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POPULATION STRUCTURE OF *TRYPANOSOMA BRUCEI* S. L. IN COTE D'IVOIRE ASSAYED BY MULTILOCUS ENZYME ELECTROPHORESIS: EPIDEMIOLOGICAL AND TAXONOMICAL CONSIDERATIONS

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ABSTRACT: Fifty-two *Trypanosoma brucei* stocks isolated in Côte d'Ivoire from sympatric locations were analyzed by cellulose acetate electrophoresis of isoenzymes. Of 13 genetic loci surveyed, 5 appeared as variable, which made it possible to delimit 12 different zymodemes. The most abundant zymodeme involved stocks isolated from both humans and pigs, which is consistent with the hypothesis that pig is a reservoir of human African trypanosomiasis in Côte d'Ivoire, as already proposed by other authors. Population genetic analysis of the isozyme data indicated a strong linkage disequilibrium, which suggests that genetic recombination is severely restricted in this sample and favors the hypothesis that the trypanosome populations surveyed are basically clonal. Nevertheless, additional studies are required to better estimate the long-term stability of these clones and the possible interference of gene exchange at an evolutionary scale. The results corroborate the hypothesis that a majority of human *T. brucei* stocks from West Africa correspond to a fairly homogeneous cluster of genotypes (*T. brucei gambiense* 'Group I', Gibson, 1986).

Since the pioneering work by Godfrey and Kilgour (1976), isozyme typing has been widely used for strain identification of *Trypanosoma brucei,* the agent of human African trypanosomiasis (HAT) (for recent review see Godfrey et al. [1990]). This made it possible to improve our knowledge on the epidemiology of HAT and on the basic biology of its causative agent.

Trypanosoma brucei mating system and population structure are still subjects of debate. The question of whether this parasite is sexual or not is epidemiologically relevant. If *T. brucei* populations undergo regular mating, this parasite's genotypes are ephemeral individual variants that cannot be used as markers for tracking epidemic outbreaks. The hypothesis that *T. brucei* is a sexual organism was first proposed by Tait (1980). Jenni et al. (1986) then successfully obtained recombinants in laboratory experiments. However, Cibulskis (1988) first proposed that gene exchange is restricted in *T. brucei,* which leads to the separate evolution of distinct parasitic lines. Tibayrenc et al. (1990) and Tibayrenc et al. (1991) then postulated that *T. brucei* is basically a clonal organism, similar to several other major parasitic protozoan species. Cibulskis (1992) later suggested that *T. brucei* undergoes clonal propagation in the short term, but that frequent genetic recombination renders the clones unstable in the long run and, hence, makes them less useful for epidemiological studies. A similar model (epidemic clonality) has been proposed recently by Maynard Smith et al. (1993) for several species of microorganisms, including *T. brucei.*

The present study is designed to address some of these problems and to clarify the epidemiology of HAT in Côte d'Ivoire. The stocks employed were mostly collected in the same localities and are well suited for epidemiological data.

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MATERIALS AND METHODS

Table I gives the geographic origin, host, and date of isolation of the stocks studied. Two main regions were surveyed, the Daloa region close to the center of the country, and the Aboisso region, at the southeast corner of the country. The Tagoura locality is very close to the town of Daloa, while the Zoupkangeu locality is located about 30 km west of Daloa. The Songan locality is located about 120 km north of the town of Aboisso. Primary isolates were intraperitoneally inoculated to either mice or *Mastomys natalensis.* Depending on the stocks inoculated, some of the experimental hosts were submitted to an immunosuppressive treatment through a single injection of 80 mg/kg cyclophosphamide (Endoxan®). Stabilates so obtained were reinoculated into Wistar rats, controlled until a parasitemia of 10^8–10^9 parasites/ml was obtained. Trypanosomes were separated from blood on a DEAE-cellulose column (Lanham and Godfrey, 1970), harvested by centrifugation, and lysed by addition of an equal volume of hypotonic enzyme stabilizer (Godfrey and Kilgour, 1976). After centrifugation at 13,500 *g,* the supernatant was recovered and stored at −70 C until used for isozyme electrophoresis.

Cellulose acetate electrophoreses (Helena laboratories, Beaumont, Texas) were carried out according to Truc et al. (1991). The 11 enzyme systems used are listed in Table II.

Specific population genetic tests (Tibayrenc et al., 1990; Tibayrenc et al., 1991) were performed with special Turbo Pascal programs in order to explore the population structure of the sample. These tests (Table III) all explore different facets of the same biological phenomenon, namely linkage disequilibrium (nonrandom association between genotypes occurring at different loci). All tests are based upon the null hypothesis that recombination occurs at random in the population under survey (panmixia). The PTAG 158 (1) and (2) (see Table I) are related to 2 subsequent isolates from the same pig. Because they exhibit the same genotype, they are likely to represent the same parasitic line and hence were counted only once in the population genetic tests.

Genetic relationships among the enzyme variants were estimated according to either standard genetic distance (average number of codon differences per gene between any 2 stocks) (Nei, 1972) or the Jaccard index of phenetic distance (Jaccard, 1908). Specific computer programs (Serres and Roux, 1986) were used to elaborate a UPGMA (unweighted pair-group method with arithmetic averages) dendrogram (Sneath and Sokal, 1973) from Jaccard's distance matrix.

RESULTS

The 11 enzyme systems surveyed allowed the study of 13 different genetic loci, because 2 systems, namely ME and NH, both revealed the activity of 2 loci (see Table II). Among these 13 loci, 11 exhibited electromorphs that could be equated to alleles (allelomorphs). The 2 loci that did not allow an allelic interpretation were *Mdh* and *Pep 2.* Of the 13 loci, 5 were

TABLE I. List of the 52 stock studied, with geographical origin, host, zymodeme determination (see Table IV), and date of isolation.

Stock	Host	Region	Zymodeme	Date
HABS.I 1006	Human	SONGAN	1	7/11/86
HABS.I 1020	Human	SONGAN	2	7/11/86
HABS.II 0740	Human	ABOISSO	2	27/01/87
HPRCT 0494	Human	DALOA	1	?
HPRCT 1065	Human	DALOA	1	?
HPRCT 1072	Human	DALOA	2	?
HTAG 107/1	Human	TAGOURA	3	10/01/86
HTAG 128/6	Human	TAGOURA	3	11/01/85
HTAG 015/5	Human	TAGOURA	3	8/03/85
HZOUK 51/5	Human	ZOUKPANGBEU	2	28/11/84
PTAG 007	Pig	TAGOURA	4	6/01/85
PTAG 014	Pig	TAGOURA	3	6/01/85
PTAG 122	Pig	TAGOURA	3	6/01/85
PTAG 123	Pig	TAGOURA	4	6/01/85
PTAG 124	Pig	TAGOURA	3	6/01/85
PTAG 125	Pig	TAGOURA	4	6/01/85
PTAG 129	Pig	TAGOURA	3	5/10/85
PTAG 130	Pig	TAGOURA	5	6/01/85
PTAG 149	Pig	TAGOURA	3	5/10/85
PTAG 151	Pig	TAGOURA	3	5/10/85
PTAG 152	Pig	TAGOURA	3	5/10/85
PTAG 153	Pig	TAGOURA	3	5/10/85
PTAG 155	Pig	TAGOURA	6	5/10/85
PTAG 156	Pig	TAGOURA	3	5/10/85
PTAG 158(1)*	Pig	TAGOURA	3	10/01/86
PTAG 158(2)*	Pig	TAGOURA	3	11/04/86
PTAG 173	Pig	TAGOURA	4	10/01/86
PTAG 174	Pig	TAGOURA	3	11/04/86
PTAG 175	Pig	TAGOURA	7	11/04/86
PZOUK 002	Pig	ZOUKPANGBEU	8	6/11/84
PZOUK 003	Pig	ZOUKPANGBEU	4	6/11/84
PZOUK 006	Pig	ZOUKPANGBEU	4	6/11/84
PZOUK 008	Pig	ZOUKPANGBEU	4	6/11/84
PZOUK 009	Pig	ZOUKPANGBEU	3	6/11/84
PZOUK 012	Pig	ZOUKPANGBEU	9	6/11/84
PZOUK 013	Pig	ZOUKPANGBEU	9	6/11/84
PZOUK 014	Pig	ZOUKPANGBEU	10	6/11/84
PZOUK 017	Pig	ZOUKPANGBEU	11	6/11/84
PZOUK 019	Pig	ZOUKPANGBEU	4	6/11/84
PZOUK 020	Pig	ZOUKPANGBEU	11	6/11/84
PZOUK 025	Pig	ZOUKPANGBEU	11	6/11/84
PZOUK 030	Pig	ZOUKPANGBEU	12	6/11/84
PZOUK 031	Pig	ZOUKPANGBEU	11	6/11/84
PZOUK 032	Pig	ZOUKPANGBEU	11	6/11/84
PZOUK 034	Pig	ZOUKPANGBEU	11	6/11/84
PZOUK 108	Pig	ZOUKPANGBEU	11	6/11/84
PZOUK 109	Pig	ZOUKPANGBEU	3	6/11/84
PZOUK 148	Pig	ZOUKPANGBEU	3	22/11/85
PZOUK 159	Pig	ZOUKPANGBEU	3	22/11/85
PZOUK 160	Pig	ZOUKPANGBEU	3	22/11/85
PZOUK 161	Pig	ZOUKPANGBEU	3	22/11/85
GZOUK 001	Tsetse fly	ZOUKPANGBEU	4	9/11/84

* Subsequent isolates from the same pig.

polymorphic, namely *Got, Me 2, NH 2, Pep 2,* and *Pgm.* The rate of polymorphism, therefore, was 5/13 = 0.38. These 5 polymorphic loci made it possible to distinguish 12 distinct enzymodemes with their corresponding isoenzyme profiles. These 12 zymodemes were distributed into 2 distinct clusters, as shown in Figure 1, which depicts the genetic similarities among the zymodemes visualized by the UPGMA dendrogram.

TABLE II. Enzyme systems studied.

Glyceraldehyde-phosphate dehydrogenase	GAPDH*	E.C.1.2.1.12
Glutamate oxaloacetate transaminase	GOT*	E.C.2.6.1.1
Glucose phosphate isomerase	GPI*	E.C.5.3.1.9
Isocitrate dehydrogenase	IDH*	E.C.1.1.1.42
Malate dehydrogenase	MDH	E.C.1.1.1.37
Malic enzyme	ME*†	E.C.1.1.1.40
Nucleoside hydrolase	NHi*†	E.C.3.2.2
Peptidase 2 (Leu-Ala)	PEP2	E.C.3.4.11 or 13
Phosphoglucomutase	PGM*	E.C.2.7.5.1
Threonine dehydrogenase	TDH*	E.C.1.1.1.10
6 Phosphogluconate dehydrogenase	6PGD*	E.C.1.1.1.44

* Enzyme systems allowing allelic reading and estimation of Nei's standard genetic distance.
† Enzyme systems showing the activity of 2 different loci: Malic enzyme: 2 loci = *Me 1* (faster) and *Me 2* (lower); nucleoside hydrolase: 2 loci = *Nh 1* (faster) and *Nh 2* (lower).

DISCUSSION

Overall genetic variability of this set of stocks is limited; maximum value for Nei's standard genetic distance is only 0.34 (with an average value of 0.16). These results are worth comparing with the ones obtained for another parasite, *Trypanosoma cruzi,* by using a comparable technical approach (Tibayrenc and Ayala, 1988). In *T. cruzi,* the standard genetic distance reaches a maximum value of 2.015, with an average value of 0.478. This result showing limited phylogenetic divergence within *T. brucei* confirms previous studies performed on other sets of *T. brucei* stocks (Truc and Tibayrenc, 1993; Mathieu-Daudé and Tibayrenc, 1994).

The results obtained from population genetic tests (see Table III) are in agreement with the general hypothesis that *T. brucei* s. l. has a basically clonal population (Tibayrenc and Ayala, 1990; Tibayrenc et al., 1991) and confirms previous results recorded from other *T. brucei* populations (Truc and Tibayrenc, 1993; Mathieu-Daudé and Tibayrenc, 1994). Indeed, the overall

TABLE III. Statistical tests used to demonstrate linkage disequilibrium or nonrandom association of gentoypes occurring at different loci (after Tibayrenc et al., 1990).

Test*	Description
d	Overrepresented, identical genotypes widespread†‡ (statistical test d1 and d2)
e	Absence of recombinant genotypes‡
f	Classical linkage disequilibrium analysis‡

* The d1 test relies on a combinatorial analysis, whereas d2, e, and f are based upon Monte Carlo simulations (with 10^4 iterations).
† Can be used qualitatively, without statistical calculations.

TABLE IV. Isozyme profiles of the 12 zymodemes recorded for the 5 variable enzyme systems, with the corresponding number of stocks (last column).*

Zd	*Me 2*	*Pep 2*	*Got*	*Nh 2*	*Pgm*	Size
1	*1/3*	II	*1/1*	*1/1*	*1/2*	3
2	*1/3*	II	*1/1*	*1/1*	*2/2*	4
3	*1/1*	I	*1/1*	*2/2*	*2/2*	21
4	*1/1*	I	*2/2*	*2/2*	*2/2*	9
5	*1/1*	IV	*2/2*	*2/2*	*1/2*	1
6	*1/1*	I	*1/1*	*2/2*	*1/1*	1
7	*2/2*	I	*2/2*	*2/2*	*1/2*	1
8	*2/2*	I	*2/2*	*2/2*	*2/2*	1
9	*1/1*	I	*2/2*	*2/2*	*1/2*	2
10	*2/2*	I	*1/2*	*2/2*	*2/2*	1
11	*1/1*	I	*1/2*	*2/2*	*2/2*	7
12	*1/1*	III	*2/2*	*2/2*	*2/2*	1
					Total	52

* Zd = zymodeme. The *Me 2, Got, Nh 2,* and *Pgm* loci are interpreted in terms of alleles, whereas the *Pep 2* locus is interpreted in terms of nonallelic genotypes.

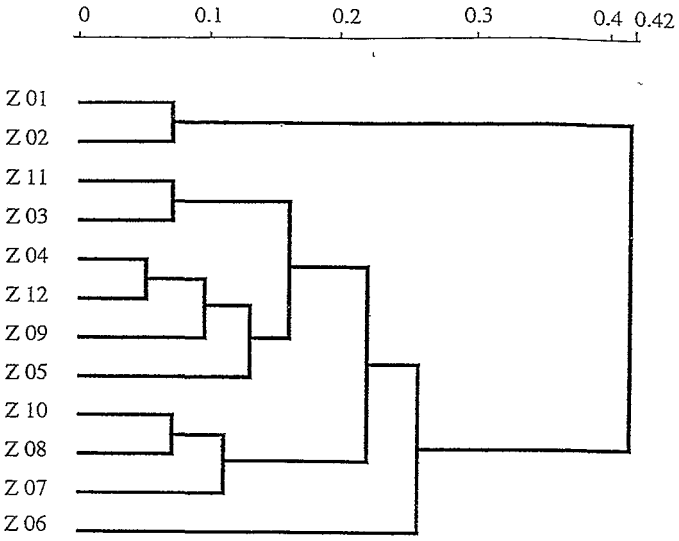

FIGURE 1. A UPGMA (unweighted pair-group method with arithmetic averages) dendrogram (Sneath and Sokal, 1973) from Jaccard's distance matrix (Jaccard, 1908), depicting the genetic similarities among the 12 *Trypanosoma brucei* zymodemes.

genotype diversity is far lower than should be expected if genetic exchanges regularly occurred. If this last hypothesis is considered as the null hypothesis, then the probability of observing zymodemes 1, 2, and 3 with a size as high or higher than actually observed (d1 test: see Table III), is 1.3×10^{-7}, 4.5×10^{-7}, and 2.8×10^{-2}, respectively. The probability of observing any genotype with the size of the dominant genotype (d2 test) is 10^{-3}. The probability of observing as few, or fewer, different genotypes than actually observed (*e*-test) is $<10^{-4}$ (no case observed out of 10^4 iterations by Monte Carlo simulation). Lastly, the probability of observing a linkage disequilibrium as high or higher than actually observed (*f*-test) is $<10^{-4}$. All these results reflect the existence of a strong linkage disequilibrium in this population.

Whatever the mating system of the organism under study, linkage disequilibrium can be generated by geographical separation and genetic drift, leading to differences of allelic frequencies among the sampling places. Nevertheless, all these stocks, except 3, were sampled in the same small region (see Fig. 1) and hence can be considered as sympatric. The samples examined can then be considered as appropriate for population genetic analyses. Moreover, linkage disequilibrium due to geographical structure generate special patterns in the geographical distribution of the overrepresented genotypes that tend to be limited strictly to given localities (Tibayrenc et al., 1991). This is not verified here because the overrepresented genotypes are sampled in different localities (see Table I). For example, both zymodemes 3 and 4 were sampled in 2 different localities, whereas zymodeme 2 was sampled in 4 different localities, 1 of which is 600 km from the others. These isolates make it possible to conclude firmly that the present population shows considerable departures from panmictic expectations.

It has been proposed that *T. brucei* natural clones are rendered unstable in the long run by frequent, although nonobligatory, genetic recombination (Cibulskis, 1992; Maynard Smith et al., 1993). To distinguish between short-term, epidemic clonality and clonal propagation that operates in the long run, Maynard Smith et al. (1993) have proposed using linkage disequilibrium tests with the genotype rather than the stock as units of analysis. The present isolates are not well suited for this approach, because only 5 variable loci could be used to delimit the zymodemes; additional genotype variability is bound to appear within each zymodeme when more discriminative methods are used. If the genotype rather than the stock is taken as a unit, the tests would need to be performed on 12 individuals (the 12 different zymodemes) only. With this procedure, the *f*-test (the only one suitable in this case, as the others are based on the use of repeated genotypes) gives nonsignificant statistical results. It is impossible to decide whether this is due to the fact that the population under examination presents an epidemic structure (Maynard Smith et al., 1993) or to insufficient sample size. It is worth noting that the same approach performed on *T. brucei* stocks analyzed with more discriminative methods (broader range of isoenzyme loci and random amplification of polymorphic DNA) showed a persistence of linkage disequilibrium even with the genotype rather than the individual taken as analysis unit (Stevens and Tibayrenc, 1995).

Clustering pattern of the stocks indicates 2 distinct groups (see Fig. 1). The first involves 7 stocks that were all isolated from humans (zymodemes 1 and 2). The second (zymodemes 3–12) is composed of 3 human stocks and 42 from nonhuman sources (from tsetse flies or pigs). The first cluster can be distinguished from the second by 3 loci (see Table IV), namely, *Me 2* (genotype *1/3*), *Nh 2* (genotype *1/1*), and Pep 2 (nonallelic genotype II). Thus, the present results roughly corroborate the classical subspecific taxonomy of the *T. brucei* complex because most of the human isolates from the present sample fall into the same cluster. This distinct group involving zymodemes 1 and 2 most probably corresponds to the *T. brucei gambiense* 'Group 1' described by Gibson (1986). This result is in agreement with previous data involving a different sampling of *T. brucei* stocks (Mathieu-Daudé and Tibayrenc, 1994). Zymodeme 3 includes 3 human isolates and is included in the second cluster, which is composed of animal isolates apart from these 3 isolates. Zymodeme 3 hence can be attributed to the very

heterogeneous *T. brucei gambiense* 'Group 2' (Gibson, 1986). In cluster 2, it is worth noting that the dominant genotype (zymodeme 3) has been recorded both from humans and pigs in the same locality, namely Tagoura (Fig. 1). This is consistent with the hypothesis that the pig is a reservoir for HAT in Côte d'Ivoire and, hence, confirms previous observations (Gibson et al., 1978, 1980; Mehlitz et al., 1982). Regarding the possible role of the pig as a reservoir, it is worth noting that apart from stocks 158 (1) and (2), several other sequential isolates have been performed on the same animals in the framework of this study (data not shown); the animals involved apparently developed no disease, although the parasites remained infective. This of course increases the risk that this animal acts as a reservoir.

In summary, the present study has fully confirmed previous epidemiologic, phylogenetic, and population genetic hypotheses on well documented sympatric *T. brucei* samples taken from the same country, namely Côte d'Ivoire. Work is under way to analyze comparable samples with more discriminative genetic techniques in order to better explore *T. brucei* clone repartition with respect to locality and host, and to estimate stability more rigorously in space and time of *T. brucei* natural clones (Cibulskis, 1992; Maynard Smith et al., 1993; Stevens and Tibayrenç, 1995).

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