Molecular diversity of *Theileria parva*: a case study of Kilosa district, Morogoro, Tanzania

L.R. Mwaijibe[1], E.D. Mwega[2], E.J. Rukambile[1], R.S. Mwakapuja[2], H.B. Magwisha[1] and E.N. Kimbita[3].

[1]Tanzania Veterinary Laboratory Agency, Central Veterinary Laboratory, P.O. Box 9254, Dar es Salaam, Tanzania;
[2]Tanzania Veterinary Laboratory Agency, Tanzania Vaccine Institute, P.O. Box 9254, Dar es Salaam, Tanzania.
[3]Sokoine University of Agriculture, Department of Microbiology, Parasitology and Immunology, P.O. Box 3012 Morogoro, Tanzania.
Email: leilagod2004@yahoo.com

SUMMARY

Theileria parva is an apicomplexan protozoan parasite which causes East Coast fever (ECF) in Eastern and Central Africa. A study was conducted between February and May 2012 in Morogoro region to determine the diversity of the *T. Parva* parasite circulating in cattle in Kilosa district. Also ECF cases reported between 2008 and 2009 at Veterinary Investigation Centres (VIC's) were analysed retrospectively to gain insight into the current status of ECF in Tanzania. A total of 100 cattle and 95 ticks were investigated. Conventional Polymerase Chain Reaction (PCR) was used to determine the diversiyof *T. parva* by amplification of surface protein (p67). The study revealed the existence of two strains of *T. parva* with 800 and 900 bp respectively. This study revealed *T. parva* allele which has never been reported to be present in the imported ECF trivalent vaccine ("Muguga cocktail") currently in use. Retrospective analysis on ECF cases of the disease indicated lower prevalence of ECF compared to the prevalence reported by other workers. These findings call for more study on the molecular epidemiology of *T. parva* circulating in cattle in the different grazing lands in Tanzania. The VICs are required to improve their diagnostic facilities and database.

Key words: Indigenous cattle, pastoralists, molecular epidemiology, genotyping.

INTRODUCTION

Theileriosis caused by *Theileria parva* is one of the important diseases constraining the development of livestock industry in Tanzania and causes mortality and performance losses particularly in highly susceptible taurine breeds and their crosses (Kivaria *et al.,* 2007). Although *T. parva* causes other forms of theileriosis including January disease in Zimbabwe and Corridor disease in South Africa, East Coast fever (ECF) which is found in East Africa countries remains the major form of theileriosis in Tanzania. Characteristic and unique DNA banding patterns have been detected in several *T. parva* stocks. Size polymorphisms displayed by *T. parva* antigen genes, PIM, p104, p150 and p67, have been used to develop several molecular tools for characterization of *T. parva* stocks, exploiting the variable regions of these genes (Geysen *et al* ., 1999; Bishop *et al* ., 2001).

Previous studies in many parts of Africa, including Kenya (Nene *et al.*, 1992), Zimbabwe (Bishop *et al.*, 1994), Uganda (Oura *et al.,* 2011) South Africa (Sibeko *et al.,* 2010) and Northen Tanzania (Mwega *et al*., 2014) reported a diversity of *T. parva* isolated in buffalo and cattle. Several genes have been investigated in search of discriminatory sequence differences between *T. parva* isolates. Among these are sporozoite antigen genes, p67, p104 and PIM (Nene *et al*., 1996; Iams *et al*., 1990; Bazarusanga *et al*., 2007).

This study reports on molecular diversity of *T. parva* parasite isolated from cattle and ticks by examining the banding pattern of the *T. parva* gene coding for (p67) in an effort to identify *T. parva* parasites that are circulating in cattle in the fieldin Kilosa district.

MATERIALS AND METHODS

Study area

Kilosa district is one of the six districts of the Morogoro region in the eastern Tanzania. The district lies between 6°S and 8°S, and 36°30'E and 38°E (Nduwamungu *et al.,* 2004). It experiences an average of eight months of rainfall (October–May), with the highest levels between February and March. The rainfall distribution is bimodal in good years, with short rains (October - January), followed by long rains (mid February - May). The mean annual rainfall ranges between 1,000 and 1,400 mm in the southern flood plain, while further north (Gairo Division) has an annual rainfall ranging from 800 to 1,100 mm. The mean annual temperature in Kilosa is about 25°C. The district is among the most important indigenous cattle area in Tanzania with the history of occurrence of ECF. Six villages were

involved in the study including Kimamba, Madoto, Parakuyo, Kwambe, Mabwegere and Mbwade.

Study design and sample size

Purposive sampling was conducted. Animals were clinically examined by observing important clinical signs such as presence of nasal discharges, lacrimation and rate of respiration. Physical examination was also performed which involved assessment of superficial lymph nodes. Animal that presented with suggestive signs were identified and sampled for future laboratory diagnosis of ECF.

Blood sampling in suspected cattle

A blood sample was collected from 100 restrained cattle by vein puncture of jugular vein. The sample was collected into EDTA vacutainer tube (Vacutainer® Dickinson B-D United Kingdom). The tubes were placed on ice in a cool box with ice packs then refrigerated before they were transferred to the Faculty Veterinary Medicine laboratories at Sokoine University of Agriculture.

Retrospective data on ECF for the period between 2008 and 2009 was accessed from Veterinary Investigation Centers which are located in seven agro-ecological zones including Eastern zone, Central zone, Western zone, Lake zone, Southern zone, Northen zone and Southern highlands zone. The epidemiological information and number of ECF cases reported by VICs Zonal Managers were obtained from Ministry of Livestock Development and Fisheries (MLDF).

Collection of *Rhipicephalus appendiculatus*

A total of 95 host-seeking (questing) *R. appendiculatus* ticks were collected from herbage by dragging a blanket along the vegetation as described by Short and Norval (1981) in the fenced, open and restricted grazing systems, respectively. The ticks were placed in 100 ml universal bottles with cotton wool dampened with sterile water. The bottles were placed in cool box maintained at approximately 0°C by ice packs to prevent death of the ticks and bacteria spoilage. At the laboratory the ticks were stored at -20°C before processing. Morphological identification of ticks was carried out by means of a stereo microscope (Olympus) using the identification keys of Walker *et al*. (2000).

DNA extraction and Screening of *T. parva*

Tick DNA samples were obtained by Proteinase-K treatment of homogenized ticks followed by phenol/chloroform extraction and ethanol precipitation as previously described (Hill and Gutierrez, 2003). Cattle genomic DNA wasextracted from whole blood using protocol as described in the Quick-g DNA^{TM} Mini Prep DNA kit (Zymo Research Corporation, CA, and U.S.A).Tick DNA was extracted as described by Hill and Gutierrez (2003). These were screened for the presence of *T. parva* DNA by Semi-nested polymerase chainreaction (Takara Thermocycler manufactured in Japan) using p104 gene as previously described by Iams *et al*., 1990. Forward primer p104F2 (5′CCACCATCTAAACCACCGTT3′) and reverse primer p104R (5′TAAGATGCCGACTATTGACACCACAA3′) for the first round and forward primer p104nF (5′ACCACCGTTTGATCCATCATTCA3′) and reverse primerp104R (5′TAAGATGCCGACTATTGACACCACAA3′) for the second round were used.

Amplification of the p67 gene from *T. parva*

Molecular typing of *T. parva* was conducted by amplification of p67 gene as described by Iams *et al*. (1990). Forward primer p67F (5′CAGGTGAAACTACATCGG3′) and reverse primer p67R (5′TACTCAAAAAAACAAACC3′) were used in amplifying p67 gene. After amplification, amplicons were resolved by electrophoresis through 1% agarose gel and visualized by fluorescence under UV light after staining with ethidium bromide (Sigma-Aldrich, USA). The 100 bp DNA molecular weight marker was used to estimate the amplicon sizes (Gutierrez, 2003).

Cattle genomic DNA samples were obtained from 1ml of whole blood using Quick-gDNA
TM Mini Prep DNA extraction kit (ZYMORESEARCH). According to manufacturer recommendations 50 µl of DNA Elusion Buffer was added to the spin column. The mixture was incubated for 5 minutes at room temperature and then centrifuged at 13,500xg for 30 seconds to elude the DNA. The eluted DNA was stored at -20°C (Gutierrez, 2003). Molecular typing of *T. parva* was conducted by amplification of p67 gene as described by Iams *et al.* (1990). Pairs of primers used in the first and second rounds of the semi-nested PCR for p104 and p67 gene. Cattle genomic DNA samples were obtained from 1ml of whole blood using Quick-g DNA TM Mini Prep DNA extraction kit (ZYMORESEARCH). According to manufacturer recommendations 50 µl of DNA Elusion Buffer was added to the spin column. The mixture was incubated for 5 minutes at room temperature and

then centrifuged at 13,500xg for 30 seconds to elude the DNA. The eluted DNA was stored at -20°C.

Data analysis

Descriptive statistics were used to determine prevalence. Comparison of prevalence between zones was conducted using (t- tests).

RESULTS

Theileria parva-positive samples were selected for amplicon size analysis of the p67 gene. The analysis showed two amplicons of different sizes present in cattle and Tick. Thirty-six percent (n=100) of animals and11.6% (n=95) of ticks were positive for p104. Two p67 PCR products of 800 and 900 bp sizes were obtained in both tick and cattle *T. parva* positive isolates. Cattle sample number 1 and tick sample number, 3, 4, 5 6 and 8 have an identical band size to that of Muguga, a *T. parva* stock from Kenya responsible for ECF (Fig 2). A hundred percent of tick *T. parva* positive isolates and only twenty percent ofcattle *T.* parva positive isolates produced bands. Eighty two percent of all tick *T. parva* isolates and 14% of cattle *T. parva* produced single amplicon of band size 900bp while 18% of tick *T.* parva isolates and 6% of cattle *T. Parva* isolates produced single amplicon of band size 800bp.

Figure 1. Amplicon profiles of the p67 gene of *T. parva* from *Rhipicephalus appendiculatus*) and cattle. M=1 kb DNA marker, Lane 1= Positive Control, Lane 2, 3,5,7,8, are samples with low parasitaemia.

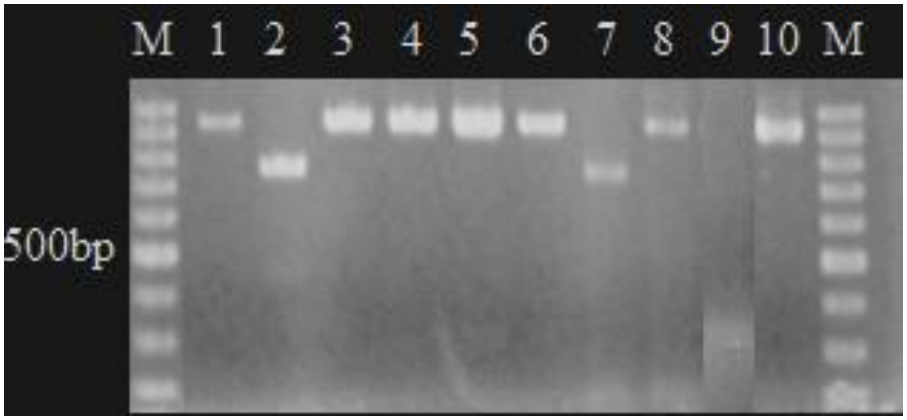

Figure 2. Amplicon profiles obtained from amplification of the central region of the p67 gene from (*R. appendiculatus*) and cattle. Lanes: M = marker Lane 1, 2 and 3 are *T. parva* isolates from cattle blood. Lane 4,5,6,7 and 8 are *T. parva* isolates from *R.appendiculatus*. Lane 9= Negative Control nuclease free water)

Prevalence of ECF infection from retrospective data

The zonal distribution of the disease showed a highest prevalence in the Eastern zone followed by Central zone and the lowest in Southern highland zone.

The prevalence of ECF was significantly higher in the Eastern zone compared to other zones (P<0.05). There was no significant difference in prevalence betweenthe Lake, Northern and Southern zone, however each individual zone showed significantly higher prevalence compared to the Southern highland zone (P<0.05) (Fig. 4).

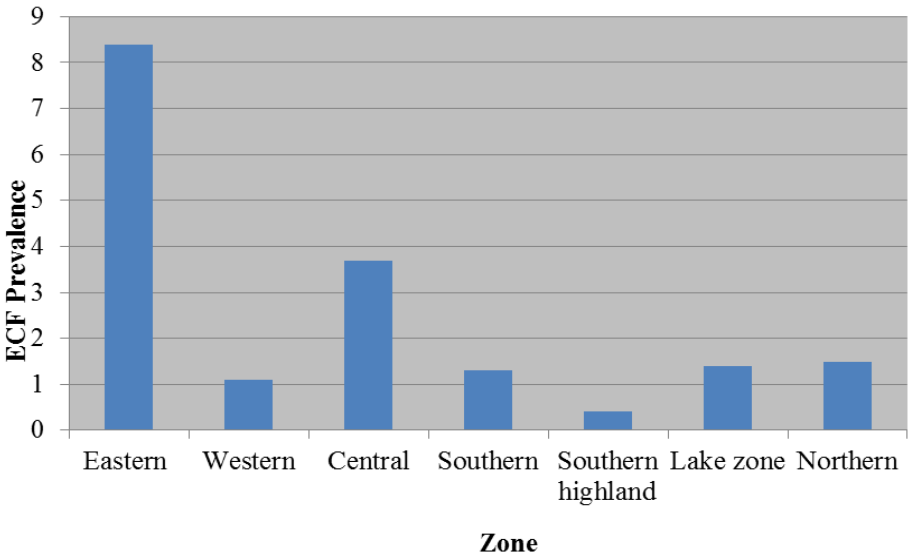

Figure 3. The prevalence of ECF for three year (2008-2010) according to the seven (7) ecological zones

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DISCUSSION

The study was carried out to characterize *T. parva* stocks isolated from indigenous cattle and ticks in Kilosa district, Tanzania. *T. parva* gene coding for surface antigenic protein p67 was used to discriminate the stocks found in indigenous cattle and ticks in this area. Sampling was done between February and April, the pasture for grazing was available therefore most of the herds were around in the study sites. The study showed low tick infestation compared to cold months of June–August according to the cattle owners' explanations. Mbassa *et al.* (2008) reported prevalence close to 100% in calves during cool months of May-July, therefore the low number of ECF cases observed in this study might be due to a problem in timing as sampling was done almost 4 months before the period of high prevalence of disease.

The data from this study indicate higher prevalence (8.4%) of the disease in Eastern zone compared to other zones and lowest (0.4%) in southern highlands. This is to be expected as climatic conditions in eastern zone are very favourable for tick survival (Elb and Anastos., 1966; Newson, 1978) allowing intensive host-tick interactions and subsequent challenge of cattle with *T. parva*. This study as in other previous studies in the East African region (Gitau *et al.*, 1997; Rubaire-Akiiki *et al.*, 2004; Bazarusanga *et al.*, 2007), demonstrated the crucial influence of agro-ecological zones on the variation in tick-borne diseases risk, both spatially and temporally.

Southern and Western Zones are traditionally not livestock areas so there are very few cattle. Probably this might be the cause of the lowest prevalence of ECF in Southern Zone. Furthermore, report from Ministry of Livestock Development and Fisheries shows that for three years no cases were reported from Mtwara rural district. This district has not been engaged in livestock keeping before.

Analysis of p67 PCR products revealed the existence of two *T. parva* alleles with band size 800 and 900 in both cattle and tick isolates which were similar with alleles reportedearlier studies done in South Africa and Northern Tanzania (Sibeko *et al.*, 2010; Mwega *et al.*, 2014). In this study no sample had more than one allele contrary to other reports which reported with up to four alleles (800 bp, 900

bp, 1000 bp, and 1100 bp), indicating a mixture of different stocks of *T. parva* since p67 is a single copy gene (Sibeko *et al.,* 2010).

Analyses of p67 PCR product profiles indicate that among the two alleles, allele with 900 bp occurred more frequently than allele with 800 bp, this is not in line with what was reported in the study which was conducted in Northern Tanzania (Mwega *et al.,* 2014). It was also found that, p67 profile similar to that of Muguga a *T. parva* stock responsible for ECF in Kenya was obtained in this study. Since live vaccination has been recently (early 2012) deployed in Kilosa, *Theileria parva* Muguga stock isolated may be derived from exotic trivalent vaccine ("Muguga cocktail"), but the possibility of being indigenous stock cannot be excluded as Muguga component present in this vaccine is clamed not to stay in vaccinated animal longer than 48 days post vaccination (Oura *et al.,* 2004), hence a low chance of becoming transmitted to ticks although further study to prove this is required.

Eighty percent blood samples which were positive for p104 they were negative on amplification of p67 gene. The p67 protein is sporozoite surface protein in which its gene is expressed during the initial stages of development of the parasite and p104 is piroplasm protein in which its gene is expressed during the final stages of the parasites. Since the initial stages of the parasite are found in lymphoid tissues like lymph nodes, when the blood is used as a source of DNA for amplification of p67 it is more likely to fail to detect some positive samples. Therefore p104 is the ideal gene to be amplified during *T. parva* screening when blood is used as the source of DNA.

Findings of *T. parva* stocks which is not in the imported ECF trivalent vaccine ("Muguga cocktail") currently in use calls for more research to establish the complete list of parasites circulating in the district and region for efficient control of ECF.

The study assumed that all VICs report all cases equally and that all ECF cases in the field were reported to VICs, which may potentially over-estimated or under-estimated the level of the disease to zones. Therefore VICs are required to improve their diagnostic facilities and database so that they can capture disease information, which is central to any future strategic control measures.

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