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# Isoenzyme characterization of *Trypanosoma brucei s.l.* stocks from different foci in the Central African Region.

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**Abstract**

In this study, 33 newly isolated stocks from the central African region, and 7 reference stocks previously identified either as *Trypanosoma brucei gambiense* (**T.b.g.**) or *Trypanosoma brucei brucei* (**T.b.b**) were examined by isoenzyme electrophoresis with 14 enzyme systems on cellulose acetate gels.
A total of 18 zymodemes were defined, with the majority of stocks belonging to zymodeme 1 which was associated with **T.b.g group 1** responsible for the chronic form of the disease in West and Central Africa. An animal stock from a domestic pig in Campo was considered as **T.b.g.** based on the superoxide dismutase (SOD) marker, though it had a unique aspartate aminotransferase (ASAT) pattern. Our results also show that the use of KIVI for diagnostic purposes is heavy, very expensive and hence not advisable.

*Key words* : *Trypanosoma brucei* ; isoenzyme characterization; genetic variability; reservoir host

**Résumé : Caractérisation d'isoenzyme de *Trypanosoma brucei s.l.* de différentes souches en Afrique Centrale.**

Dans cette étude, 33 nouvelles souches de trypanosomes de la région d'Afrique centrale et 7 souches de références décrites antérieurement comme **T.b.g.** ou **T.b.b.** ont été caractérisées par électrophorèse d'isoenzymes à l'aide de 14 systèmes enzymatiques sur gel d'acétate de cellulose. Au total, 18 zymodèmes ont été définis , la majorité des souches appartenant au zymodème 1 assimilé à **T.b.g groupe 1** responsable de la forme chronique de la maladie en Afrique Centrale et Occidentale. Une souche animale isolée sur un porc domestique à Campo a été considérée comme **T.b.g** sur la base de son phénotype SOD, ceci en dépit de son phénotype unique pour l'ASAT. Nos résultats montrent également que l'utilisation du KIVI comme outil diagnostic de masse est lourde, très peu rentable et déconseillée.

*Mots clés* : *Trypanosoma brucei*, caractérisatrion isoenzymatiques, variabilité génétique, réservoir.

## Introduction

Trypanosomiasis or sleeping sickness is a parasitic disease caused by microscopic blood parasites, trypanosomes, which affects both animals and man (Bruce, 1895). These parasites belong to the genus *Trypanosoma*. Human African Trypanosomiasis (HAT) occurs widely in different foci within tropical Africa.
Three morphologically identical subspecies had been described within the *Trypanosoma brucei* species complex (Hoare, 1972) on basis of their geographical distribution, host specificity and course of disease as follows :

- *T. b. gambiense* (**T.b.g.**): the agent for HAT in West and Central Africa, causing a chronic infection in man,
- *T. b. rhodesiense* (**T.b.r.**): typical of East Africa, man infective and causing an acute disease,
- *T. b. brucei* (**T.b.b.**): sympatric with both **T.b.g.** and **T.b.r.**, and infecting both domestic and wild animals.



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This gross classification however, appears no longer valid as it could not stand the test of time: **T.b.r.** is known to have an animal reservoir (Tait *et al.*, 1985; Paindavoine *et al.*, 1989 ; Stevens & Godfrey, 1992) ; stocks from West African domestic animals have been found resistant to human serum lytic test (Rickman & Robson, 1970) suggesting infectivity to man (Gibson *et al.*, 1978). The use of enzyme electrophoresis for intrinsic characterization of trypanosomes has revealed identical electrophoretic patterns for both animal and human stocks suggesting the presence of an animal reservoir (Godfrey & Kilgour, 1976; Mehlitz *et al.*, 1982; Scott *et al.*, 1983; Gibson *et al.*, 1980 ; Truc *et al.*, 1991, Truc & Tibayrenc, 1992).

Accepting the definition of **T.b.g.** as all *T. brucei* stocks isolated from man in Central and West Africa, Gibson (1986) used isoenzyme profiles based on several enzymes (zymodemes) to divide **T.b.g.** into 2 groups. A zymodeme is a set of stocks having exactly the same patterns for all the enzyme systems studied (WHO, 1978).

**T.b.g. group 1** to which most isolates belong shows limited isoenzyme variability, high resistance to human serum, low virulence to rodents and is attributed to the agent responsible for the chronic form of the disease in man. **T.b.g. group 2**, however, shows greater overall isoenzyme heterogeneity and no associated isoenzyme markers (Godfrey & Kilgour, 1976; Tait *et al.*, 1984). This group includes stocks of both human and animal origin. The more recently defined «bouaflé strain» representing **T.b.b.** from West African animals which also includes man-infective forms (Stevens & Godfrey, 1992) falls under this latter group.

Intrinsic characterization of **T.b.g.** has in the past been limited by field sampling (Dukes *et al.*, 1989). This obstacle has been highly improved upon by the development of the «Kit for In-Vitro Isolation» (KIVI) of trypanosomes. Originally designed to facilitate the field sampling of **T.b.g.** stocks from patients (Aerts *et al.*, 1992), the KIVI has equally proven its potential value in the diagnosis of gambian sleeping sickness (Truc *et al.*, 1992), since samples from apparently aparasitaemic individuals have given positive KIVI cultures (Truc *et al.*, 1994). This suggests that the KIVI can be used both for diagnosis and the isolation of parasites.

Several analytical methods exist for the characterization of trypanosomes. These include RAPD (Random Amplified Polymorphic DNA, a PCR based method), RFLP (Restriction Fragment Length Polymorphism) used for the characterization of parasite DNA (Gibson, 1994). Multilocus isoenzyme analysis which involves the study and comparison of several enzyme systems between different stocks in an attempt to estimate their relatedness to a common ancestor allows for a quick and easy comparison of many characters since each enzyme band is considered as a separate character (Gibson *et al.*, 1978). The comparison of data when a high number of loci are studied has been highly facilitated by the introduction of computer analysis (WHO, 1978). Though it is argued that the genes coding for proteins studied by this technique may be occupying only a few chromosomes in the parasite genome and that the ploidy of most of the genome is still doubtful (Mathieu-Daude *et al.*, 1995), it still remains the most valuable tool at present for the characterization of trypanosomes. We used this technique to study *Trypanosoma brucei* s.l. stocks isolated especially from foci in the Central African sub-region. Our main goal is to bring out genetic differences between the parasites in order to improve on their taxonomic status. This study will also enable us to appreciate the importance of animal reservoirs in the maintenance of foci and the circulation of stocks between foci.

## Materials and Methods

*Origin of biological material*

Stocks have been isolated from foci in the Central African Republic (RCA), Equatorial Guinea (GE), Chad (TC), and Cameroon (CM). We have 2 non-referenced stocks from the Sangha focus in the Congo Brazzaville (CG). We also have 26 East African **T.b.g.** stocks from Uganda, some of which are resistant to arsobal®.

*Collection of samples & isolation of stocks*

152 KIVI's (17 from domestic animals) were sampled from serological suspects in the Fontem

*Table I*

Details on stocks studied.

| Focus (Country) | Stock | Date Isolation) | Host | Zym | Status | Ref |
|---|---|---|---|---|---|---|
| Fontem (CM) | A005 | 1989 | Man | 1 | T.b.g.* | A |
| Campo(CM) | 133C | 1998 | Man | 1 | T.b.g. | H |
| Campo(CM) | 959C | 1999 | Man | 1 | T.b.g. | H |
| Campo(CM) | 2434C | 1998 | Man | 1 | T.b.g. | H |
| Campo(CM) | 2903C | 1998 | Man | 1 | T.b.g. | H |
| Campo(CM) | 3126C | 1998 | Man | 1 | T.b.g. | H |
| Campo(CM) | 3359C | 1998 | Man | 1 | T.b.g. | H |
| Campo(CM) | 3738C | 1998 | Man | 1 | T.b.g. | H |
| Campo(CM) | 3769C | 1998 | Man | 1 | T.b.g. | H |
| Campo(CM) | 4282C | 1998 | Man | 1 | T.b.g. | H |
| Mbini(GE) | 13/97D | 1998 | Man | 1 | T.b.g. | H |
| Mbini(FE) | 14/97D | 1998 | Man | 1 | T.b.g. | H |
| Mbini(GE) | 17/97D | 1998 | Man | 1 | T.b.g. | H |
| Mbini(GE) | 18/97D | 1998 | Man | 1 | T.b.g. | H |
| Bouenza(RDC) | MALOUNDA | 1989 | Man | 1 | T.b.g* | B |
| Fontem(CM) | 5776F | 1998 | Man | 2 | T.b.g. | H |
| Campo (CM) | 3205C | 1998 | Man | 3 | T.b.g. | H |
| Campo (CM) | 3392C | 1998 | Man | 3 | T.b.g. | H |
| Bodo (TC) | DESIRE | 1998 | Man | 3 | T.b.g. | H |
| Bodo (TC) | NATONDJI | 1998 | Man | 3 | T.b.g | H |
| Obo (RCA) | BIBIANA | 1998 | Man | 4 | T.b.g. | H |
| Obo (RCA) | MBADI | 1998 | Man | 4 | T.b.g | H |
| Obo (RCA) | MARY | 1998 | Man | 4 | T.b.g. | H |
| Mbini (GE) | 11/97D | 1998 | Man | 5 | T.b.g. | H |
| Mbini (GE) | 15/97D | 1998 | Man | 5 | T.b.g | H |
| Mbini (GE) | 16/97D | 1998 | Man | 5 | T.b.g. | H |
| Campo (CM) | MIBENE | 1997 | Man | 6 | T.b.g. | H |
| Campo (CM) | 2700C | 1998 | Man | 7 | T.b.g | H |
| Campo (CM) | 3524C | 1998 | Man | 7 | T.b.g. | H |
| Vavoua (CI) | TH2 | 1978 | Man | 8 | T.b.bouaflé* | C |
| Fontem (CM | JUA | 1979 | Man | 9 | T.b.g.* | D |
| Sangha (CG) | Boleko1132 | 1996 | Man | 10 | T.b.g. | H |
| Sangha (CG) | Ngombe2298 | 1996 | Man | 10 | T.b.g. | H |
| Campo (CM) | PO3A | 1998 | Pig | 11 | T.b.g. | H |
| Kogo (GE) | LEONTIO | 1998 | Man | 12 | T.b.g. | H |
| Kogo (GE) | GENEVIEVE | 1998 | Man | 12 | T.b.g. | H |
| Campo (CM) | 3232C | 1998 | Man | 13 | T.b.g. | H |
| Matuga (KE) | M253 | 1990 | Goat | 14 | T.b.b.* | E |
| Kogo (GE) | ALFONSO | 1998 | Man | 15 | T.b.g. | H |
| Boma (RDC) | SW94/87 | 1987 | Pig | 16 | T.b.b.* | F |
| Mbini (GE) | 12/97D | 1998 | Man | 17 | T.b.g. | H |
| Zambia | TRPZ166 | 1982 | Sheep | 18 | T.b.b. * | G |

* = *Reference stock* *KE = Kenya* *RDC = République Démocratique du Congo*

A = Dukes et al., 1989. E =Godfrey *et al.*, 1990. H = Oceac, 1998.

B = Truc & Tibayrenc (1993) F = Truc, 1991.

C = Mehlitz *et al.*, (1986) G = Mathieu Daudé F., 1991.

focus known to harbour trypanosomes that lack the LiTat 1.3 antigen used in the normal CATT test (Dukes *et al.*, 1992; Asonganyi *et al.*, 1994). 13 domestic animals were also sampled in Campo, 2 of which were parasitologically confirmed. Elsewhere, samples were drawn only from parasitologically confirmed sleeping sickness patients. The KIVI was used in its dual capacity as a diagnostic tool and an isolation medium for trypanosomes (Aerts *et al.*, 1992; Truc *et al.*, 1997).

In brief, 5 ml of venous blood are drawn from each patient (or suspect) using syringes containing the anticomplementary anticoagulant,

sodium polyanetholesulphate (PAS) (5%) (Le Ray *et al.*, 1970), and inoculated into the KIVI vial under sterile conditions. Transported to the laboratory, they are maintained at 27°C and followed up for trypanosomes at least twice a week by microscopical examinations for at least 45 days (McNamara *et al.*, 1995; Truc *et al.*, 1992). Details on stocks studied are presented in Table I.

*Parasite culture/harvest & sample preparation*
The positive KIVI samples are grown as procyclics by massive culture in Cunninghan's medium (Cunningham, 1977) supplemented with 20% foetal calf serum and antibiotics. Parasite pellets are then obtained by centrifugation (2500rpm/4°C/15 min.) followed by 2 washes in PBS (pH 7.2). Samples are prepared using lysis buffer as described by Truc & Tibayrenc (1993). The supernatant which constitutes the enzyme extract is stored in aliquots of 12 ml at -80°C.

*Electrophoresis*
14 enzyme systems corresponding to 18 loci were chosen in this study (Table II). Electrophoretic analysis were carried out on cellulose acetate gel plates (Helena Laboratories, Beaumont, Texas). Staining and electrophoretic procedures were adapted from Truc *et al.* (1991), Truc & Tibayrenc (1993). A total of 35 non referenced stocks and 7 others identified as **T.b.b.** or **T.b.g.** by previous authors (Table I) were examined.

## Results

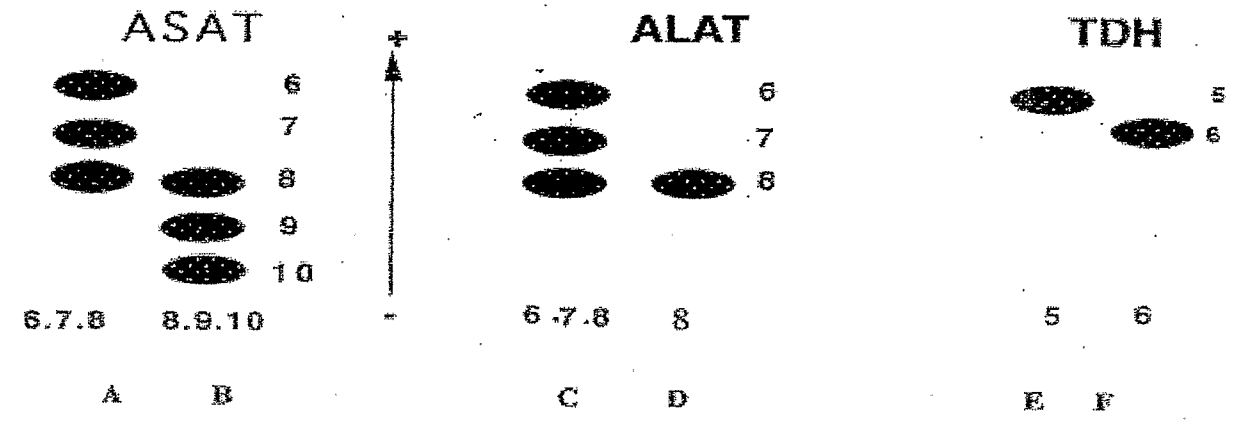
*In vitro isolation and parasite culture*
222 KIVI's were received in our laboratory. 35 were found positive with trypanosomes. Two of these were contaminated with fungi and thus, could not be isolated. A total of 33 new stocks (including one from a domestic pig in Campo) were isolated, giving a yield of 14.86%.

*Isoenzyme characterization*
3 of the newly isolated stocks from the Obo focus (RCA) showed a slow single-banded ALAT pattern while all the rest, had a characteristic ALAT triplet. The ASAT triplet was also common to all stocks though intensity of bands varied. The animal stock (PO3A) here was, however, clearly distinct by its unique phenotype (figure 1). The SOD system, was also invariable for all newly isolated stocks.
In all, 10 of the 14 enzyme systems (corresponding to 11 loci) were variable: ALAT, ASAT (GOT), TDH, IDH, PEP-2, NHi, NHd, ME, PGM and SODb. The polymorphism rate was, therefore, 0.61 (number of variable loci / total number of loci studied). 18 zymodemes (Zym) were defined, the majority of stocks belonging to zymodeme 1 (table I).

*Table II*
Enzyme systems studied.

| Enzyme system | Abbreviation | Code |
|---|---|---|
| Alanine aminotransferase | ALAT | EC 2.6.1.2 |
| Aspartate aminotransferase | ASAT (GOT) | EC 2.6.1.6 |
| Glucose phosphate isomerase | GPI | EC 5.3.1.9 |
| Glucose-6-phosphate dehydrogenase | G6PDH | EC 1.1.1.49 |
| Isocitrate dehydrogenase | IDH (ICD) | EC 1.1.1.42 |
| Malate dehydrogenase | MDH | EC1.1.1.37 |
| Malic enzyme | ME | EC1.1.1.40 |
| Nucleoside hydrolase (inosine) | NHi | EC 3.2.2.1 |
| Nucleoside hydrolase (deoxyinosine) | NHd | EC 3.2.2.1 |
| Peptidase (substrate l-leucyl-l-alanine) | PEP2 | EC 3.4.11 or 13.-. |
| 6-Phosphogluconate dehydrogenase | 6PGDH | EC 1.1.1.44 |
| Phosphoglucomutase | PGM | EC 2.7.5.1 |
| Superoxide dismutase | SOD | EC 1.15.1.1 |
| Threonine dehydrogenase | TDH | EC 1.1.1.103. |

*Figure 1.*
Banding patterns for some enzyme systems.




*The bands were atributed arbitrary numbers begining with the furthest band from the cathodic end. A - F represent the different phenotypes for the systems shown: B (ASAT triplet) unique to PO3A; D ( slow ALAT pattern): found only in RCA stocks.*

## Discussion

Samples from Fontem and 85% of domestic animals from Campo, unlike in other cases, were drawn from serological suspects who were all negative for parasitological examinations. Here, the KIVI was used as a diagnostic tool. This explains the overall low KIVI yield in positive cultures (14,86%). The yield would otherwise be 54,24% excluding KIVI samples from serological suspects. This suggests that the use of KIVI for diagnostic purposes would be very heavy and expensive. McNamara *et al* (1995) made a similar observation following an evaluation study of the KIVI in Northern Uganda. It is therefore, not advisable to use the KIVI for large scale diagnostic campaigns.

However, the fact that one stock (5776F) was still isolated from this lot shows the limits of available parasitological diagnostic techniques in detecting 100% of patients, and raises the question of screening threshold for sampling suspects by KIVI. Two particularly interesting cases from Campo focus were, however, observed: 3232c & SOMABc, negative for all parasitological examinations but highly positive by serological tests; both gave positive KIVI cultures. This suggests that the screening threshold for sampling suspects by KIVI should be reviewed in order to limit cost and reduce the load of work as well.

A high circulation of animal trypanosomes among domestic animals had been reported in the Fontem focus (Asonganyi *et al.*, 1990). A recent prospection in december 1998 resulted in the isolation of 18 animal stocks from domestic pigs by KIVI out of 32 samples taken (56%). This high prevalence of animal trypanosomes may account for the high level of false positive humans recorded, who may have received animal strains from infected tsetse flies responsible for the presence of specific antibodies, but that failed to survive in human blood.

The ALAT and ASAT enzyme systems had been described as characteristic of **T.b.g.** though no particular patterns were limited to **T.b.g.** alone (Godfrey & Kilgour, 1976; Mehlitz *et al.*, 1982; Stevens *et al.*, 1992). The ALAT triplet, the slow single banded ALAT pattern and the ASAT (GOT) triplet had been described by previous authors respectively as ALAT 1, ALAT 11 and ASAT 2 , all characteristic of **T.b.g.** (Godfrey *et al.*, 1987). This ALAT 11 pattern previously seen in Kenyan and Zairian **T.b.g.** (Stevens *et al.*, 1992) was found in stocks from the Obo focus (RCA), along the RDC (former Zaïre) border. This suggests that it could be the same zymodeme that has spread across the border from RDC.

More recently, some SOD patterns were found

useful in defining **T.b.g.** zymodemes, and in differentiating animal and human trypanosomes (Stevens *et al.*, 1992; Truc *et al.*, 1994, 1977). However, this enzyme system was invariable for all our newly isolated stocks including PO3A, which we could not consider as animal trypanosomes though it was isolated from a domestic pig in the Campo focus (CM). This stock should be a variant of **T.b.g.** in the domestic pig. Truc and Tibayrenc (1993) had also identified animal stocks from this region by isoenzymes as **T.b.g.** Two zymodemes (Zym 1 & Zym 3) grouped stocks of different origins (TC, CM, GE & CG). This observation clearly shows that stocks belonging to different zymodemes could be found within a given focus, and that stocks from different foci may have identical enzyme patterns. It is likely that these stocks spread from a common ancestor to different regions thanks to human movements. Truc and Tibayrenc (1993) based on the occurence of identical zymodemes in different foci to explain the spread of THA in Congo Brazzaville and RDC.

Zymodeme 1 includes the majority of our stocks, all of human origin, and two reference stocks (A005, MALOUNDA) identified by previous authors as **T.b.g.** (Table I). These stocks equally exhibit the typical ALAT, ASAT and SOD patterns described previously for **T.b.g.** We, therefore, associate this zymodeme with **T.b.g. group 1** (Gibson, 1986) which corresponds to the typical *Trypanosoma brucei gambiense* causing a chronic disease in man.

The other **T.b.g.** zymodemes with greater overall isoenzyme heterogeneity and no associated isoenzyme markers correspond to **Tbg group 2**. This is a heterogeneous group that also includes animal stocks resistant to human serum and/or showing identical electrophoretic patterns with human stocks (Gibson, 1986).

Our study is, however, still going on and at present, we have 17 newly isolated stocks from domestic pigs in the Fontem focus, 22 new human stocks from Bipindi (an apparently new extension of the Campo focus), and 26 other **T.b.g.** stocks from Uganda. We hope to throw more light on the epidemiology and specific status of these parasites after the characterization of these stocks.

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contre les Endémies en Afrique Centrale

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PM 253
3 JUIN 1999
Santé

ISSN 0255-5352