Mixed infections of trypanosomes in tsetse and pigs and their epidemiological significance in a sleeping sickness focus of Côte d'Ivoire

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SUMMARY

In a sleeping sickness focus of Côte d'Ivoire, trypanosomes were characterized in humans, pigs and tsetse using various techniques. Out of 74 patients, all the 43 stocks isolated by KIVI (Kit for In Vitro Isolation) appeared to belong to only one zymodeme of *Trypanosoma brucei gambiense* group 1 (the major zymodeme Z3). The only stock isolated on rodents belonged to a different, new, zymodeme (Z50), of *T. b. gambiense* group 1. From 18 pigs sampled in the same locations as the patients, PCR showed a high proportion of mixed infections of *T. brucei s. l.* and *T. congolense* riverine-forest. Zymodemes of *T. brucei s. l.* from these pigs were different from those found in humans. From a total of 16 260 captured tsetse (*Glossina palpalis palpalis*), 1701 were dissected and 28% were found to be infected by trypanosomes. The most prevalent trypanosome was *T. congolense* riverine-forest type, followed by *T. vivax*, *T. brucei s. l.* and *T. congolense* savannah type, this latter being associated to the forest type of *T. congolense* in most cases. Mixed infections by 2 or 3 of these trypanosomes were also found. Use of a microsatellite marker allowed us to distinguish *T. b. gambiense* group 1 in some of the mature infections in tsetse. Differences in infection rates and in trypanosome genotypes according to the host might indicate that the pig may not be an active animal reservoir for humans in this focus.

Key words: sleeping sickness, Côte d'Ivoire, *Trypanosoma*, tsetse, microsatellite, animal reservoir, mixed infection.

INTRODUCTION

The protozoan parasite *Trypanosoma* (*Trypanozoon*) *brucei* is the aetiological agent of Human African Trypanosomosis (HAT, or sleeping sickness) in man, and nagana in animals. The human disease is a major public health problem in Africa (WHO, 1998), and the economic impact of the animal disease is a severe constraint for the development of this region. In addition to *Trypanozoon*, 2 other subgenuses are involved in nagana, *Nannomonas* (*T. congolense*) and *Duttonnella* (*T. vivax*), all being transmitted to the mammalian host by an infected tsetse during its bloodmeal.

In West Africa, the main vector is *Glossina palpalis*, and the pathogenic trypanosome for humans is *T. b. gambiense*. A major problem in the epidemiology of human disease is that *T. b. gambiense* is hardly differentiable from the 'animal' trypanosome *T. b. brucei*. Moreover *T. b. gambiense* has been found so scarcely in tsetse that our knowledge of transmission still suffers many gaps (Frezil & Cuisance, 1994), including the importance of an animal reservoir for the gambiense disease (Van Hoof, Henrard & Peel, 1937; Gibson *et al.* 1978; Mehlitz *et al.* 1982). In addition, controversy has occurred on the subspecies classification of *T. brucei s. l.* The only group which can be considered as a taxonomic unit is the one defined as *T. b. gambiense* group 1 (Gibson, 1986), which represents 80% of all West and Central African human isolates. Other trypanosome strains isolated from humans in West and Central Africa which did not belong to this group were classified in a heterogeneous *T. b. gambiense* group 2, or Bouafle group (Godfrey *et al.* 1990).

The development of molecular techniques applied to trypanosomosis, like DNA probes or PCR (Kukla *et al.* 1987; Moser *et al.* 1989; Majiwa *et al.* 1993) brought new insights on the epidemiology of the disease, first on animal trypanosomes with the discovery of many more multiple infections of different trypanosome groups in tsetse than previously suspected (Majiwa *et al.* 1994; Solano *et al.* 1995; Masiga *et al.* 1996). In the human disease, molecular studies to identify trypanosomes in tsetse and mammalian host remain scarce (but see McNamara *et al.* 1995; Morlais *et al.* 1998), certainly due to the lack of molecular markers able to distinguish 'human' and 'non human' trypanosomes within

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T. brucei s. l., although markers have been found in *T. b. rhodesiense* that can do this differentiation (Welburn *et al.* 2001; Radwanska *et al.* 2002). Here, in order to better understand tsetse/trypanosomes relations, we tried to characterize, by parasitological techniques and recently developed molecular markers, the trypanosomes found in the same place at the same time in tsetse, human host (patients), and one of the most suspected of potential animal reservoirs, the pig.

MATERIALS AND METHODS

The HAT focus of Bonon is located in Central West Côte d'Ivoire (6°55′N–6°W). Between 2000 and 2001, a medical survey was done on humans, followed by a survey on pigs located where the patients had been diagnosed. An entomological survey was also implemented at each season of the year in the area (see below).

Survey on humans and pigs; trypanosome isolation and molecular techniques

An exhaustive medical survey was implemented by the National Control Program of HAT to detect HAT cases, preceded by an exhaustive individual census of the entire population of the area (Solano *et al.* 2003). Sleeping sickness cases were detected by a classical procedure involving CATT test on blood and plasma (Card Agglutination Test for Trypanosomosis, Magnus *et al.* 1978), followed by mini-Anion Exchange Centrifugation Technique (mAECT, Lumsden *et al.* 1979), or lymph node examination when adenopathy was present. From each patient, 5 ml of blood were inoculated in a KIVI kit for trypanosome isolation (Aerts *et al.* 1992), followed by multiplication in axenic culture medium (Cunningham, 1977) according to the protocol described by Truc *et al.* (1992). The pellets of parasites were conserved in liquid nitrogen until use.

From a subsample of 16 of these patients (the first 16 who gave their consent), the blood was also inoculated in mice for another procedure of trypanosome isolation (mice isolation, MI). In detail, 0·5 ml of blood, from a heparinized tube, was injected intraperitoneally into 2 mice. The mice were inoculated with cyclophosphamide (Endoxan, 300 mg/kg) 2 days after blood injection, and then every 5 days. The mice were followed up every 3 days, from the 6th day after inoculation for a period of 2 months, by microscopical examination of tail blood. When parasitaemia became high (10^8 trypanosomes/ml), blood from the mouse was inoculated in rats. When the parasitaemia in the rat reached 10^8 trypanosomes/ml, a cardiac puncture was performed, and trypanosomes from the blood were filtered by using a macro-anion exchange column. The pellets of parasites were conserved into liquid nitrogen until required.

Blood was also taken from 18 pigs from the medical survey area. For each pig blood sample, parasitological diagnosis was performed by microscopical examination of the buffy coat (HCT, Murray *et al.* 1977), and each blood sample was inoculated both to KIVI and to mice for trypanosome isolation as described above.

MultiLocus Enzyme Electrophoresis (MLEE) was implemented to identify trypanosome zymodemes in humans and pigs. Proteins were extracted from the pellets of parasites previously grown by KIVI and MI according to the method of Truc, Mathieu-Daude & Tibayrenc (1991). Stocks were characterized by MLEE on cellulose acetate plates (Helena®) and 10 enzymatic systems representing 13 loci were revealed: ALAT (EC 2.6.1.2), GOT (EC 2.6.1.1), Nhi (EC 3.2.2.1), Nhd (EC 3.2.2.1), ME (EC 1.1.1.40), PEP-2 (EC 3.4.11), MDH (EC 1.1.1.37), IDH (1.1.1.42), PGM (EC 2.7.5.1), SOD (EC 1.15.1.1) (see Jamonneau *et al.* 2002 for details). For the analysis procedure, a UPGMA dendrogram was built starting with the Jaccard genetic distances (d, Jaccard, 1908) calculated from MLEE results to determine the relationships between stocks (Sneath & Sokal, 1973). Reference stocks of *T. b. gambiense* group 1 and Bouafle group were included, as well as stocks of *T. congolense*-like groups for the UPGMA comparison.

PCR with satellite and microsatellite markers was implemented on a subsample of 10 pigs to identify the trypanosome species/group which had been detected. A simple 1% Chelex treatment derived from the method of Walsh, Metzger & Higuchi (1991) was used to obtain DNA for PCR reactions (see Solano *et al.* 1999 for details). The following primers were used: TBR1-2 (Moser *et al.* 1989), specific for *T. brucei s. l.*, TCF 1-2 (Masiga *et al.* 1992), specific for *T. congolense* West African Riverine Forest (*T. congolense* F), TCS 1-2 (Majiwa *et al.* 1993), specific for *T. congolense* Savannah (*T. congolense* S). *T. vivax* primers were not used in pigs because of known refractoriness of the pig for this trypanosome (Kageruka, 1987). In addition, on all the trypanosome pellets (from humans and pigs grown by KIVI and MI), another primer pair was used: TRBPA 1-2, the product of which, a 149 bp fragment, has been reported to be specific for *T. b. gambiense* group 1 (Herder *et al.* 2002; Truc *et al.* 2002). A negative control was included at each chelex procedure and within each PCR reaction.

Entomological survey, tsetse dissection, sample collection

Following the medical survey, the key sites (home, water supply points, working places) of the dwellings of each patient was recorded by use of a Global

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Positioning System (GPS) and subsequently 320 Vavoua traps (Laveissière & Grébaut, 1990) were settled in the area frequented by the patients. The captures were done in November 2000 (end of rainy season), January 2001 (cold dry season), April 2001 (end of hot dry season), and July 2001 (beginning of rainy season). Each trap remained for 4 days, with cages changed daily, and tsetse count, sex ratio, and dissection done daily. For the dissection, the mouthparts were first removed to prevent contamination from the midgut, and dissecting instruments were cleaned between each organ dissection with sodium hypochlorite, followed by rinsing in sterile water. Each organ (mouthparts, salivary glands, midgut) was put into a separate Eppendorf tube containing 30 μl of sterile distilled water.

For the PCR, we used the same 1% Chelex procedure as described above, and the same PCR primers as for the pigs (TBR 1-2, TCS1-2, TCF 1-2, TRBPA 1-2). In addition, primers specific for *T. vivax* were also used (TV1-2, Masiga *et al*. 1992).

RESULTS

Humans

Out of 15 227 people who came to the survey, 74 patients were found to have HAT, which gave a prevalence of 0·48% (0·47-CI_{95}-0·49).

From each of the 74 KIVI inoculated, 43 (58·1%) were successfully grown and trypanosomes were isolated. They all were shown to belong to the major zymodeme 3 of *T. b. gambiense* group 1 by MLEE (as Sique reference stock, see Fig. 1). From the 16 human stocks inoculated in mice, only 1 was successfully isolated (1 Human/MI). It was shown to have a different genotype (zymodeme 50) from all those isolated by KIVI. Using the diagnostic microsatellite marker TRBPA 1-2, the 149 bp band specific for *T. b. gambiense* group 1 was observed in all these samples, whatever the isolation method.

Pigs

By HCT, 4 pigs were found to be infected with trypanosomes. Out of 18 pigs, 13 *T. brucei* stocks were isolated by KIVI (72%), from which 8 were also isolated by MI (see Table 1). By PCR using satellite sequences on a subsample of 10 pigs, mixed infections involving *T. brucei s. l.* and *T. congolense* F were found in 9 pigs. In the 10th pig only *T. brucei s. l.* was found. No *T. congolense* S was detected in any of the pigs. All the *T. congolense* F which were identified by PCR were no longer present after isolation by KIVI or MI.

By MLEE, all the stocks isolated from pigs by KIVI and MI were different (but very close) to those isolated from humans and belonged to distinct *T. brucei s. l.* zymodemes, namely Z44 to Z49 (see Fig. 1). Details concerning the reference stocks have been reported by Jamonneau *et al*. (2003), from which Fig. 1 was modified by adding the human stock isolated by MI (1 Human/MI). When a stock was isolated simultaneously by the two techniques from each single pig, 2 different zymodemes of *T. brucei* were observed (already published by Jamonneau *et al*. 2003). Using TRBPA1-2, the 149 bp band specific for *T. b. gambiense* group 1 was detected in 9 trypanosome stocks isolated by KIVI (see Table 1) and in 1 stock isolated by MI (sample no. 6).

Tsetse

Parasitological results. During the 4 tsetse surveys between 2000 and 2001, a total of 16260 tsetse belonging to *G. p. palpalis* were caught, giving an apparent density of 3·19 flies/trap/day. From these, 1701 flies could be dissected. The parasitological infection rate obtained by dissection followed by microscopical examination was 28·1% [26·1-CI_{95}-30·1] (i.e. 478 flies were seen harbouring trypanosomes in either the midgut, the salivary glands, or the mouthparts).

Only 1 tsetse individual was found to be infected by trypanosomes in the salivary glands, which gives a *T. brucei s. l.* infection rate of 0·06% as determined by dissection. From the infected tsetse, 52·9% were infected in the midgut only, 27% in the mouthparts only (presumably *T. vivax*), and 19·6% had a concomitant infection of the midgut and the mouthparts (presumably *T. congolense*).

PCR results on infected tsetse. Because of logistical constraints, only 382 out of the 478 infected flies could be analysed by PCR. For each infected tsetse, PCR was implemented on mouthparts, salivary glands, and midgut. From these, 143 infections were due to *T. congolense* F (37·4%) which was the most prevalent, followed by *T. vivax* (95 occurrences, 24·9%), *T. brucei s. l.* (52 occurrences, 13·6%), and *T. congolense* S (16 occurrences, 4·2%). A total of 143 tsetse (37·4%) positive for trypanosomes by microscopy did not give any PCR signal (note that the total number of infections is more than 382 because of mixed infections).

Extended to all the dissected flies, the infection rates by species or groups of trypanosomes would give the following: 10·5% for *T. congolense* F, 6·9% for *T. vivax*, 3·8% for *T. brucei s. l.*, 1·1% for *T. congolense* S as determined by PCR (see Fig. 2).

From the 382 dissected tsetse analysed by PCR, 59 were infected by more than one trypanosome. The more frequent associations were: *T. congolense* F and *T. brucei s. l.* (16), and *T. congolense* F and *T. vivax* (16), followed by *T. congolense* F and *T. congolense* S (11), and *T. brucei s. l.* and *T. vivax* (8). *T. congolense* S and *T. vivax* was the less common association (1 case). In some tsetse, 3 different trypanosomes were observed (6 cases), even 4 (in 1 tsetse).

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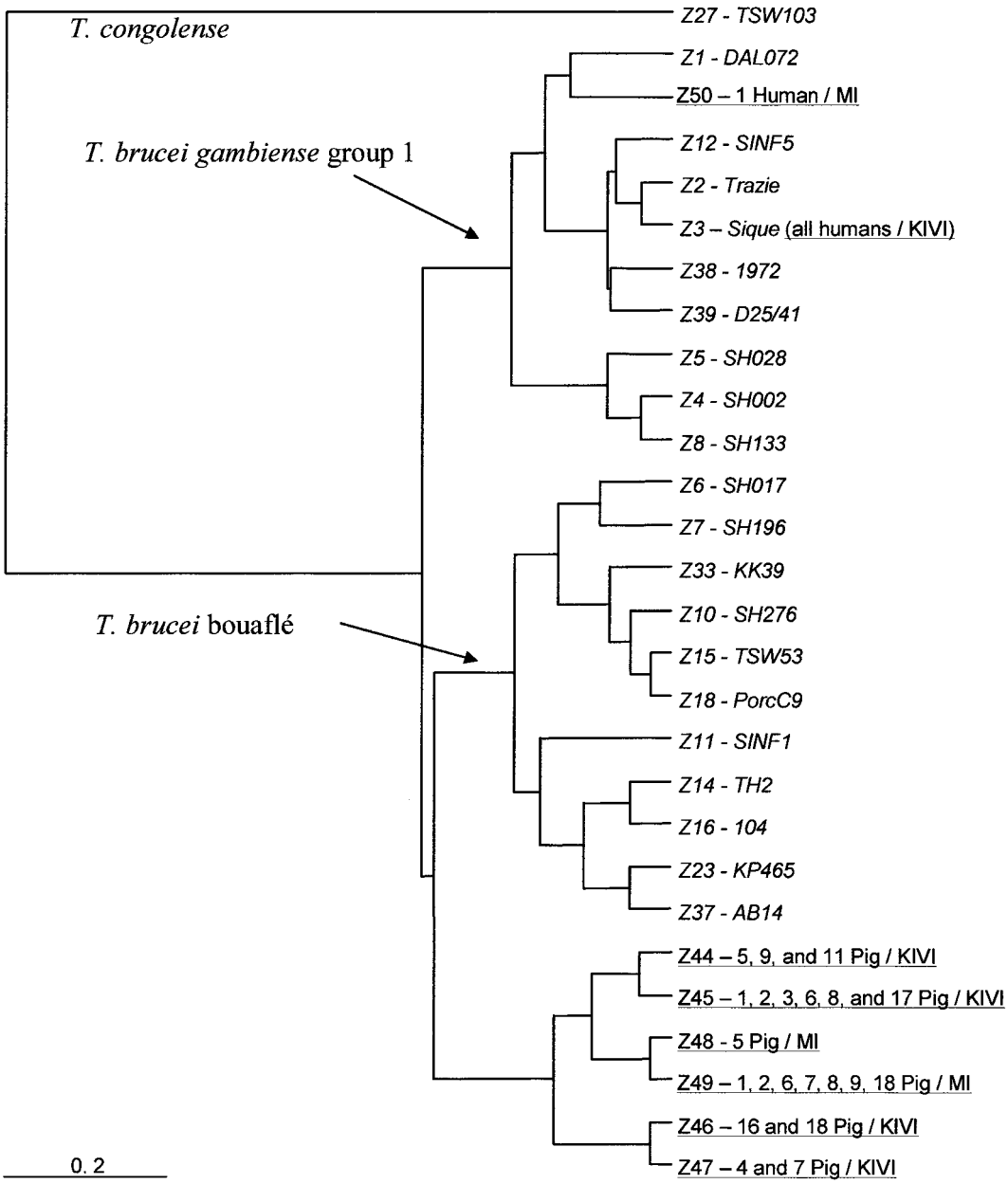

Fig. 1. Dendrogram showing genotypes obtained by MLEE on trypanosomes isolated from humans and pigs by mice inoculation and KIVI. The reference stocks are in italics and are preceded by their zymodeme number. The stocks obtained in the present study are underlined, are also preceded by their zymodeme number, and are followed by the host of origin (ex: sample Z48 19 MI/pig, is sample number 19, isolated from pig by mice inoculation, and belongs to zymodeme 48).

It has to be noted that of 16 infections with *T. congolense* S, it was found 15 times with another trypanosome. From 52 tsetse infected with *T. brucei s. l.*, 31 (59·6%) also harboured another trypanosome group.

Mature infections

For *T. congolense*, an infection was scored as mature if trypanosomes were found by PCR in either the proboscis and midgut concomitantly, or in the proboscis alone. In the latter case, we assume a disappearance of old *T. congolense* infections in the midgut, as previously described (Jordan, 1964).

For *T. congolense* F, 93 out of the 143 infections (65%) were mature. For *T. congolense* S, 12 out of 16 (75%) were mature. Hence, the mature infection rates for these trypanosomes in this study are: 6·8% for *T. congolense* F, and 0·8% for *T. congolense* S (see Fig. 2). Among the 12 mature infections of *T. congolense* S, all were mixed with other trypanosomes: in 10 tsetse, it was associated with mature infections of *T. congolense* F (among which, one also with *T. brucei s. l.*, and one with *T. brucei s. l.* and *T. vivax*), in one tsetse it was with *T. vivax*, and in the latter with *T. brucei s. l.* and *T. vivax*.

For *T. brucei s. l.*, an infection was considered as mature if both salivary glands and another organ

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Table 1. Results of trypanosome detection and isolation in pigs by various techniques

(From 18 pigs, the search for trypanosomes was done first on the field using a parasitological technique (HCT, 2nd column). Blood was taken and returned to the lab, PCR was implemented for various species/groups of trypanosomes (3rd column) on some of the pigs (N.D.: not done). Mice and KIVI (4th and 5th columns) were also inoculated on the field and culture performed in the lab for trypanosome stocks which grew (see text for details). PCR/TRBP was then implemented on isolated stocks to check for the 149 bp allele specific for *T. b. gambiense* group 1 (6th column).)

Sample no.	HCT on the field	PCR result on blood	mice isolation	KIVI isolation	(PCR/TRBPA) on KIVI stocks
1	N.D.	**Tb**; **Tcf**	**yes**	**yes**	**Tbg1**
2	Neg.	**Tb**; **Tcf**	**yes**	**yes**	**Neg.**
3	**T+**	**Tb**; **Tcf**	no	**yes**	**Tbg1**
4	Neg.	**Tb**; **Tcf**	no	**yes**	**Tbg1**
5	**T+**	**Tb**	**yes**	**yes**	**Tbg1**
6	**T+**	**Tb**; **Tcf**	**yes**	**yes**	**Tbg1**
7	Neg.	**Tb**; **Tcf**	**yes**	**yes**	**Neg.**
8	Neg.	**Tb**; **Tcf**	**yes**	**yes**	**Tbg1**
9	**T+**	**Tb**; **Tcf**	**yes**	**yes**	**Tbg1**
10	Neg.	**Tb**; **Tcf**	no	no	N.D.
11	Neg.	N.D.	no	**yes**	**Neg.**
12	Neg.	N.D.	no	no	N.D.
13	Neg.	N.D.	no	no	N.D.
14	Neg.	N.D.	no	no	N.D.
15	Neg.	N.D.	no	no	N.D.
16	Neg.	N.D.	no	**yes**	**Tbg1**
17	Neg.	N.D.	no	**yes**	**Tbg1**
18	Neg.	N.D.	**yes**	**yes**	**Neg.**

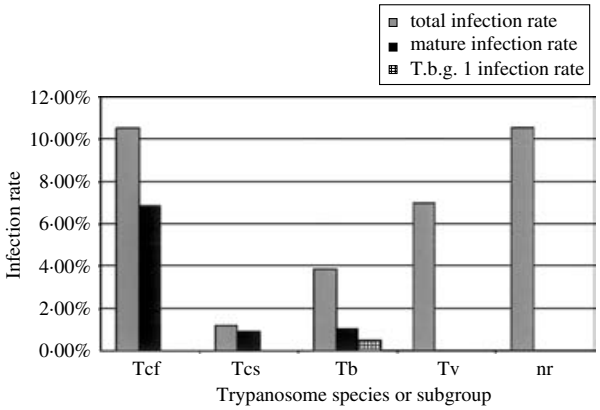

Fig. 2. PCR infection rates by species or groups of trypanosomes. Results obtained by PCR on the infected tsetse, extended to all dissected tsetse. Tcf: *Trypanosoma congolense* 'West African riverine forest' type; Tcs: *T. congolense* 'Savannah' type; Tb: *T. brucei s. l.*; Tv: *T. vivax*; nr: 'not recognized', i.e. infections detected by microscopy which did not give any amplification signal by PCR with the primers used. Tbg: *T. brucei gambiense* group 1. See text for the definition of 'mature infection rate', according to trypanosome species.

(proboscis and/or midgut) were found simultaneously positive by PCR. Of the 52 tsetse found infected with *T. brucei s. l.*, 14 had such mature infections. This would give a total mature infection rate of *T. brucei s. l.* of 1% (see Fig. 2). On these 14 tsetse having mature *T. brucei s. l.* infections, we used the *T. b. gambiense* group 1 specific primer set (TRBPA1-2, see Fig. 3). Seven of them showed the specific 149 bp band of *T. b. gambiense* group 1 at least in the salivary glands (Table 2). This would give a 0·5% infection rate of mature *T. b. gambiense* group 1 as determined by PCR.

Out of these 7 *T. b. gambiense* group 1 infections in the salivary glands, only one was a mixed infection involving another trypanosome (*T. congolense* F). Whereas of the 7 *T. brucei s. l.* infections that were not recognized as *gambiense*, 5 were mixed with other trypanosomes (see Table 2).

DISCUSSION

This study carried out in the HAT focus of Bonon in Côte d'Ivoire was performed to more clearly understand trypanosomes/tsetse/human and animal relations by characterizing trypanosomes found in the same place at the same time in these various potential or actual components in HAT epidemiology.

First, in humans, as expected using KIVI, only 1 zymodeme of *T. b. gambiense* group 1 was observed by MLEE, the predominant zymodeme Z3. Jamonneau *et al.* (2002) already reported this strong monomorphism, which was subsequently attributed to the selective nature of KIVI (Jamonneau *et al.* 2003). Here indeed, the only stock isolated from a patient by MI (Z50) was found to be genetically different by MLEE from all those isolated by KIVI (Z3), although both were shown to belong to *T. b. gambiense* group 1 by the TRBPA 1-2 marker. As isolation and culture stages probably select genotypes, the real genetic diversity of *T. b. gambiense* in

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Table 2. PCR results on tsetse having mature *Trypanosoma brucei* infections

(See text for definition of mature *T. brucei* infections; + positive; − negative. For PCR results, i.e. with PCR using TBR1-2, TCF1-2, TCS1-2, TV1-2 primer pairs, positive = presence of the band at the expected size. For TRBPA1-2 primer pair, positive = presence of the 149 bp fragment specific for *T. b. gambiense* group 1. *T.c.f.*: *T. congolense* 'West African riverine forest' type; *T.c.s.*: *T. congolense* 'Savannah' type; *T.b.*: *T. brucei s. l.*; *T.v.*: *T. vivax*. *T.b.g.* group 1: *T. brucei gambiense* group 1. For instance, tsetse no. 3 is infected by *T. brucei s. l.*, *T. congolense* F and *T. vivax*. Tsetse no.6 is infected by *T. b. gambiense* group 1.)

Sample no.	Sex	PCR TBR1-2	PCR TCF1-2	PCR TCS1-2	PCR TV1-2	PCR TRBPA	Interpretation
1	F	+	−	−	−	−	*T.b.*
2	F	+	−	−	−	−	*T.b.*
3	F	+	+	−	+	−	*T.b./T.c.f./T.v.*
4	M	+	+	−	−	−	*T.b./T.c.f.*
5	F	+	+	−	−	−	*T.b./T.c.f.*
6	F	+	−	−	−	+	*T.b.g.* group 1
7	F	+	−	−	−	+	*T.b.g.* group 1
8	F	+	−	−	−	+	*T.b.g.* group 1
9	F	+	−	−	−	+	*T.b.g.* group 1
10	F	+	−	−	−	+	*T.b.g.* group 1
11	F	+	+	−	−	+	*T.b.g.* group 1/*T.c.f.*
12	F	+	−	−	−	+	*T.b.g.* group 1
13	F	+	−	−	−	−	*T.b.*
14	F	+	−	+	+	−	*T.b./T.c.s./T.v.*

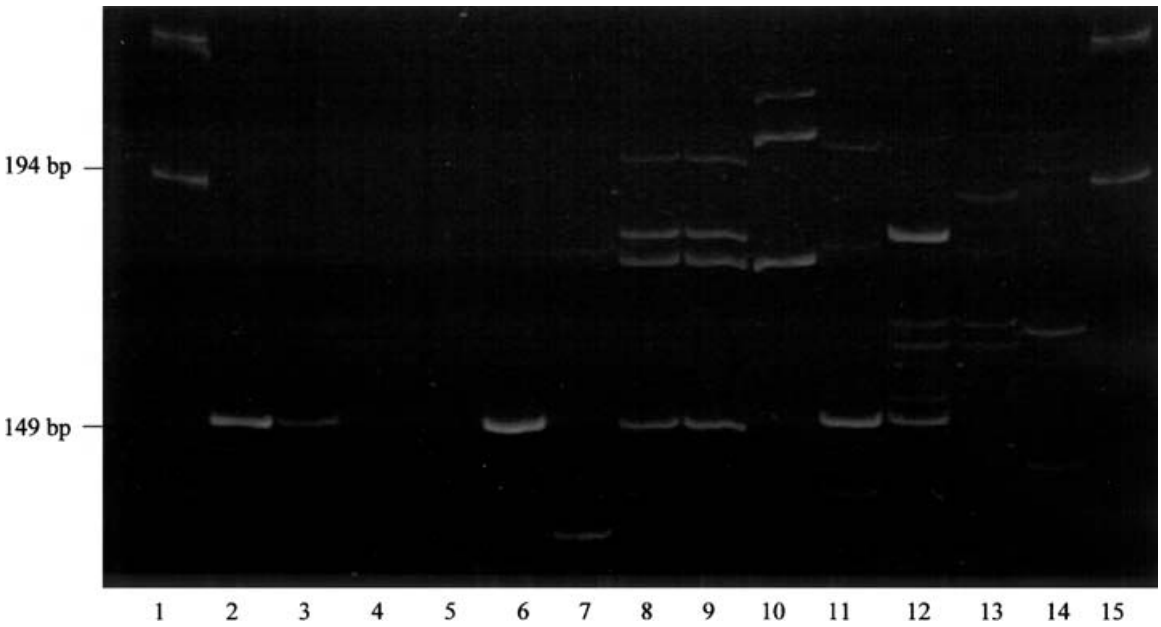

Fig. 3. Acrylamide gel with TRBPA showing the 149 bp fragment diagnostic of *Trypanosoma brucei gambiense* group 1 in reference stocks and in salivary glands and midguts from field-caught tsetse. Lanes 1 and 15: molecular marker (M4, Eurogentec), Lane 4: negative control, Lane 8: *Trypanosoma brucei gambiense* group 1 reference stock (Jua, Truc *et al*. 1991). Lane 9: *T. b. gambiense* group 1 reference stock (Peya, Truc *et al*. 1991). Lane 10: *T. b. brucei* reference stock (Stib215, Gibson *et al*. 1978). Lanes 2 and 3: salivary gland and midgut of a tsetse infected by *T. b. gambiense* group 1 in these two organs, Lanes 5, 6 and 7: salivary gland, midgut, and proboscis of a tsetse infected by *T. b. gambiense* group 1 in the midgut only, Lanes 11, 12 and 13: salivary gland, midgut, and proboscis of a tsetse infected by *T. b. gambiense* group 1 in the salivary glands and midgut, Lane14: midgut of a tsetse in which *T. b. gambiense* group 1 was not found.

humans must be higher than previously observed. For instance, multiple infections by different genotypes of *T. brucei s. l.* have recently been reported in patients (Truc *et al*. 2002; Jamonneau *et al*. 2004), as well as in tsetse (Letch, 1984). This strongly suggests that direct identification of trypanosomes within blood, without involving isolation and culture stages should be used in the future, provided that sensitive and specific molecular markers are available.

In our sample of 18 pigs, a high trypanosome prevalence was observed (72·2%), in accordance with previous studies (Mehlitz, 1986). The high

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prevalence of mixed infections of *T. congolense* F and *T. brucei s. l.* in pigs also confirms previous results (Mehlitz, 1986; Noireau *et al.* 1986), with additional information here on the *T. congolense* molecular group. We also noted that on pigs infected with these two trypanosome species, following a culture step (with both KIVI and MI) *T. brucei s. l.* always outcompeted *T. congolense* F which systematically disappeared.

Jamonneau *et al.* (2003) recently showed by MLEE that zymodemes of *T. brucei s. l.* on a single pig were different according to the isolation method used (KIVI and MI). In the present work, in contrast to other studies (Gibson *et al.* 1978; Mehlitz *et al.* 1982), zymodemes found in pig by MLEE were all different (albeit very close) to those found in humans whatever the isolation method used. By PCR with TRBPA 1-2, the specific 149 bp fragment of *T. b. gambiense* group 1 appeared in some of the stocks, confirming the close relationship between stocks from pigs and from humans.

These results suggest a very high diversity of genotypes of trypanosomes at the intra- (e.g. within *T. brucei s. l.*) and inter-specific level (e.g. *T. brucei s. l.* and *T. congolense*) circulating in pigs in this HAT focus, the majority of them remaining uncharacterized. It appears also that various genotypes circulate in humans, but that the ones which were characterized differ from those found in pigs, although again many genotypes of *T. brucei s. l.* in humans are still unknown (all those not isolated by MI nor by KIVI).

In tsetse, trypanosome infection rates observed using microscopical examination were higher than generally reported in West Africa (Squire, 1954; Lefrançois *et al.* 1998), even in Côte d'Ivoire (Croft *et al.* 1984; Nekpeni *et al.* 1991). An important number of infections seen by microscopical examination could not be characterized by PCR, as already reported (Solano *et al.* 1995; Lefrançois *et al.* 1998; Morlais *et al.* 1998). This may be attributed to the fact that the range of primers used in this study could not identify all the trypanosomes known to develop in the tsetse gut, e.g. *T. simiae*, *T. varani*, Tsavo *T. congolense* (Hoare, 1972; Majiwa *et al.* 1993), and also to PCR inhibition due to elements present in the midgut (Ravel *et al.* 2004).

T. congolense F was the most prevalent trypanosome in *G. p. palpalis*, as expected in this transitional forest-savannah area around a peridomestic habitat (see also Morlais *et al.* 1998) where pigs are abundant, and also small ruminants in which *T. congolense* F can develop. The higher prevalence of *T. congolense* F in *G. p. palpalis*, and its common association with *T. brucei s. l.* in mixed infections strenghtens the idea that there may be a predominant pig/tsetse/pig cycle in this area, also suggested by the reported bloodmeal preferences of *G. p. palpalis* for the pig (Sané, Laveissiere & Meda, 2000).

T. congolense S was weakly present in *G. p. palpalis* and, when present, was in most of the cases, associated to its closely related *T. congolense* F. In Toumodi, which is located in the same country at the same latitude as Bonon but in a savannah area, the reverse was observed in *G. longipalpis*, i.e. few *T. congolense* F, but always associated with *T. congolense* S which was the most prevalent (Solano *et al.* 1995). It may be that some evolutive mechanisms of association between these 2 trypanosome groups exist that help them to succeed in their transmission cycle in adverse ecological conditions (Solano *et al.* 2001).

Looking at mature infections, with parasitological detection only 1 salivary gland was found infected with trypanosomes out of 1701 dissected tsetse, which has been classically reported (Hoare, 1972). The use of PCR permitted a higher detection rate for *T. brucei s. l.* mature infections (in 14 tsetse) (see also Ravel *et al.* 2003). In addition, using a primer pair specific for the detection of *T. b. gambiense* group 1, this latter was found in 7 of the 14 mature infections. Eventually, the mature *T. b. gambiense* group 1 infection rate in tsetse was 0·5%, which is very close to the human prevalence of the disease in this focus (0·48%).

The great number of mixed infections involving 2, 3 or even 4 trypanosome groups in a single tsetse confirms previous results (Masiga *et al.* 1996; Woolhouse *et al.* 1996; Lehane *et al.* 2000), and supports the idea that tsetse can be sequentially infected on the field, at least for *T. congolense* and *T. vivax* (Squire, 1954). This might be different for trypanosomes infecting humans, which would infect tsetse mostly at their first bloodmeal (Van Hoof, 1937; Maudlin & Welburn, 1994), thus explaining a lower infection rate in tsetse for these trypanosomes. In addition, we observed in the present study that from the 7 tsetse harbouring a mature infection with *T. b. gambiense* group 1, only one was infected with another trypanosome, which might mean that either an established *T. b. gambiense* infection will prevent, in most cases, other trypanosomes from reaching a mature stage (when the first bloodmeal is on an animal), or that a tsetse infected by *T. b. gambiense* on a human (which occurs not frequently) will feed afterwards more readily on a human. In both cases, it can be assumed that a man/tsetse/man cycle, although occurring much less frequently than a pig/tsetse/pig cycle in this area, can mostly explain by itself the close and low prevalences of *T. b. gambiense* in tsetse and humans in this focus. Then, looking at the high infection rate of *T. brucei s. l.* in pigs, it has to be deduced that most of these trypanosomes will probably not be transmitted to humans. In this study, this is strenghtened by the fact that zymodemes found in pigs were all found to be different from those found in humans, although some of them belonged to *T. b. gambiense* group 1. However, this latter point has to be taken with caution, given that most of the

genotypes circulating were not identified because of selection by culture.

Although being quite speculative, these scenarii would suggest that in the field in this area, there are few chances that a human could be infected by trypanosomes originating from pigs via a tsetse.

An interesting outcome of this study is the ability to identify *T. b. gambiense* group 1 trypanosomes in tsetse, although it is not known if the *T. b. gambiense* group 1 identified by TRBPA 1-2 in tsetse and in pigs are infective for humans. Another outcome is that the study was conducted in the different hosts of the pathogenic system at the same place and at the same time, and it shows a very high diversity and intense circulation of multiple different trypanosome genotypes of different species and subspecies among the different hosts. It also appears important to study the circulation of animal trypanosomes from which interactions with 'human' trypanosomes may help to understand the transmission of the human disease. We are aware that these results only involve this focus and might not be extended to others, and also that our sample of pigs was not sufficient to reach definitive conclusions. In the future, higher numbers and different species of animals should be used. Direct identification of trypanosomes will hopefully be performed without culture steps to avoid sampling bias, because many genotypes of *T. brucei* have never been isolated and could change the conclusions drawn so far.

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