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Full Length Research Paper

Analysis of the domestic animal reservoir at a micro-geographical scale, the Fontem sleeping sickness focus (South-West Cameroon)

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To better understand the epidemiology of sleeping sickness in two Human African Trypanosomiasis (HAT) sub foci (central and northern sub foci) of the Fontem focus where diversity in the prevalence of *Trypanosoma bucei gambiense* was reported in domestic animals and man, 397 domestic animals were sampled in eight villages. Parasitological tests revealed trypanosomes in 86 (21.60%) animals. The CATT test was positive in 254 (64%) animals with the lowest value in dogs. The PCR test revealed *T. b. gambiense* in 11.55% of pigs, 3.45% of goats and 15.38% of sheep. The *T. b. gambiense* infection rates were not significantly different between the two sub foci. However, *T. b. gambiense* was found in animals from all villages of the Northern sub focus while only animals from Menji and Nsoko (Central sub focus) revealed this infection. The detection of *T. b. gambiense* in animals of the central sub focus was in line with results of medical surveys where HAT patients were detected in the same villages. The absence of patients in the northern sub focus despite the circulation of *T. b. gambiense* in animals from all villages of this sub focus since several years is surprising and needed more investigations.

Key words: Domestic animals, *T. b. gambiense,* sleeping sickness, animal reservoir.

INTRODUCTION

Under the combined effects of socio-political, economic, environmental and genetic factors, there has been a recrudescence of sleeping sickness in many historic foci in the Central African region (Cattand, 1994; Grébaut et al., 2001; Kaba et al., 2006; Brun et al., 2009). About 70,000 new cases are reported recently, with three quarters coming from the Democratic Republic of Congo (DRC) and Angola (Seed, 2001; WHO, 2007). Many hypotheses including the genetic diversity of trypanosomes and tsetse flies as well as the presence of an animal reservoir were suggested to explain the reactivation and the maintenance of the disease in the various foci. Genetic studies have shown that *Trypanosoma brucei* s.l. has a flexible mode of reproduction, and that genetic exchanges between *T. brucei* isolates can occur in the *Glossina* vector.

These exchanges can be at the origin of new *T. brucei* genotypes which introduce a genetic diversity that lead sometimes to the emergence of different epidemiological

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Abbreviations: PCR, Polymerase chain reaction; **HAT,** Human African Trypanosomiasis.

profiles of HAT (Tait et al., 1984; Paindavoine et al., 1989; Gibson, 2001; MacLeod et al., 2001; Njiokou et al., 2004).

In West and Central Africa, investigations on the animal reservoir of the Gambian sleeping sickness has shown that *T. brucei gambiense* infects a variety of domestic and wild animals (Molyneux, 1973; Mehlitz et al., 1982; Herder et al., 2002; Njiokou et al., 2006). The role of some animals in the epidemiology of HAT has been shown experimentally since cyclical transmission of *T. b. gambiense* in pig and cattle for example does not affect its virulence and its pathogenicity for humans (Van Hoof et al., 1942; Moloo et al., 1986). In spite of such experimental finding, definitive evidence that these animals play an important role in the maintenance or the resurgence of HAT still not yet well elucidated. Such evidence would help to define efficient control strategies by integrating the control of animal reservoirs. Indeed, domestic animals have a crucial importance as they live close to humans, and enjoy close social relationship with them (Laveissière et al., 2000).

In Cameroon, investigations on the animal reservoir using a combination of parasitological, immunological and PCR based methods have shown that domestic and wild animals harbour *T. b. gambiense* DNA and antibodies against *T. b. gambiense* Litat 1.3 antigen in almost all HAT foci (Njiokou et al., 2006, 2010) . At this macro-geographical scale, animal infection rates by *T. b. gambiense* differed significantly among the foci, reflecting the level of the transmission of HAT in these localities. The Fontem sleeping sickness focus of the South West region of Cameroon seems to have a particular status because medical surveys performed by the national sleeping sickness control team detect HAT patients only in the Central sub focus, whereas preliminary studies on animal reservoirs showed that pigs were infected by *T. b. gambiense* in both Central and Northern sub foci (Nkinin et al., 2002; Simo et al., 2006). Furthermore, the Fontem focus is a remote zone, isolated from the other Cameroonian HAT foci (Doumé, Campo and Bipindi) currently investigated.

To better understand the epidemiology of sleeping sickness in different HAT sub foci of the Fontem focus, we undertook to study the domestic animal reservoir status at a micro-geographical level, notably the villages of the Central and the Northern sleeping sickness sub foci of the Fontem focus, in order to precise the implication of those animals in the transmission cycle of HAT in this focus.

MATERIALS AND METHODS

Study zone

This study was carried out in Fontem (5°40'12 N, 9°55'33 E), a sleeping sickness focus in the South West region of Cameroon. Known since 1949, this focus is subdivided in three sub foci: the North, the Centre and the South sub foci (Figure 1) . The Fontem focus was previously amongst the most active foci in Cameroon (Asonganyi and Ade, 1994).

It remains active with very few cases (8 patients out of > 16 000 persons examined; OCEAC, MINSANTE, unpublished data) detected only in the Centre sub focus. Preliminaries studies on animal reservoir revealed *T. b. gambiense* in pigs from the centre and North sub foci (Nkinin et al., 2002; Simo et al., 2006) . The main population activities in this focus are agriculture, palm oil extraction, animal husbandry and poultry farming at a small scale. The Fontem focus is characterized by a tropical humid climate with varied topography of hills and valleys through which several high speed rivers flow (Asonganyi et al., 1990).

Collection of samples

Sampling of the domestic animals was done in July 2006 and June 2007 in eight villages: Besali, Bechati, Folepi and Agong in the Northern sub focus and Nsoko, Fossung, Menji and Azi in the central sub focus (Figure 1). Geographic coordinates of each of these villages were recorded using a Global Positioning System (Table 1). The objective of the study was explained to the local authorities and villagers of the study zone. After obtaining their approval, villagers were asked to catch and/or keep their domestic animals. In each village, all domestic animals that had spent at least 3 months in the study zone were selected. From each animal, about 5 ml of blood was collected into EDTA coated tubes. Bleeding was performed from the jugular vein in goats, pigs and sheep and from the saphena vein in dogs. Blood samples were then processed and analysed using direct and indirect methods to detect trypanosomes (0MS, 1986).

Direct methods of detection of trypanosomes

Parasitological tests: A drop of each blood sample was used to make a thick blood film (TBF), which was further, examined for the presence of trypanosomes under light microscope at 1,000X magnification.

The detection of trypanosomes using Capillary Tube Centrifugation (CTC) was performed according to the method described by Woo (1970). Briefly, about 70 µl of each blood sample was taken up in a heparinised capillary tube and its extremities were sealed with potty. After centrifugation at 11,000 rpm for 5 min, trypanosomes were detected at the red blood cell/platelet boundary at 100X magnification.

Polymerase chain reaction (PCR)

DNA was extracted from blood samples using (DNeasy Tissue Kit) (QIAGEN) . Initially, 1 ml of blood was mixed with 1 ml of sterile water and vortexed before centrifuging at 14,000 rpm for 10 min. The supernatant was discarded and the pellet containing parasites was re-suspended in 200 µl PBS. Then, the DNA was extracted following manufacturer's instructions. The DNA extract was stored at -20°C until use.

The detection of *T. brucei* s.l. was carried using the TBR1/TBR2 primers (Masiga et al., 1992). PCR reactions were performed in 20 µl (final volume) of mixture containing 10 mM Tris-HCl (pH9), 50 mM KCl, 1.5 mM $MgCl_2$, 15 pmol of each primer, 200 µM of each dNTP, 0.4 Units of Taq DNA polymerase and 5 µl of DNA solution (or 5 µl of water for negative controls). Amplification was done according to the protocol described by Masiga et al. (1992) . The amplification program contains an initial denaturation step at 94°C for 3 min 30 s, followed by 40 amplification cycles composed of a

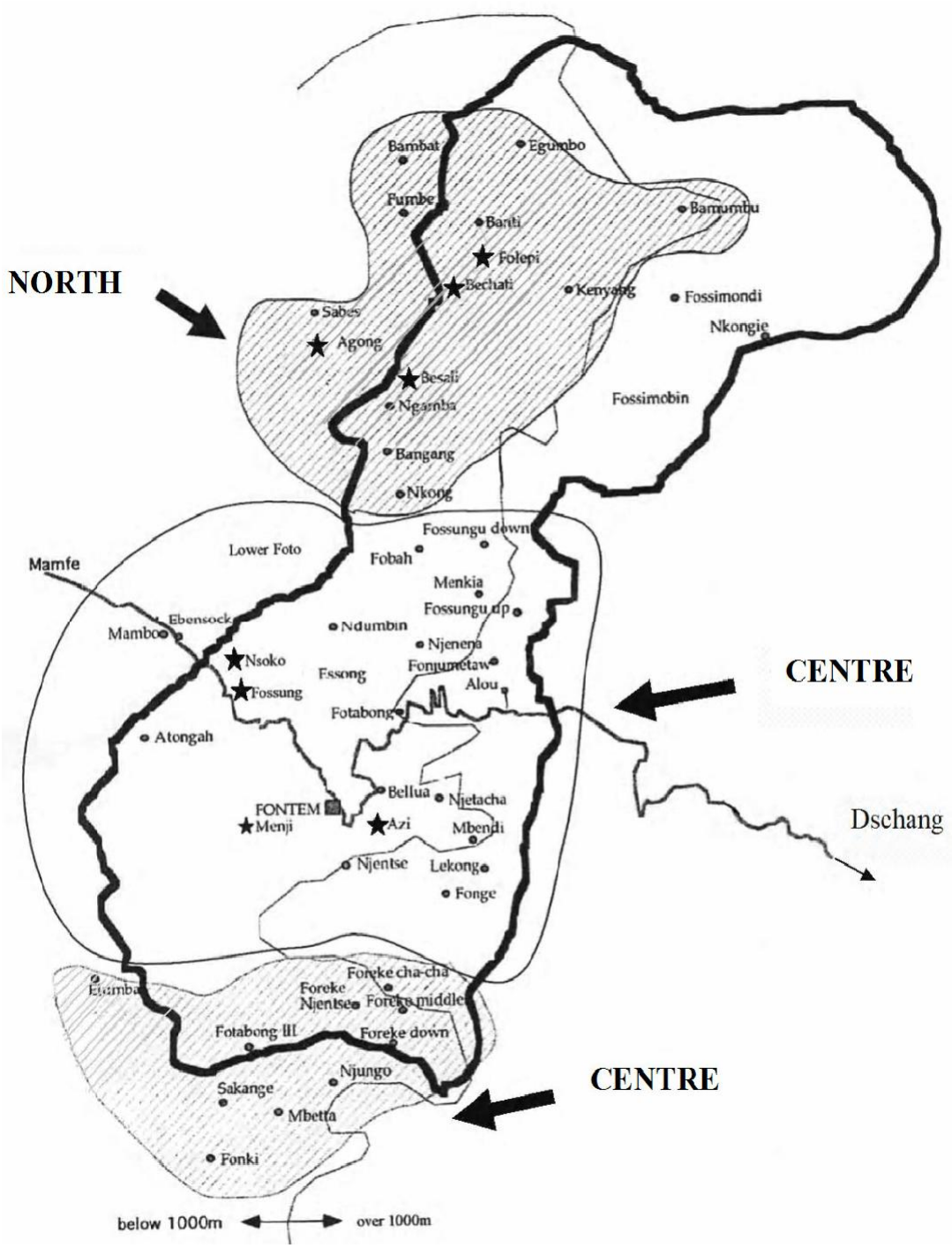

Figure 1. Map of Fontem focus (Simo et al., 2006).

( ): Villages where animals were sampled.

denaturation step at 94°C for 30 s, an annealing step at 58°C for 30 s and an extension step at 72°C for 1 min. A final extension was performed at 72°C for 5 min. The PCR products were resolved on 2% agarose gel containing ethidium bromide and visualised under UV light.

All samples positive for *T. brucei* s.l. were submitted to a second PCR as described by Herder et al. (2002). During this second amplification, primers TRBPA1/TRBPA2 (Herder et al., 2002) which amplify a DNA sequence of 149 bp characteristic of *T. b. gambiense* group 1 were used. The PCR conditions were identical to those described above, except that the annealing step was performed at 62°C. The PCR products were resolved on 10%

Table 1. Geographic coordinates of the villages where animals were sampled.

Sub foci	Villages	Geographic coordinates	
		Latitude	Longitude
North	Besali	05°38'044''N	009°54'790''E
	Bechati	05°40'060''N	009°55'187''E
	Folepi	05°40'074''N	009°56'041''E
	Agong	05°39'295''N	009°53'750''E
Centre	Menji	05°29'257''N	009°51'194''E
	Nsoko	05°31'670''N	009°49'671''E
	Fossung	05°30'878''N	009°50'024''E
	Azi	05°28'798''N	009°52'816''E

polyacrylamide gel. Electrophoresis was done at 90 V for 17 h in TBE X1. The gels were stained in ethidium bromide, washed in water and the bands were identified under UV light.

Indirect methods of detection of trypanosomes

Card agglutination test for trypanosomiasis (CATT): The immunological test was performed on plasma using CATT 1.3 test (Card agglutination test for trypanosomiasis) as described by Magnus et al. (1978). Briefly, the blood was centrifuged at 3000 rpm for 5 min, and 50 µl of each plasma was mixed with a drop of CATT 1.3 reagent (lyophilised bloodstream *T. b. gambiense* forms with LiTat 1.3 antigen expressed on their surface and suspended in phosphate buffer) and then shaken at 60 rpm for 5 min on an orbital agitator. Samples presenting agglutination were concluded as positive (Magnus et al., 1978). The remaining blood sample was stored at 4°C for molecular ana.

Statistical analysis

Statistical analysis was done using the programme XLSTAT-PRO version 2009.3. The ^{2} test was used to compare the antibody rates, the *T. brucei* s.l. and *T. b. gambiense* infection rates as well as the unidentified trypanosome infection rates. The comparisons were done according to the village, the sub foci and to the animal species.

The concordance between the different tests was assessed by comparing, for a given test, the percentage of positive samples to that of negative ones, according to the percentage of positive samples obtained with the other test, using the ^{2} test. The threshold for significance was set at 5%.

RESULTS

During the field trips performed in July 2006 and June 2007, 397 animals including 225 pigs, 87 goats, 65 sheep and 20 dogs were sampled in the Fontem focus: 184 were from Central sub focus and 213 from Northern sub focus. Detailed results concerning the number of animals, the species and the villages where these animals were sampled are reported in Tables 2 and 3.

Parasitology

Out of the 397 animals sampled, Thick Blood Film and Capillary Tube Centrifugation tests revealed trypanosomes in 86 (21.66%) of them. The four animal species sampled here were found with trypanosome infections, with significant different infection rates (Table 2). Animals from all the studied villages harboured trypanosomes with significant different infection rates.

In addition, in the Central sub focus, animals from Menji were significantly more infected compared to other villages. In the contrary, the trypanosome infection rates were not statistically different between the animals sampled in:

(1) The villages of the Northern sub focus (Table 3),
(2) The Northern (19.25%) and the Central (24.46%) sub foci (Table 4).

Serology

The CATT 1.3 test was positive in 254 (64%) animals. All the animal species harboured antibodies against the LiTat 1.3 antigen. The antibody rates were statistically different between the four animals species sampled in the Northern sub focus and when considering the entire focus (Tables 2). In addition, no significant difference was observed between the antibody rates in animals from the two HAT sub foci (Table 4) . Identical results were obtained when comparing the antibody rates between villages of each sub focus and between pigs, goats and sheep in the entire focus (Table 3).

PCR

The PCR test identified 140 (35.26%) animals harbouring *T. brucei* s.l . This trypanosome species was found in the four animal species as well as in all villages of the

Table 2. Number and percentage of positive animals for different tests, by animal species.

Animal species	Zones	Number examined	TBF/CTC (%)	CATT (%)	TBR (%)	TBG (%)
Pigs	North	91	24 (26.37)	53 (58.24)	50 (54.95)	14 (15.38)
	Centre	134	35 (26.12)	94 (70.15)	42 (31.34)	12 (08.95)
	Total 1	225	59 (26.22)	147 (65.33)	92 (40.88)	26 (11.55)
Goats	North	46	08 (17.39)	30 (65.21)	20 (43.48)	0.0 (0.00)
	Centre	41	09 (21.95)	28 (68.29)	05 (12.20)	03 (07.32)
	Total 2	87	17 (19.54)	58 (66.67)	25 (28.73)	0.3 (03.45)
Sheep	North	58	06 (10.34)	40 (68.96)	17 (29.31)	10 (17.24)
	Centre	07	01 (14.28)	03 (42.85)	02 (28.57)	0.0 (0.00)
	Total 3	65	07 (10.77)	43 (66.15)	19 (29.23)	10 (15.38)
Dogs	North	18	03 (16.67)	05 (27.78)	04 (22.22)	0.0 (0.00)
	Centre	02	0.0 (0.00)	01 (50.00)	0.0 (0.00)	0.0 (0.00)
	total 4	20	03 (15.00)	06 (30.00)	04 (20.00)	0.0 (0.00)
Total	North	213	41 (19.24)	128 (60.09)	91 (42.72)	24 (11.26)
	^{2}		6.11	10.37	12.92	11.78
	P		0.10	0.016	0.005	0.008
	Centre	184	45 (24.45)	126 (68.47)	49 (26.63)	15 (8.15)
	^{2}		1.37	2.61	6.63	0.95
	P		0.71	0.45	0.08	0.81
	All	397	86 (21.66)	254 (63.97)	140 (35.26)	39 (9.82)
	^{2}		8.05	10.60	7.82	9.20
	P		0.045	0.014	0.05	0.027

TBF/CTC: Thick blood film and capillary tube centrifugation, TBR: *Trypanosoma brucei* s.l., TBG: *Trypanosoma brucei gambiense,* CATT: Card agglutination test for trypanosomiasis.

Fontem HAT focus. The infection rates by this trypanosome species differs significantly between animal species and villages of the northern sub focus (Tables 2 and 3). Similar results were observed in the entire focus (Table 4).

T. b. gambiense was detected in 39 (9.82%) animals: 26 (11.55%) in pigs, 10 (15.38%) in sheep and 3 (03.45%) in goats (table 2). In the two sub foci, no *T. b. gambiense* infection was found in dogs. The infection rates differed significantly between animal species, pigs being the most infected. In the Northern sub focus, only pigs and sheep were found positive, and the infection rates differed significantly between these animal species. In the Central sub focus, pigs and goats carried *T. b. gambiense* infections, but the infection rates did not differ significantly (Table 2). Analysis by village revealed *T. b. gambiense* infections in animals of all villages of the northern sub focus.

No significant difference was observed between the *T. b. gambiense* infection rates in the villages of this sub focus. In the Central sub focus, only animals from Menji and Nsoko were found with *T. b. gambiense* infections with significantly differences according to the villages (Table 3) . No significant difference was observed between the *T. b. gambiense* infection rates of the two sub foci (Table 4).

Concordance test

Out of the 86 animals that carried trypanosome infections, 19 were positive for the two parasitological tests (Thick Blood Film and CTC). Moreover, 53 and 14 trypanosome infections were revealed only by CTC and Thick Blood Film, respectively. The Chi square test between CTC and TBF was significant (^{2} = 32.11; $P < 0.0001$) in favour of negative samples, showing that results of CTC and TBF are not linked.

A total of 24 animals were positive for both CATT test and *T. b. gambiense*-PCR, whereas 230 were only positive for the CATT test and 15, only positive for PCR. The Chi square test between CATT and PCR was significant (^{2} = 334.14; $P < 0.0001$) in favour of negative samples; suggesting that positive CATT tests are not linked absolutely to the presence of *T. b. gambiense* in animals.

Table 3. Number and percentage of positive animals for different tests, by study area.

Study sites	No animals	TBF/CTC (%)	CATT (%)	TBR (%)	TBG (%)
North					
Besali	67	11 (16.42)	38 (56.72)	19 (28.36)	11 (16.42)
Bechati	90	17 (18.89)	57 (63.33)	45 (50.00)	10 (11.11)
Folepi	37	10 (27.02)	22 (59.46)	17 (45.95)	01(02.70)
Agong	19	03 (15.79)	11 (57.89)	10 (52.63)	02 (10.52)
Sub-total	213	41 (19.25)	128 (60.09)	91 (42.72)	24 (11.27)
$\div^2$		1.93	0.75	8.51	4.50
P		0.58	0.86	0.036	0.21
Centre					
Menji	86	32 (37.21)	66 (76.74)	28 (32.56)	12 (13.95)
Nsoko	38	06 (15.79)	21 (55.26)	07 (18.42)	03 (07.89)
Fossung	21	03 (14.28)	12 (57.14)	06 (28.57)	0.0 (0.00)
Azi	39	04 (10.25)	27 (69.23)	08 (20.51)	0.0 (0.00)
Sub-total	184	45 (24.46)	126 (68.48)	49 (26.63)	15 (08.15)
$\div^2$		14.54	7.05	3.64	9.19
P		0.002	0.07	0.30	0.027
Total		86 (21.66)	254 (64.00)	140 (35.26)	39 (09.82)
$\div^2$	397	19.20	10.40	23.44	13.93
P		0.008	0.16	0.001	0.052

No: Number of, TBF/CTC: Thick blood film and capillary tube centrifugation, TBR: *Trypanosoma brucei* s.l., TBG: *Trypanosoma brucei gambiense,* CATT: Card agglutination test for trypanosomiasis.

Table 4. Number and percentage of positive animals for different tests, between the two sub-foci.

Sub focus	No animals examined	TBF/CTC (%)	CATT (%)	TBR (%)	TBG (%)
North	213	41 (19.24)	128 (60.09)	91 (42.72)	24 (11.26)
Centre	184	45 (24.45)	126 (68.47)	49 (26.63)	15 (08.15)
$\div^2$		1.57	3.01	11.19	1.082
P		0.20	0.083	0.001	0.29

No: Number of, TBF/CTC: Thick blood film and capillary tube centrifugation, TBR: *Trypanosoma brucei* s.l., TBG: *Trypanosoma brucei gambiense,* CATT: Card agglutination test for trypanosomiasis.

DISCUSSION

The parasitological tests showed that 21.66% of animals carry trypanosomes. This result is in line with those reported previously in domestic animals of the Fontem focus (Nkinin et al., 2002; Simo et al., 2006) as well as in animals of the other HAT foci of West and Central Africa (Scott et al., 1983; Noireau et al., 1986; Asonganyi et al., 1990). No significant difference was found between the trypanosome infection rates in domestic animals sampled in the two HAT sub foci as well as in villages of the northern sub focus whereas there was a significant difference between villages of the Central sub focus and when comparing villages of the entire focus. These results suggest that, although the relative homogeneity in the ecological and bioclimatic conditions (same climate, vegetation, topography and unique vector), epidemiological considerations such as human activities are slightly different between villages. CTC identified many positive animals compared to thick blood film confirming its high sensitivity. A high discordance was observed between the two tests, suggesting their complementarity.

The high proportion of animals found positive for the CATT test confirms the result of Nkinin et al. (2002) in the Fontem focus and those of Njiokou et al. (2010) in other HAT foci of Cameroon. This indicates the presence of antibodies directed against the LiTat 1.3 antigens of *T. b. gambiense* in these animals. However, the high antibody rates obtained here does not corroborate the low prevalence (11.89%) of *T. b. gambiense* revealed by PCR. Indeed, the analysis of CATT and PCR-*gambiense* results revealed a high discordance between these two

tests; suggesting that many CATT tests are positive in animals that do not carry *T. b. gambiense* infection. It is probably the case of animals harbouring other trypanosomes of *T. brucei* complex species or *T. congolense* as reported by Noireau et al. (1986). Animals positive to PCR-*gambiense* and negative for the CATT test are probably infected by *T. b. gambiense* isolates which do not express the LiTat 1.3 antigenic variant as already reported in the Fontem focus (Dukes et al., 1992; Asonganyi; Ade, 1994; Kanmogne et al., 1996). It is also possible that some of these animals were recently infected (less than 10 days) and the *T. b. gambiense* antibodies were not produced at time of sampling. Similar results were obtained during experimental infections of pigs by *T. b. gambiense* (Penchenier et al., 2005). Moreover, serological analysis showed that the antibody rate is not significantly important in pigs than in sheep and in goats. However, analysis of blood meals of *Glossina palpalis palpalis* in the same area revealed that 55% were from pigs, 23% from human and very little from other domestic animals (Njitchouang, pers. Comm.). These results can be explained by the fact that pigs are reared within a maximum of 12 months while other domestic animals are maintained for many years in the villages and accumulate infections (Njiokou et al., 2010).

The percentage of animals infected by *T. brucei* s.l. (35.66%) and *T. b. gambiense* (9.82%) are comparable to those reported by Simo et al. (2006) in the Fontem focus, and superior to the values obtained by Njiokou et al. (2010) in the four HAT foci of southern Cameroon. These results confirm a high circulation of trypanosomes of *T. brucei* s.l. complex and that of *T. b. gambiense* in domestic animals in Fontem, despite the relative low prevalence of sleeping sickness in humans compared to other HAT foci like Bipindi and Campo (Penchenier et al., 1999; Grébaut et al., 2001). The presence of *T. brucei* s.l. and *T. b. gambiense* respectively in 4 and 3 animal species examined confirms a direct affinity between these hosts and trypanosomes of the *T. brucei* s.l. complex, and suggesting their direct implication in the epidemiology of sleeping sickness (Nkinin et al ., 2002 ; Simo et al., 2006 ; Njiokou et al., 2010).

Looking at *T. b. gambiense* infections in animals between the two HAT sub foci, no significant difference was observed. This result is surprising given the results of medical surveys that revealed HAT patients in the central sub focus and no patient in the northern sub focus. The identification of *T. b. gambiense* in animals in two villages (Menji and Nsoko) of the central sub focus are in line with results obtained during medical surveys where HAT patients were detected in these two villages during the last decade (National Trypanosomiasis control program unpublished data). This indicates an active transmission of HAT in both man and animal in these villages; confirming several disease transmission cycles including man, pig and goats in the central sub focus. In the northern sub focus where *T. b. gambiense* was identified in animal of all villages, no HAT patient was detected during these last decades. However, *T. b. gambiense* infections were identified in animals from this sub focus since several years (Nkinin et al., 2002); indicating a circulation of this trypanosome sub species and also a predominant animal transmission cycle in all villages of the northern sub focus. Furthermore, the presence of human blood meals in tsetse flies captured in this sub focus indicates a contact between tsetse flies and man. This contact is illustrated by the high positivity of inhabitants of this sub focus to immunological test like CATT 1.3 (Magnus et al., 1978) and Latex *T. b. gambiense* (OCEAC, unpublished data). These results show clearly that human of the Northern sub focus are in contact with *T. b. gambiense*. However, the absence of HAT patients in this sub focus is difficult to explain given the fact that transmission conditions found in this sub focus are similar to those of the central sub focus. Moreover, the genetic characterization of *T. b. gambiense* isolated in pigs and human of these sub foci showed considerable genetic homogeneity (Nkinin et al., 2002; Njiokou et al., 2004). One interesting aspect that needs to be addressed in order to improve knowledge on the epidemiology of HAT in these sub foci requires investigations on human susceptibility to *T. b. gambiense* infection. In West Africa, diversity in the clinical evolutions associated to human genetic factors has been recently suspected (Jamonneau et al., 2004; Garcia et al., 2006).

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