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Characterization of West African *Trypanosoma (Trypanozoon) brucei* isolates from man and animals using isoenzyme analysis and DNA hybridization

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Abstract. A total of 18 West African *Trypanosoma (Trypanozoon) brucei* stocks isolated from man and animals were characterized using isoenzyme analysis with isoelectric focusing (IEF) and DNA hybridization. They were compared with four *T. (T.) brucei* isolates from East and West Africa that had previously been analysed and well defined. All experiments were carried out with cell lysates of procyclic trypanosomes produced in vitro. The different stocks could be separated into two distinct groups according to their isoenzyme and DNA patterns. The homogeneous group of *T. b. gambiense* was characterized by zymodeme A and highly specific DNA-banding patterns (type G) always associated with stable human serum resistance. The non-*gambiense* group (consisting of *T. b. rhodesiense* and *T. b. brucei*) was determined by a great variation in these markers. Our results clearly indicate the existence, of *T. b. rhodesiense*-like parasites in West African patients. Due to their lack of human serum resistance, the four characterized animal isolates can be referred to as *T. b. brucei*.

The species *Trypanosoma (Trypanozoon) brucei* consists of three different tsetse-transmitted subspecies according to their diverse pathogenicity, host range and geographical distribution: *T. b. rhodesiense* and *T. b. gambiense*, which are pathogenic to man, causing the acute or chronic form of African sleeping sickness, and *T. b. brucei*, the infectivity of which is restricted to game and domestic animals, causing nagana within the entire tsetse belt. *T. b. gambiense* occurs in West and Central Africa, being preferentially transmitted by tsetse flies of the *palpalis* group, whereas *T. b. rhodesiense* is generally believed to exist in East Africa, being mainly transmitted by *Glossina morsitans* subspecies.

However, these classical criteria are unreliable for an identification of the parasites, as divergences from the three subspecies types may be observed. Being indistinguishable by morphological characteristics, the three subspecies can now be differentiated by biological and biochemical means. The blood incubation infectivity test (BIIT) (Rickman and Robson 1970) and the in vitro test for human serum resistance developed by Jenni and Brun (1982) can be used to separate the human infective subspecies *T. b. rhodesiense* from *T. b. brucei*, which is lysed by the trypanocidal factor in normal human serum (Barth 1989). Moreover, isoenzyme analysis by starch gel electrophoresis (Godfrey 1979; Gibson et al. 1980) or by isoelectric focusing (IEF) (Ebert 1982; Betschart et al. 1983) and DNA hybridization with nuclear DNA (Pays et al. 1983, 1984; Paindavoine et al. 1986) are further tools for an accurate subspecies characterization of trypanosome isolates from man and animals. By applying these technologies to epidemiological research in West African sleeping sickness, the existence of an animal reservoir for *T. b. gambiense* has been proven (Gibson et al. 1978; Mehlitz et al. 1982; Zillman et al. 1984; Paindavoine et al. 1986).

The aim of the present work was to characterize West African *T. (T.) brucei* isolates by isoenzyme analysis with IEF and, in a second step, to compare these data with the results obtained with the DNA hybridization technique.

Materials and methods

Trypanosome stocks. The characterized *T. (T.) brucei* stocks (Table 1) were derived from man and animals in highly endemic

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Table 1. Origin of characterized trypanosome stocks

Stabilate designation	Date of cryopreservation	Host	Locality (country)
TH1/ 78E (020)	17.10.78	Man	Koudougou (I.C.)
TH3/ 78E (020)	20.10.78	Man	Koudougou (I.C.)
TH17/ 78E (020)	19.10.78	Man	Koudougou (I.C.)
TH64/ 78E (020)	27.10.78	Man	Koudougou (I.C.)
TH137/ 78E (020)	30.10.78	Man	Koudougou (I.C.)
TH162/ 78E (021)	03.10.78	Man	Sietinfla (I.C.)
TH141/ 78E (022)	30.10.78	Man	Suefla (I.C.)
TH31/ 78E (025)	07.11.78	Man	Bazi (I.C.)
TH152/ 78E (026)	15.12.78	Man	Carrefour (I.C.)
TH115/ 78E (027)	02.11.78	Man	Koetinga (I.C.)
TH170/ 78E (027)	01.08.79	Man	Koetinga (I.C.)
TH1/ 78E (031)	01.11.78	Man	Hosp. Daloa (I.C.)
TH2/ 78E (031)	15.12.78	Man	Hosp. Daloa (I.C.)
TSW73/ 78E (022)	23.10.78	Pig	Suefla (I.C.)
TSW180/78E (028)	31.10.78	Pig	Dégbézéré (I.C.)
TD52/ 78E (021)	20.02.79	Dog	Sietinfla (I.C.)
TH Gamey Dolo/80 Lib	12.12.80	Man	Gbao (L.)
TGP2/80 Lib	22.07.80	*Glossina palpalis*	Bindin (L.)
Reference stocks:			
TH DAL 069/78E	07.11.79	Man	Koetinga (I.C.)
TH114/78E (020)	28.11.78	Man	Koudougou (I.C.)
STIB 704	20.01.82	Man	Ifakara (T.)
STIB 247 – L	03.12.71	Kongoni	Serengeti (T.)
STIB 723 CAE	Hybrid clone, Jenni et al. (1986)		

I.C., Ivory Coast; L., Liberia; T., Tanzania

areas of the Ivory Coast (Mehlitz et al. 1981) and Liberia (Zillmann et al. 1984). They were grown in untreated or immunosuppressed *Mastomys natalensis* until cryopreservation. The bloodstream-form stabilates were kindly provided by Dr. D. Mehlitz of the Tropical Institute, Hamburg. For comparative purposes, two West African and two East African *T. (T.) brucei* isolates (Table 1), which have previously been characterized elsewhere (Mehlitz et al. 1982; Paindavoine et al. 1986; Jenni et al. 1986), were used as reference stocks.

Propagation of bloodstream-form parasites. Bloodstream-form stabilates were intraperitoneally (i.p.) inoculated into immunosuppressed *M. natalensis* 24 h after treatment with cyclophosphamide (300 mg/kg). Bloodstream forms were harvested by cardiac puncture and cryopreserved in liquid nitrogen or used for in vitro transformation to procyclic culture forms.

Transformation of bloodstream forms to procyclic culture forms. Two different methods of transformation were used, depending on the virulence and pleomorphism of the corresponding parasite population:

A. Bloodstream forms could be directly transformed to procyclic culture forms in vitro according to Brun and Schönenberger (1979, 1981), with the following modifications: the medium used was Minimum Essential Medium (MEM 109-4) with 20% heat-inactivated fetal bovine serum (FBS), and the TCA-cycle intermediates, citrate and *cis*-aconitate, were used in 3-m*M* concentrations. Transformation was carried out in 10-ml plastic tubes or in T-25 tissue-culture flasks at 27° C.

B. Whenever bloodstream-form trypanosomes could not be directly adapted to procyclic forms under in vitro conditions, teneral *G. m. morsitans* or *G. m. centralis* were fed on an infected *M. natalensis*. The origin of the fly pupae and maintenance of infected flies were as described by Richner and Jenni (1986).

At 1 week after infection, the flies were dissected under sterile conditions according to the modified method of Brun and Schönenberger (1979). The insect surface was sterilised by dipping into 70% ethanol. Thereafter, the flies were thoroughly washed in semi-defined medium (SDM-79) (Brun and Schönenberger 1979) and the abdomen was dissected. The midgut was removed and opened in a petri dish containing 2 ml SDM-79 with 15% FBS and 50–100 µg/ml gentamycin. Procyclic forms were transferred into a T-25 tissue-culture flask containing 5 ml of the same medium, gassed with 5% CO_2 in air and incubated at 27° C. As soon as the midgut forms started to grow, the FBS content could be reduced to 10% and gentamycin, to a concentration of 10 µg/ml. Stabilates were normally prepared 4 weeks after initiation of the in vitro cultures.

Preparation of procyclic cell lysates. For isoenzymatic analysis a total of about 10^9 procyclic culture forms, grown in stirred glass flasks at 27° C, were washed three times in phosphate-buffered saline-glucose (PSG) 6:4 (pH 8.0) (Lanham and Godfrey 1970) and lysed as described by Betschart et al. (1983). The lysates were immediately frozen as 5-µl beads in liquid nitrogen and the protein concentrations were estimated using the Peterson (1977) modification of the Lowry method.

Isoenzyme analysis of procyclic culture forms. Isoelectric focusing (IEF) was carried out as described by Richner and Jenni (1986) and Betschart et al. (1983). The Servalyt Precotes pH 3–10 (Serva, Heidelberg) were prefocused to 150 Vh (volt-hours)

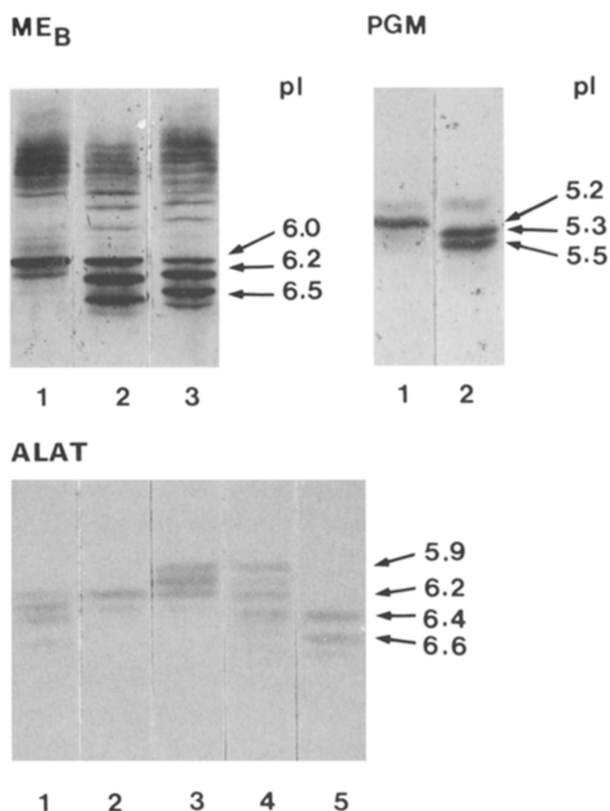

Fig. 1. Schematic illustration of the different isoenzyme patterns found in analyzed *T. (T.) brucei* stocks and their corresponding pI range. All designations of isoenzyme patterns or zymodemes are based on analysis of procyclic culture forms with IEF

before the samples were applied. Focusing was stopped when 2200 Vh had been reached. The incubation mixtures for the different enzyme reactions were prepared according to Gibson et al. (1978).

DNA hybridization with nuclear DNA from procyclic culture forms. The isolation of nuclear DNA from at least 2×10^9 trypanosomes and its digestion by the restriction endonuclease Pst I (5 units Pst I/μg DNA) was carried out as described by Paindavoine et al. (1986). The specific DNA content was determined by measuring the optical density (OD) at 260 nm. The separation of the DNA fragments by agarose gel electrophoresis and subsequent Southern transfer (Southern 1975) were carried out as described by Paindavoine et al. (1986).

The two probes used in these experiments, AnTat 1.1 and AnTat 1.8, had been prepared from cloned *T. b. brucei* cDNAs synthesized from variable surface antigen mRNAs (Pays et al. 1980). They were labelled with ^{32}P by nick-translation according to Rigby et al. (1977) just before use. Hybridization reaction was done as described by Pays et al. (1980). Autoradiography took place at −70° C over 18–72 h with an intensifying screen.

Results

The results obtained from the isoenzyme analysis are summarized in Fig. 1 and Table 2. Of the five enzymes tested, only PGM showed no variations among all West African stocks analysed. ALAT and ME_B showed a higher degree of variation (Fig. 1). The results of all isoenzyme profiles were used to group the stocks according to the classification used by Mehlitz et al. (1982). A detailed description of this grouping has been given by Richner (1987).

Stock TH162/78E (021), including its two clones, and the reference stock TH114/78E (020) were the only human isolates from the Ivory Coast that showed atypical isoenzyme patterns (zymodemes G and C, respectively). All other human isolates from the Ivory Coast (including the reference stock TH DAL 069/78E) belonged to a highly homogeneous group of organisms represented by zymodeme A. The Liberian stock TH Gamey Dolo/80 Lib, isolated from man, presented divergencies from zymodeme A in ALAT and PEP2, associated with zymodeme D. All animal isolates were characterized by different zymodemes, constituting a very heterogeneous group.

The trypanosome isolates could clearly be divided into two distinct groups according to their

Table 2. Enzyme profiles of procyclic culture forms analysed by isoelectric focusing (IEF)

Stabilate designation	Enzyme profiles:		
	ALAT	ME_B	PGM
TH1/ 78E (020)	1	2	2
TH3/ 78E (020)	1	2	2
TH17/ 78E (020)	1	2	2
TH64/ 78E (020)	1	2	2
TH137/ 78E (020)	1	2	2
TH162/ 78E (021)	2	1	2
TH162/ Clone 1	2	1	2
TH162/ Clone 2	2	1	2
TH141/ 78E (022)	1	2	2
TH31/ 78E (025)	1	2	2
TH152/ 78E (026)	1	2	2
TH115/ 78E (027)	1	2	2
TH170/ 78E (027)	1	2	2
TH1/ 78E (031)	1	2	2
TH2/ 78E (031)	1	2	2
TSW73/ 78E (022)	2	3	2
TSW180/78E (028)	3	1	2
TD52/ 78E (021)	4	1	2
TH Gamey Dolo/80 Lib	5	2	2
TGP2/80 Lib	1	1	2
Reference stocks:			
TH DAL 069/78E	1	2	2
TH114/ 78E (020)	2	3	2
STIB 247-L	2	1	1
STIB 704	2	1	1

For a summary of enzyme profiles, see Fig. 1

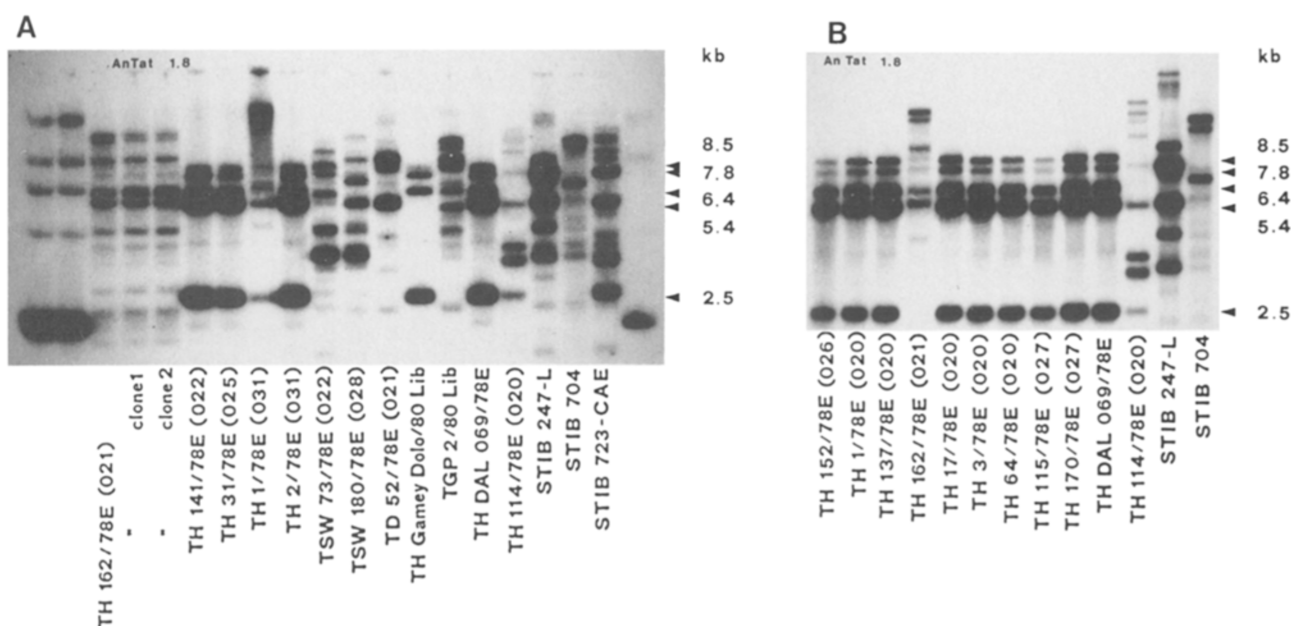

Fig. 2A, B. Hybridization patterns of AnTat 1.8 VSG probe on Pst I-digested nuclear DNA from 23 different *T. (T.) brucei* stocks

DNA hybridization patterns (Fig. 2). They could be designated as *gambiense* (G) or non-*gambiense* (NG) (Table 3), when other criteria such as geographical origin, natural host (Table 1), resistance to human serum in vitro and zymodemes were also considered.

Group 1: T. b. gambiense, G

This very homogeneous group of organisms (the majority of the analysed West African human isolates belonged to this group) was characterized by a typical hybridization pattern with the DNA probe AnTat 1.8, corresponding to five major Pst-I fragments of 8.5, 7.8, 6.4, 5.4 and 2.5 kb (Fig. 2A and B). A slight variation from this pattern could be observed in *T. b. gambiense* TH Gamey Dolo/80 Lib, which lacked the 5.4- and 8.5-kb fragments. Stock TH1/78E (031) showed a completely atypical pattern with probe AnTaT 1.8. The DNA of all stocks showing the five-band pattern after digestion with Pst I and hybridization with AnTat 1.8 as well as that of stock TH1/78E (031) did not react with the non-*gambiense*-specific probe AnTat 1.1 (data not shown).

Group 2: T. b. non-gambiense, NG

The members of this group showed highly variable DNA hybridization patterns with AnTat 1.8 in terms of the number, size and labelling intensity of the different fragments (Figs. 2A and B), as well as with AnTat 1.1 (data not shown). Whereas *T. b. gambiense* stocks can unambiguously be discriminated from non-*gambiense* isolates such as *T. b. brucei* and *T. b. rhodesiense,* the latter two subspecies could not be differentiated by DNA hybridization techniques. TH162/78E (021), including clones 1 and 2, and the reference stock TH114/78E (020) were the only West African human isolates that belonged to group 2 according to their DNA type. On the other hand, all analysed animal isolates showed the typical non-*gambiense* characteristics.

Discussion

Procyclic culture forms of West African trypanosome isolates proved to be an excellent source of test material for both isoenzyme analysis and DNA hybridization. Midgut forms can easily be grown in vitro in large amounts and seem to be a real alternative to blood-stream forms, especially in the case of low-virulence stocks, thereby saving many laboratory animals. Of course, many characteristics of the parasite are altered during its transformation (including certain isoenzyme patterns; Betschart and Richner, unpublished results), but when all experiments are conducted with the procyclic vector stage, comparative analyses are legitimate.

Using starch gel electrophoresis, Godfrey and

Table 3. List of results including the in vitro test for human serum resistance, isoenzyme analysis and DNA hybridization

Stabilate designation	HSRT in vitro	Zymodeme[a]	DNA type
TH1/ 78E (020)	R	A	G
TH3/ 78E (020)	R	A	G
TH17/ 78E (020)	R	A	G
TH64/ 78E (020)	R	A	G
TH137/ 78E (020)	R	A	G
TH162/ 78E (021)	S	G	NG
TH162/ Clone 1	S	G	NG
TH162/ Clone 2	S	G	NG
TH141/ 78E (022)	R	A	G
TH31/ 78E (025)	R	A	G
TH152/ 78E (026)	R	A	G
TH115/ 78E (027)	R	A	G
TH170/ 78E (027)	R	A	G
TH1/ 78E (031)	R	A	G[b]
TH2/ 78E (031)	R	A	G
TSW73/ 78E (022)	S	C	NG
TSW180/78E (028)	S	AF	NG
TD52/ 78E (021)	S	AH	NG
TH Gamey Dolo/80 Lib	R	D	G
TGP2/80 Lib	S	E	NG
Reference stocks:			
TH DAL 069/78E	R	A	G
TH114/78E (020)	R/S	C	NG
STIB 247-L	S	F	NG
STIB 704	R	F	NG

[a] According to the classification of Mehlitz et al. (1982)
[b] *T. b. gambiense* according to hybridization pattern with probe AnTaT 1.1 but not with AnTaT 1.8

HSRT, human serum resistance test in vitro; R, human-serum-resistant; S, human-serum-sensitive; G, *T. b. gambiense*; NG, non-*gambiense*

Kilgour (1976) were the first to describe ALAT I as a typical *T. b. gambiense* marker. This finding was later confirmed by Gibson et al. (1978, 1980), Mehlitz et al. (1982) and Zillmann et al. (1984), who showed that *T. b. gambiense* stocks were associated with ALAT I, mainly in combination with ASAT II. Tait et al. (1984) considered the enzyme variant PEPC-O to be a reliable *T. b. gambiense* characteristic, which could be strictly correlated with human serum resistance and DNA type G. *T. b. gambiense* stands out as a real sub- or sibling species within the *T. brucei* complex, this position being determined by its extremely conservative characteristics according to many different biological and biochemical criteria. Alternatively, *T. b. rhodesiense* can be considered to be a set of variants of *T. b. brucei* rather than representing a proper subspecies (Tait et al. 1984, 1985).

In the present comparative analysis, IEF was used to characterize procyclic culture forms of *T. (T.) brucei* so as to identify *T. b. gambiense* by specific isoenzyme patterns. Malic enzyme (E.C. 1.1.1.4) type 2 could presumably be considered to be a new *T. b. gambiense*-specific marker based on IEF results. Most of the *T. b. gambiense* stocks isolated from man showed this enzyme pattern in combination with the patterns of ALAT I and PGM 2. According to this and other studies (Tait et al. 1984; Paindavoine et al. 1986), there is strong evidence for a coexistence of *T. b. gambiense* with human pathogenic non-*gambiense* stocks (which can be referred to as *T. b. rhodesiense*) in highly endemic West African areas. These human isolates of the non-*gambiense* type exhibit great phenotypic and genotypic similarities with East African *T. b. rhodesiense* and *T. b. brucei* stocks and are characterized by highly variable isoenzyme and DNA patterns, virulent behaviour within the vertebrate host, instability with regard to human serum resistance under laboratory conditions and wide host and vector ranges (unpublished results).

Representatives of these non-*gambiense* organisms, such as TH114/78E (020), can form hybrids during simultaneous cyclical transmission with *T. b. brucei* (Jenni et al. 1986). An exchange of genetic material, thus far demonstrated between non-*gambiense* isolates and evident by hybrid isoenzyme and DNA patterns, could be an explanation for their rapid evolutionary diversification.

It is evident that *T. b. gambiense* is associated with a stable resistance to human serum, zymodeme A (excepting TH Gamey Dolo/80 Lib) and DNA type G. Other biological characteristics integrated into this study, such as the preference for a certain tsetse fly species or subspecies as an effective vector, revealed a high vector specificity for *T. b. gambiense* stocks (Richner et al. 1988).

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