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Full Length Research Paper

Estimation of the *Theileria parva* entomological inoculation rate (EIR) by means of tick burden and proportion of infected questing ticks in three different farming systems in Rwanda

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A field study was carried out to determine the patterns of transmission of *Theileria parva* infection (expressed as entomological inoculation rate (EIR)) between cattle and *T. parva* infected *Rhipicephalus appendiculatus* ticks in three different farming systems in Rwanda. The EIR was measured for a given period of time as a product of the tick burden feeding on an animal and the proportion of them that are *T. parva* infected. A total of 750, 600 and 150 questing ticks were collected in restricted, free-range and fenced farming systems, respectively. A PCR-RFLP method was used to discriminate between *T. parva* and *Theileria taurotragi* species co-infecting *R. appendiculatus*. Data on tick numbers infecting animals were obtained from a survey carried out in the three farming systems in similar conditions in the previous year. The results showed higher EIR (9 infected ticks/animal/week) in the restricted farming system as a result of higher tick numbers (233 ticks/animal) and high infection rate in ticks (3.98%) than in the fenced (high infection rate (4.37%) but acaricide suppressed tick burden (28 ticks per animal)) and in the free grazing (high tick number (292 ticks/animal) but low infection rate (0.17%)) suggesting an epidemiologically critical situation in animals kept under restricted conditions. The study showed clearly that tick load is not the only epidemiological determinant in ECF, differential managerial methods applied lead to variable epidemiological situations in a given ecological area.

Key words: *Theileria parva*, tick numbers, infection rate, EIR, farming system.

INTRODUCTION

East Coast fever (ECF) caused by a protozoan parasite *Theileria parva* and transmitted by *Rhipicephalus appendiculatus* tick is considered to be the greatest limitation for improving cattle production in Rwanda (Bazarusanga, 1999; Bazarusanga et al., 2007a). Data on prevalence, infestation burden and seasonal dynamics of ixodid ticks are important variables in the epidemiology and control of East Coast fever (ECF). In Rwanda, *R. appendiculatus* is the most abundant tick on cattle (Paling and Geysen, 1981; Bazarusanga et al., 2007b).

The numbers of *R. appendiculatus* ticks on the host is an indicator of the possible intensity of transmission of *T. parva* infection (Lessard et al., 1990). However, the overall *T. parva* transmission is mainly determined by the proportion of infected *R. appendiculatus* in this population that successfully attach to the host. Data obtained from

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field ticks show variation in prevalence of infection (Walker et al., 1981; Moll et al., 1986; Kariuki et al., 1995; Watt et al., 1997) as a function of variable vector / host contact conditions. Differences in husbandry practices influencing host-tick interactions result in different epidemiological situations of *T. parva* infection (Gitau et al., 2000; Rubaire-Akiiki et al., 2004).

In Rwanda, tick load infesting animals varies depending on the management method. Paling et al. (1991) reported a mean daily *R. appendiculatus* tick burden ranging from 20 to over 150 per animal. A number of farming systems from free-range to restricted grazing or fencing is practiced. Zero-grazing is currently being promoted to prevent contacts between ticks and cattle. However, the incidence of ECF remains high even in zero-grazed farming units, probably due to ticks present in the cut and carried forage (Gitau et al., 1994).

Estimation of the infection rates in vector populations is essential in the calculation of the intensity of transmission of vector-borne diseases and in evaluating the effect control programs may have on the transmission of these pathogens. Transmission intensity of *T. parva* at a given site is estimated by the entomological inoculation rates (EIR). This parameter gives the number of infective ticks attaching on an animal and can be determined as a product of the number of ticks feeding on cattle for a given period of time and the proportion of them that are *T. parva* infected(Geysen, 2000). The prevalence of infection in host-seeking ticks is expected to be low (Moll et al., 1986; Ogden et al., 2003). Precise estimation of tick infection prevalence therefore requires the examination of large numbers of ticks. The routine method based on the detection of infective sporozoites of *T. parva* in stained individual tick salivary glands (Young and Leitch, 1982; Voigt et al., 1995) is cumbersome, particularly when large numbers of ticks must be analyzed. In addition, this method does not allow the discrimination between *T. parva* and *Theileria Taurotragi* which frequently co-exist in *R. appendiculatus* (Norval et al., 1992).

The PCR methods are as sensitive as the standard staining method (Chen et al., 1991; Watt et al., 1997) but have the advantage of being highly specific and subject to pooling of samples. A PCR-based pooling method has been successfully used to detect *Onchocerca volvulus* infection in pools of the *Simulium* vector population (Katholi et al., 1995). To estimate the infection prevalence from the proportion of positive pools, however, pooling must be random and the PCR assay must be able to detect a single infected *R. appendiculatus* in a pool containing large numbers of uninfected ticks (Katholi et al., 1995).

The objectives of this study was to estimate the *T. parva* infection prevalence in host-seeking *R. appendiculatus* ticks collected from the grass and to estimate the *T. parva* entomological inoculation rate(EIR), from the estimated infection prevalence in questing ticks and the tick burden on cattle in three farming system in Rwanda.

MATERIALS AND METHODS

Study environment and farming systems

The study was conducted in the eastern low (AEZ 4) and central high plateaus (AEZ 2) of Rwanda (Figure 1) where the highest *R. appendiculatus* tick loads are observed on cattle (Bazarusanga et al., 2007b). Most of the animals in the study areas are of Ankole type kept under different management systems. The eastern low plateau is subdivided in a non-project and a project zone: (1) The non-project zone is located in the southern part and a free-range farming system is the rule. Adult animals move from pasture to pasture over very long distances depending on water and feed availability whereas calves are kept indoors for almost their first year of life. Acaricides are infrequently applied (once every 2 to 3 months) and the tick infestation is high. (2) A more structured husbandry system is found in the project zone in the northern part of the eastern plateau and all age categories (calves and adult cattle) are kept on permanently fenced farms. A weekly acaricide application is practiced. (3) The densely populated central high plateau has a marked land shortage for livestock and agriculture activities. The majority of cattle owners are small holder farmers keeping a limited number of cattle. Restricted grazing on small land pastures is the rule and tick control is practiced at variable frequencies.

Tick abundances

Data on tick numbers used in this study were derived from a cross-sectional tick survey conducted in the wet season in April and May 2003 as previously described by Bazarusanga et al. (2007b). Half-body tick counts were performed on 12, 15 and 18 randomly selected animals in the fenced, free-range and restricted grazing systems, respectively on the eastern low (AEZ 4) and central high (AEZ 2) plateaus (Figure 1).

Questing *R. appendiculatus* collections

In the wet season in April and May 2004, a total of 150, 700 and 750 adult questing ticks were collected from pasture through blanket dragging techniques as earlier described (Short and Norval. 1981) in the fenced, open and restricted grazing systems, respectively. To increase the chances of collecting a high number of questing ticks, visited sites in each farming system were selected in locations where higher numbers of ticks were found on cattle during the previous tick survey. After collection, the ticks were immediately put in 70% alcohol. Once at the laboratory, the ticks were thoroughly washed with sterile water to remove the alcohol, air-dried and kept at –20°C in sealed plastic bags containing silica gel.

Pooling of ticks and DNA extraction

Parasite DNA was extracted from 5 pooled ticks using a modified Boom (1990) method. Each tick was cut into two pieces using a sterile scalpel blade. Half-tick pieces of 5 whole ticks were pooled together in a 1.5 ml tube containing 250 µl of lysis buffer (60 mM Tris-HCl, pH 7.4, 60 mM EDTA, 10% Tween 20, 1% Triton X-100, 1.6 M Guanidine-HCl), 250 µl of RODI (reverse osmosis de-ionised) water and 50µl of Proteinase K (Sigma, 20mg/l) and left for over-night incubation at 56°C in a shaking thermomixer. A volume of 4 µl of Diatomaceous Earth (Sigma) was then added. The mixture was

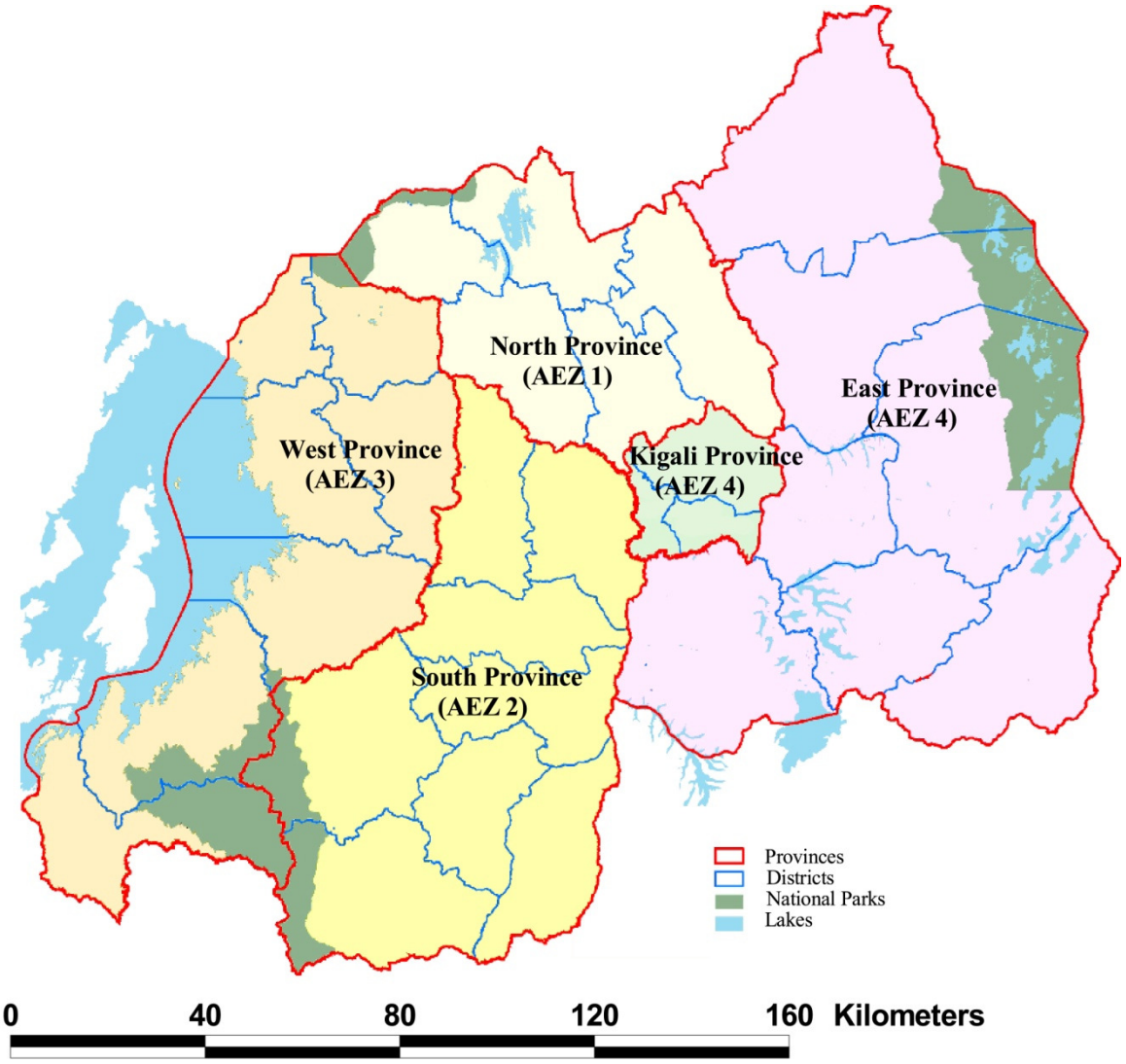


Figure 1. Subdivision of the major agro ecological zones (AEZs) in Rwanda.

incubated for 1 h at 37°C in the thermomixer, followed by short centrifugation for 20 s and the supernatant discarded. The resultant pellet was washed with 900 µl of 70% ethanol (v/v) at 4°C, centrifuged for 20 s and the supernatant discarded. The rinsing with ethanol was repeated and the pellet was washed with 900 µl acetone and dried for 20 s in a thermoblock at 50°C. To the dried pellet, 90 µl of TE buffer (10 mM tris, 1 mm EDTA, and pH 8) was added and the mixture was incubated for 20 min at 60°C under shaking. The final product was subjected to short centrifugation for 40 s before a 50 µl volume of supernatant was extracted and transferred to a new tube.

PCR and RFLP analysis

Extracted DNA was used as a template in a semi-nested PCR amplification of the *Theileria* spp. Cox III mitochondrial locus. Pairs of primers CoxIII F/CoxIII R and CoxIII nR/CoxIII F (Figure 2) were used in the first and second round of a semi-nested PCR, respectively. A 5 µl volume of DNA was used as template in a final 25 µl PCR reaction volume. Reaction mixtures in the first round contained 1 µl of Y sub (Yellow Sub™ GENEO BIO Products, Germany), 3.33 µl of milli-Q water, 12.5 µl of buffer (20 mM tris-HCL, pH 8.4, 100 mM KCl, 0.2% Triton X-100, 1.6 mM $MgCl_2$), 2 µl of dNTP's (Deoxyribo-nucleotide triphosphate), 0.4 µl of each primer (25 umol/µl) and 0.37 µl of *Taq* polymerase (Silverstar DNA polymerase, Eurogentec, Belgium). Each sample was overlaid with fine mineral oil and amplification took place in a heating block of a programmable thermocycle (PTC-100™, MJ Research) as follows: denaturation at 92°C for 30 s, annealing temperature of 59°C for 45 s and 1 min elongation at 72°C. The amplification cycle was repeated 39 times. The second round of the semi-nested PCR reaction was carried out in a total volume of 25 and 0.5 µl of the first round was added to the reaction mixture comprising 24.5 µl of master mix: 1 µl of Y sub (Yellow Sub™ GENEO BIO Products, Germany), 7.95 µl of milli-Q water, 12.5 µl of buffer (20 mM tris-HCL, pH 8.4, 100 mM KCl, 0.2% Triton X-100, 1.6 mM $MgCl_2$), 2 µl of dNTP's (Deoxyribo-nucleotide triphosphate), 0.4µl of each primer (25 umol/µl) and 0.25µl of *Taq* polymerase (Silverstar DNA polymerase, Eurogentec, Belgium). The amplification conditions were as described in the first round, although an annealing temperature of 56°C was used and the amplification cycle was

Atgcg–aaacagcgcacaatcata–tttaaa**gtac**attaatataattaacatatttgaaactctatatctgtt––ttata**gtac**aggattagata
ccc––tggaatatattgattccacatataagaactttattataatgtatgtaaatcaatatttattatacggaactacacttaaatatttaagtgttgg
tgaatttttttatgaacagtttaacaatatttataaatagtattagagagataatgacttctacaactatggtaat**gtac**gctatatttggaatgtttata
ttttcagaaatattagtgttttcgacatttatatggggatattttcatttaagattatcgaatccaattttactagctgaattaaatgtagaagcctatctt
caaatctctgacgttttaaatactggaagtattttagtatcaataattcttcatagagttcaagaaagtgccaactttgaaacagacttttttatgga
acaattgctattaatagggtttatatttttatcgttacaaaatgatgaatattcattaattctatcttatgtgaacaattattggatgacattatatttttca
ttttaacaggacttcattcgttacatgtatgtgccggaggcatttttt**gtac**taatacagtcatatttttatgaaggtgatggatctcaaagagatgaa
gaattcaatgctggagt**gtac**tggcattttgtagaaatgat–––ttggatagctttaacaatgttg–––ttatttctagcttaa

Figure 2. *Theileria parva* Cox III gene showing the annealing sites (CoxIII F: –aaa…ata–…; CoxIII R:––tta…ccc –– and CoxIII nF: –––ttg…ttg–––) and *RsaI* restriction sites (Bold).

repeated 24 times.

Standard detection with ethidium bromide staining was used after electrophoresis of the amplified samples together with a 100 bp molecular weight marker. Positive samples were further analyzed to discriminate *T. parva* and *T. taurotragi* infections. Restriction was carried out in a final volume of 15 µl, consisting of 4 µl DNA and 11 µl of RFLP mix containing 0.3 µl of restriction enzyme (Biolabs, New England), 9.2 µl of milli-Q water and 1.5 µl of buffer. The *RsaI* restriction enzyme was used and incubation was done overnight at 37°C. A 4 µl volume of the restriction product of each sample was mixed with 2 µl of loading buffer and placed on PAGE gel in an electrophoresis tank at 100 V for 2 h 40 min. The gel was then incubated in sybr Green for 40 min before photography using UV light and green filter.

Statistical analysis and EIR estimation

Statistical analysis was done in Stata 9 (StataCorp2006. Stata Statistical Software: Release 9.2. Texas: Stata Corporation). The pool prevalence was defined as the proportion of pools of ticks that were positive at *T. parva* PCR analysis. The pooled tick prevalence data was analyzed using the farming systems as discrete explanatory variable in a logistic regression. Tick abundance was analyzed using the same explanatory variable in a negative binomial regression. The linear estimators and the standard errors were estimated for each farming system in both regressions. They were used to define, for each farming system, two separate normal distributions from which paired but independent random values were sampled 100,000 times. The exponents of the values sampled from the distributions defined by the negative binomial regression estimators were used to build half-body tick burden distributions. Whole-body tick burden distributions were constructed by summing paired (but independent) values from half-body tick load distributions. Distributions of infection prevalence in tick pools were obtained by transforming values sampled from the distributions defined by the logistic regression estimators. A probability formula was used to transform the estimated pool infection prevalence in individual prevalence:

$$P_i = 1-(1-P_p)^{1/n}$$

Where, Pi is standing for individual prevalence, P_p for pool prevalence and n for number of ticks in pools.

This transformation relies on two assumptions: samples belonging to a pool are independent and one positive sample in the pool makes the pool positive. Paired (but independent) transformed tick burden and infection prevalence values were then multiplied to generate an EIR distribution. The EIR was expressed as the number of infected *R. appendiculatus* that attach to individual animal over an average tick feeding period (approximately one week). Finally, percentiles 2.5, 50 and 97.5 were estimated for the individual prevalence of infection in ticks, the tick burden and the EIR in each of the 3 farming systems.

RESULTS

From the total 300 pools tested, 56 (18.67%) gave a positive *T. parva / T taurotragi* amplicon, showing a 682 bp expected length (Figure 3). Higher number of positive pools was found in restricted (26%) and in fenced (23.33%) than the free-range (8.33%) farming systems. The RFLP method was further used to discriminate between *T. parva* and *T. taurotragi* DNAs. Table 1 shows the length of the expected *RsaI*-restriction fragments and Figure 4 the species-specific profiles (*T. parva*: 3 bands at 376, 187 and 89 bp; *T. taurotragi*: 2 bands at 640 and 30 bp). *T. parva* was the predominant infection in the fenced and the restricted farming systems whereas *T. taurotragi* was the most prevalent infection in the free-range farming system (Table 2).

The pool prevalence and confidence intervals were calculated (Figure 5B) and the total infection prevalence ranged between 1.72 and 5.18%. The free-range system had significantly lower *T. parva* infection in ticks than the fenced ($p = 0.002$) and the restricted ($p = 0.001$) farming systems but the *T. parva* infection prevalence did not differ between the fenced and the restricted farming systems (Figure 5C).

The fenced farming system had significantly lower mean tick burden per animal than the free-range ($p < 0.001$) and the restricted ($p < 0.001$) whereas no significant difference was found between the free-range

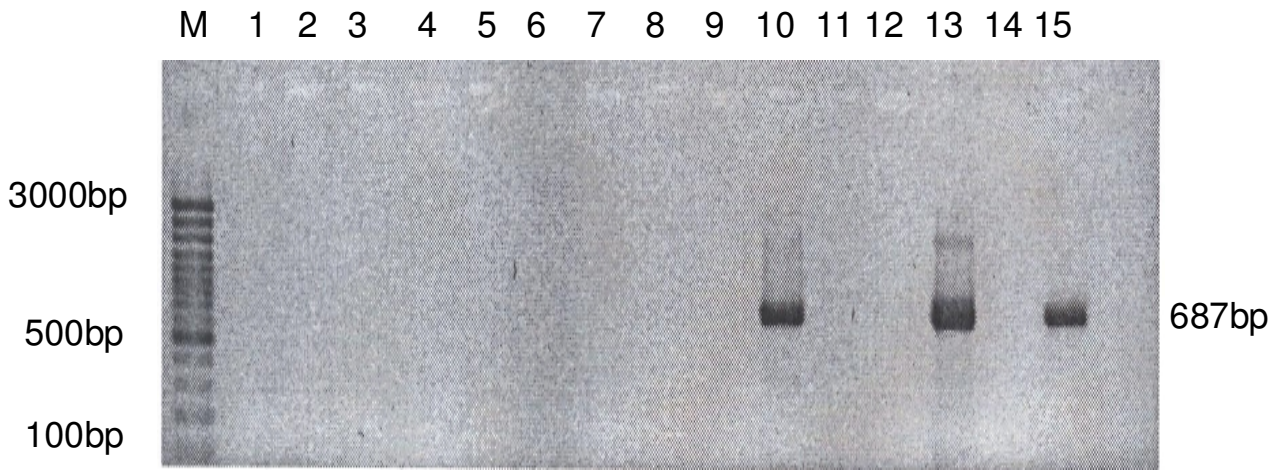


Figure 3. Cox III positive sample (lane 10) as compared to Cox control amplicon in the first round (lane 13) and the second round (lane 15) of a semi-nested PCR. M is a 100 bp marker.

Table 1. *Rsal* restriction sites and the resulting fragment length.

Number of *Rsal* restriction fragments	Fragment length (bp)	Restriction sites	
		From *Rsal*	To *Rsal*
1	376	264	640
2	187	77	264
3	47	30	77
4	42	640	...
5	30	...	30

and the later farming system (Figure 5A). The EIR obtained in the three farming systems differed among them (Figure 5D). The EIR was lower in the fenced farming system (1 infected tick/animal/week) than in the restricted (9 infected ticks/animal/week) but higher than in the free-range (1 infected tick/animal/2 weeks) farming systems.

DISCUSSION

Suitable ecological conditions for tick development and survivals prevail in the plateaus of Rwanda and the year to year variation in tick numbers is not significant (Paling and Geysen, 1981; Paling et al., 1991; Bazarusanga et al., 2007b). The aim of this study was to estimate the EIR from tick numbers infesting animals and infection prevalence in field ticks. Previous reports on analysis of infection rates in field ticks were based on individual tick data, a time consuming and costly approach when large numbers need to be examined. The technique described here provides a simple way to estimate the prevalence of *Theileria* infection in pools of unfed, host-seeking *R. appendiculatus* ticks collected from the field. Based on the algorithm model developed by Katholi et al. (1995), the prevalence of *Theileria* infection in *R. appendiculatus* was estimated.

The prevalence of *T. parva / T. taurotragi* ranged between 1.7 and 5.2% in different farming systems. The infection rates in ticks was previously reported to vary from 0% and as high as 25% (Gitau et al., 2000). Our results are consistent with the *Theileria* infection rates detected by the salivary gland staining method in individual field *R. appendiculatus* earlier reported to be 1.8% in Rwanda (Paling et al., 1991) and 2.6% in Kenya (Moll et al., 1986). While there is no simple method for discriminating between *T. parva* and *T. taurotragi* infected salivary glands (Young et al., 1980; Voigt et al., 1995), the PCR-based methods could allow the differentiation between *Theileria* parasites co-infecting *R. appendiculatus.*

The *T. parva* infection prevalence detected by the pooling method was low and varied between 0.2 and 4.4% in different farming systems but was in line with the 2.7% prevalence detected in individually PCR-tested *R. appendiculatus* collected from field sites in neighboring Tanzania (Ogden et al., 2003; Swai et al., 2006). The infection level in the tick population is influenced by the

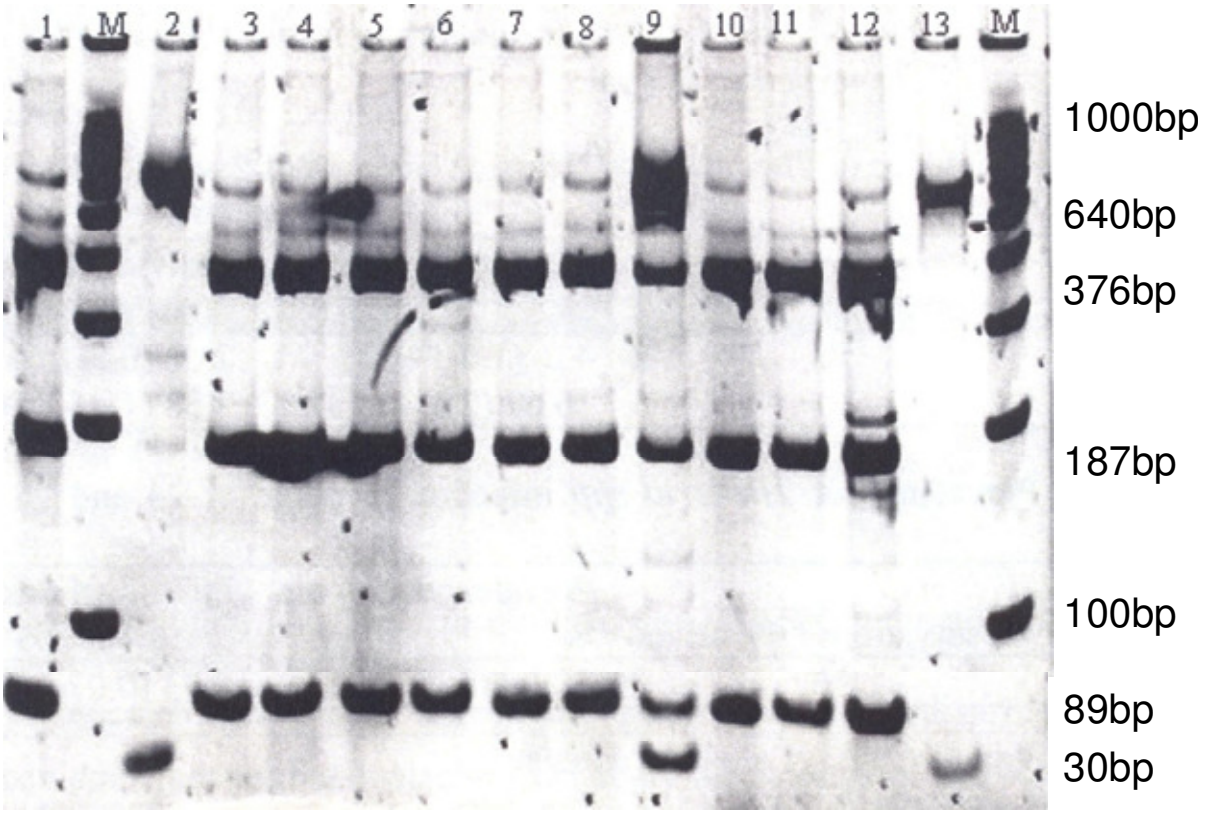


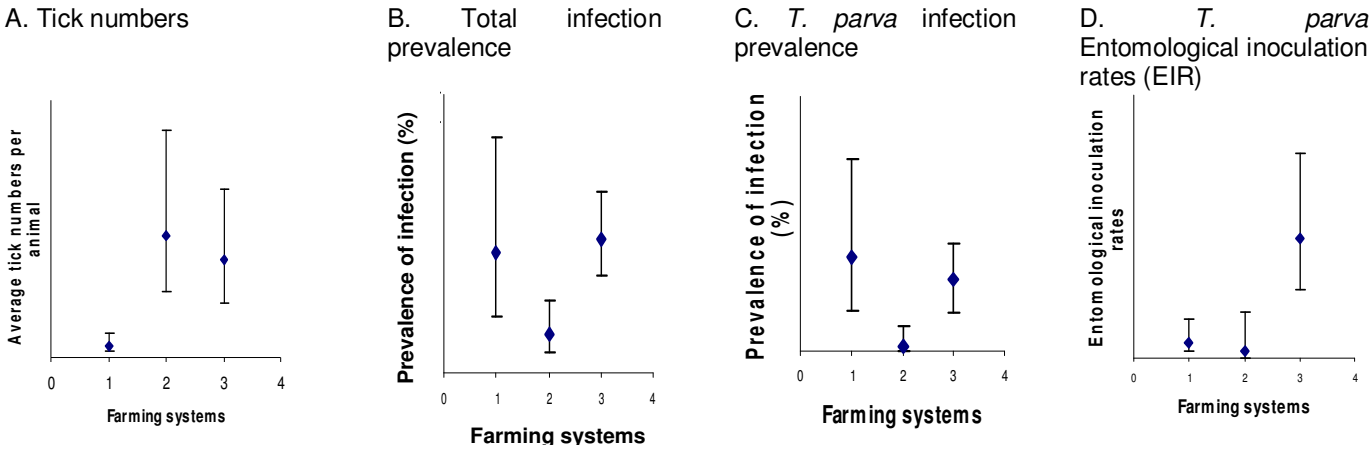
Figure 4. Species-specific RFLP profiles of Cox PCR positive amplicon (682bp long) on Sybr Green stained PAGE gel using *Rsa*I restriction enzyme. *T. parva* and *T. taurotragi* profiles were sized against 1000 base pair markers (M). *T. parva* positive profiles (lanes 1; 3-8 and 10-12) show 3 bands (low = 89 bp; medium = 187 bp and high = 376 bp) whereas the two bands (low = 30 bp and high = 640 bp) characteristic of *T. taurotragi* are found in lanes 2 and 13. Mixed *T. parva/T. taurotagi* infections (lane 9) are characterized by the 5 bands (3 bands for *T. parva* and 2 bands for *T. taurotragi).*

Table 2. Total number of positive pools in the total tested per grazing system.

Farming system	Total number of pools	Positive pools	*T. parva* (single infection)	*T. taurotragi* (Single infection)	*T. parva/T. taurotragi* (mixed infections)
Restricted	150	39 (26%)	27 (18%)	8 (5.33%)	4 (2.66%)
Free-range	120	10 (8.33%)	1 (0.83%)	9 (7.50%)	0
Fenced	30	7 (23.33%)	6 (20%)	1 (3.33%)	0
Total	300	56 (18.67%)	34 (11.33%)	18 (6%)	4 (1.33%)

presence of clinically diseased or carrier animals in the field (Norval et al., 1992). In a study conducted in Tanzania, Swai et al. (2006) have shown that low prevalence prevails in endemic conditions where most of infected ticks acquire infection from carrier animals. It is also known that host-to-tick transmission from carrier cattle is low (Medley et al., 1993) and the *T. parva* carrier state is common in animals in Rwanda (Paling and Geysen, 1981; Bazarusanga, 1999, Bazarusanga et al., 2007a, Bazarusanga et al., 2008).

In this study, the proportion of *T. parva* infected ticks was much lower in the free-range than in the restricted and the fenced farming systems. The difference in tick infection rates between the various farming systems can be explained by the fact that in the free-range system, young animals are kept indoors in their first year of life to prevent contact with ticks. Under such management, the ticks feed only on low parasitaemic adult carriers. Our results are consistent with a 5 to 6 fold lower PCR-based detected parasitaemia previously reported in adult carriers



1	28 (17-46)	5.18(2.41-10.17)	4.37 (1.92-9.09)	1.20 (0.46-2.96)
2	292 (188-455)	1.72 (0.92-3.15)	0.17 (0.02-1.16)	0.49 (0.06-3.52)
3	233 (155-349)	5.84 (4.26-7.84)	3.89 (2.27-5.57)	9.06 (5.21-15.60)

Figure 5. Tick numbers, infection rates and entomological inoculation rates (EIR) in the three farming systems (1: Fenced; 2: Free-range; 3: Restricted).

than calves in the free-range farming system (Bazarusanga et al., 2008)

Alternatively, the lower infection rates could be also a result of ticks feeding on hosts other than cattle in the free-range pastoral system. This could probably explain the high proportion (9/10) of *T. taurotragi* infected ticks found in the free-range management system. In this farming system, the pastoral system practiced will tend to cause variation in cattle host availability for feeding ticks. This will result in reduced transmission efficiency in that most parasites will die off in the infected ticks. The decline in *T. parva* infection levels in *R. appendiculatus* over time has been demonstrated by Ochanda et al. (2003).

In contrast, the system of fencing or confining animals will result in increasing contacts between ticks and cattle of all age categories, giving ticks many possibilities of feeding on high parasitaemic young animals (Purnell et al., 1974; Bazarusanga et al., 2008). This is in agreement with the reported positive correlation between infection rates and parasitaemia in cattle (Young et al., 1996). An interesting observation in the fenced farming system was the evidence that *T. parva* transmission remained significant although the cattle were dipped weekly. This finding is somewhat surprising, as one would predict that the level of transmission and challenge will be low. A similar high *T. parva* transmission has been reported in weekly dipped herds in Ouganda (Oura et al., 2007) suggesting that control of ECF in eastern Africa is hardly achievable by tick control alone.

When looking at the EIR calculations, the restricted farming system had much higher EIR than the free-range and the fenced farming systems. This is not surprising given the high tick counts and infection rates in field ticks found in the restricted managerial system. The higher challenge of *T. parva* infection detected in the restricted animals is likely to increase the risk of clinical ECF since the severity of the disease is dose-dependent (Barnett, 1957).

A similar situation would be observed in the fenced farming system but the reduced contact between animals and infected ticks through weekly acaricide application in this farming system lead to low levels of infection challenge and probably low clinical cases. In the free-range farming system, a significantly lower EIR was found although the highest tick numbers was collected on cattle. The majority of animals would receive low *T. parva* challenge leading to reduced number of diseased animals.

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