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Recombinant DNA probes reveal simultaneous infection of tsetse flies with different trypanosome species

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The utility of recombinant DNA probes in the detection of natural trypanosome infection of tsetse flies has been assessed in Lambwe Valley, near the shores of Lake Victoria, Kenya. The tsetse flies were surveyed during two different seasons in 1988. Three different probes used each contained highly repetitive DNA sequences specific for a species or subspecies of trypanosomes of the *Nanomonas* subgenus. A fourth probe contained repetitive sequences common to trypanosome species of the *Trypanozoon* subgenus. Mixed mature or immature infections were detected in a variety of combinations in different individual tsetse flies. Such infections were detected in both the guts and mouthparts of some tsetse flies. Simultaneous natural infection of tsetse with the savannah type *Trypanosoma congolense* and Kilifi type *T. congolense*, *T. congolense* and *Trypanosoma brucei* or *T. congolense* and *Trypanosoma simiae* were demonstrated.

The probes have thus been used to demonstrate the presence in Lambwe Valley, south-western Kenya, of a type of *T. congolense* first observed among trypanosome isolates obtained from sentinel cattle exposed to natural infection on a ranch at Kilifi on the Kenya coast. This type of *T. congolense* appears not to be confined to the coastal region nor to any particular species of tsetse flies and may contribute significantly to livestock morbidity in other areas of eastern Africa. In the Kilifi area, *T. congolense* was found primarily in *Glossina austeni*; in Lambwe valley, it was found in *Glossina pallidipes*.

Key words: DNA probes; Epidemiology; *Trypanosoma*: Tsetse infection

Introduction

In epidemiological studies of disease caused by the trypanosomes, it is important to known the genetic identity of the causative organisms, and the extent of their natural distribution in both the vectors and hosts in the affected areas. To obtain this information, the different trypanosome species infecting the tsetse fly vectors, wild and domestic animals, and human beings in an area have to be accurately determined. For purposes of effective disease control, it is essential that the geographic distribution of, and phylogenetic relationships amongst, the trypanosomes be accurately determined. This would allow for the assessment of the contribution of each type of trypanosome to the disease problem.

This study was undertaken to evaluate the possible use of a set of highly repetitive DNA sequence probes in the detection and identification of trypanosome species infecting a natural population of tsetse flies in an area. The study focused on the trypanosome species that comprise the subgenus *Nannomonas*, specifically *Trypanosoma congolense* and *Trypanosoma simiae*, because, although these trypanosomes are morphologically similar, they show marked diversity in their morphological forms, pathological manifestations of the diseases they cause in different mammalian hosts, and in their ability to grow in

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Abbreviations: PSG, phosphate saline glucose; SSC, standard saline citrate; SDS, sodium dodecyl sulfate.

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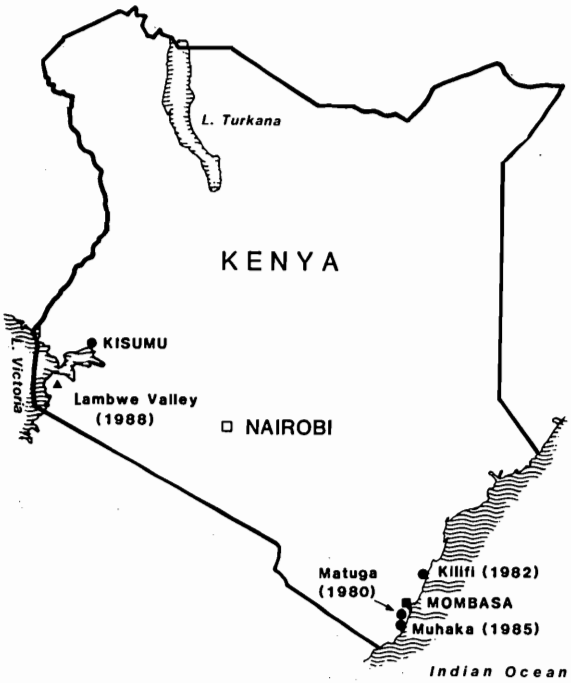

Fig. 1. Map of Kenya showing locations of Kilifi, Lambwe Valley, Matuga and Muhaka where trypanosomes have been observed that possess the highly repetitive DNA sequences contained in the probe pgNIK-450. All the locations except Lambwe Valley are in the coastal region of Kenya.

various laboratory rodents [1–3]. Lambwe Valley, near the shores of Lake Victoria, Kenya (Fig. 1), was chosen for the study because of the high tsetse density in the area and their relatively high rate of infection with trypanosomes. Furthermore, Lambwe Valley is geographically and ecologically different from the other areas from which *Nannomonas* trypanosomes had been obtained during previous surveys [4,5].

Materials and Methods

Tsetse fly trap locations. Tsetse surveys were performed during two different seasons: from 11 to 13 March 1988, in the rainy season, and from 17 to 19 September 1988, in the dry season. The tsetse were caught using biconical traps [6] set up in or around Ruma thicket, located within the Ruma National Park at Lambwe Valley.

The tsetse flies were sorted into females and males, teneral and non-teneral. The non-teneral were each assigned a number then dissected under ordinary Leitz dissection microscopes (Leitz, Wetzlar, FRG). The dissected organs of each fly were separated into mouthparts and guts, and the organs in PSG (57 mM Na_2HPO_4/3 mM NaH_2PO_4/45 mM NaCl/55 mM glucose, pH 8.0 were placed on microscope slides for examination under the light microscope. The dissected organs of each infected fly were recovered separately in a total volume of 400 μl of PSG and then thoroughly mixed by vortexing in a 1.5 μl microfuge tube. 100 μl of the suspension was loaded onto a piece of Hybond-N nylon membrane (Amersham, U.K.) contained in the Minifold apparatus (Schleicher and Schuell, USA). Thus, a suspension from each infected fly was distributed equally into 8 spots, 4 containing mouthparts and 4 containing gut suspensions. After all the samples had soaked onto the filter, each slot was rinsed with 500 μl of 0.5 M NaOH/1.5 M NaCl before the filter was dislodged from the Minifold. The filters were subsequently baked at 80°C for at least 2 h.

Trypanosome DNA manipulations. DNA from trypanosomes was prepared following published procedures [7]. The DNA was ordinarily digested with 20-fold excess of restriction enzyme and separated according to size in agarose gels following conventional procedures [7]. Southern blot transfer of such DNA fragments were performed as described [8].

The nucleotide sequence of the recombinant plasmid clones, each containing a unit repeat of a repetitive DNA sequence, was determined on both stands by the dideoxynucleotide chain termination method [9] using a Sequenase kit (United States Biochemical Corp. U.S.A.).

Recombinant DNA probes. Initial identification, cloning and verification of the species-specific DNA probes that distinguish the *Nannomonas* trypanosomes have been described [10,11]. Designations and trypanosome species-specificities of these recombinant plasmids are summarised in Table I. Recombinant plasmid pgDR1 is a portion of *ingi*, or TRS, elements and has been described [12,13]. These elements occur primarily in the *Trypanozoon* trypanosomes and are absent from the *Nannomonas* trypanosomes [13].

TABLE I

Recombinant plasmids containing DNA probes used in this study

Designation	Trypanosome specificity	Enzymes that generate a unit repeat	Reference
pgNRE-372	*T. congolense* (Savannah type)	*Mbo*I, *Aha*III, *Eco*RII, *Bgl*I, *Hpa*II	[10]
pgNIK-450	*T. congolense* (Kilifi type)	*Aha*III, *Alu*I, *Mbo*I	[10]
pgNS-600	*T. simiae*	*Mbo*I, *Eco*RI	[11]
pgDRI	*Trypanozoon*	*Mbo*I	[12]

All the recombinant plasmids were radioisotope-labelled by the random priming procedure [14]. Specific activities obtained were higher than 1×10^8 cpm μg^{-1} of DNA.

Nucleic acid hybridization. These were performed exactly as has been described [11] except where indicated otherwise. High stringency post-hybridization washes to remove the non-specifically bound probe were performed twice for 1 h each time with 0.1 × SSC (15 mM NaCl/1.5 mM Na_3-citrate, pH 7.4), 0.1% SDS at 65°C; low-stringency washes were performed twice for 15 min each time with 6 × SSC, 0.1% SDS at 30°C. After washing, the membranes were exposed to film with intensifying screens at −70°C for 1–4 days.

Results

The repetitive DNA sequence probes are trypanosome species-specific. The nucleotide sequence composition of a unit repeat of each of the recombinant DNA probes pgNRE-372 and pgNS-600 is shown in Fig. 2A and B respectively. pgNRE-372 is 372 bp long and is similar to that independently identified by Gibson et al. [15], except for a two-base insertion in our isolate. pgNS-600 is comprised of 520 bp. The two show no significant sequence similarity, have no significant open reading frames and do not give rise to transcripts detectable by Northern blot hybridizations.

When used as hybridization probes under stringent conditions, the DNA sequence hybridize specifically with DNA isolated from trypanosomes of the same species, irrespective of genotype or antigenic repertoires of the trypanosome [11,16,17]. Because all the probes were identified and subsequently used under highly stringent and therefore selective conditions, we sought to determine the conditions of hybridization under which their specificities would be maintained.

```
                                                                    60
GATCAAATTT CGAGCGAGAA CGGGCACTTT GCGATTTTCC CAAAATTCAC CTTTTTGGGC
                                                                   120
CCAAATGGCA AAAACCGGTT TTTTTGAAAA TGGTCAAAAA TGTCAAAAAC GCAAAAATTC
                                                                   180
GAAAAACGCG TATTTGGCAC GTATTTTTCG TTTTCGGGCC TATTTGACGC GCATAGTGAT
                                                                   240
TTTTCAAAAT TTTGCAAAAA ATTGTGTCAA AAACTTTTTC TAATTTTTGC AAATTTTTGA
                                                                   300
AAATTTTTTT GTAAAAAAAT ATTTTTTTTT GACTTTTTCG GCCAAAATTT TTTCTGTTCC
                                                                   360
AAAAATGGTT GTGCGGGATT TGTTTGTCCC TAATTTTCAT CGAAAACGCC GAAATCCCTT

TTAAAAATGA TC
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A

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                                                                    60
GATCGACTCC GGGCGACCGT TCTGACCCGA CCATGGGCTC AGGGGGGGTA CCGTACACGC
                                                                   120
CCATTTTGGC CATGACCCCC CCAGGCCGGT CGGCAGTCAC CGGTCAAAAA CGCATTTTCG
                                                                   180
GGCAAAAAAA ATATTTTTAC GTATTGACGC GCGCTGCGAT ATCCAGCCAG TATATCGGTT
                                                                   240
TTCAGCCAAT TTGGACACCC CTACCGGCCC AACCGGACCC GCCTTGTGCA CCACGGATTT
                                                                   300
GAAGGGTCGC GGTCCAAAAT CCCAAAAATC GCAAAATCGA CCCCCGACAA TTCTGACCCG
                                                                   360
ATTTGCTCAT AGAGATGGCC GAGTTCGCGC TTTAATCGAA ATCCGTTCGA AAAACGCATG
                                                                   420
TGCCTAGCGC CGGAACGGGC CATACCGCAC CTTGGGGGAA AATGCCAAAT GGCCGTTTTG
                                                                   480
GGCCTAAGGC CGTTTTTCCC GTCACAGAAC ATGCCGAGAC CTCGTCTCAA ACGGATTTAA
                                                  525
ATGCGTAATG CCACGGTCCG CCGCATGAAG AAACACTAGC TGATC
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B

Fig. 2. Complete nucleotide sequences of single unit of the repeats comprising the recombinant DNA probes pgNRE-372, shown in panel (A)and pgNS-600, shown in panel (B). The *Sau*3A sites, GATC, marking the beginning and end of each unit repeat are underlined.

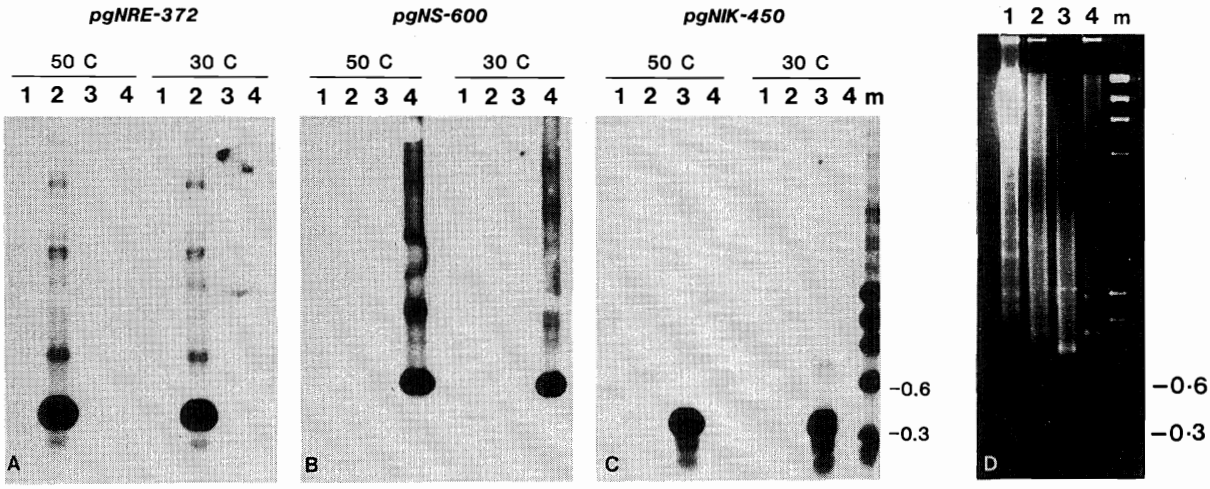

Fig. 3. Specificity of the recombinant DNA probes at varying stringencies. 2 μg of DNA from four different trypanosomes each representing a genotypic group or species was digested with the restriction enzyme *Dra*I, resolved by electrophoresis in an agarose gel and photographed (panel D). Cut filter strips with blots of the DNA were separately hybridized with one of the probes indicated in each of the panels (A) pgNRE-372; (B) pgNS-600; (C) pgNIK-450. Post-hybridization washes were performed with 6 × SSC at the temperatures indicated in °C. In each panel DNA from the different trypanosomes was loaded in the lanes as follows: 1, *T. vivax* ILDat 1.2; 2, Savannah type *T. congolense* ILNaR 1; 3, Kilifi type *T. congolense* ILNat 6.1 and 4, *T. simiae* KETRI 2431/1. Lane m contains molecular weight markers, a mixture of *Hin*dIII digest of λ DNA and *Hae*III digest of ϕX174 DNA.

Fig. 3 shows that under ordinary hybridization and post-hybridization conditions the *Nannomonas* repetitive DNA sequences hybridized only with DNA from trypanosomes of the same species. The recombinant plasmid pgDR1 hybridizes weakly to *Trypanosoma vivax* DNA under relaxed post-hybridization wash conditions [13]. That no hybridization was observed, even at low stringencies of post-hybridization washes (30°C, 6 × SSC), between the repetitive DNA sequences from the morphologically identical Savannah type *T. congolense* and Kilifi type *T. congolense* trypanosomes emphasizes the genomic diversity of populations of *T. congolense* and validates the potential utility of these two probes in the identification of the two different populations of *T. congolense*.

Since neither of the two *T. congolense*-specific DNA probes (pgNRE-372 and pgNIK-450) hybridizes with *T. simiae*, and the *T. simiae* repetitive DNA probe (pgNS-600) does not hybridize with either of the two types of *T. congolense* [11], we infer that these probes can collectively be used to identify at least three of the major types presently thought to comprise the *Nannomonas* subgenus of trypanosomes.

The probes reveal mixed infections in tsetse flies. Fig. 4A shows representative strips of a filter membrane on which suspensions of organs from the infected tsetse flies were placed for hybridization with the different repetitive DNA probes. Fig. 4B shows autoradiographs obtained from the filters (shown in Fig. 4A) hybridized with the four different probes.

The flies included in all the analyses had been shown by microscopic examination to have trypanosome infection at least in the gut. Therefore the lack of hybridization signal at a spot hybridized with a particular probe indicated the absence of trypanosomes detectable by that probe. Such spots were taken as negative and formed the baseline, any signals above which were scored as positive. The criterion was used throughout in the interpretation of all the data obtained from dot-blot hybridizations. From the data shown, it is evident that the probes used could detect trypanosome infection in tsetse flies in a majority (87%) of the cases. The infection in only one (Fig. 4B, lane 1) of the 8 flies on this filter did not react with any of the four probes used. Additionally, the probes revealed that the majority of the flies examined in this filter had been infected with more

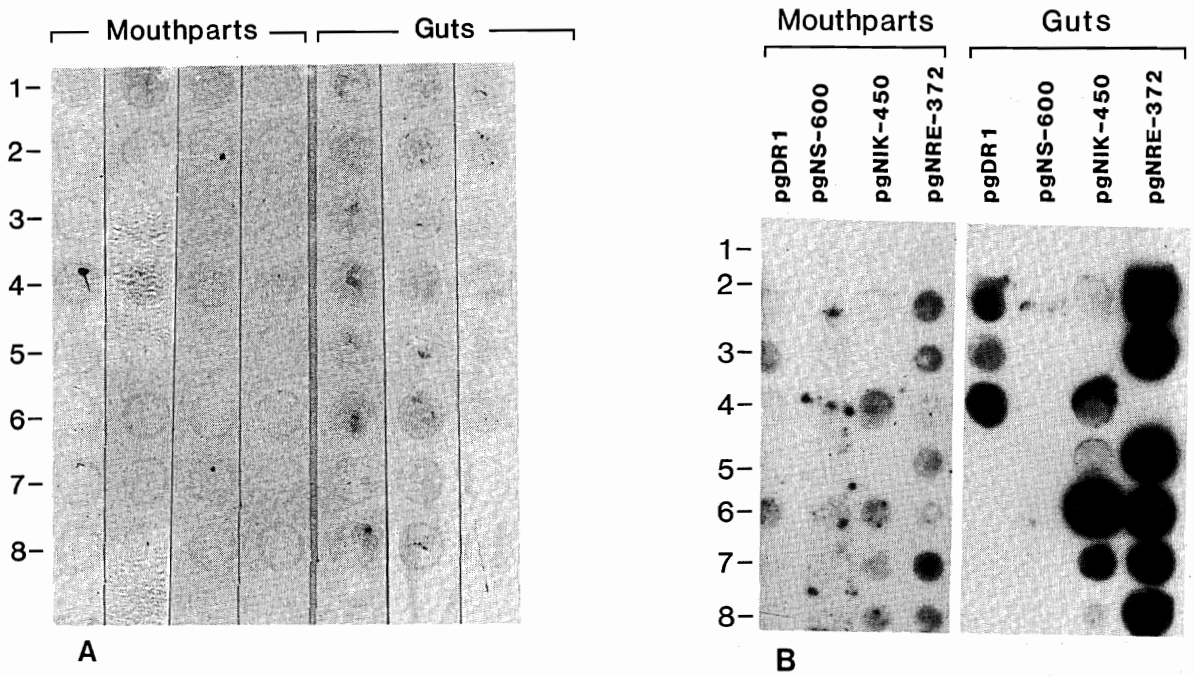

Fig. 4. Detection of mixed infections in tsetse fly mouthparts and guts. Suspensions of infected tsetse organs were spotted on nylon filter paper, which was cut in strips and the strips hybridized with separate probes. A photograph of the re-assembled filter strips is shown in panel A. Autoradiograph of the same filter strips after hybridization with the probes, indicated above each strip, is shown in panel B.

than one trypanosome species (Fig. 4B, lanes 2–8); the trypanosomes were detected in both the guts and the mouthparts. In one fly (Fig. 4B, lane 6), *T. brucei* type trypanosomes were detected only in the mouthpart suspensions, but not in the gut.

It is apparent that the fly whose parts were placed in slots marked 2 had savannah type *T. congolense,* Kilifi type *T. congolense* and *T. brucei* infections. Infection with the two types of *T. congolense* was detectable both in the mouthparts and in the gut of this fly. The fly at slots marked 3 had *T. congolense* and *T. brucei* infections detectable in both the mouthparts and the gut. The flies at slots 5, 6, 7 and 8 had infections that would be considered mixed. None of the flies sampled in March 1988 had parasites detectable by pgNS-600, the *T. simiae* probe. The majority of the 600 flies dissected during this survey had infections confined primarily to the mouthparts and thus indicative to *T. vivax*. They were not analysed with the four DNA probes.

During the sampling of tsetse flies in September 1988, infection of the tsetse with *T. simiae* was detected in 11% of the 74 flies confirmed by microscopy to have *Nannomonas* type infection. This information is summarised in Table II.

TABLE II

Trypanosome species detected in tsetse flies sampled in September, 1988

	N	% of total
Number dissected	1086	
Number examined with four probes	74	6.8
Number in which infection was detected by the four probes	27	2.5
Infections not detected by any of the four probes	47	4.3
Infections[a] with:		
T. simiae	8	0.74
T. congolense (Kilifi type)	9	0.83
T. congolense (Savannah type)	23	2
T. brucei	10	0.9

[a]The presence of infections was determined by microscopy. Many of the infections were found to be mixed, thus the total number of infections revealed by the four probes [50] is greater than the number of flies [27] in which infections were detected.

TABLE III

Presence of trypanosome infections undetectable by the four probes used

Tsetse number	Probe used							
	Gut				Mouthparts			
	372	450	600	ingi	372	450	600	ingi
45								
46	+			+				
47								
48								
49	+							
50								
51								
52								
53								
54								
55	+							
56								
58								
59	+	+		+	+	+		
60	+			+	+			+
61	+	+		+				
62	+	+			+			
63	+	+			+	+		+
64	+	+			+	+		
65	+	+			+	+		
66		+				+		
67	+				+			
68	+				+			
69	+	+			+	+		

Only those flies which appeared to definitely have *Nannomonas* type-infection were spotted. The guts and mouth-parts were spotted separately. Number of flies spotted, 24 (100%); number of infections detected, 14 (58%); number with undetected infections, 10 (42%). Fly number 46 was identified by microscopic examination as positively possessing a *T. congolense* infection.

The probes do not reveal all the different trypanosome species infecting tsetse flies. The primary objective of this study was to evaluate the possibility of using recombinant DNA probes to identify trypanosomes of the *Nannomonas* or *Trypanozoon* subgenuses present in naturally infected tsetse flies; therefore, only those flies with infections in their gut were examined with the probes. However several tsetse flies, at least 6 during March and 47 during September surveys, were observed in which trypanosomes present in the infections could not be revealed by any of the four DNA probes used. Parts of an infected fly illustrating such a case are shown in Fig. 4, position 1; data on the other flies with such infections are summarised in Tables II and III.

Discussion

In the epidemiological studies of diseases caused by parasitic organisms, it is imperative to know accurately the genetic identity of the causative organisms and the extent of their geographic distribution. This task could be facilitated by the use of species-specific DNA probes. We and others have shown previously that under laboratory conditions such probes have the potential for use in the specific identification of trypanosome species infecting tsetse flies [16,17]. The possible use of the probes in the detection of natural infection of tsetse flies in a defined area has been evaluated in this study.

The probes are specific, sensitive and identify unequivocally the respective trypanosome types when present in an individual infected fly. Be-

cause of the specificity of the probes (Fig. 3), it has been possible to demonstrate the simultaneous infection of tsetse flies with trypanosomes of different species. This has hitherto been impossible to demonstrate without the subinoculation of the trypanosome isolates into laboratory rodents, a procedure with the disadvantage that the trypanosomes which grow slowly or do not grow at all in the rodents would be outgrown or eliminated.

In the representative dot-blot hybridization shown in Fig. 4B, the tsetse fly at position 1 was scored negative for all the trypanosomes that would be revealed by any of the probes used. The tsetse at position 3 was scored positive for *T. brucei* and Savannah type *T. congolense*, but negative for both *T. simiae* and Kilifi type *T. congolense*. In the mixed infections, the relative proportions of trypanosomes appeared to differ in the mouthparts and in the gut. In the mouthparts of the tsetse at position 4, there were more of the Kilifi type *T. congolense* than *T. brucei*. In the tsetse at position 6, *T. brucei* was detected primarily in the mouthparts and not in the gut. The presence of trypanosomes in the vector mouthparts indicated that the parasites had reached the metacyclic stage and therefore had the potential for transmission to the mammalian hosts.

The presence of mixed infections in tsetse implies either the prevalence of such infections in livestock and other animals in the area, or frequent feeding of the tsetse flies on animals infected with the different trypanosomes. The present study did not address either of these possibilities.

Of the mixed infections observed during the study, the most surprising were those composed of the two genetic types of *T. congolense* [10,18,19]. The *T. congolense* in which the predominant repetitive DNA sequence is that contained in the probe pgNIK-450 was first observed among isolates obtained by ILRAD from sentinel cattle exposed to natural infection on a ranch in Kilifi, Kenya (Fig. 1) [4,10]. Similar *T. congolense* were subsequently found among isolates obtained from livestock in other localities within the Kenya coast [18,19]. It has been suggested that this type of *T. congolense* may be ecologically or geographically confined to the coastal region of Kenya because of the species of tsetse flies prevalent there [5]. Our data do not support this suggestion: in the coastal region of Kenya, *G. austeni* was the main tsetse species found in the area where sentinel cattle were exposed to natural infection; the only female *G. pallidipes* caught there was not infected [4,21]. In Lambwe Valley, all the tsetse caught and dissected were *G. pallidipes*. No *G. austeni* was found. That the *G. pallidipes* in Lambwe Valley were infected with both types of *T. congolense* (Fig. 4) suggests that the vector is not a significant barrier to the possible transmission of these trypanosomes to mammalian hosts.

The finding of trypanosomes in Lambwe Valley that have a repetitive DNA sequence similar to that in the *T. congolense* isolates from the coastal region of Kenya implies that the trypanosomes in the two regions with different ecology and inhabited by different species of tsetse flies, may be the same parasites. The data at hand do not rule out the unlikely possibility that the flies which seem to be infected by both types of *T. congolense* were infected with only one type of *T. congolense* which contains both kinds of repetitive DNA.

During the tsetse infection survey carried out in March 1988, no trypanosomes were found that were detectable by pgNS-600, the probe specific for *T. simiae*. During the second survey eight tsetse flies, representing 0.74% of the dissected and 11% of the infected flies, had infections detectable by this probe (Table II). All were present as mixed infections. These observations could be explained in part by any of the following: (1) Tsetse infection by *T. simiae* may have been very low in Lambwe Valley at the time of the first survey and insufficient numbers of flies examined to find those infected with the parasite (600 and 1086 flies were dissected during the March and September surveys, respectively). (2) Some *T. simiae* infecting tsetse in Lambwe Valley may have a repetitive DNA sequence different from that contained in the probe pgNS-600 used in this study. This proposition is plausible given the diversity among the *Nannomonas* trypanosomes; however, the probe used detects *T. simiae* parasites from both East and West Africa (refs. 11 and 17, and Majiwa and Gibson, unpublished observations).

(3) Tsetse trap locations were different during the two surveys. During the first survey there had been minimal tsetse control measures in Lambwe Valley, and consequently tsetse traps were set up in many locations in the periphery and centre of the Ruma thicket; during the second survey, tsetse control measures undertaken during the previous rainy season had considerably reduced the number of tsetse flies. No flies were caught in the periphery of the thicket, and all those examined during this survey had been caught in the centre of the thicket. This is where flies infected with *T. simiae* would most probably be found.

The DNA probes used here offer the possibility of determining the species of trypanosomes infecting tsetse flies by direct detection of the parasites in the fly. However, the two probes used for the two types of *T. congolense* do not reveal other type of *T. congolense* [17]. Such trypanosomes include those isolated from humid riverine zones of West Africa, and those that may have been present in some of the infected flies in Lambwe Valley (Tables II and III).

The combination of such probes with the polymerase chain reaction (PCR) [22] technology should facilitate studies aimed at determining the distribution of the different types of *Nannomonas* trypanosomes and evaluation of the contribution of each type to livestock morbidity and mortality in Africa.

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