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ORIGINAL PAPER

Livestock trypanosomosis in Uganda: parasite heterogeneity and anaemia status of naturally infected cattle, goats and pigs

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Abstract The prevalence and pathogenic effects of trypanosomosis were determined in cattle, goats and pigs reared in Kasese, Jinja and Rakai districts, Uganda; presence of trypanosomes was detected by buffy coat technique (BCT). The overall prevalence of trypanosomosis in cattle was 7.6 % (144/1,891), 0.7 % in goats (4/573) and 2.3 % in pigs (9/386). Internal transcribed spacer 1 (ITS1) of ribosomal DNA polymerase chain reaction was utilised to identify trypanosomes to species level and revealed infections in 108 of the 144 trypanosome-positive cattle while all infected goats and pigs gave amplicons. *Trypanosoma vivax* was the most prevalent trypanosome species in cattle in single and mixed infections compared to infections involving *Trypanosoma congolense* and *Trypanosoma brucei*; in pigs, eight were mixed infections with one single *T. vivax* infection. No predominant trypanosome species was detected in goats. Anaemia, the main trypanosomosis pathological feature, was investigated by determining packed cell volume (PCV). Mean PCV values by *t* test in infected individuals were significantly lower than non-infected individuals ($P<0.05$) for all animal species. However, the proportion of anaemic animals was not significantly different in infected and non-infected individuals. In addition, the percent of infected animals by Fisher's exact test depended on district of origin and species but not sex. These findings show that trypanosomosis is a major cause of anaemia in livestock in endemic areas. Cattle were the major animal species affected by trypanosomosis; similar genotypes of trypanosomes were detected in the three animal species. BCT was more effective than ITS1 rDNA detecting trypanosomes in naturally infected cattle.

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Introduction

Animal trypanosomosis, also known as *nagana*, is one of the major diseases hindering livestock productivity on the African continent. In sub-Saharan Africa, trypanosomes are tsetse-borne and their distribution coincides with that of their vector. Livestock disease is caused by *Trypanosoma vivax*, *Trypanosoma congolense*, *Trypanosoma simiae*, *Trypanosoma brucei brucei* and *Trypanosoma suis*. In addition to the tsetse-borne species, African mammals harbour non-pathogenic trypanosomes namely *Trypanosoma theleiri* and *Trypanosoma ingens* commonly found in domestic and wild animals. This disease not only results in severe losses in the productivity of domestic livestock due to reduced capacity to do work, poor growth, weight loss, low milk yield, infertility and abortion but also impairs the development of animal agriculture in zones that constitute 41 % of the land but which carry only 26 % of the ruminant population (FAO 1998).

In most regions of Uganda, trypanosomosis is endemic; however, changes in land use patterns and settlements coupled with control strategies involving treatment of animals

mainly cattle with trypanocides and spraying with acaricides result in changes of dynamics of the disease, necessitating constant research about disease dynamics. Although investigations about nagana have been going on for decades, the bulk of research in animal trypanosomosis has been done in cattle and relatively little attention has been paid to other domestic animal species. Unlike cattle, sheep and goats are kept in a very broad range of agro-ecological zones where they contribute considerably to the rural economies as a source of meat, milk, manure and readily disposable income. In Uganda, natural trypanosomosis in pigs have been reported (Katunguka 1996; Waiswa et al. 2003; Biryomumaisho et al. 2009). In addition, pigs have been shown to harbour human infective *T. brucei rhodesiense* (Ng'ayo et al. 2005; Simukoko et al. 2007).

It has been believed that goats are highly resistant to trypanosomosis, that caprine trypanosomosis is only sporadic, and that the disease in goats is of little economic consequence (Griffin 1978). However, current epidemiological information indicates that goats can play an important role in the dissemination of the disease. Thus, goats naturally infected with *T. congolense*, *T. vivax* or *T. brucei* and presenting clinical disease are regularly observed in many sub-Saharan countries in Africa. Regional differences in the prevalence of caprine trypanosomosis exist but can be high in some areas (Kramer 1986). In general, caprine trypanosomosis is more common in East than in West Africa, mainly attributed to differences in feeding preferences between riverine and savannah species of *Glossina*; the latter being more inclined to feed on goats (Smith and Sherman 1994). Breed differences in susceptibility to natural infections in goats (Masiga et al. 2002) have also been reported.

Animal diseases can be identified basing on clinical signs and knowledge of prevalent diseases in a particular area. However, the clinical signs of trypanosomosis are so varied and the disease occurs in varied ecological conditions. Scarcity of parasites in affected animals and occurrence of asymptomatic carriers hinder early detection (van Meirvenne and Le Ray 1985; Nantulya 1990). Serological methods have in turn been used to overcome this problem; however, these methods cannot distinguish current from previous infections (Gonzales et al. 2003). Polymerase chain reaction (PCR) technique has largely been utilised to increase on sensitivity and has further been employed to assign trypanosomes to their respective taxonomic and sub-taxonomic levels (Desquesnes and Davilla 2002). The most useful targets are those that are presented in multicopy form such as mini-exon, kinetoplastic mini-circles and minisatellites compared to single copy sequences which are more difficult to amplify. Masiga et al. (1992) developed primers for specific detection of *T. congolense* subtypes basing on minichromosomes of the nuclear DNA, which contain satellite DNA; later, Ventura et al. (2002) used mini-exon genes for detection of *T. vivax*. Kinetoplastic mini-circle DNA sequences have been useful for *Trypanosoma evansi* detection (Artama et al. 1992). The main disadvantage of these techniques was several reactions needed to be made to completely identify trypanosomes to species level. A suitable internal transcribed spacer (ITS) primer set for trypanosomes (KIN 1 and KIN 2) was developed by McLaughlin et al. (1996) and subsequently was evaluated by Desquesnes et al. (2001) but the detection of *T. vivax* with these primers was less sensitive. This led to the development of new internal transcribed spacer 1 (ITS1) rDNA-based primers (CF and BR) and their subsequent evaluation by Njiru et al. (2005). These primers showed 100 % homology with *T. vivax* rDNA sequence in GenBank (accession number U22316).

The aim of the present study, therefore, was to determine the prevalence and major causative agents of bovine, caprine, and porcine trypanosomosis in Uganda. The study further evaluated the sensitivity of ITS1 rDNA PCR when compared with microhaematocrit centrifugation technique as gold standard in natural trypanosome infections. The severity of infection in natural trypanosome infection was assessed by inferring on the status of anaemia of trypanosome-infected animals.

Materials and methods

Collection of livestock blood samples and screening for trypanosomes

Cattle, goats and pigs were bled from the districts of Kasese, Jinja and Rakai, Uganda (Fig. 1). In each district, two subcounties were selected. The animals of all ages and sex were bled with sterile gauge 19 disposable needles and 10 ml syringes, and blood was evacuated into 5 ml vials containing ethyldiaminetetraacetic acid (EDTA) as anticoagulant. In cattle and goats, the jugular vein was used; when not accessible, the middle coccygeal vein/artery was utilised. In larger pigs (more than 50 kg), blood was obtained from the marginal ear vein, and in smaller pigs, from the anterior vena cava or the cutaneous abdominal vein. Of this blood, 20 μl was spotted onto Whatman FTA cards for subsequent DNA extraction for later use in the ITS1 rDNA speciation study. Capillary tubes were subsequently centrifuged at 1,020×*g* for 5 min and the presence of trypanosomes detected by observation of parasite motion just above the buffy coat. Packed cell volume (PCV) was measured in percent by placing a centrifuged capillary tube on a microhaematocrit reader and mean readings rounded off to the nearest percentage whole number.

Extraction of DNA from blood spotted on Whatman FTA cards

Trypanosome DNA was obtained from dried Whatman FTA cards' whole blood spots; three or four circles each 2 ml in

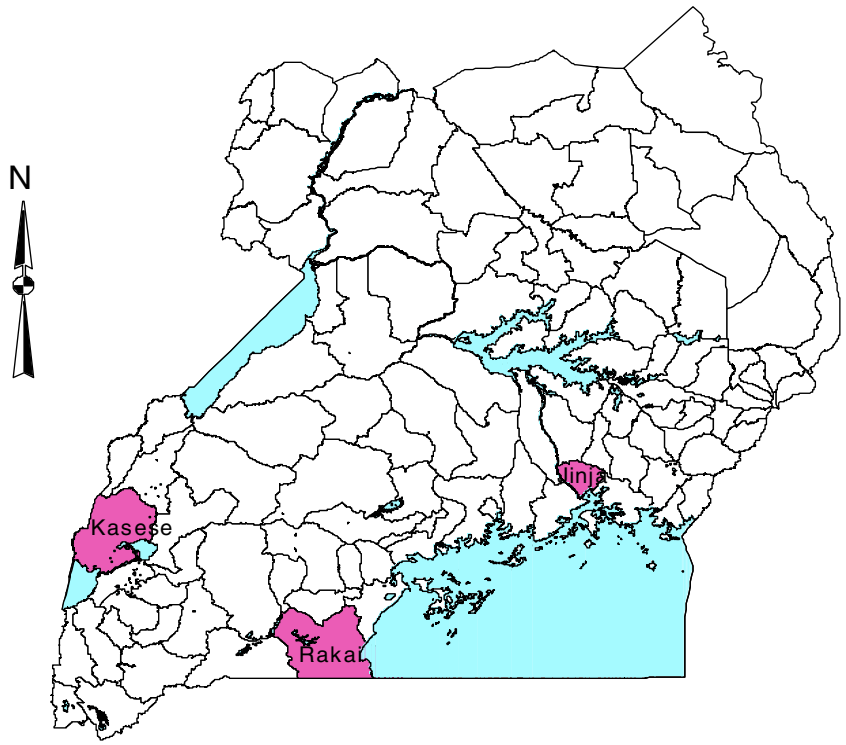

Fig. 1 Map of Uganda showing geographical location of districts (*in pink*) where livestock trypanosomosis survey was carried out. The *blue areas* are water bodies

diameter were punched out and DNA extracted using QIAamp® mini kit (2003). The circles were placed in 1.5 ml microfuge tubes, covered with cell lysis buffer and incubated at 85 °C for 10 min. Genomic DNA was extracted by adding proteinase K and the extract was incubated at 56 °C for 1 h. The DNA was precipitated with ethanol and subsequently eluted in 110 μl of buffer AE (10 mM Tris-Cl; 0.5 mM EDTA, pH9.0). Small aliquots of DNA were stored at −20 °C until required for use as template DNA for testing individual DNA extracts for infecting trypanosome species by ITS1 rDNA PCR.

Amplification of the rDNA intergenic sequence by the polymerase chain reaction (ITS1-PCR)

The ITS1 rDNA amplification reactions were performed in a PTC-100 Peltier thermal cycler; 25 μl PCR mix contained 2.5 μl of 10× PCR buffer (15 mM $MgCl_2$, 0.25 μl of each 10 mM dNTP solution), 0.5 μl containing 10 mM (10× mixture) of the forward and reverse ITS1 rDNA primers, 0.125 μl of HotStar Taq DNA polymerase and 5 μl of the DNA extracted from FTA blood spots. The ITS1 rDNA primers used were: forward (CF), 5′-CCGGAAGTTCACC GATATTG and reverse (BR), 5′-TTGCTGCGTTCTTCAAC GAA. The thermal cycler was programmed as follows: lid preheating at 96 °C for 15 min followed by 38 cycles each consisting of denaturation step at 94 °C for 30 s, annealing at 56 °C for 1 min and extension at 72 °C for 90 s. The mixture was incubated at 72 °C for 10 min for the final extension step. The products were temporarily kept at 4 °C in the thermocycler pending electrophoresis in a 1.5 % agarose gel containing 0.5 μg/ml ethidium bromide and flooded with 1× TBE buffer. Electrophoresis was done at the following conditions: 55 V, 250 mA and run for 30 min.

The positive control trypanosome DNA

The *T. brucei*-positive control DNA was extracted from the procyclic form of cloned stock 927 (*T. brucei* TREU 927/4 PAL/KE/70/EATRO 1534). *T. congolense* and *T. vivax* DNA was extracted from strains IL 3000 and IL 2569, respectively, and kindly provided by Dr. Phelix Majiwa.

Authorization to use animals in this research

Permission to use animals in this research was granted by the Research and Higher Degrees Committee of the Faculty of Veterinary Medicine, Makerere University, Uganda when the research proposal for PhD studies from which this manuscript was derived was approved in June 2006.

Statistical analysis

All data were analysed with R statistical package; box plots presenting median PCV values for different animal species were made; mean PCV differences in animals in which trypanosomes were detected compared to trypanosome-free animals were compared with Student *t* test while proportions of anaemic animals in infected and non-infected animals were determined with exact Fisher's test.

Geographical location of districts in Uganda where blood samples were obtained

The location of districts where blood samples of cattle, goats and pigs which were used in this research is shown in Fig. 1. The districts are wide apart and all located in trypanosomosis-endemic area.

Results

Trypanosome prevalence in cattle, goats and pigs

Overall, 1,891 cattle were bled of which 144 tested positive for trypanosomes by buffy coat technique (BCT) 144/1,891 =7.6 % prevalence in all the districts of Kasese, Jinja and Rakai. By individual districts, trypanosome prevalence in Kasese was 2.9 % (17/584), Jinja 11 % (87/807) and 4.4 % (22/500) in Rakai. The area of origin of cattle was a significant high risk factor for trypanosomosis ($P<0.05$). In the three districts, 573 goats were bled of which only four were trypanosome-positive, hence prevalence of 0.7 %. None of the pigs bled in Kasese was trypanosomes-positive; no pigs were located in Rakai District and trypanosomes were detected in samples of pigs from Jinja District in 9 of 253 pigs or 2.3 % of pigs. Results for speciation by ITS1 rDNA are shown in Table 1.

Table 1 ITS 1 rDNA speciation of trypanosomes in cattle, goats and pigs by district of origin

District	Numbers of animals positive for trypanosomosis							
	Tb	Tc	Tv	Tbc	Tbv	Tcv	Tbcv	NA
Cattle								
Kasese	4	2	11	1	6	2	2	6
Jinja	5	3	19	3	25	0	9	22
Rakai	5	2	8	0	1	0	0	8
Subtotal	14	7	38	4	32	2	11	36
Goats								
Kasese	0	1	0	0	0	0	0	0
Jinja	0	0	1	1	0	1	0	0
Rakai	0	0	0	0	0	0	0	0
Subtotal	0	1	1	1	0	1	0	0
Pigs								
Kasese	0	0	0	0	0	0	0	0
Jinja	0	0	1	4	0	3	0	1
Rakai	–	–	–	–	–	–	–	–
Subtotal	0	0	1	4	0	3	0	1
Total	14	8	40	9	32	6	11	37

Tb T. brucei, *Tc T. congolense*, *Tv T. vivax*. *Tcv* mixed infection of *T. congolense* and *T. vivax*, *Tbcv* mixed infection of *T. brucei*, *T. congolense* and *T. vivax*, *NA* no amplicon obtained

Sensitivity and positive predictive value of ITS1 rDNA PCR technique in livestock natural trypanosome infection

BCT was taken as "gold standard" for establishment of presence of trypanosomes in an animal host. Of the 144 FTA blood spots from cattle confirmed positive, 108 yielded amplicons, giving a sensitivity of 75 % [(108/144)×100] and negative predictive value of 25 %. In goats, however, the sensitivity of ITS1 rDNA PCR was 100 %; goats [(4/4)×100] and in pigs [(9/9)×100] and positive predictive value of 100 % in both cases.

Speciation of trypanosomes with ITS1 rDNA PCR

Trypanosomes were assigned species with ITS1 rDNA PCR and results are presented in Table 1. Species of trypanosomes were assigned basing on corresponding bands with positive control DNA (Fig. 2). In cattle, *T. vivax* was the most encountered trypanosome species both in single and mixed infections. In samples from Kasese District for cattle, there were four single *T. brucei* infections, while in Jinja and Rakai districts, each had five. In all the three districts, there were fewer *T. congolense* single infections: two in Kasese and Rakai and only three from Jinja. Among the single infections, *T. vivax* was the highest: 19 from Jinja, 11 from Kasese and 8 from Rakai. Among the double trypanosome infections, the combination of *T. brucei* and *T. vivax* occurred most. Among the triple (*T. brucei* + *T. congolense* + *T. vivax*) mixed infections, Jinja had 9, Kasese 2 and none from Rakai District. In cattle from the three districts, at least 83 samples had *T. vivax*, *T. congolense* (24) and *T. brucei* (61).

The speciation for trypanosome DNA extracted from pig blood from Jinja District is shown in Fig. 2b. Eight of the nine pigs in which PCR was done with primers targeting the ITS1 rDNA gene had *T. vivax* infection. *T. brucei* + *T. congolense* and *T. vivax* mixed infection were detected in four pigs, *T. congolense* + *T. brucei* in a single pig and *T. vivax* single infection in a single pig.

In the goats, Fig. 2c and illustrated in Table 1, *T. congolense* single infection was detected in one goat from Kasese. Each of the Jinja samples had the following trypanosomes: *T. vivax*, *T. congolense* + *T. vivax* (2) and *T. vivax* + *T. brucei* (3). None had a triple mixed trypanosome infection.

Anaemia status of trypanosome-infected animals

In all animal species (Fig. 3a), infected animals had significantly lower PCV values than animals in which trypanosomes were not detected: for cattle (b) (P value=0.029) as well as for pigs (c) (P value=0.042) and goats (d) (P value= 0.026 by t test for comparison of means). However, the proportion of anaemic animals was not significantly

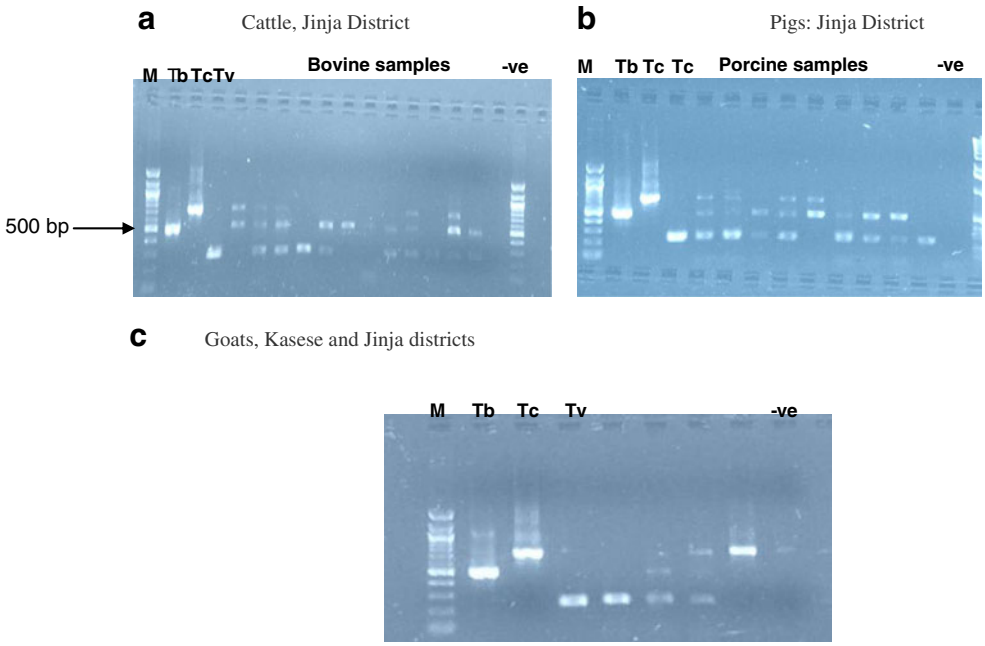

Fig. 2 Typical banding pattern of ITS1 rDNA PCR in cattle (**a**), pigs (**b**) and gel (**c**) for goats

different in infected and non-infected individuals by comparison of proportions with exact Fisher's test.

When area of origin was considered, trypanosome-infected animal percentages depended significantly on the

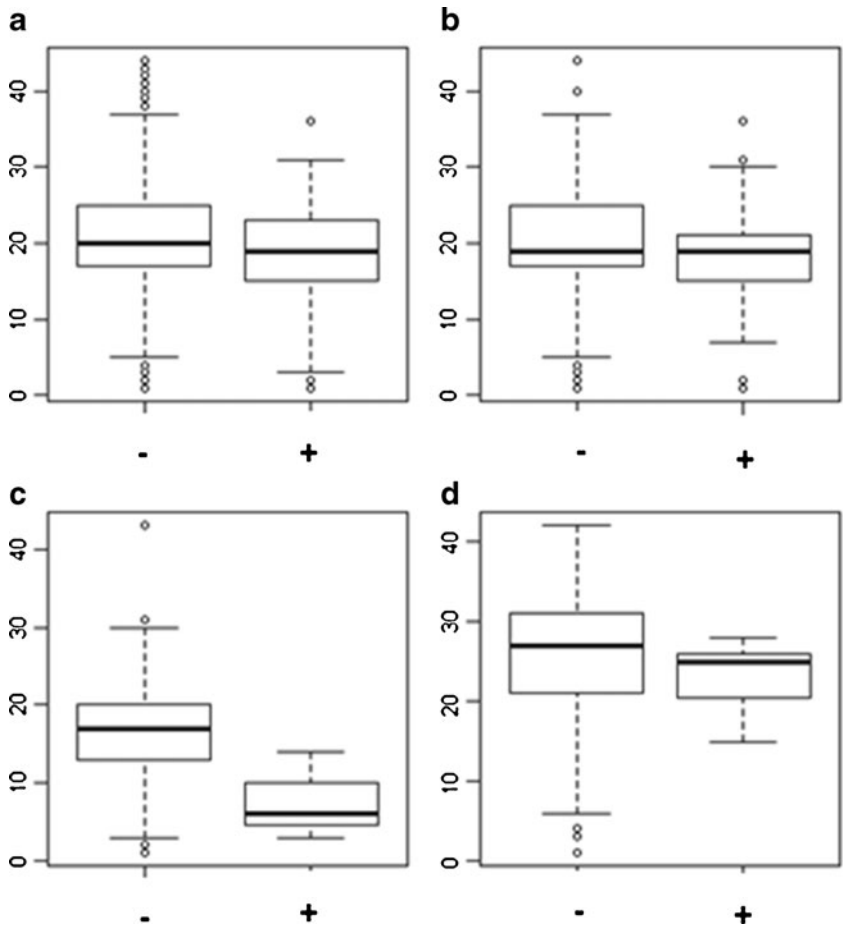

Fig. 3 Median PCV values in trypanosome-positive (+) and negative (−) animals. **a** Pooled PCV values of all animals; **b** for cattle; **c** for goats and **d** for pigs. For all panels, PCV of infected animals was significantly lower than in non-infected animals ($P<0.05$) although the proportion of infected animals was small

district of origin and by species ($P<0.05$) but not on sex of the animals by Fisher's exact test (Fig. 4).

Discussion

The overall prevalence of trypanosomosis was 7.6, 2.8 and 0.7 % in cattle, pigs and goats, respectively, as determined with buffy coat method. The data on trypanosomosis prevalence vary with geographical location and can be explained among other factors by the rate hosts are fed on by the tsetse flies (Rogers 1988), the amount of carbon dioxide produced by the host (Torr et al. 2006) or short range tsetse olfactory and visual stimuli (Warnes 1995) and the behaviour of the host (Torr and Mwangiro 2000). Because cattle have large body sizes, they are likely to produce more carbon dioxide trails and hence more likely to attract tsetse flies more than pigs or goats (Simukoko et al. 2007). Combining these factors implies that cattle are more likely to get tsetse fly bites than pigs and goats. Failure to detect trypanosomes in goats in Rakai and only one in Kasese may partly be explained by behaviour: in those areas, paddock and extensive grazing systems are practiced thus allowing goats to be largely mobile, sometimes over wide areas.

Anaemia is recognised as the most important pathological feature of trypanosomosis in both experimental and domesticated animals. All trypanosome-positive animals had significantly lower PCV ($P<0.05$) than animals in which trypanosomes were not detected. However, the proportion of anaemic animals in individual species was not significantly different in trypanosome-infected and non-infected animals. The percentage of trypanosome-infected cattle depended significantly on district of origin and species but not on sex of the animals (Fig. 4). The confounding factors for anaemia in the study areas include poor state of nutrition in animals, presence of intercurrent infections mainly gastrointestinal helminths and stressful conditions of trekking for search of pasture and water. Similar observations have been made by Fajinmi et al. (2011).

In the present study, the 63 cattle that were trypanosome-positive but not anaemic can be explained as new infections that had not progressed to chronicity, a state associated with anaemia development. Moreover, there is a tendency to occasionally treat cattle against trypanosomosis when compared to other livestock. However, all trypanosome-infected pigs and goats were anaemic, confirming that these were likely to be chronic infections and re-affirms these animal species receive less attention in trypanosomosis control programmes. Presence of infection in cattle and more affected animals not being anaemic can further be explained by differences in virulence of different trypanosome species. Classically, *T. congolense* is considered to be more pathogenic in

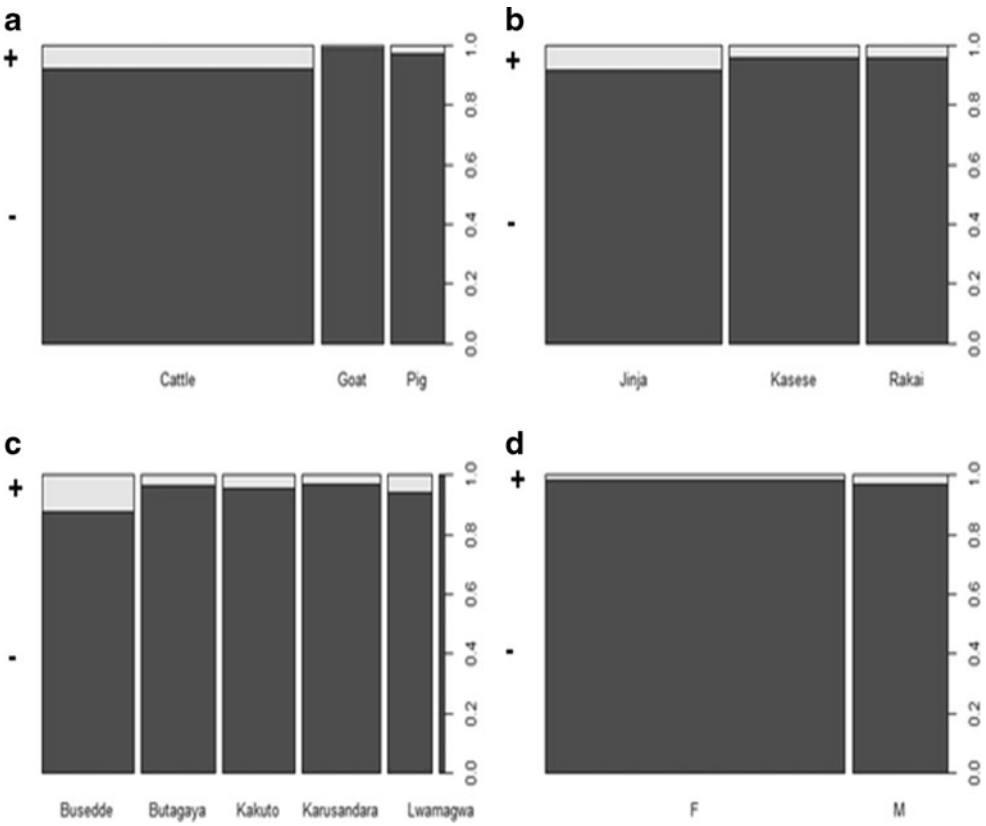

Fig. 4 The clear section shows the proportion of parasitized individuals among anaemic and non-anaemic individuals by animal species (**a**), district of origin (**b**), sub-county of origin (**c**) and sex of the animal (**d**). The proportion of anaemic and non-anaemic individuals is shown by the width of the two columns: cattle, $P=0.029$; goats, $P=0.026$; pigs, $P=0.042$ by *t* test

Eastern and Central Africa, while *T. vivax* is known to be more pathogenic in Western Africa (Eisler et al. 2004) although severe haemorrhagic *T. vivax* outbreaks have been reported in Uganda (Magona et al. 2008) and other African countries (Assoku and Gardiner 1989; Kimeto et al. 1990). Notably, *T. vivax* was the predominant trypanosome species in single or mixed infections in this study.

Using the buffy coat technique, 144 cattle were found trypanosome-positive; however, when the same samples were analysed with ITS1 rDNA PCR, only 108 were found to be positive. This is surprising as PCR-based reactions are known to be more sensitive than parasitological techniques with sensitivity of about 50 % (Picozzi et al. 2002). However, the sensitivity of molecular tools depends on degree of parasitaemia in trypanosome-infected animals because the chance in a less parasitized animal to have detectable trypanosome DNA template is less. In many natural infections, trypanosomosis takes a chronic course that is characterised by low parasitaemias thus making both techniques less efficient in detecting natural trypanosome infections. The amount of DNA detected in a sample depends on the type of sequence target: for instance, in *T. evansi*-infected mice, the minimum amount of DNA from blood during peak parasitaemia that was detectable by PCR was 0.001 ng with the ESAG6/7 and TBR1/2 primer. But when using TBR1/2 primers derived from purified parasites, 0.000001 ng (1×10^{-14} g) was detected (Fernandez et al. 2009). Using *T. brucei*, 927 purified DNA (Biryomumaisho 2009) showed that ITS1 rDNA detected a minimum of 3.5×10^{-14} g equivalent to DNA derived from 0.35 of a trypanosome and these are in agreement with the TBR1 results with *T. evansi* and Masiga et al. (1992) who showed that one trypanosome yields 0.1 pg (1×10^{-13} g) of DNA.

This study has highlighted the relative importance of goats and pigs to cattle in the epidemiology of animal trypanosomosis in Uganda. Notable was the high incidence of trypanosomosis in pigs reared in Jinja District: parts of this district are endemic to sleeping sickness. Basing on results from this study, it can be concluded that although goats and pigs can be affected by trypanosomes, the low proportion of affected animals indicates their decimal role in the epidemiology of the disease in livestock. However, their importance as reservoirs of trypanosomosis could be important especially when the cattle population is depleted as happens in wars and related calamities.

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