Full Length Research Paper

Acquisition and transmission of *Theileria parva* by vector tick, *Rhipicephalus appendiculatus*

Satoru Konnai[a,*], Saiki Imamura[a], Chie Nakajima[a], William Harold Witola[a], Shinji Yamada[a], Martin Simuunza[b], Andrew Nambota[b], Jun Yasuda[c], Kazuhiko Ohashi[a], Misao Onuma[a]

[a]*Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan.*

[b]*Epidemiology Section, Disease Control Department, School of Veterinary Medicine, University of Zambia, P.O. Box 32379, Lusaka, Zambia.*

[c]*Veterinary Teaching Hospital, Faculty of Agriculture, Iwate University, Morioka, Iwate 020-8550, Japan.*

Correspondent footnote: *Corresponding author; mailing address, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan. Phone: +81-11-706-5215. Fax: +81-11-706-5217. E-mail: konnai@vetmed.hokudai.ac.jp

Abstract

In order to investigate the transmission dynamics of *Theileria parva* (*T. parva*) by the brown ear tick, *Rhipicephalus appendiculatus* (*R. appendiculatus*), under experimental conditions, detection of *T. parva* in ticks and cattle was performed by a quantitative real-time PCR assay. A calf inoculated with a *T. parva* mixture became PCR-positive for *T. parva* infection on day 8 post-inoculation, and subsequently, nymphal ticks were introduced and maintained to feed on the infected calf for 6 days. Engorged nymphs were collected daily and allowed to molt into adults, and overall, 70.8% (121/171) of the adult ticks acquired the *T. parva* infection. Furthermore, the *T. parva* infection rate in ticks under field conditions was monitored by real-time PCR in *R. appendiculatus* ticks collected from a traditionally-managed pastoral land of Zambia, on which Sanga breed cattle are traditionally reared and the area has endemic East Coast fever (ECF). A total of 70 cattle were randomly selected in the same area and 67 (95.7%) were found to be serologically positive for *R. appendiculatus* tick antigen (RIM36). Twenty-nine (43.3%) of the 67 serologically positive cattle were real-time PCR-positive for *T. parva*, although no piroplasms could be detected in the blood smears. Unexpectedly, out of 614 *R. appendiculatus* nymphal and adult ticks collected by flagging vegetation, 4.1% were positive for *T. parva* DNA. However, since the rate of transmission of *T. parva* from infected cattle to ticks and vice versa and the serological evidence of exposure to *R. appendiculatus* ticks in naturally-exposed cattle were relatively high, it would be wise in such a case to consider vector control as well as vaccination against ECF as control measures.

Key words: *Theileria parva*; real-time PCR; Detection; Monitoring; Transmission;

1. Introduction

Theileria parva (*T. parva*) is haemoprotozoan parasite transmitted by the tick *Rhipicephalus appendiculatus* (*R. appendiculatus*), causing East Coast fever (ECF) of cattle in several African countries, including Zambia (Billiouw et al., 2002; Geysen et al., 1999; Minjauw et al., 1997). The disease causes high mortality and morbidity, and is considered to be an important constraint to the improvement of the livestock industry in Africa.

T. parva is an intracellular protozoan parasite that infects bovine lymphoid cells in its schizont stage and erythrocytes in its piroplasm stage. The piroplasm stage of *T. parva*, which resides in the erythrocytes, is infective to *R. appendiculatus* ticks. *T. parva* is acquired by immature ticks during blood feeding, and transmission of *T. parva* is strictly trans-stadial as the parasite is transmitted only by the nymphal and adult stages after acquiring infections during feeding as larvae or nymphs, respectively. In tick-borne hemoprotozoan parasite infections, the level of parasitemia in the reservoir hosts at the time of tick feeding is critical for efficient acquisition of the pathogens by the ticks (Eriks et al., 1993; Ueti et al., 2005). Interestingly, Ueti et al. (2005) have demonstrated the ability of the vector tick *Boophilus microplus* to acquire and transmit *Babesia equi* following feeding on chronically infected horses with low-level parasitemia. Ticks (7.1-50%) that acquired *B. equi* from chronically infected horses,

as well as those (22%) fed during the acute phase of infection, successfully transmitted the parasite to naive horses. The results unequivocally demonstrated that chronically infected horses with low-level parasitemia are competent mammalian reservoirs for tick transmission of *B. equi*. Nevertheless, the parasitemia levels required for the ticks to be able to acquire *T. parva* upon feeding on reservoir hosts are unknown.

Recently, Ogden et al. (2003) described that experimental transmission rate of *T. parva* to ticks was higher when ticks fed on an acutely-infected calf as nymphs. In contrast, transmission rate of *T. parva* to ticks was lower when ticks fed on carriers with low-level parasitaemia that had been infected by the simultaneous inoculation of a lethal dose of *T. parva* stabilates (cryopreserved sporozoites) and injection of a dose of long-acting oxytetracycline (Marcotty et al., 2002). In addition, Young et al. (1996) reported that several factors could influence the ability of the vector tick to acquire *T. parva* and the transmission of *T. parva* from infected-ticks to cattle. It seems likely the covariate piroplasm parasitaemia was the most important for abundance and intensity in ticks and prevalence in ticks. Interestingly, they indicated the evidence that high levels of prevalence, abundance and intensity were associated with levels parasitaemia from the regression coefficients associated with piroplasm parasitaemia. These observations led to speculations that the level of parasitemia could affect the rate of acquisition of *T. parva* by ticks. However, there is only limited information concerning the correlation between levels of parasitemia in hosts and the ability of ticks to acquire *T. parva* infection because of the lack of reliable methods for precise quantification of the parasitaemia.

In the present study, we undertook to determine some of the factors that could be affecting the transmission rate of *T. parva* between ticks and cattle, both under experimental and field conditions, by using the quantitative real-time PCR assay targeting the *T. parva* single-copy gene encoding the 104 kDa antigen (*p104*).

2. Materials and methods

2.1 Tick acquisition feeding on T. parva-infected cow and tick transmission feeding

Four Friesian calves aged 1-year old were used. *T. parva* negative calves were maintained under a strict acaricidal control regime at the University of Zambia (UNZA), School of Veterinary Medicine Experimental Animal Facility. For experimental infection, a calf was inoculated with 5 ml of a 1:50 dilution of the *T. parva* mixture (Muguga, Kiambu 5 and Serengeti transformed). The *T. parva* mixture, originally obtained from the Center for Ticks and Tick borne Diseases, Malawi, was used in this study. *T. parva* free ticks were obtained from a colony of *R. appendiculatus* maintained on rabbits at the School of Veterinary Medicine, UNZA. Five hundred nymphal ticks were introduced and maintained on a calf's ears with the help of ear bags at day 9 after inoculation of the *T. parva* mixture. Six days after the tick introduction, engorged ticks that had dropped from the calf were collected and maintained at 20°C with >95% relative humidity.

Forty unfed adult ticks (20 male and 20 female) that molted from nymphs that had fed on the calf were regarded as experimentally infected ticks. These were in turn

made to feed on clean (uninfected) calves in order for the calves to acquire the *T. parva* infection from the ticks. Blood samples were collected daily starting from day zero post-tick challenge and used for the PCR and microscopic (Giemsa staining) monitoring of the infection.

2.2 Field samples

R. appendiculatus ticks were collected by flagging vegetation with a cotton flannel in the Shibuyunji area of Lusaka, central Zambia, where ECF is known to be endemic. A total 614 *R. appendiculatus* ticks (369 nymphs and 245 adults) were collected and maintained as described above until DNA extraction. A total of 70 traditionally-managed Sanga cattle without history of vaccination against ECF were randomly selected in the Shibuyunji area and venous blood samples collected for DNA extraction, serum extraction and for the preparation of thin blood smears for the determination of parasitaemia by Giemsa staining.

2.3 DNA extraction

Tick DNA samples were obtained by Proteinase-K treatment of homogenised ticks followed by phenol/chloroform extraction and ethanol precipitation as previously described (Hill and Gutierrez, 2003). Bovine genomic DNA samples were obtained from 0.5 ml of whole blood samples of cattle using the Wizard™ Genomic DNA Purification kit (Promega, Madison, WI) as previously described (Konnai et al., 2005). Purified genomic DNA concentration was measured by optical density (OD) at 260nm

and stored at 4°C.

2.4 Nested PCR and real-time quantitative PCR

Nested PCR was performed using a thermal cycler (System 9700, Applied Biosystems). The primary reaction mixture used 100 ng of DNA sample as template in a total volume of 30 μl. The reaction mixtures contained 10 mM Tris-HCl, 50mM KCl (pH8.3), 0.1% Triton-X100, 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate (dNTP) at a concentration of 120 μM, 2.5 units of rTaq polymerase (Takara, Ostu, Japan), and each primer at a concentration of 0.5 μM. The outer primers were *T. parva p104* primers IL3231; 5'-ATTTAAGGAACCTGACGTGACTGC-3' and IL755; 5'-TAAGATGCCGACTATTAATGACACC-3' which produce a 496-bp product as described previously (Kaba et al., 2005; Skilton et al., 2002). *T. parva p104* gene is highly conserved within the *T. parva* strains (Skilton et al., 2002). Cycling conditions involved an initial 5 min denaturation at 95°C, followed by 30 cycles, each consisting of a 30 s denaturation at 94°C, a 30 s annealing at 65°C, and 1 min extension at 72°C. The 30 cycles were followed by a 5 min extension at 72°C. Samples from which no products were amplified after the initial PCR were subjected to re-amplification by nested PCR. The reaction mixture for the nested amplifications used 3 μl of the primary PCR products as the template in a total volume of 30 μl. The reaction mixture for each nested amplification contained 10 mM Tris-HCl, 50 mM KCl (pH8.3), 0.1% Triton-X100, 1.5 mM $MgCl_2$, each dNTP at a concentration of 120 μM, 2.5 units of rTaq polymerase, and each primer at a concentration of 0.5 μM. The inner primers

were *T. parva* p104 primers IL4234; 5'-GGCCAAGGTCTCCTTCAGAATACG-3' and IL3232; 5'-TGGGTGTGTTTCCTCGTCATCTGC-3' which produced a 277-bp fragment. Nested cycling conditions were as described above for the primary amplification.

The real-time quantitative PCR targeted the single-copy *T. parva p104* gene and was based on Light Cycler technology. Real-time PCR amplifications were performed in a Light Cycler™ (Roche Diagnostics, Mannheim, Germany) as previously described (Konnai et al., 2005). Three microliters (100 ng) of template DNA were added to a 17 µl reaction mixture containing PCR buffer, each of the oligonucleotide primers (at a final concentration of 0.2 µM) and 10 µl of SYBR Premix Ex Taq™ (TAKARA, Tokyo, Japan). Cycling reactions were performed under the following conditions: 10 s at 95°C, followed by 50 cycles of 5 s at 95°C, 20 s at 65°C and 10 s at 72°C. A melting curve program involving heating to 60-99°C at a rate of 0.1°C/s with a final cooling to 40°C was used. Ten-fold serial dilutions of plasmid DNA bearing the *p104* gene were prepared in the range of 10^{1} to 10^{-7} pg (limited detection dose) and used for creating a quantification standard curve. The concentrations of parasite DNA in the test samples were worked out from the established standard curve. As an internal control, the *R. appendiculatus* tick β-actin gene or bovine β-globin gene were amplified in each test sample using primer pairs ACT-108: 5'-TGGATCGGCGGCTCCATCCT-3'/ACT-rev-A: 5'-GAAGCACTTGCGGTGGACAATG-3'(da Silva et al., 2005) and PC03: 5'-ACACAACTGTGTTCACTAGC-3'/PC04:

5’-CAACTTCATCCACGTTCACC-3’(Konnai et al., 2005) for *R. appendiculatus* β-actin and bovine β-globin, respectively. Results were expressed as weight of *T. parva* genome per 100 ng of template DNA derived from 0.5 ml of bovine whole blood. To control for variables in the quality of tick samples, data were expressed as *T. parva* genome/tick genome ratio. PCR products were purified with the Geneclean III Kit (Q-BIOgene, USA) and DNA sequencing was performed by the CEQ 2000 DNA analysis system (Beckman Coulter, Inc., Fullerton, CA).

2.5 Western blot analysis

To determine exposure of the Sanga cattle to *R. appendiculatus* tick infestation, serum samples of the cattle were used in Western blotting assays. The *R. appendiculatus* immuno-dominant molecule 36 (RIM 36) (Bishop et al., 2002) was prepared as a recombinant protein as previously described (Imamura et al., 2002). *R. appendiculatus* adults were obtained from a tick colony at the Hokkaido university. Salivary glands from partial fed 30 ticks on rabbits were dissected in PBS and homogenized within TRIzol (Invitorogen, Carlsbad, CA, USA) and the reverse transcriptase (RT) reaction was performed with 1 μg of purified total RNA. The *R. appendiculatus* RIM 36 cDNA were amplified by PCR using adult tick cDNA as template and the primers 5’-GGATCCATGAAGGTCTTCGTCGCTGTC-3’, containing the ATG translation start codon and *EcoR*I restriction site, and 5’-CTCGAGTTAGATTGCAACGTGTTCCTGTACTTG-3’, containing the TAA translation stop codon and *Xho*I restriction site. The conditions of PCR were an initial

4-min incubation at 94°C, followed by 35 cycles of incubation at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, with the final extension at 72°C for 10 min. The amplified product was digested and ligated into the expression vector pET32a (Novagen, Inc., Madison, WI), which produces the desired recombinant product linked to TRX and subcloned into *Escherichia coli* AD494. Expression of the protein product by transformed *E. coli* was induced over 4 h with 0.5 mM isopropyl-1-thio-beta-D-galactoside. Expressed proteins were affinity-purified on nickel-charged columns under the native conditions according to the instructions of the manufacture (Novagen). The purified recombinant protein was electrophoresed by SDS-PAGE and blotted onto nitrocellulose membrane strips. The strips were blocked in 3% skim milk in PBST and incubated for 1 h with test serum diluted 1:500 in PBST. Serum from a calf experimentally infested with ticks was used as positive control serum. After 3 washes of 10 min each, the strips were incubated with secondary antibody (peroxidase-conjugated goat anti-bovine IgG) at 1:1000 dilution for 1 h at 25°C. After 4 washes of 10 min each, positive signals were generated with 3,3-diaminobenzidine tetrahydrochloride and cobalt chloride.

2.6 Statistical analysis

Data were analyzed by one-way analysis of variance followed by Student *t*-test. Differences between groups were considered significant if probability values of $p<0.05$ were obtained. All statistical analyses were performed with the statistical software Statcel2 (OMS, Saitama, Japan).

3. Results

3.1. Transmission rate of T. parva in R. appendiculatus ticks

A calf was experimentally inoculated with the *T. parva* mixture, and *T. parva* DNA load was monitored by real-time PCR. The calf became PCR-positive 8 days post-inoculation though it was still aparasitaemic (Fig. 1A). On day 9, nymphal ticks were introduced, and maintained on the calf until they engorged. There were no detectable piroplasms on blood smears made from the calf's blood and no observable clinical signs of ECF during the 6 days of tick feeding. Total DNA was extracted from 171 (90 male and 81 female) randomly selected molted adult ticks and used for the real-time PCR assay for detection of *T. parva* (Table 1). Overall, 70.8% (121/171) of all ticks placed on the calf acquired *T. parva* infection. There was no difference in infection acquisition rate between male (63.3%) and female (79.0%) ticks that fed on the calf.

3.2. Monitoring of T. parva in tick-exposed calves

Forty unfed adult ticks (20 male and 20 female) infected with *T. parva* were fed to repletion on naïve calves. Though *T. parva* infection could be detected by real-time PCR as early as day 11 post-tick exposure, by microscopic examination of Giemsa stained smears *T. parva* could only be detected in peripheral blood starting from day 16 post-tick exposure (Fig. 1B, C and D). The increase in parasitaemia levels from days

16 through to 22 post-tick exposure correlated to the increase in *T. parva* DNA load as monitored by microscopic examination and real time PCR, respectively. Further the *T. parva* DNA load increase corresponded to the onset of the ECF clinical signs and the acute stage of infection. The clinical signs of the calves became severe, and body temperatures were over 40°C from day14 post-exposure, during which time the *T. parva* DNA load exceeded 10^{-4} pg. One of the calves developed dyspnoea and died on day 22 post-exposure when *T. parva* DNA load had reached 10^{1} pg (Fig. 2B).

3.3. Detection and quantification of T. parva in field samples

A total of 614 unfed, host-seeking *R. appendiculatus* ticks were collected and individually analyzed for the presence of *T. parva* DNA. Out of 614 ticks, 25, including 17 nymphs and 8 adult ticks, were found infected (Table 1). All the 614 ticks analyzed tested positive for tick β-actin, which was used as control for template DNA loading. Quantitative real-time PCR showed that the 25 *T. parva* positive ticks had parasite DNA load ranging from 10^{-5} to 10^{-3} pg per tick and when this was expressed as a *T. parva* genome/tick genome ratio (Table 1). The *T. parva* DNA load in the ticks was found not to be significantly different between nymphs and adults, and naturally-infected and experimentally-infected ticks.

Of the 70 bovine sera samples analyzed, 67 (95.7%) were positive for the RIM 36 antibody by Western blot analysis, indicating serologically that the cattle had exposure to *R. appendiculatus* ticks (Table 2). Of the 70 naturally-exposed cattle, twenty-nine (41.4%) were found to be positive for *T. parva* infection by real-time PCR analysis with

a *T. parva* DNA load in the range of 0.000001433 to 5.094 per 100 ng of template DNA sample (Table 2). All the 29 cattle were also positive for the RIM 36 antibody as detected by Western blot analysis. Twenty-three (79.3%) of the PCR-positive cattle had no detectable piroplasms on blood smears (Fig. 2). Only cattle that had a *T. parva* DNA load of more than 10^{-2} pg had microscopically detectable piroplasms, consistent with the results obtained from the experimentally infected calves (Fig. 2).

4. Discussion

The *T. parva* infection rate in adult *R. appendiculatus* ticks feeding on low parasitaemic, *T. parva* carrier cattle has been observed to be lower than in ticks feeding on calves with acute, high parasitaemic infections (Marcotty et al., 2002; Ogden et al., 2003). These findings suggest that the level of parasitemia in the infected animals contribute to the efficiency of acquisition of *T. parva* infection by ticks. In the present study, to assess this possibility, we monitored the *T. parva* DNA load in ticks and calves under experimental as well as field conditions by using quantitative real-time PCR in order to determine factors that could be affecting tick-mediated *T. parva* transmission.

The nymphal ticks fed on calf with low *T. parva* DNA load 10^{-6} to 10^{-3}pg /100ng of bovine DNA acquired *T. parva*, with detection of *T. parva* in 70.8% of molted adult ticks. Moreover, we detected *T. parva* DNA in ticks (nymphs and adults) collected in the field from an area where most of the animals tested had no piroplasms on blood smears, though positive by PCR, which was consistent with the data obtained under experimental conditions. We observed that nymphal ticks experimentally fed on calf

with very low parasitaemia (only detectable by PCR) acquired *T. parva* infection at a higher rate than previously observed (Marcotty et al., 2002). These findings high-light the inadequacy associated with microscopy-based *T. parva* screening compared to PCR. To be able to detect piroplasms by microscopy, the *T. parva* DNA load (as determined by real time PCR) needed to be at least 10^{-3} pg under both experimental and field conditions. Although we could not compare the sensitivity directly to other methods, the detection limit of real-time PCR might correspond to other PCR-based methods reported previously having sensitivity levels of up to one piroplasm in 10^7 erythrocytes (Watt et al., 1998).

In the present study, *T. parva* transmission trials using *R. appendiculatus* as vector ticks indicated that the ticks had the ability to acquire *T. parva* infection from carrier animals with low levels of parasitaemia. In addition, the ticks that acquired *T. parva* from the calf during the low *T. parva* DNA load of infection successfully transmitted the parasite to naive calves. Indeed, one of the calves developed dyspnoea and died on day 22 post-exposure when *T. parva* DNA load had reached 10^1 pg. A limited number of naturally-infected ticks were quantified, yet as expected, there was no difference in *T. parva* DNA load between naturally-infected ticks (0.0026±0.0022) and ticks that were fed on the experimentally-infected calf (0.0033±0.0022). Although the number of infected adult or nymph ticks required to effectively transmit *T. parva* under natural conditions still remains unknown, this result indicates one of the risks of transmission of *T. parva* from carrier animals with low levels of parasitaemia to naive calves via the ticks having the high ability to acquire *T. parva*. Similar *T. parva* DNA loads were

observed among naturally infected ticks collected from an ECF endemic area with most of the animals having low level parasitaemia. Considering that a high percentage (95.7%) of the animals were found to be serologically positive to *R. appendiculatus* exposure, it would be wise to undertake tick control measures as well as vaccination against ECF in such a scenerio.

The average prevalence rate (41.4%) of *T. parva* infection among Sanga cattle in Zambia was generally similar to that described elsewhere in Africa. *T. parva* prevalence rates among indigenous and crossbred cattle in Uganda, Tanzania and Kenya have been reported to be 7-65%, 23.4% and 27-100%, respectively, while the prevalence rates among ticks collected from the same areas of Tanzania and Kenya have been reported to be 2.6% and 2.3-14.2%, respectively (Ogden et al., 2003; Oura et al., 2004; Watt et al., 1998). We observed that the prevalence rate of *T. parva* infection among host-seeking *R. appendiculatus* collected from the field was on average 4.1 %, which was quite similar to that observed in Kenya and Tanzania. Interestingly, in spite of the high prevalence of *T. parva* in Sanga cattle, the low prevalence was observed in the questing ticks. It seems likely the several factors could influence the ability of the vector tick to acquire *T. parva* and the transmission of *T. parva* from infected-ticks to cattle (Young et al., 1996). There remain many unknowns that may influence the association between real-time PCR positive cattle and ticks and transmission, including variables in the number, viability, and infectivity of sporozoites within the salivary glands, pathogen and *T. parva* strain differences, and duration of tick feeding in the field, that require additional investigation.

Currently, vaccination against EFC involves the simultaneous inoculation of a lethal dose of *T. parva* stabilates (cryopreserved sporozoites) and a long-acting oxytetracycline dose in several African countries, including Zambia (Uilenberg, 1999). It is likely that the resulting carrier cattle after the immunization could act as the main reservoirs of *T. parva* (Marcotty et al., 2002; Kariuki et al., 1995; Mutugi et al., 1991). Since the rate of transmission of *T. parva* from infected cattle to ticks and vice versa and the serological evidence of exposure to *R. appendiculatus* ticks in naturally-exposed cattle were relatively high, it would be wise in such a case to consider vector control as well as vaccination against ECF as control measures.

Acknowledgments

The encyclopedic *T. parva* detection knowledge of Dr. Richard Bishop, International Livestock Research Institute, and Dr. Vishvanath Nene, The International Institute for Genomic Research, are gratefully appreciated. We wish to thank Mr. Chota Amos and Mr. Mwelwa Chembensofu, for technical assistance. This research was sponsored by Grants-in-Aid for Scientific Research from the Japan Society the Promotion of Science.

References

Billiouw, M., Vercruysse, J., Marcotty, T., Speybroeck, N., Chaka, G., Berkvens. D.,

2002. *Theileria parva* epidemics: a case study in eastern Zambia. Vet. Parasitol. 107, 51-63.

Bishop, R., Lambson, B., Wells, C., Pandit, P., Osaso, J., Nkonge, C., Morzaria, S., Musoke, A., Nene, V., 2002. A cement protein of the tick *Rhipicephalus appendiculatus*, located in the secretory e cell granules of the type III salivary gland acini, induces strong antibody responses in cattle. Int. J. Parasitol. 32, 833-842.

da Silva Vaz I. Jr., Imamura, S., Nakajima, C., de Cardoso, F. C., Ferreira, C. A., Renard, G., Masuda, A., Ohashi, K., Onuma, M., 2005. Molecular cloning and sequence analysis of cDNAs encoding for *Boophilus microplus*, *Haemaphysalis longicornis* and *Rhipicephalus appendiculatus* actins. Vet. Parasitol. 127, 147-155.

Eriks, I. S., Stiller, D., Palmer, G. H., 1993. Impact of persistent *Anaplasma marginale* rickettsemia on tick infection and transmission. J. Clin. Microbiol. 31, 2091-2096.

Geysen, D., Bishop, R., Skilton, R., Dolan, T. T., Morzaria, S., 1999. Molecular epidemiology of *Theileria parva* in the field. Trop. Med. Int. Health. 4, A21-A27.

Hill, C. A., Gutierrez, J. A., 2003. A method for extraction and analysis of high quality genomic DNA from ixodid ticks. Med. Vet. Entomol. 17, 224-227.

Imamura, S., da Silva Vaz I. Jr., Sugino, M., Ohashi, K., Onuma, M., 2005. A serine protease inhibitor (serpin) from *Haemaphysalis longicornis* as an anti-tick vaccine. Vaccine. 23, 1301-1311.

Kaba, S. A., Musoke, A. J., Schaap, D., Schetters, T., Rowlands, J., Vermeulen, A. N., Nene, V., Vlak, J. M., van Oers, M. M., 2005. Novel baculovirus-derived p67 subunit vaccines efficacious against East Coast fever in cattle. Vaccine. 23,

2791-2800.

Kariuki, D. P., Young, A. S., Morzaria, S. P., Lesan, A. C., Mining, S. K., Omwoyo, P., Wafula, J. L., Molyneux, D. H., 1995. *Theileria parva* carrier state in naturally infected and artificially immunised cattle. Trop. Anim. Health Prod. 27,15-25.

Konnai, S., Usui, T., Ikeda, M., Kohara, J., Hirata, T., Okada, K., Ohashi, K., Onuma, M., 2005. Imbalance of tumor necrosis factor receptors during progression in bovine leukemia virus infection. Virology. 339, 239-248.

Marcotty, T., Brandt, J., Billiouw, M., Chaka, G., Losson, B., Berkvens, D., 2002. Immunisation against *Theileria parva* in eastern Zambia: influence of maternal antibodies and demonstration of the carrier status. Vet. Parasitol.110, 45-56.

Minjauw, B., Otte, J., James, A. D., de Castro, J. J., Sinyangwe, P., 1997. Effect of different East Coast Fever control strategies on fertility, milk production and weight gain of Sanga cattle in the Central Province of Zambia. Exp. Appl. Acarol. 21, 715-730.

Mutugi, J. J., Young, A. S., Kariuki, D. P., Tameno, J. M., Morzaria, S. P., 1991. Epidemiological observations on theileriosis following field immunisation using infection and treatment. Trop. Anim. Health Prod. 23, 75-82.

Ogden, N. H., Gwakisa, P., Swai, E., French, N. P., Fitzpatrick, J., Kambarage, D., Bryant, M., 2003. Evaluation of PCR to detect *Theileria parva* in field-collected tick and bovine samples in Tanzania. Vet. Parasitol. 112, 177-1183.

Oura, C. A., Bishop, R. P., Wampande, E. M., Lubega, G. W., Tait, A., 2004. Application of a reverse line blot assay to the study of haemoparasites in cattle in

Uganda. Int. J. Parasitol. 34, 603-613.

Skilton, R. A., Bishop, R. P., Katende, J. M., Mwaura, S., Morzaria, S. P., 2002. The persistence of *Theileria parva* infection in cattle immunized using two stocks which differ in their ability to induce a carrier state: analysis using a novel blood spot PCR assay. Parasitology. 124, 265-276.

Ueti, M. W., Palmer, G. H., Kappmeyer, L. S., Statdfield, M., Scoles, G. A., Knowles, D. P., 2005. Ability of the vector tick *Boophilus microplus* to acquire and transmit *Babesia equi* following feeding on chronically infected horses with low-level parasitemia. J. Clin. Microbiol. 43, 3755-3759.

Uilenberg, G., 1999. Immunization against diseases caused by *Theileria parva*: a review. Trop. Med. Int. Health. 4, A12-A20.

Watt, D., Kiara, H., Sparagano, O. A., 1998. A PCR-based field evaluation of Theileria infections in cattle and ticks in Kenya. Ann. NY. Acad. Sci. 849, 69-77.

Young, A.S., Dolan, T.T., Morzaria, S.P., Mwakima, F.N., Norval, R.A., Scott, J., Sherriff, A., Gettinby, G., 1996. Factors influencing infections in *Rhipicephalus appendiculatus* ticks fed on cattle infected with *Theileria parva*. Parasitology. 113, 255-266.

Figure legends

Fig. 1. Monitoring of the *T. parva* DNA load in experimentally infected calves. (A: *T. parva* mixture inoculated calf. B, C and D: *T. parva*-infected ticks exposed calf. Each line represent *T. parva* DNA load (full circle) and body temperature (empty circles). Arrow in A indicates the tick exposure period. Arrows in C and D donate treatments with oxytetracyclines. Plus or minus on the X-axis indicate results of detection of piroplasm by microscopic examination (+: positive, -: negative).

Fig. 2. Quantification of the *T. parva* DNA load in naturally-exposed Sanga cattle. Results of detection of the *T. parva* DNA correspond to Table 2. Individual dots indicate $PCR^{+}Blood\ smear^{+}$ (full circle) and $PCR^{+}Blood\ smear^{-}$ (empty circles) ticks, respectively. The results are shown as the concentrations of *T. parva* DNA obtained by dividing the concentrations of the PCR products from 100ng template DNA.

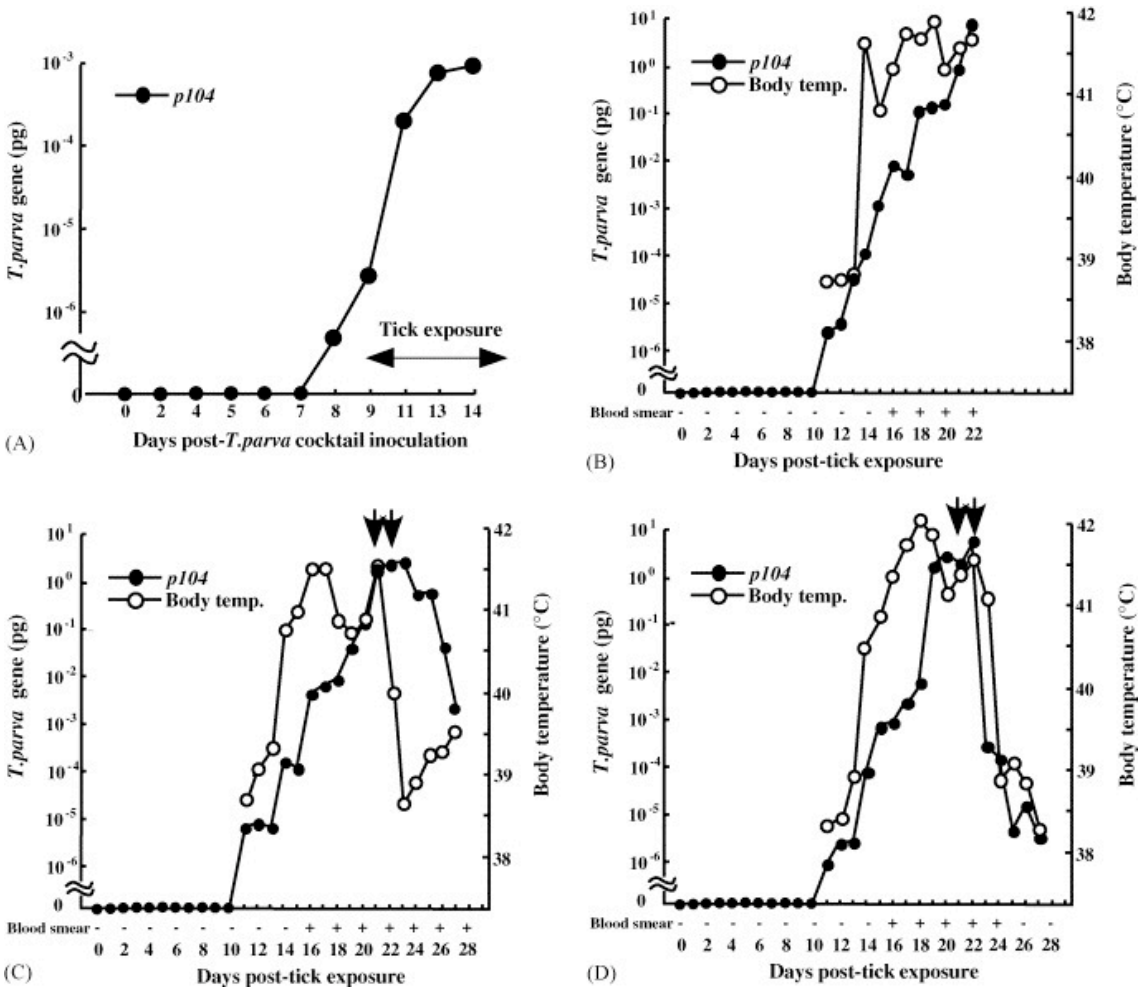
(A)
p104
T.parva gene (pg)
Tick exposure
Days post-T.parva cocktail inoculation
(B)
p104
Body temp.
T.parva gene (pg)
Body temperature (°C)
Blood smear
Days post-tick exposure
(C)
p104
Body temp.
T.parva gene (pg)
Body temperature (°C)
Blood smear
Days post-tick exposure
(D)
p104
Body temp.
T.parva gene (pg)
Body temperature (°C)
Blood smear
Days post-tick exposure

Fig. 1

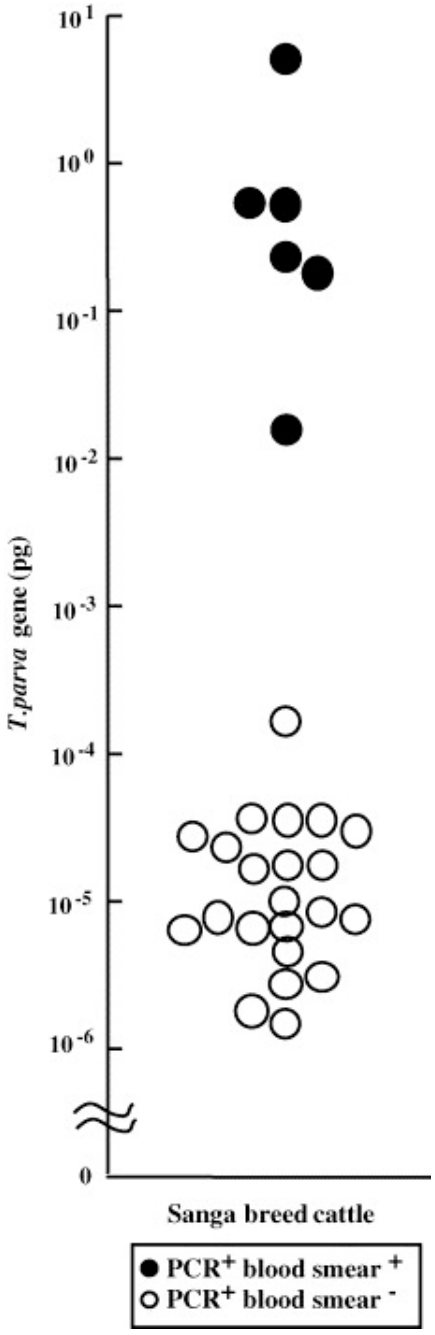
10 1
10 0
10 -1
10 -2
10 -3
10 -4
10 -5
10 -6
0
T.parva gene (pg)
Sanga breed cattle
PCR+ blood smear +
PCR+ blood smear -

Fig. 2

Table 1
PCR detection of *T. parva* DNA in experimentally infected and field-collected *R. appendiculatus* ticks

Situation	Life stage	No. of ticks examined	No. (%) of positive	*T. parva* load (p104/β-actin)
Experimentally-infected	Adult	171	121 (70.8%)	0.0033 ± 0.0022
	(Male)	(90)	57 (63.3%)	0.0030 ± 0.0024
	(Female)	(81)	64 (79.0%)	0.0035 ± 0.0024
Field-collected	Nymph	369	17 (4.6%)	0.0025 ± 0.0019
	Adult	245	8 (3.3%)	0.0030 ± 0.0026
	(Male)	(128)	4 (3.1%)	0.0029 ± 0.0019
	(Female)	(117)	4 (3.4%)	0.0031 ± 0.0034
	Total	614	25 (4.1%)	0.0026 ± 0.0022

Table 2
Detection of *R. appendiculatus* antibody and *T. parva* DNA in Sanga breed cattle in Shybuyuni-ji area in Zambia

No. of cattle examined	No. (%) of positive			
	tick antibody (anti-RIM36)	*T. parva*		
		Blood smear	nested PCR	Real-time PCR
70	67 (95.7%)	6 (8.6%)	29 (41.4%)	29 (41.4%)