Natural infection of cattle and tsetse flies in South Africa with two genotypic groups of *Trypanosoma congolense*

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(*Received 21 November 2008; revised 18 December 2008; accepted 19 December 2008; first published online 2 March 2009*)

SUMMARY

The polymerase chain reaction was used to detect trypanosomes in samples collected from cattle, wild animals and tsetse flies in KwaZulu-Natal Province, South Africa. A total of 673 samples from cattle and 266 from tsetse flies in the study area located near the Hluhluwe-Umfolozi Game Reserve were analysed. Both *Trypanosoma congolense* and *T. vivax* were found as single or mixed infections in cattle and tsetse flies. Moreover, the *T. congolense* in the infections were found to comprise 2 genotypic groups: the Savannah-type and the Kilifi-type, which were present either as single or mixed infections in cattle and in tsetse flies.

Key words: *Trypanosoma congolense*, tsetse, mixed infection, KwaZulu-Natal, genotypic groups.

INTRODUCTION

Tsetse-transmitted trypanosomosis is limited to sub-Saharan Africa, where its occurrence is coincident with vegetation suitable for habitation by the tsetse fly. The southernmost boundary of the tsetse belt is located in the KwaZulu-Natal Province, South Africa.

Only 4 species of tsetse, namely *Glossina morsitans morsitans, G. pallidipes, G. austeni* and *G. brevipalpis*, are known to have been present in South Africa. However, *G. m. morsitans* was eliminated in the late 1890s after rinderpest decimated cattle and wildlife upon which these flies thrive (Du Toit, 1954). In the 1950s, the South African government undertook aggressive campaigns, involving aerial spraying, to control tsetse populations and so eradicated *G. pallidipes* (Du Toit, 1954). Currently, only *G. brevipalpis* and *G. austeni* remain, confined to the north-eastern part of KwaZulu-Natal Province (Kappmeier *et al*. 1998). It was observed that the incidence of livestock trypanosomosis, caused by *T. brucei brucei, T. congolense* and *T. vivax* here, occurs in cycles, with peaks followed by long breaks that allow livestock farming (Du Toit, 1954).

Increased interest in game farming – the holding of wild animals on reserves for either meat or hunting – and tourism in southern Africa has led to the establishment of trans-frontier game parks with unrestricted movement of wildlife across national boundaries. Given the role of wildlife in maintaining tsetse flies and trypanosomes, the control of trypanosomosis needs to be targeted and more accurately monitored, to accommodate competing interests of game farming and smallholder livestock farming in the region. Studies were therefore conducted using molecular tools to investigate the population structure of trypanosomes prevalent in KwaZulu-Natal Province, South Africa. Evidence was found for the frequent occurrence of mixed infections of livestock and tsetse flies with multiple species of trypanosomes, and the presence of at least 2 genotypic groups of *T. congolense*. These observations have practical implications for control of trypanosomosis in livestock owned by communities near the game parks.

MATERIALS AND METHODS

Study area

The study was conducted in the KwaZulu-Natal Province, South Africa. Blood samples were collected from cattle at Boomerang commercial farm and 14 different communal diptanks, the locations of which are shown on the map in Fig. 1.

Sampling

A total of 673 samples from cattle and 266 from tsetse flies (>80% of which were *G. brevipalpis*) were analysed. The cattle, predominantly Nguni breed, sampled at the communal diptanks are owned by smallholder farmers resident in Hluhluwe, KwaZulu-Natal, whereas those at Boomerang,

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doi:10.1017/S0031182009005587 Printed in the United Kingdom

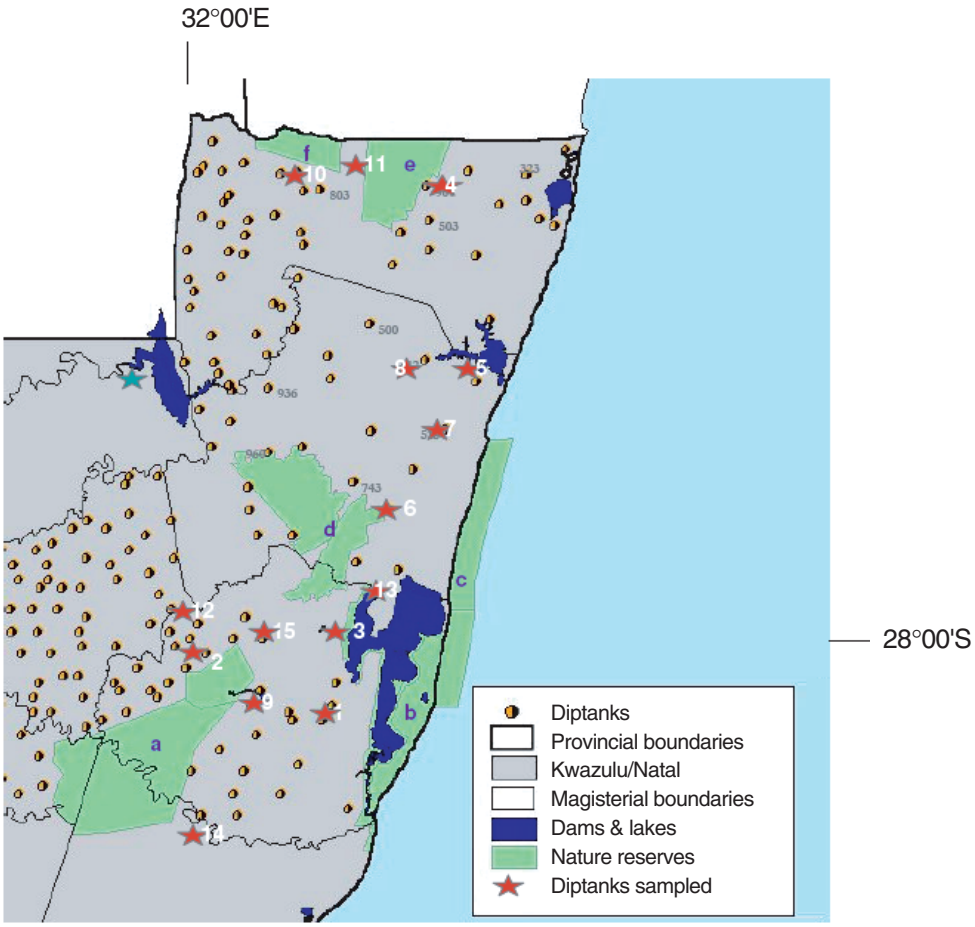

Fig. 1. Map of the study area, showing location of the commercial farm and communal diptanks, indicated with red stars. Boomerang commercial farm is marked 1; Qakweni diptank, 2; Mahlambinyathi, 3; Manzabomvu, 4; Mbazwana, 5; Mkhumbikazana, 6; Mpini, 7; Mseleni, 8; Mvutshini, 9; Ndumo, 10; Nhlanjwana, 11; Nhlwati, 12; Nibela, 13; Ocilwane, 14 and Ekuphindisweni, 15. The nature reserves are: a, Hluhluwe-Umfolozi Game Reserve; b, Greater St Lucia Wetland Park; c, St Lucia Marine Reserve; d, Mkuzi Game Reserve; e, Tembe Elephant Park; and f, Ndumo Game Reserve. The latitude and longitude co-ordinates of the area are shown on the right and top respectively.

a mixture of Nguni and Brahman breeds, belong to a commercial farmer.

When deemed necessary, because the animal was found to be positive for trypanosomes by microscopic examination of the buffy coat made from its blood or because it was suspected to be suffering from *nagana* (the local name for livestock trypanosomosis), approximately 500 μl of the blood from each such animal sampled was injected into a mouse. Buffy coats were made from blood of the target animals and then analysed by polymerase chain reaction (PCR) to determine the species of trypanosomes in the infection.

Stabilates of 5 *T. congolense* isolates from African buffaloes at Hluhluwe-Umfolozi Game Reserve were kindly provided by Ms Nkuna, University of Pretoria.

Tsetse flies were caught using H-traps baited with 4-methyl phenol, acetone and octanol (Kappmeier, 2000) erected at Hluhluwe-Umfolozi, Hells Gate and Charter's Creek game reserves and at the Boomerang farm. To avoid or reduce mortality of the flies caught, the traps were visited early in the mornings and late in the afternoons to collect the tsetse flies, which were subsequently transported to the ARC Tsetse Research Station, Kuleni, KwaZulu-Natal. They were kept covered with a dark cloth in the laboratory until they were dissected on the same or the following day. Before dissection, the flies were immobilized by cooling at 4 °C for approximately 10 min. Dissection was carried out in phosphate-buffered saline in 2% glucose (PSG) under a dissection microscope.

The flies were divided into 2 groups. From the first group, midguts and proboscides were dissected and placed separately on microscope slides for examination under a compound microscope at 400× magnification. When trypanosomes were seen in either of these tsetse organs, the proboscis was completely recovered from the slide and placed in a 0·2 ml tube. After maceration and complete suspension in PSG

buffer, it was injected into a mouse. For the second group of flies, the organs were dissected and separately blotted onto FTA elute cards (Whatman Biosciences Ltd, Brentford, UK). For analysis, a 3 mm radius disk was punched from the spot where each sample was applied, the punched disk washed and the DNA in it eluted following the manufacturer's instructions (Whatman, Biosciences Ltd, Brentford, UK).

Monitoring parasitaemia in mice

Mice injected with blood or a suspension of infected tsetse organs were monitored for the presence of trypanosomes by microscopic examination of a thick smear of tail blood on a microscope slide. This was done daily from the third day post-infection. Buffy coats made from the mice were placed on FTA elute cards and the DNA from these eluted according to the manufacturer's instructions (Whatman, Biosciences Ltd, Brentford, UK). The eluted DNA was kept frozen at −20 °C until use.

Amplification reactions

To detect trypanosome DNA, 2 rounds of amplification were done as described by Geysen *et al*. (2003). In the first round, the primer set 18ST nF2 (CAA CGA TGA CAC CCA TGA ATT GGG GA) and 18ST nR3 (TGC GCG ACC AAT AAT TGC AAT AC) were used. In the second round, 18ST nF2 was used with 18ST nR2 (GTG TCT TGT TCT CAC TGA CAT TGT AGT G) in a hemi-nested amplification. In all cases, DNA of *T. congolense* IL3000 and *T. vivax* IL2160 were included as reference.

The first amplification reaction was carried out in a final volume of 25 μl, containing 10 mM Tris-HCl, pH 8·3, 50 mM KCl, 1·5 mM $MgCl_2$, 200 μM of each dNTP, 0·8 μM of each of the primers and 0·5 U *ExTaq* polymerase (Takara Bio Inc.), and 2·5 μl of the eluted DNA. The thermocycling parameters were as described by Geysen *et al*. (2003).

To detect a particular species of trypanosome, the species-specific primers, TCN1 (TCG AGC GAG AAC GGG CAC TTT GCG A) and TCN2 (ATT AGG GAC AAA CAA ATC CCG CAC) for Savannah-type *T. congolense* (Majiwa *et al*. 1994), *and* TCK1 (GTG CCC AAA TTT GAA GTG AT) and TCK2 (ACT CAA AAT CGT GCA CCT CG) (Masiga *et al*. 1992) for Kilifi-type *T. congolense*, were used. The amplifications were carried out in 25 μl, containing 10 mM Tris-HCl, pH 8·3, 50 mM KCl, 1·5 mM $MgCl_2$, 100 μM of each dNTP, 0·4 μM of each of the primers and 0·5 U *ExTaq* polymerase (Takara Bio Inc) and 2·5 μl of the eluted DNA. The tubes were placed in a thermocycler and incubated at 94 °C for 4 min in an initial denaturation step, followed by 30 cycles of 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C.

The PCR products were separated by electrophoresis in agarose gels, stained with ethidium bromide and then photographed.

Sections of the agarose gels containing the desired PCR products from the hemi-nested amplification were excised and the DNA fragments therein purified using the QIquick gel extraction kit (Qiagen, UK). The DNA was subsequently digested to completion with a combination of *Msp*1 and *Eco*571, and the digests separated by electrophoresis in a 10% polyacrylamide gel, stained with silver then photographed.

RESULTS

Savannah-type and Kilifi-type T. congolense *infect cattle in KwaZulu-Natal*

Figure 2 shows that PCR products of 2 different sizes are obtained when a set of primers that target a segment of the trypanosomal 18S rRNA gene are used in the amplification of trypanosome DNA in buffy coat samples collected from cattle in KwaZulu-Natal. One group of samples (Fig. 2, lanes 1, 4–6) yielded a PCR product of approximately 750 bp, which is identical in size to that observed in the DNA of IL3000 (Fig. 2, lane 8), a known Savannah-type *T. congolense* (Majiwa *et al*. 1985). The others yielded a PCR product of approximately 800 bp (Fig. 2, lanes 2 and 7), which is similar in size to what is expected from this gene locus in the Kilifi-type *T. congolense*, as predicted from its sequence (Accession number TCU22317) in GenBank.

Data obtained from analysis of samples from 673 cattle, isolates from 5 buffaloes and 140 tsetse flies are summarized in Tables 1 and 2. Of the 673 samples from cattle, 125 (18·6%) were positive by PCR. Thirty of these infections were due to *T. vivax*, 5 were due to concurrent infection of the cattle with *T. vivax* and Savannah-type *T. congolense*, 63 were due to Savannah-type *T. congolense*, 22 due to Kilifi-type *T. congolense* and 5 were due to mixed infection with Savannah- and Kilifi-type *T. congolense*. PCR analysis of the 5 buffalo isolates showed that they were all Savannah-type *T. congolense*.

Twenty of the 140 dissected tsetse flies were found to be infected with trypanosomes and 19 of the infections were mature since the trypanosomes were detected in the proboscides. Organs from each of the 20 tsetse flies found to be infected with trypanosomes were injected, each into a separate mouse. PCR analyses of trypanosomes from these mice revealed that the tsetse were infected with the Savannah-type *T. congolense* (Table 1). It is known, however, that inoculation of immuno-competent mice with infected vector or host tissues is inefficient in capturing some trypanosome species like *T. vivax*.

Table 1. Summary of data obtained upon analysis by PCR of samples from cattle and tsetse flies

(K denotes Kilifi-type and S, Savannah-type.)

Source	No. of samples analysed	No. of positive samples (%)	*T. vivax*	Mixed *T. vivax* and S *T. congolense*	S *T. congolense*	Mixed S and K *T. congolense*	K *T. congolense*
Cattle	673	125 (18·6)	30	5	63	5	22
Tsetse flies	140	20 (14)	—	—	20	—	—

Table 2. Summary of data from PCR analyses of samples collected from cattle at different diptanks

(S, denotes Savannah-type and K, Kilifi-type.)

Location	No. of samples	No. of +ve samples (%)	Trypanosome species detected
Ekuphindisweni	38	2 (5·2)	*T. vivax, T. congolense* (*S*)
Mahlambinyathi	60	39 (65)	*T. vivax, T. congolense* (*S*)
Mvutshini	36	33 (91·7)	*T. congolense* (*S*)
Nhlwati	45	5 (11)	*T. vivax*
Qakweni	56	4 (7)	*T. congolense* (*S, K*)
Ocilwane	33	0	—
Nibela	21	0	—
Manzibomvu	50	0	—
Mbazwana	49	0	—
Mkhumbikazane	49	0	—
Mpini	16	0	—
Mseleni	51	0	—
Ndumo	43	4 (9·3)	*T. congolense* (*S, K*)
Nhlajwane	44	4 (9·1)	*T. congolense* (*K*)
Boomerang – Weaners	43	29 (67)	*T. congolense* (*S, K*)
Boomerang – Adults	40	5 (12·5)	*T. congolense* (*S, K*)

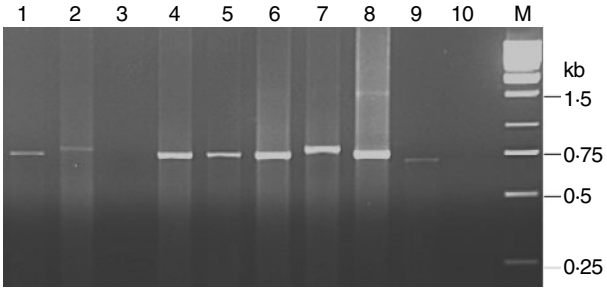

Fig. 2. Photograph of a 1·5% agarose gel showing resolution of PCR products from amplification of DNA eluted from FTA cards. The primers used (18ST nF2 and 18ST nR3) target a segment of the 18S rRNA gene. In lanes 1–7 are PCR products obtained from buffy coats of individual cattle numbered 1–7; in lane 8 is the PCR product from DNA of *Trypanosoma congolense* IL3000; lane 9, *T. vivax* IL2160; and lane 10, no target DNA. Lane M contains the 1 kb DNA size marker.

Tsetse flies in KwaZulu-Natal are infected with both Savannah-type and Kilifi-type T. congolense

The observation that the Savannah- and the Kilifi-type *T. congolense* infect cattle in some parts of KwaZulu-Natal (Fig. 2 and Tables 1 and 2) prompted an investigation to determine whether these two types of *T. congolense* occur either as single or mixed infections in the tsetse flies in the area. In Fig. 3 are shown representative data from PCR analyses of organs of the tsetse flies caught at Boomerang commercial farm, Charter's Creek Game Reserve and Hell's Gate Game Reserve (see Fig. 1 for locations), using oligonucleotide primers which are specific to either of the two types of *T. congolense*.

The tsetse flies were found to be infected with the 2 types of *T. congolense* in different combinations: both genotypes in the midgut and proboscis of a single tsetse fly (Fig. 3, lane 1 in panels A and B); the 2 genotypes in the midgut of a single fly (Fig. 3, lane 2 m in panels A and B), but only 1 of them in the proboscis of the same fly (Fig. 3A, lane 2 p); and 1 genotype in the midgut (Fig. 3A, lane 3 m) and the other in the proboscis (Fig. 3 lane 3 p, panel B) of a single tsetse fly.

Data from these analyses are summarized in Table 3. The majority (55%) of the infections were due to Savannah-type *T. congolense*; 33% of the infections comprised Savannah-type and Kilifi-type *T. congolense*. Of the tsetse fly samples analysed by PCR, 89% were positive.

DISCUSSION

Molecular reagents were used in the present study to gather information on trypanosome species or

Table 3. Summary of data from analysis by PCR of samples from tsetse flies caught at specific locations in the study area

Trap location	Boomerang	Charter's Creek	Hell's Gate
Infection found			
Kilifi-type *T. congolense* alone	1	0	0
Savannah-type *T. congolense* alone	37	16	16
Both Kilifi-type and Savannah-type *T. congolense*	1	17	23
Non-infected	11	3	1
Total number examined	50	36	40
% of samples positive by PCR	78%	91·6%	97·5%

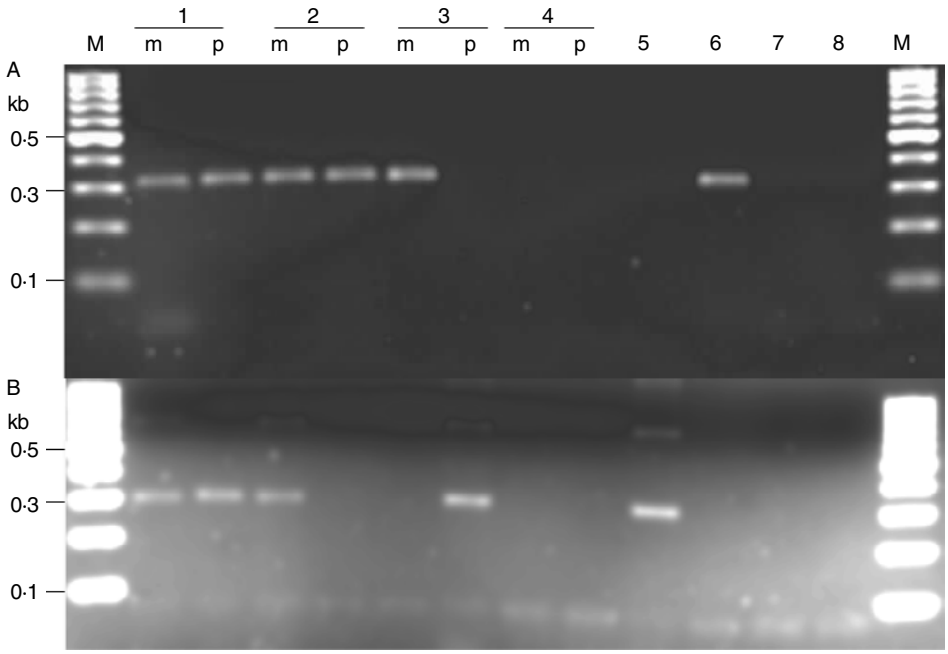

Fig. 3. Photograph of a 1·5% agarose gel containing resolved PCR products from the amplification of DNA eluted from FTA cards on which were blotted either the midgut or proboscides of tsetse flies. The primer pair used on samples in (A) is specific for Savannah-type *Trypanosoma congolense*, and the pair used on those in (B) is specific for Kilifi-type *T. congolense*. In lanes indicated with an 'm' are products from the midguts of individual tsetse flies, whilst in lanes indicated with 'p' are products from the proboscides of the same tsetse flies. Products from a non-infected tsetse fly from the ARC-OVI tsetse colony are in lanes 4. Lane 5 contains products from DNA of OVIKZNTT/7098/07, shown to be a Kilifi-type *T. congolense*; and lane 6, DNA of IL3000, a known Savannah-type *T. congolense*; lane 7, *T. vivax* DNA; lane 8, no DNA. Lanes M contain the 100 bp DNA size marker.

types circulating among tsetse flies, wildlife and livestock in KwaZulu-Natal.

The finding of both *T. congolense* and *T. vivax* in the tsetse-infested areas here confirms recent observations made with the traditional methods of parasite identification (Van den Bossche *et al*. 2006). Although the sampling was limited in the current study, it was surprising that *T. vivax* was found only in samples collected from cattle at 3 diptanks (Ekuphindisweni, Mahlambinyathi and Nhlwati) near the Hluhluwe-Umfolozi Game Reserve, where as *T. congolense* was more widespread, being detected in samples collected from cattle at 6 different diptanks (Ekuphindisweni, Mahlambinyathi, Mvutshini, Ndumo, Nhlajwane and Qakweni) around the Hluhluwe-Umfolozi Game Reserve and at Boomerang. Furthermore, *T. congolense* was also found in buffaloes and in tsetse flies within the study area. Both *T. congolense* and *T. vivax* were present as mixed infections in cattle at 2 diptanks in the study area. It is surprising that *T. brucei* was not detected in any of the samples examined, given the prevalence of trypanosome infections in both livestock and wildlife, known reservoirs of this trypanosome species. There are several plausible explanations: the sampling was not comprehensive enough to include *T. brucei* infections; both *G. austeni* and *G. brevipalpis* are poor vectors of *T. brucei*; or,

indeed, *T. brucei* is absent from this southernmost tsetse belt.

Microscopic examination of blood smears from infected animals or dissected organs of the tsetse fly vectors cannot differentiate mixed infections with *Nannomonas*, because trypanosomes in this subgenus are morphologically and developmentally identical. By using Savannah- and Kilifi-type *T. congolense*-specific primers in PCR, it has been possible to demonstrate infections of tsetse flies and cattle with the 2 genotypes of *T. congolense* known to have different pathogenicity in cattle (Bengaly *et al*. 2002). That these 2 genotypic groups of *T. congolense* persist as mixed infections in tsetse flies indicates that opportunities exist for them to hybridize. However, natural occurrence of such an event remains speculative since no evidence has been found for it.

The Savannah-type *T. congolense* was found at Mahlambinyathi, Mvutshini, Ndumo and Qakweni diptanks and at the Boomerang farm, whereas Kilifi-type *T. congolense* was found at Nhlanjwane, Ndumo and Qakweni diptanks and at the Boomerang farm.

Trypanosoma congolense was previously known to occur in this area (Van den Bossche *et al*. 2006) and an assumption has been made that the population is a single homogeneous genotypic group. Mixed infections of tsetse flies with different trypanosome species, subspecies or types appears to be common (Majiwa and Otieno, 1990; MacLeod *et al*. 1999; Lehane *et al*. 2000; Jamonneau *et al*. 2004; Kubi *et al*. 2005; Malele *et al*. 2003; Adams *et al*. 2008). However, mixed infections comprising different genotypic groups of *T. congolense* have not been reported before.

This is the first study to document the presence of Kilifi-type *T. congolense* in South Africa. Initially found among isolates collected from cattle at Kilifi on the Kenya coast (Majiwa *et al*. 1985), the Kilifi-type *T. congolense* has subsequently been found in the interior of Kenya, Uganda and Tanzania (Majiwa and Otieno, 1990; Nyeko *et al*. 1990; Mugittu *et al*. 2001; Adams *et al*. 2008) and now in South Africa. There is no reason to presume that they are confined to any particular region of Africa.

Limited studies on the relative pathogenicity of the different types of *T. congolense* suggest that the Kilifi-type *T. congolense* may not be pathogenic to cattle (Bengaly *et al*. 2002). Should this hold true, then different control strategies could be adopted in controlling animal trypanosomosis in areas where only the less pathogenic types of *T. congolense* exist. Such an approach may be more acceptable when considering the competing, often conflicting, interests among game farmers, conservationists of natural biodiversity and livestock farmers. The finding of both types of *T. congolense* in KwaZulu-Natal necessitates a determination of their relative distribution in the area, the contribution of each type to livestock morbidity and the interactions, if any, between the two when both are present in individual tsetse vectors or animal hosts.

We acknowledge ILRI for supplying us with trypanosome DNA; Peter Van den Bossche and Rhulani Nkuna for trypanosome isolates; Olivier Matthee, Jerome Ntshangase and Danie de Klerk for assistance with field work; the Department of Science and Technology and the Department of Agriculture for financial assistance. We thank our colleagues Kirsten Gillingwater, Charles Katsande and Arthur Spicket for critical reading of the manuscript.

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