTRODUCTION

Theileria and *Babesia* species are among the major piroplasms of cattle and small ruminants (Uilenberg, 1995; Gubbels *et al.*, 1999; Oura *et al.*, 2004; Oura *et al.*, 2005). Theileriosis and babesiosis cause significant economic losses in tropical and sub-tropical regions of the world (Uilenberg, 1995; Kursat *et al.*, 2004; Jongejan and Uilenberg, 2004). The recent drive to improve Ugandan local cattle breeds through importation of high yielding dairy breeds for cross breeding has often been challenged by high mortalities due to ticks and tick-borne diseases (Loria *et al.*, 1999; Georges *et al.*, 2001). As a result, cross breeding programs have not yielded the expected benefits, in part, due to theileriosis and babesiosis.

Tick-borne piroplasm prevalence studies so far carried out in Uganda have used serological techniques (Katende *et al.*, 1998; Rubaire-Akiiki *et al.*, 2004; Rubaire-Akiiki *et al.*, 2006; Kabi *et al.*, 2008). The main weaknesses of serological tick-borne disease diagnosis have been reported as cross reactivity, low specificity and sensitivity and being poor at detecting low piroplasm levels in carrier animals (Papadopoulos *et al.*, 1996; García-Sanmartín *et al.*, 2006). The aim of this study was to determine the prevalence of different *Theileria* and *Babesia* species in cattle from Kashaari county-Mbarara district (Uganda) (Fig. 1) using RLB. We further explored how the prevalence of these important cattle tick-borne hemoparasites varies with cattle age group, breed, management system and location (sub county) of the farm. To understand the best predictor(s) of infection with *Theileria* and *Babesia* species, we ran a logistic regression analysis and established maximum likelihood estimates associated with age, breed, management system and sub county of origin with regard to tick-borne hemoparasite prevalence hence demonstrating that management system is the best predictor of cattle infection with both *Theileria* and *Babesia* species. This study has therefore improved on the accuracy of the prevalence data on *Theileria* and *Babesia* species in this cattle keeping part of the country by use of reverse line blot hybridization assay; a very highly sensitive and specific molecular tool (Gubbels *et al.*, 1999; Georges *et al.*, 2001; Bekker *et al.*, 2002; Oura *et al.*, 2003; Nagore *et al.*, 2004a; Nagore *et al.*, 2004b; García-Sanmartín *et al.*, 2006). Such accurate tick-borne pathogen prevalence data is essential in

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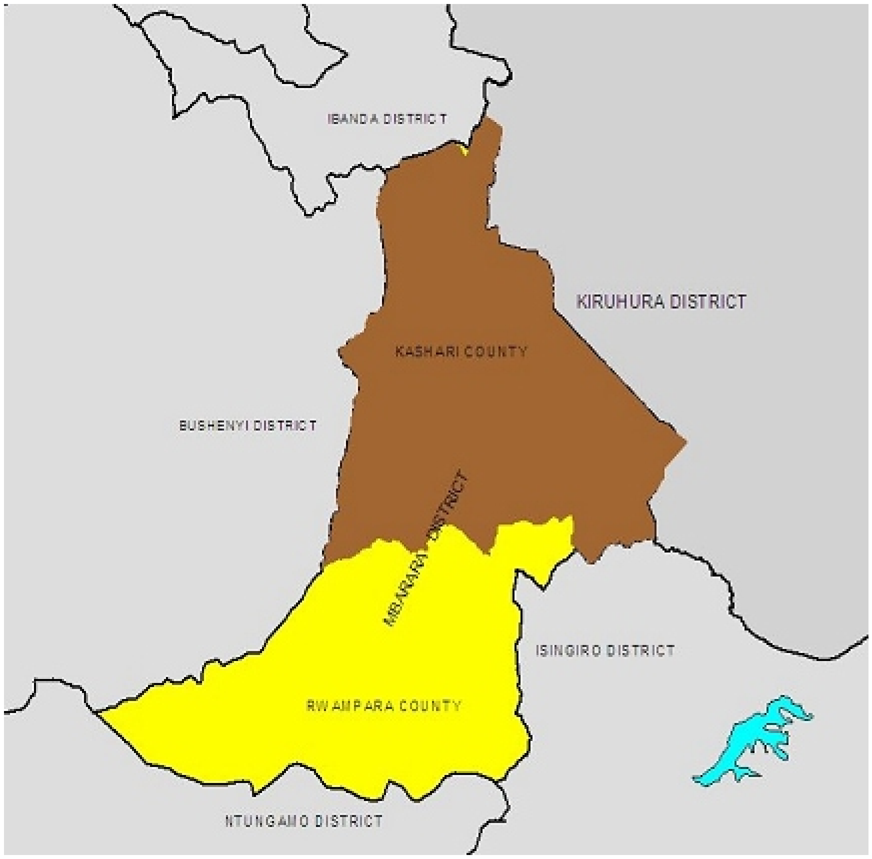

Fig. 1: Map of Mbarara district showing the locatioon of kashaari county

formulation of control strategies especially basing on future use of multivalent subunit vaccines (Morzaria *et al.*, 2000; Oura *et al.*, 2003). Future use of multivalent subunit vaccines is envisaged to be one of the most needed approaches in tick-borne disease control especially having realized that live and attenuated vaccines have been faced by an array of setbacks (Delafuente *et al.*, 2002). Such targeted tick-borne disease control programs are very important especially in the leading livestock production areas of western Uganda and other parts of Africa.

MATERIALS AND METHODS

Study area: This study was carried out in Kashaari county-Mbarara district (Fig. 2) in southwestern Uganda from January to the end of March 2008. The study area was purposely selected because cattle keeping is a major activity in the livelihood of the people in the area. The mean minimum and maximum annual temperatures are 14.6 and 26.3ºC, respectively with annual rainfall of 822 mm occurring in 114 rainy days in the year shared in two rainy seasons (March–May and September–December). The general climatic conditions are favorable for dairy production (Faye *et al.,* 2005). At the time of the study, Kashaari county (Fig. 2) had an estimated cattle population of 102,143 in 3752 herds.

Sampling and sample size determination: Six of the 9 sub counties of Kashaari county were selected using computer generated random numbers (sub counties were Biharwe, Bubaare, Kakiika, Kashare, Rubaya and Rwanyamahembe). The animals sampled were stratified according to age, breed, management system and sub county of origin as prevalence of TBs was to be analyzed against these variables. The used expected prevalence of tick-borne diseases as taken from literature (Oura *et al.*, 2003, Oura *et al.*, 2004; Oura *et al.*, 2005) was 50 %, N (population size) =102,143 heads of cattle. Sample size was calculated using Win Episcope 2.0 Software at accepted error of 5%, at 95% level of confidence. 1ML of jugular blood was collected into EDTA coated vaccutainer tubes. Blood samples were transported on ice to the laboratory for further storage and processing. In the laboratory, blood samples were stored at -20ºC until required for PCR. In total 363 blood samples were

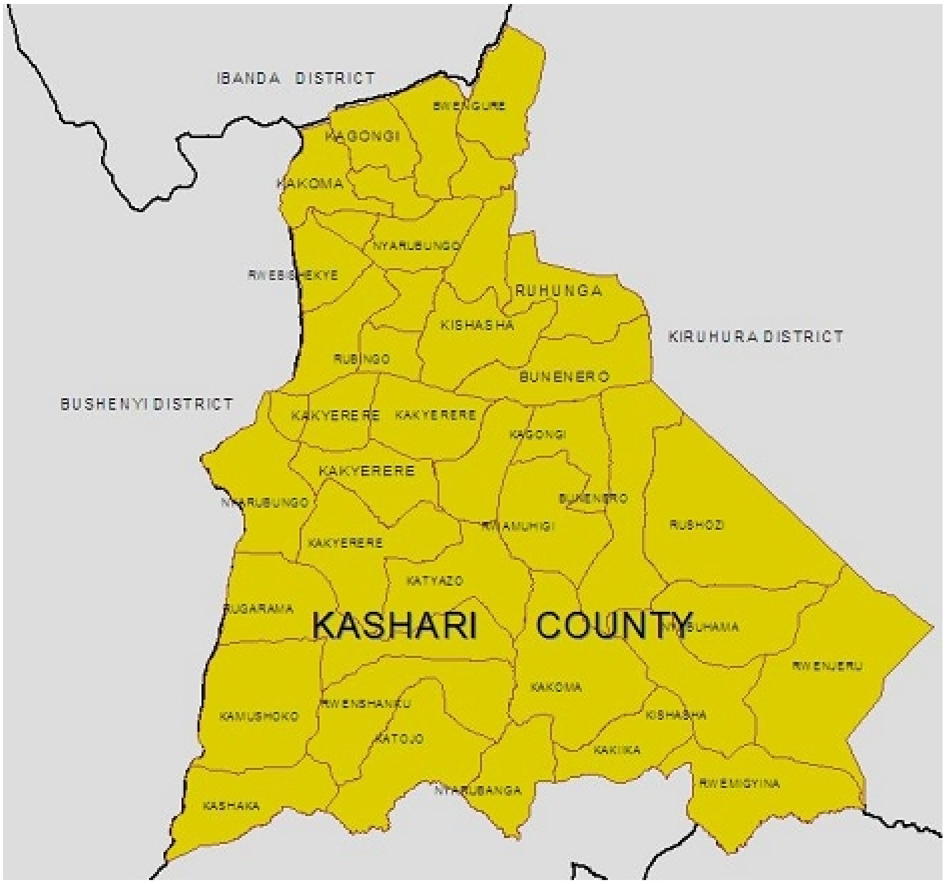

Fig. 2: Map of Kashaari county

analyzed by RLB. Each of the sampled farmers was requested to complete a questionnaire so as to get information about the farmers' biodata, livestock kept, management systems used by different farmers, problems that farmers face in livestock production and assessment of livestock productivity.

DNA extraction, PCR and RLB: DNA extraction was done as earlier described by d'Olveira *et al.* (1995) and DNA Stored at -20ºC until needed for PCR. PCR was completed according to Gubbels *et al*. (1999) and as modified by Oura *et al.* (2004). To determine which *Theileria* and *Babesia* species were present in any of the blood samples collected, the PCR products from each of the samples were allowed to hybridize with *Theileria* and *Babesia* species-specific oligonucleotides (Table 1) commercially appended onto the reverse line blot biodyne C membrane. A single pair of primers, RLB-F2 (5'-GACACAGGGAGGTAGTGACAAG-3') and RLB-R2 (biotin-5'-CTAAGAATTTCACCTCTGACAGT-3') was used to amplify a 430- to 490-bp fragment of the 18S SSU rRNA gene spanning the V4 region conserved for both *Theileria* and *Babesia* species.

Statistical analysis: Data were entered and managed in Epidata version 3.1 (Lauristen, 2000) and exported to statistical package for social scientists -SPSS version 16.0 for uni-variable and multivariate analysis. To establish the association between the prevalence of *Theileria* and *Babesia* species and; age, cattle breed and management system, the Chi-square test was used. The level of significance was set at 0.05 to achieve 95% confidence. Discrete data from the questionnaires were summarized into means and standard deviations while continuous variables were summarized into percentages and confidence intervals.

RESULTS

Current tick control situation and farmers tick-control perspectives: Forty one percent (15/37) of the farmers all from Kakiika and Kashare practiced extensive management with communal watering places. The rest of the farmers (49%) practiced restricted (paddocked) grazing with watering places at each farm. Breeds kept include crossbreeds of Ankole long-horned cattle and Holstein Friesians (50%), pure long -horned Ankole cattle

Table: 1. Oligounucleotide probe sequences that were hybridized onto reverse line blot membrane

Oligonucleotide probe	Sequence	Reference
theilere/babesia catch-all	TAATGGTTAATAGGARCRGTTG	GUBBELS *et al.* (1999)
Babesia felis	TTA TGC GTT TTC GGS CTG GC	EU-FP-6 Inco-DEV project (ICTTD-3), 2006.
Babesia divergens	ACT RAT GTC GAG ATT GCA C	Gubbels *et al.* (1999)
babesia microti	GRC TTG GCA TCW TCT GGA	EU-FP-6 Inco-DEV project (ICTTD-3), 2006.
Babesia bigemina	CGT TTT TTC CCT TTT GTT GG	Gubbels *et al.* (1999)
Babesia bovis	CAG GTT TCG CCT GTA TAA TTG AG	
Babesia rossi	CGG TTT GTT GCC TTT GTG	EU-FP-6 Inco-Dev
Babesia canis canis	TGC GTT GAC GTT TTG AC	Project (ICTTD-3), 2006.
Babesia canis vogeli	AGC GTG TTC AGA TTT GCC	
Babesia major	TCC GAC TTT GGT TGG TGT	
Babesia bicornis	TTG GTA AAT CGC CTT GGT C	
Babesia caballi	GTG TTT ATC GCA GAC TTT TGT	
Theileria annulata	CCTCTGGGGTCTGTGCA	Georges *et al.* (2001)
Theileria parva	TTCGGGTCTCTGCATGT	Gubbels *et al.* (1999)
Theileria mutans	CTTGCGTCTCCGAATGTT	
Theileria taurotragi	TCTTGGCACGTGGCTTTT	
Theileria velifera	CCTATTCTCCTTTACGAGT	
Theileria buffeli/orientalis	GGCTTATTTCGGWTTGATTTT	
Theileria sp. buffalo	CAGACGGAGTTTACTTTGT	EU-FP-6Inco-Dev Project
Theileria sp. kudu	CTG CAT TGT TTC TTT CCT TTG	(ICTTD-3), 2006
Theileria sp. sable	GCT GCA TTG CCT TTT CTC C	
Theileria bicornis	GCG TTG TGG CTT TTT TCT G	
Theileria equi	TTC GTT GAC TGC GYT TGG	
Theileria lestoquardi	CTT GTG TCC CTC CGG G	

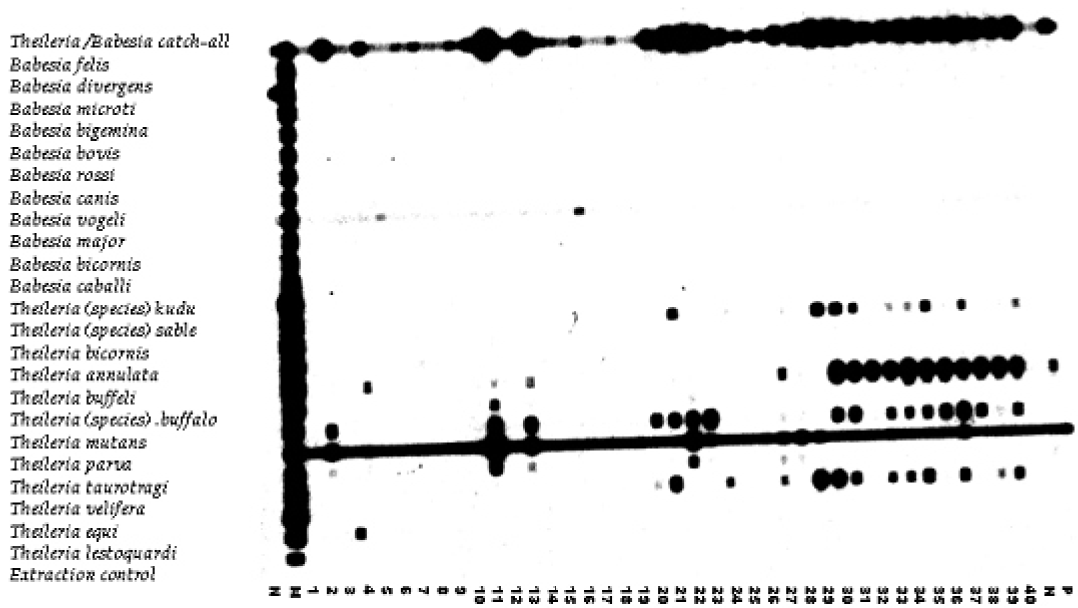

Fig. 3: RLB x- ray representation of some of the PCR products obtained from hemoparasite samples

(46.9%) and exotic Holstein Friesians (3%). Eighty six percent (32/37) of the farmers keep their calves indoors up to 6 months of age and thereafter; leave them to graze together with their dams. Organophosphates and synthetic pyrethroids were the acaracides commonly used. On average, farmers sprayed animals four times a month. Eighty seven percent (87%) of the farmers sprayed their animals at the same rate throughout the year.

Reverse line blot results: Representative results of the hyper ECL film reverse line blot membrane after exposure are as in Fig. 3.

Prevalence of bovine *Theileria* and *Babesia* species: Of the 363 cattle sampled, 74 (20.4, 95% Confidence interval-CI, 16.2-24.6 %) of them were positive with *Theileria* and *Babesia* species. The prevalence of

Theileria species was 19.8% (CI = 95%, 15.7-23.9%). The most and least prevalent *Theileria* species detected were *T. parva* and *T. bicornis* at (24.0%; CI = 95%, 19.6-28.4%) and (3.8%; CI = 95%, 1.8-5.8%) respectively. Sixty eight percent (68%; CI = 95%, 65.5-70.5%) of the animals were dually infected with *Theileria* species. *Babesia vogelli* was the only *Babesia* species detected in 3 animals (0.6%; CI = 95%, -0.4-1.6%) and these animals were also co-infected with *Theileria* species. Very high prevalences of *Theileria* and *Babesia* species was recorded in the sub counties of Kakiika and Kashare at 63.4% (CI = 95%, 58.4-68.4%) and 62.9% (CI = 95%, 57.9-67.9%) respectively. Apparently, no animal from Biharwe sub county was positive for any of the *Theileria* and *Babesia* species. There was a very strong association (X^2 = 163.3, p<0.01; 5df) between *Theileria* and *Babesia* species prevalence and sub county of origin with 92% (CI = 95%; 89.2-94.8%) of the positive cases from Kashare and Kakiika sub counties.

The number of infected animals generally increased with increasing age. Young cattle (9- 24 months) had the highest prevalence of *Theileria* and *Babesia* species of 32.7% (CI = 95%; 27.87-37.57). Calves (≤5.9 months) had the lowest prevalence of *Theileria* and *Babesia* species of 7.8% (CI = 95%, 5.04-10.56%).

There was a very strong association (X^2 = 10.5, p = 0.03; df = 3) between *Theileria* and *Babesia* species prevalence and different cattle age groups with 83.8% (CI = 95%, 80.0-87.6%) of the infected cattle above 9 months of age.

Cross-bred cattle had the highest prevalence of *Theileria* and *Babesia* species of 28.0% (CI = 95%; 23.37-32.63%) while pure exotic cattle had the lowest prevalence of 8.0% (CI = 95%; 5.21-10.79%). The prevalence of *Theileria* and *Babesia* species was strongly associated (X^2 = 13.2, p = 0.001, 2df) with cattle breed with 70% (CI = 95%, 65.3-74.7%) of all animals positive with either *Theileria* or *Babesia* species being cross bred cattle. The prevalence of *Theileria* and *Babesia* species varied across cattle breed in the order of; cross-bred>Ankole long-horned cattle>pure exotic cattle)

Extensively managed animals had the highest prevalence of *Theileria* and *Babesia* species of 19.0% (CI = 95%; 15.0-23.0%) while the semi-intensively and intensively managed cattle had very low prevalences of 0.8 (CI = 95%; -0.1-1.7%) and 0.6% (CI = 95%; -0.4-1.6%) respectively.

There was a very strong association (X^2 = 9.1, p = 0.11, df = 2) between the prevalence of *Theileria* and *Babesia* species and the management system. The prevalences among different cattle age groups, breeds, management systems and sub county of origin are summarized in Table 2.

Table 2: Prevalence of *Theileria* and *Babesia* species in cattle from Kashaari county (Uganda)

Variable	Number (n) positive N = 363	Percentage positive (95% CI)
Individual species prevalence		
None	289	79.6 (75.5-83.8)
All *Theileria* species	71	19.8 (15.7-23.9)
B. vogelli	3	0.6 (-0.4-1.6)
T. parva	43	24 (19.6-28.4)
T. mutans	33	18.4 (14.4-22.4)
T. velifera	25	13.7 (10.2-17.2)
T. taurotragi	24	14 (10.4-17.6)
T. buffeli	23	12.6 (9.2-16.0)
T. ssp. *(sable)*	19	10.4 (7.3-13.5)
T. ssp. *(buffalo)*	7	4.4 (2.3-6.5)
T. bicornis	5	3.8 (1.8-5.8)
Sub-county		
Kakiika	26	63.4 (58.4-68.4)
Kashare	39	*62.9 (57.9-67.9)
Bubaare	5	7.0 (4.4-9.6)
Rubaya	3	6.8 (4.2-9.4)
Rwanyamahembe	1	1.3 (0.1-2.5)
Biharwe	0	0 (0)
Age group		
Adult cattle (above24 months)	44	20.0 (15.88-24.12)
Young cattle (9-24 months)	18	32.7 (27.87-37.57)
Calves 2(6-8.9 months)	8	22.2 (17.92-26.48)
Calves 1(0-5.9 months)	4	7.8 (5.04-10.56)
Breed of animal		
Cross-bred	51	28.0 (23.37-32.63)
Ankole-long horned	21	12.0 (8.65-15.35)
Exotic	1	8.0 (5.21-10.79)
Management system		
Extensive	68	19.0 (15.0-23.0)
Semi-intensive	3	0.8 (-0.1-1.7)
Intensive	2	0.6 (-0.4-1.6)

*: Three animals co-infected with *B. vogelli*

To study the influence/effect of age, breed and management system on prevalence of TBs in cattle, a 3 step multivariate /Backward Regression (BR) analysis (elimination and selection strategy) of the above 3 variables was carried out and age was eliminated. The breed of animals and management system were retained as the best predictors of infection with *Theileria* and *Babesia* species under the current study conditions. The maximum likelihood estimates of binary logistic model of factors for prediction of prevalence of bovine *Theileria* and *Babesia* species are presented in Table 3.

DISCUSSION

The observation that majority (92%) of farmers who had fenced off farms watered their animals communally is an indication that farmers either still lack information about the benefits of restricted/paddocked grazing with regard to tick-borne diseases and other livestock disease control or have no capital input for this farm activity. This means that the majority of animals were freely exposed to ticks and tick-borne parasites at these communal watering points. Similarly, in belief that high grade cross breeds are more susceptible to tick-borne diseases than cross-1 and 2, the latter are not well cared for in terms of tick-borne disease control which explains why crosses were seen to be strongly associated

Table 3: Maximum likelihood estimates of binary logistic model of factors for prediction of prevalence of bovine TBs in Kashaari County

Independent variable	Dependent variable			
	Infection with *Theileria* and *Babesia* species			
Predictors of *Theileria* and *Babesia* species	Coefficient (bi)	SE (bi)	P	OR (95% CI)
Constant	-0.745	1.130	0.001	0.475
Breed	0.933	0.290	0.044	2.543 (1.440-4.489)
Management system	-2.083	1.036	0.510	0.125 (0.016-0.950)

(X^2 =13.2, p = 0.001, 2df) with a higher prevalence of *Theileria* and *Babesia* species compared to pure breeds. On average farmers sprayed animals four to eight times per month. Farmers that had long been involved in cross breeding program were spraying twice a week. Farmers with Ankole long-horned cattle, their cross breeds and pure exotic breeds still apply acaracides at the same rate across all breeds and this may in effect wipe out the endemic stability to tick-borne diseases that has long been noted in most local breeds (Norval *et al*., 1992; Perry and Young, 1995). However, farmers with crossbred cattle and local Ankole cattle have been noted to give crossbred cattle less attention compared to those with pure Frisians and crossbred cattle, for they believe that crossbred cattle are somehow resistant. This resistance is not as effective as it is in local breeds, which have long been exposed to ticks and TBDs (Norval *et al*., 1992; Perry and Young, 1995; Minjauw and McLeod, 2003). This partial tick control attention offered to cross bred cattle and therefore higher exposures to tick infestation partly explains why a higher prevalence of *Theileria* and *Babesia* species was strongly associated (X^2 = 13.2, p = 0.001, 2df) with cross bred cattle.

Communal watering of animals is associated with movement of animals from one farm to another, which facilitates pasture infestation and makes it possible for ticks to attach on cattle from different herds and hence transmit *Theileria* and *Babesia* species. This partly explains why sub counties that practice this type of cattle husbandry had the highest *Theileria* and *Babesia* species prevalence compared to others. This is further supported by the observed highest prevalence of *Theileria* and *Babesia* species in animals under the extensive system of management. In addition, the lesser intensity of tick control in communal husbandry explains the observed high prevalence of *Theileria* and *Babesia* species where it is practiced.

In agreement with Oura *et al*., (2004), cross bred cattle were more associated with high *Theileria* and *Babesia* species prevalence (X^2 = 13.2, p = 0.001, 2df) compared to Ankole-long horned cattle and pure Frisians. The pure Frisians sampled were under intensive management where they were sprayed twice a week with synthetic pyrethroid acaracides. Thus intensification and frequent spaying of pure Friesians explains the very low prevalence in this category of cattle. It is noted that the difference in prevalence among different cattle breeds is not genetic but relates more to difference in frequencies of acaracide application.

The observed increase of infection with age is mainly due to restriction of calf movement by keeping them indoors, which is practiced by 86.5% of the farmers. This practice results in postponement of infection for several months (Rubaire-Akiiki *et al*., 2006; Katunguka-Rwakishaya and Rubaire-Akiiki, 2008) resulting in the observed upward trend of infection with increasing cattle age. This phenomenon may as well be explained by the age dependent immunity to *Theileria* and *Babesia* species infection that has earlier been explained for theileriosis (Bruce *et al*., 1910; Norval *et al*., 1992) and babesiosis (Mahoney and Ross, 1972).

The practice of keeping calves indoors up to 6 months of age has a negative effect of interrupting endemic stability establishment and could result in high losses due to tick-borne diseases. Considering the fact that hummoral immunity is not protective (Morzaria *et al*., 2000) against ECF as it was initially thought (Bruce *et al*., 1910; Norval *et al*., 1992) and that calves are kept indoors up to 6 months of age, farmers who can afford need to advocate for ECF vaccination before calves are let onto pastures. Better still, controlled exposure of calves to ticks should be allowed for calves to develop acquired immunity to different tick-borne diseases and most importantly to ECF. Short of this, high mortalities due to ECF will occur in such naïve calves let on pasture at 6 months of age.

In this study, the majority (68%) of the animals were dually infected with *T.parva* and other *Theileria* species. Dual *T.parva* and other *Theileria* species infections reflect the multi-vectorial potential of *Rhipicephalus* species (Minjauw and McLeod, 2003). *Theileria* and *Babesia* species and the proportions in which they were detected strongly agree to those in literature (Oura *et al.,* 2004). However the prevalence of *T.parva* in this study was found to be 24%, a far lower percentage to *T. parva* prevalence in south western Uganda of 54% reported by Oura *et al.* (2005), which could be explained by the increased effort in the control of this disease by farmers in south western Uganda. This is supported by information that at the time of the Oura *et al.* (2005) study, farmers were applying the acaracides once a week and had poor crushes making acaracide application less effective. In the current study, farmers sprayed their cattle at least once a week and most of the farms had good crushes. These management changes in regard to tick control are likely to be due to improved service delivery by the National Agricultural Advisory Service (NAADS), which was operational in all the sub counties sampled, and the availability and affordability of acaracides to and by the majority (89%) of farmers.

Out of the 363 cattle sampled, only 3 (0.6%) were positive for *B. vogelli*, which is in agreement with other studies (Oura *et al.,* 2004). *Babesia* sp. are transmitted mainly by *Boophilus* sp., which are one-host ticks and spend most of the time in their life cycle on a single host making them easily killed by acaracides. In Kashaari, farmers apply acaracides weekly and some times twice a week (Mugisha *et al.,* 2005), a regime set purposely to control three-host ticks like *Rh. appendiculatus* that transmit ECF. Since one-host ticks (*Boophilus* sp.) would need longer application intervals, the bi/weekly acaracide application is responsible for very low vector populations and hence the negligible *Babesia* infections observed. Geographical Information Systems based models to predict the distribution of different tick-species and the diseases they vector in east Africa have as well indicated few or no *Boophilus* species in south western Uganda (Kruska *et al.,* 2003), further validating these findings.

Cattle breed and management system were shown to be the most important predictors of infection with *Theileria* and *Babesia* species. There was 2.5 times risk of infection associated with cross bred cattle (OR = 2.5, 95% CI; 1.44-4.49) compared to that of local and exotic breeds. The risk of infection with TBs was in the order of; cross-bred>Ankole long-horned cattle>pure exotic cattle).

Much as management system was associated with a lower risk of infection with *Theileria* and *Babesia* species (0R = 0.125, 95%CI; 0.016-0.95) than that associated with breed, it is apparent that management system was a very important predictor of infection with TBs. Regardless of breeds, management practices of rate of acaracide application, restriction of calf movement up to 6 months of age, restricted grazing (paddocking) and zero grazing were the most important parameters that determined the risk of infection with *Theileria* and *Babesia* species.

Reverse line blot hybridization assay was able to pick infections in animals whose PCR products showed no fragment separation at 1-2% agarose gel electrophoretic separation. In such animals, the hemoparasite load was very low to be picked by such non species-specific PCR. This demonstrated very high sensitivity of reverse line blot hybridization. Reverse line blot hybridization is therefore a very sensitive and specific (due to species specific oligonucleotides used) diagnostic test needed for such epidemiological studies like this.

It is therefore recommended that; longitudinal studies be carried out to study the fluctuations in incidence of different tick-borne diseases in cattle. This will cater for differences in tick-borne parasites in cattle attributable to fluctuations in tick densities in different seasons. Secondly, more studies should be carried out to determine the prevalence of *Anaplasma* and *Eihrichia* species in this area to help in tick-borne disease control decision-making.

ACKNOWLEDGMENT

The authors wish to acknowledge the Belgian Embassy (in Uganda) through the Belgian Technical Cooperation (BTC) and EPIGENVAC/ICTD III under the coordination of Dr. Margaret Saimo-Kahwa for their financial support leading to completion of this work. We are also highly indebted to Prof. George William. Lubega; The Principal Investigator in The Molecular Biology Laboratory (MOBILA) at Makerere University who provided laboratory space, consumables and technical guidance leading to the completion of this study.

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