Mixed populations of Trypanosoma brucei in wild Glossina palpalis palpalis

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Abstract

In many previous characterization studies of *Trypanozoon*, isolates have been subpassaged numerous times in laboratory rodents until a quantity of trypanosomes sufficient for analysis has been obtained. In addition to the numerous biochemical effects of such a process on the parasite, it appears probable that adaptation to an unnatural host may also serve to filter out less virulent populations from mixed infections, leading to an underestimate of the true level of genetic diversity. By the early cloning of trypanosomes from susceptible captive flies infected from the primary isolate – the midgut of a wild tsetse – the present study provides evidence of the range of genetically different *Trypanosoma brucei* populations which may coexist within the midgut of individual tsetse flies in nature. The three primary isolates from tsetse yielded one, five and nine genetically distinct populations. Cloned populations were confirmed as *T. brucei* using the polymerase chain reaction, and were characterized by karyotype analysis and multilocus isoenzyme electrophoresis. These data allowed a limited assessment of the level of genetic variability in natural populations of *T. brucei*.

Introduction

Despite several studies of naturally occurring mixed infections of *Trypanosoma (Trypanozoon) brucei* in mammalian hosts (Scott, 1981; Mehlitz et al., 1982), studies of mixed populations of trypanosomes in wild caught tsetse flies have been less frequently reported (Letch, 1984; Godfrey et al., 1990; Majiwa and Otieno, 1990). Given the importance of the tsetse fly as the probable site of genetic exchange in trypanosomes (Jenni et al., 1986), this represents a potentially serious gap in our understanding of the disease, particularly when considering genetic variability within *Trypanozoon*. Furthermore, in previous studies of genetic variation within natural infections of *T. brucei* from tsetse (Letch, 1984; Godfrey et al., 1990), parasites were inoculated directly into laboratory rodents; clones were then made from samples taken at intervals of several days during the parasitaemic cycle. Thus, certain populations which were less well adapted for growth in experimental mice may already have been overgrown, and perhaps eliminated, by the time the first samples were taken.

The present study addresses this problem by cloning and growing procyclic trypanosomes derived from susceptible laboratory flies infected from the primary isolate – the midgut of a wild tsetse. The cloned populations were confirmed as *T.brucei* using the polymerase chain reaction (PCR), and were characterized by karyotype analysis and multilocus isoenzyme electrophoresis (MLEE). These data allowed a limited assessment of the level of genetic variability occurring naturally in *T. brucei*. Results are discussed in relation to other studies on reproductive processes in field populations of *Trypanozoon* (Tait, 1980; Gibson, 1990; Tibayrenc et al., 1990; Cibulskis, 1992; Stevens and Welburn, 1993).

Materials and methods

Trypanosome origins. The origins of the three stocks and the 29 clones made from them are presented in Table **1**, together with WHO codes and information on the three standard stocks.

Trypanosomes were isolated from *Glossina palpalis palpalis* caught in Challier traps in and around the village of Kouassi-Perita, near Bouafié, central Côte d'Ivoire in June 1989 (Mehlitz and Schares, personal communication); a detailed description of the area is provided by Mehlitz et al., (1981). Parasites were isolated from tsetse midguts (Mehlitz and Schares, personal communication) and transferred to supplemented Cunningham's medium (Cunningham, 1977) as described by Dukes et al. (1989); isolates were cryopreserved before despatch to Europe. Stabilates of the three primary isolates were kindly collected and provided by the research teams of Professor D. Mehlitz, Free University of Berlin and Dr. I. Maudlin, Tsetse Research Laboratory (TRL), Bristol.

Procyclic cloning and growth in culture. Stabilates were thawed and washed by centrifugation in SM culture medium (Cunningham, 1977). Trypanosomes were then resuspended in 1.0 ml SM, half of which was removed to one well of a 24 well culture plate, and incubated at 27 °C in 5 % CO_2 enriched air; the remainder was fed to laboratory *G. morsitans morsitans* (TRL colony FX9) as described previously (McNamara and Snow, 1991). Where trypanosomes failed to grow directly from the primary isolate they were re-isolated into culture medium from the midguts of laboratory flies. To minimise the potential loss of less virulent trypanosome strains from any mixed infections, midgut cultures in the first stage of exponential

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Table 1 Isolate details.

WHO Code	Short Code	Host	Cloned populations	Reference
GPAP/CI/89/KP 13	KP 13	*G.p.palpalis*	5 (N°s 1–5)	
GPAP/CI/89/KP 14	KP 14	*G.p.palpalis*	9 (N°s 1–9)	
GPAP/CI/89/KP 33	KP 33	*G.p.palpalis*	15 (N°s 1–10, 12–16)	
Standard stocks:				
GPAP/CI/82/KP 2–2 cl. 38	KP 2	*G.p.palpalis*	–	Letch, 1984
MHOM/CI/78/TH 1–037	TH 1	Man	–	Mehlitz et al., 1982
MHOM/SD/82/BIYAMINA cl. B	BIYAMINA	Man	–	Godfrey et al., 1987

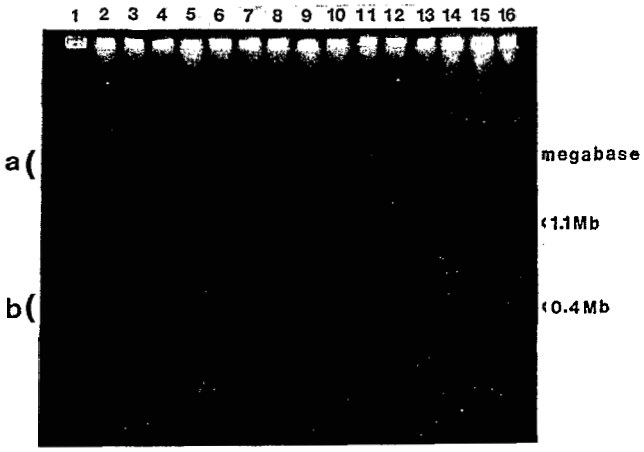

Fig. 1 Molecular karyotypes of cloned trypanosome populations of isolate GPAP/CI/89/KP 33; uncloned parental stock, lane 2; clones 1–14, lanes 3–16. Two karyotypes can be distinguished by the presence or absence of chromosome bands in the megabase (a) and 400 kb (b) regions of the gel. Marker shown in lane 1.

growth were diluted with fresh SM to give a trypanosome concentration of 10^3–10^4 ml^{-1}; clones were made by direct observation of single parasites in a 0.5 µl drop of medium in a well of a microtitre plate. Immediately after the presence of a single organism had been confirmed by a second observer the well was flooded with 100 µl of SM; the process was repeated until up to 35 clones had been prepared. Plates were incubated at 27 °C in 5 % CO_2 enriched air, and, if cloning was successful, the trypanosomes were subcultured into progressively larger volumes of medium.

Thereafter, cultures were maintained at 10^6 trypanosomes ml^{-1}, at 28 °C for 6–10 days (Dukes et al., 1989). Once a culture exceeded 2×10^9 trypanosomes in 120–150 ml of medium, it was harvested (Gray et al., 1987). Finally, trypanosomes were lysed, and centrifuged to separate the water soluble enzymes from the trypanosome debris. The supernatant containing the enzymes was removed and stored as beads in liquid nitrogen (Gibson et al., 1980), while the solid material was retained for DNA extraction.

PCR primer analysis. Total trypanosome DNA was prepared according to standard methods (Van der Ploeg et al., 1982). Oligonucleotide primers designed to anneal specifically to the satellite DNA of a particular species (Moser et al., 1989; Masiga et al., 1992) were used to check that all populations were *T. brucei*. Populations were also checked with *T. congolense* (Savannah type) and *T. simiae* PCR primers; reaction conditions were as described by Masiga et al. (1992).

Karyotype analysis. Trypanosomes grown in culture were lysed *in situ* in agarose blocks at a final concentration of 2×10^9 ml^{-1} (Van der Ploeg et al., 1984). Chromosome sized fragements of DNA were size fractionated in a 1 % agarose gel using an LKB Pulsaphor system with a hexagonal electrode array. The program used had five phases (900 s, 15 h; 300 s, 15 h; 210 s, 10 h; 180 s, 10 h; 40 s, 10 h) and a running time of 60 h at 130 V. Gels were stained with ethidium bromide and photographed by ultraviolet transillumination (Fig. 1).

Enzyme range. Electrophoresis was carried out on cellulose acetate plates (CAE) (Mathieu-Daudé, 1991; Ben Abderrazak et al., 1993). The following 15 enzyme systems were examined: EC 1.6.-.-, Diaphorase (DIA); EC1.1.1.10, Threonine dehydrogenase (TDH); EC1.1.1.37, malate dehydrogenase (MDH); EC 1.1.1.40, "malic" enzyme (ME); EC 1.1.1.42, isocitrate dehydrogenase (ICD); EC 1.1.1.44, phsophogluconate dehydrogenase (6PGD); EC 1.1.1.49, Glucose-6-phosphate dehydrogenase (G6PDH); EC 1.2.1.12, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH); EC 2.6.1.1, aspartate aminotransferase (ASAT); EC 2.7.5.1, phosphoglucomutase (PGM); EC 3.2.2.1, two nucleoside hydrolases using different substrates; the enzyme utilizing inosine was coded NHI (previously labelled as NH; Gibson et al., 1980; Godfrey et al., 1990) and that utilizing deoxyinosine, NHD; EC 3.4.11.-. or .13.-, Leucine aminopeptidase (LAP); EC 3.4.11.-. or .13.-, peptidase, substrate: L-leucyl-L-alanine (PEP_B); EC 5.3.1.9, glucose phosphate isomerase (GPI).

Numerical analysis. A similarity matrix was produced according to Jaccard's method (1908) using a computer program (Stevens and Cibulskis, 1990). Similarity values were calculated using all isoenzyme bands for each enzyme, between each pair of populations; parental stocks were not included as they were, by definition, composites of the cloned populations under study. A dendrogram was constructed by the *unweighted pair-group method using arithmetic averages* (UPGMA; Fig. 2).

Results

Cloning of stocks

None of the cultures initiated directly from the primary isolates grew; only those stocks which were successfully re-isolated from laboratory flies were cloned. Furthermore, despite standardized procedures and conditions, e. g. cultures in the same early growth stage, cloning efficiencies varied between 14 % (GPAP/CI/89/KP 14) and 48 % (GPAP/CI/89/KP 33).

PCR primers

Analysis of parasite DNA with PCR primers (Moser et al., 1989; Masiga et al., 1992) confirmed all stocks, both parental and cloned populations, as *T. brucei*,

Controls, populations tested with the *T. congolense* (Savannah type) and *T. simiae* probes, were negative.

Molecular karyotype analysis

The three uncloned populations, GPAP/CI/89/KP 13, GPAP/CI/89/KP 14 and GPAP/CI/89/KP 33, had distinct karyotypes. However, only KP 33, which yielded two distinct karyotypes, produced clones with different chromosome banding patterns (Fig. 1).

Isoenzymes: zymodemes

The isoenzyme banding patterns obtained were coded according to Mathieu-Daudé (1991) and Truc et al. (1991). Using the fifteen enzyme systems, interpreted as 17 loci (see *interpretation*), 17 zymodemes were recognised in the 35 populations analysed (Fig. 2), including the standard stocks. Sample populations, including parental stocks, were classified in 15 zymodemes; cloned populations were classed in 14 zymodemes. Full details of these isoenzyme data are available on request from J. R. Stevens.

The following seven loci were invariant and monomorphic for the 35 populations (including three standard populations) studied: GPI, ME_A, NHI_A, NHI_B, NHD, TDH, MDH. Among the 32 sample populations an eighth enzyme, LAP, was also invariant and monomorphic. The parental population of KP 13 and all five cloned populations were enzymatically identical, and were placed in zymodeme 4. The nine cloned populations of KP 14 were classified in five zymodemes; the parental stock was classified in the same zymodeme (Z5) as two of it's cloned populations. The fifteen cloned populations of KP 33 were classified in nine zymodemes, while the parental stock was placed in a separate zymodeme (Z10); one cloned population was placed in Z4 with all populations of KP 13.

Due to the large number and selection of enzyme systems examined in the current study, the direct matching of zymodemes with those described in previous studies (Godfrey et al., 1990; Stevens et al., 1992; Truc and Tibayrenc, 1993) was not possible. Indeed, as Tibayrenc and Ayala (1988) point out, delineation of zymodemes is highly dependent upon the methods used, and upon the range of isoenzyme markers under study. This was particularly true for comparisons made with earlier studies which used thin-layer starch gel electrophoresis (Gibson et al., 1980; Letch, 1984; Tait et al., 1984).

Isoenzymes: interpretation

For four of the fifteen enzyme systems, a genetic interpretation of isoenzyme patterns was not possible; thus, the numerical analysis was phenetic. However, an allelic interpretation of eleven enzymes, equating to thirteen loci, was possible, allowing analysis to be based on a total of 17 putative loci. Such an interpretation also permitted further investigation of the genetic relationships between populations.

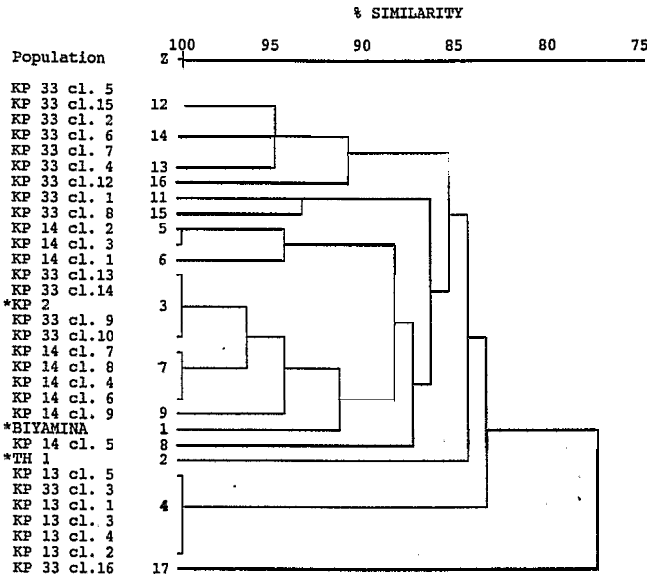

Fig. 2 Dendrogram constructed by UPGMA from a Jaccard similarity coefficient matrix. Cloned populations are shown, together with the three standard stocks (marked*) which equate to the recognised subspecies of *T. brucei*; KP 2, *T. b. brucei*; TH 1, *T. b. rhodesiense*; BIYAMINA, *T. b. gambiense*; KP 2 and BIYAMINA are also cloned stocks. The zymodeme (Z) into which a population is classified is shown at the end of each branch.

Previous studies of enzyme polymorphisms in *Trypanozoon* show homozygous and heterozygous patterns typical of monomeric and dimeric enzymes in a diploid organism (Gibson et al., 1980;Tait, 1980; Stevens and Godfrey, 1992; Truc and Tibayrenc, 1993). Given that trypanosomes are diploid, practical allelic interpretation of isoenzyme pattern followed the method described by Ferguson (1980) and Ben Abderrazak et al. (1993). Single band patterns were interpreted as homozygotes for all enzymes. Double and triple band patterns were interpreted as heterozygotes, associated with monomeric and dimeric enzymes respectively; alleles were allocated on the basis of the positions of the upper and lower bands in a pattern. Only consistently reproducible bands were included, and samples were rerun electrophoretically to elucidate the validity of weak shadow bands and poorly separated multiple patterns.

Genetic interpretation of most isoenzymes was straightforward; however, ME and NHI produced two distinct sets of band patterns, not easily explained as the product of a single locus. Consequently, ME and NHI were interpreted as two loci and the following nomenclature was adopted: ME_A, ME_B and NHI_A, NHI_B.

Numerical analysis

The degree of genetic variation present within the cloned populations from the three original sample isolates is shown in the dendrogram (Fig. 2); the high degree of variation present in two of the isolates (GPAP/CI/89/KP 14 and GPAP/CI/89/KP 33) is of particular note. However all populations, clones and standards, are greater than 78 % similar, though such a result

must be interpreted with regard to similarities between the standard stocks (Stevens and Godfrey, 1992).

Four populations from KP 33 are isoenzymically identical to KP 2, while over half of the populations of KP 14 are 95 % similar; KP 2 has been unambiguously classified as West African *T. b. brucei* in several previous studies (Letch, 1984; Kaukas et al., 1990; Mathieu-Daudé, 1991). This provides a strong indication that the populations of KP 14 and KP 33 may be classified as *T. b. brucei*, strain group *bouaflé*.

The same populations which are 95 % similar to the *T. b. brucei* standard, KP 2, are also 92 % similar to the *T. b. gambiense* standard, BIYAMINA. However, the high level of isoenzymic similarity between *T. b. brucei* and *T. b. gambiense* has been well documented and will be considered further (see Discussion). Cloned populations are, at maximum, only 85 % similar to the *T. b. rhodesiense* standard, TH1.

All populations of KP 13 are isoenzymatically identical, and are also identical to a single population (Clone 3) of KP 33. This group of populations were a maximum of 84 % similar to the other stocks analysed.

Discussion

In previous characterization studies of *Trypanozoon* parasites (Gibson et al., 1980; Tait et al., 1984; Godfrey et al., 1990; Stevens et al., 1992; Truc and Tibayrenc, 1993), most stocks were isolated into rodents and then subpassaged numerous times until a quanity of parasites sufficient for characterization was obtained. Such a process may affect any number of characteristics of the trypanosomes under study, e.g. adaption to host (Fairbairn, 1933; Dukes et al., 1989),antigenic type (Lumsden and Herbert, 1975), and virulence (Gibson et al., 1980). However, one of the most serious consequences of rodent subpassage is the filtering out of less virulent populations from mixed infections (Letch, 1984), which leads in turn, to an underestimate of the true level of genetic variation.

The present study provides evidence of the range of genetically different *T. brucei* populations which may coexist within the midgut of individual tsetse flies in nature, the three primary isolates from tsetse yielding one, five and nine genetically distinct populations when subjected to extensive early cloning. Moreover, this degree of genetic variation was, in at least one instance, reflected by the presence of two distinct trypanosome karyotypes in the same fly; such a variation in the structure of the genome implies significant genetic differences. In the few previous studies addressing this problem, Letch (1984) found two genetically distinct populations of *T. b. brucei* in a single tsetse fly, while Godfrey et al. (1990) identified mixed populations in seven out of 73 isolates from tsetse. However, most were found by chance, with no deliberate attempt to identify and separate mixtures soon after the initial isolation. Indeed, of more than 900 isolates characterized by isoenzymes (Godfrey et al., 1990), only 26 were described as mixed populations; of these, only two were shown to contain mixtures of three populations, including one from a tsetse.

Scott (1981) and Mehlitz et al. (1982) found two isoenzymically distinct populations of *T. brucei* spp. in each of two isolates from village pigs examined in Côte d'Ivoire, while Schutt and Mehlitz (1981) demonstrated the long-term coexistence of strains of *T. b. brucei* and *T. b. gambiense* in an experimental pig, and the particular difficulty in detecting the presence of certain less virulent populations. Majiwa and Otieno (1990) demonstrated the presence of mixed infections in tsetse flies using DNA probes, while Moloo et al. (1982) and Gibson (1989) have shown experimentally that tsetse infected with *T. brucei* spp. can also be infected with trypanosomes of other subgenera and subspecies. However, no studies of mixed infections with closely related strains have so far been undertaken. The current study indicates, therefore, that the occurrence of mixed populations of *T. brucei* in individual tsetse flies may be more common in nature than previously supposed, suggesting a need for further study of this aspect of the host-parasite interaction.

Such a result, together with evidence from a range of previous studies, could have important consequences for genetic exchange (Tait, 1980) between populations of trypanosomes in tsetse. The detection of nine and five closely related, but distinct, populations within two of the three primary isolates suggests a degree of variation not easily explained without some form of exchange of genetic material (Gibson, 1990), particularly when the importance of the tsetse fly as a potential site of trypanosome gene exchange is considered (Jenni et al., 1986). However, as several researchers have indicated, the simple detection of genetic variants provides little indication of the underlying mechanisms in the absence of quantitative data (Tibayrenc et al., 1990; Cibulskis, 1992). Furthermore, differences between the predominantly endemic form of trypanosomiasis found in this region of West Africa (Godfrey et al., 1990) and the often epidemic form found in East Africa eg. Uganda (Stevens and Welburn, 1993), suggest that conclusions drawn from these results should be limited to trypanosomiasis in the western area.

In a broader context, our findings for tsetse flies are in agreement with the results of several studies which suggest that naturally occurring mixed infections with more than *one species* of trypanosome are not uncommon. In drug trials conducted by Unsworth and Birkett (1952), all untreated cattle had mixed infections at the end of a 45 day trek south into the tsetse zone from northern Nigeria, while Killick-Kendrick and Godfrey (1963) showed that more than 60 % of infections in Nigerian livestock were mixed.

The level of genetic variation within a sample can be quantified using a suitable index of diversity (Stoddart, 1983; Hoffmann, 1986). However, the method of data collection employed in the current study, i.e. the repeated sampling of the same primary isolate in order to maximize the detection of genetic variants (repeats of zymodemes being sampled from the same population), invalidates the use of such measures. Thus while indices may be calculated they will be of little value for these data. However, the dendrogram (Fig. 2) does provide a measure of the genetic variation observed.

Nine cloned populations from KP 14 and KP 33 were 92% similar to the *T. b. gambiense* standard, while others, including some populations from parental stocks KP 14 and KP 33, were much less similar; all cloned populations of KP 13 were only 84% similar. Thus, despite the degree of separation, the placement of cloned populations in relation to standard stocks of *T. b. brucei, T. b. gambiense* and *T. b. rhodesiense* appeared logical, although the placement of certain populations in relation to the *T. b. gambiense* standard highlights the anomalies which may be encountered when differentiating West African *T. brucei* spp. by isoenzymes (Godfrey et al., 1990; Mathieu-Daudé, 1991; Stevens and Godfrey, 1992). Indeed, it may well be that the degree of separation between the cloned populations and the standard stocks is a reflection of the enzyme range used, rather than an exact measure of the genetic separation of the parasite groups.

Whatever, the isolation of individual populations from mixed infections by cloning and *in vitro* culture, before the less virulent components are overgrown, appears to give a more accurate picture of the natural genetic variation present in *Trypanozoon*.

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