IDENTIFICATION OF TRYPANOSOMES IN WILD ANIMALS FROM SOUTHERN CAMEROON USING THE POLYMERASE CHAIN REACTION (PCR)

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Summary:

One possible explanation of the maintenance of many historical foci of sleeping sickness in Central Africa could be the existence of a wild animal reservoir. In this study, PCR was used to detect the different trypanosome species present in wild animal captured by hunters in the southern forest belt of Cameroon (Bipindi). Trypanosomes were also detected by a parasitological method (Quantitative buffy coat: QBC). Parasite could not be isolated in culture medium (Kit for *in vitro* isolation: KIVI). Specific primers of *T. brucei* s.l., *T. congolense* forest type, *T. congolense* savannah type, *T. vivax*, *T. simiae* and *T. b. gambiense* group 1 were used to identify parasites in the blood of 164 animals belonging to 24 different species including ungulates, rodents, pangolins, carnivores, reptiles and primates. Of the 24 studied species, eight were carrying *T. b. gambiense* group 1. Those parasites pathogenic to man were found in monkeys (*Cercocebus torquatus* and *Cercopithecus nictitans*), in ungulates (*Cephalophus dorsalis* and *C. monticola*), in carnivores (*Nandinia binotata* and *Genetta servalina*) and in rodents (*Cricetomys gambianus* and *Atherurus africanus*). 13 species (54 %) were carrying *T. brucei* s.l. identified as non-gambiense group 1.

KEY WORDS : sleeping sickness, wild animal, reservoir, PCR, *T. b. gambiense*, forest belt, Cameroon.

Résumé : IDENTIFICATION PAR PCR DES TRYPANOSOMES CHEZ LES ANIMAUX SAUVAGES DU SUD-CAMEROUN

Une explication possible du maintien de nombreux foyers historiques de la maladie du sommeil en Afrique centrale pourrait être l'existence d'un réservoir animal sauvage. Dans cette étude, la PCR a été utilisée pour identifier les différentes espèces de trypanosomes hébergées par des animaux sauvages capturés par des chasseurs dans la forêt du Sud-Cameroun (Bipindi). Les trypanosomes ont également été détectés par une technique parasitologique (QBC : Quantitative buffy coat). Les parasites n'ont pas pu être isolés sur milieu de culture (KIVI : Kit for in vitro isolation). Des amorces spécifiques de T. brucei *s.l.,* T. congolense *type forêt,* T. congolense *type savane,* T. vivax, T. simiae *et* T. b. gambiense *groupe 1 ont été utilisées pour identifier les trypanosomes dans le sang de 164 animaux sauvages appartenants à 24 espèces différentes et comprenant des ongulés, des rongeurs, des pangolins, des petits carnivores, des reptiles et des primates. Sur les 24 espèces étudiées, huit étaient porteuses de* T. b. gambiense *groupe 1. Ces parasites potentiellement pathogènes pour l'homme ont été trouvés chez des singes (*Cercocebus torquatus *et* Cercopithecus nictitans*), chez des ongulés (*Cephalophus dorsalis *et* C. monticola*), chez des petits carnivores (*Nandinia binotata *et* Genetta servalina*) et chez des rongeurs (*Cricetomys gambianus *et* Atherurus africanus*). 13 espèces (54 %) étaient porteuses de* T. brucei *s.l. identifié comme non-gambiense groupe 1.*

MOTS CLÉS : *maladie du sommeil, animaux sauvages, réservoir, PCR,* T. b. gambiense*, forêt, Cameroun.*

Despite the numerous medical surveys which have significantly reduced the incidence of sleeping sickness, eradication has never been obtained and recently, most of the historical foci are in resurgence. A number of hypothesis have been advanced in an attempt to explain the persistance of West African trypanosomiasis including the existence of reservoir hosts and the presence of asymptomatic human carriers (Molyneux, 1980). The existence of wild animal reservoir for *T. brucei rhodesiense* has been demonstrated by inoculating a volunteer with trypanosomes isolated from a bushbuck (*Tragelaphus scriptus*) (Heisch *et al.*, 1958). Similarly, the possible existence of an animal reservoir for gambian sleeping sickness has also been investigated in West Africa. However, the isolation of *T. b. gambiense*-like trypanosomes have largely been ignored because of the avirulence of these organism both in man and in domestic or wild animals (Molyneux, 1973). Several workers have shown that domestic animals are capable of acting as suitable reservoir hosts (Van Hoof, 1947; Gibson *et al.*, 1978; Mehlitz *et al.*, 1982). Previous studies carried out in West and Central Africa have shown that wild mammals can harbour trypanosomes including *T. brucei s.l.* (Burridge *et al.*, 1966; Allsopp, 1972; Mehlitz, 1982; Komoin-Oka *et al.*, 1994; Truc *et al.*, 1997a, 1997b). Besides, *T. b. gambiense* is capable of

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infecting a wide range of wild animals under experimental conditions (Frézil & Carnevale, 1976).
In the past, the major difficulty in demonstrating that *T. b. gambiense* also infects mammals other than man was the lack of techniques capable to differentiate trypanosomes in naturally infected hosts (Molyneux, 1973). The development of new molecular markers has enable the detection of *T. brucei* s.l. subspecies in naturally infected hosts and vectors (Herder *et al.*, 1997; MacLeod *et al.*, 1999; Biteau *et al.*, 2000).
In this study, PCR (Polymerase chain reaction) was used to identify trypanosome species harboured by wild animals from the south cameroonian region. Trypanosomes were also detected by QBC (Quantitative buffy coat; Bailey & Smith, 1992), a parasitological technique which does not allow the distinction between the different subspecies. Parasites were also isolated on KIVI (Kit for *in vitro* isolation; Aerts *et al.*, 1992) culture medium.

MATERIALS AND METHODS

Study area

The sleeping sickness focus of Bipindi (3° 06' N, 10° 30' E) is situated in south province of Cameroon, approximately 75 km off the coast. It is a rainforest area characterized by a typical equatorial climate with four seasons. The main activity is extensive peasant agriculture characterized by burned ground farming with numerous encampments. Hunting is also an important resource for self consumption and for sale.

Collection of samples

Sampling of blood from wild animals was done in the villages of Lambi and Bidjouka (Bipindi) during the rainy season in July and in October 1999. The animals caught by trapping or firearm were brought back to the village. We did not have any incentive action on the hunters since only the animals already killed and intended for sale were sampled. Moreover, the hunters were not informed of our coming since we had met them on the markets (meat points of sale). When it was possible, an aseptic sample of blood was then taken to inoculate KIVI medium. A second sample was taken on EDTA tube for QBC and PCR tests. The QBC analyses were carried out within two hours after sampling; thin blood smears were then visualised on samples positive by QBC.

DNA isolation

Samples were treated with Ready AMP™ genomic purification kit (Promega, Madison, WI, USA) as described by Penchenier *et al.* (1996). Supernatants containing single stranded DNA were stored at 4° C or used directly as template for PCR amplification.

PCR analysis

Specific primers for *T. brucei* s.l. (TBR1 & 2; Moser *et al.*, 1989), *T. congolense* "forest type" (TCF1 & 2; Masiga *et al.*, 1992), *T. congolense* "savannah type"(TCS1 & 2; Majiwa *et al.*, 1994), *T. vivax* (TVW1 & 2; Masiga *et al.*, 1992), *T. simiae* (TSM1 & 2, Masiga *et al.*, 1992) and *T. b. gambiense* "group 1" (TRBPA1 & 2; Herder *et al.*, 1997) when positive with TBR1 and 2, were used to amplify DNA extracted from animal blood.
PCR reactions were performed in 25 µl of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1 % Triton X100, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 20 picomoles of each primer, 5 µl of template DNA and one unit of *Taq* DNA polymerase (Promega). Amplifications were carried out in a thermocycler (Techne Gene E) programmed for 40 cycles of 30 seconds at 92° C, 30 seconds at 60° C and one minute at 72° C for TRBPA and with an annealing temperature of 55° C for *T. brucei* s.l. (TBR) and 60° C for the others (*T. congolense* forest and savannah type, *T. simiae* and *T. vivax*). Amplification products were resolved on 1.5 % agarose gel, or on 4 % agarose/10 % acrylamide gel for the procyclic acidic repetitive protein (PARP) gene (TRBPA: Fig. 1).

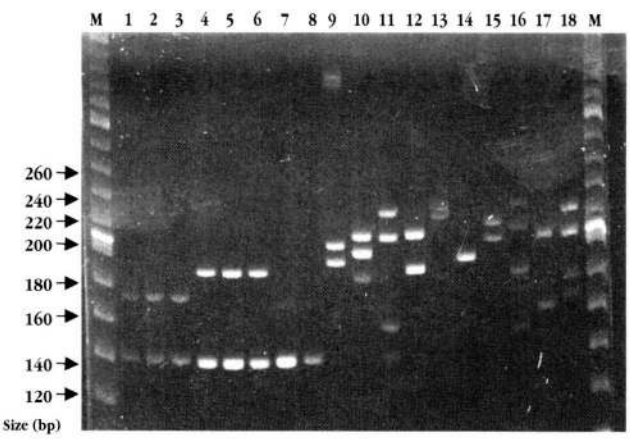

Fig. 1. – Results of the PCR amplification using TRBPA primers on different trypanosome isolates.
M: Molecular weight marker (MWM) 100/20 base pair (bp) ladder. Lane 1 to 8: *T. b. gambiense* group 1; lane 9 to 12: *T. b. gambiense* non group 1; lane 13: *T. b. rhodesiense*; lane 14 to 18: *T. b. brucei*.

RESULTS

164 animals belonging to 24 species were sampled: 54 (33 %) primates, 45 (27.4 %) ungulates, 39 (23.8 %) rodents, five (3 %) reptiles, 10 (6.1 %) pangolins and 11 (6.7 %) carnivores (Table I). Trypanosomes were observed in three out of 50 QBC tests

Common name	Species name	Total tested	Species of trypanosome					
			TB	TBG1	TCRF	TCS	TV	TSM
Brush-tailed porcupine	*Atherurus africanus*	20	5	2	–	2	2	2
Giant rat	*Cricetomys gambianus*	17	2	3	–	1	1	–
Sun squirrel (red-legged)	*Heliosciurus rufobrachium*	2	1	–	–	–	–	–
	Rodents Sub-Total	**39**	**8**	**5**	**–**	**3**	**3**	**2**
Duiker (blackstiped)	*Cephalophus dorsalis*	7	2	1	–	–	1	–
Blue Duiker	*Cephalophus monticola*	30	3	1	–	1	2	–
Duiker (ogilby's)	*Cephalophus ogilbyi*	1	–	–	–	–	–	–
Duiker (yellow-backed)	*Cephalophus sylvicultor*	2	–	–	–	1	–	–
Duiker (peter's)	*Cephalophus callipygus*	1	–	–	–	–	–	–
Sitatunga	*Tragelaphus spekei*	3	–	–	–	–	–	–
Royal antelope	*Neotragus pygmaeus*	1	–	–	–	–	–	–
	Ungulates Sub-Total	**45**	**5**	**2**	**–**	**2**	**3**	**–**
Mangabey (white-eyelid)	*Cercocebus torquatus*	1	–	1	–	–	–	–
Moustached monkey	*Cercopithecus cephus*	13	1	–	–	1	1	–
Mona monkey	*Cercopithecus mona*	3	–	–	–	–	1	–
Greater white-nosed monkey	*Cercopithecus nictitans*	16	2	2	–	–	2	–
Mandrill	*Mandrillus sphinx*	4	–	–	–	–	–	–
Dwarf guenon	*Miopithecus talapoin*	12	2	–	–	–	3	–
Galago	*Perodicticus potto*	2	1	–	–	–	1	–
Golden Potto	*Arctocebus calabarensis*	3	1	–	1	–	–	–
	Primates Sub-Total	**54**	**7**	**3**	**1**	**1**	**8**	**–**
Long-tailed pangolin	*Manis tetradactyla*	5	1	–	–	–	–	–
Tree pangolin	*Manis tricuspis*	5	1	–	–	–	1	–
	Pangolins Sub-Total	**10**	**2**	**–**	**–**	**–**	**1**	**–**
Palm civet (two spotted)	*Nandinia binotata*	8	1	2	–	–	1	–
Small-spotted genet	*Genetta servalina*	2	–	1	–	1	1	–
African civet	*Viverra civetta*	1	–	–	–	–	–	–
	Carnivores Sub-Total	**11**	**1**	**3**	**–**	**1**	**2**	**–**
Monitor lizard	*Varanus niloticus*	5	–	–	–	–	1	–
	Reptiles Sub-Total	**5**	**–**	**–**	**–**	**–**	**1**	**–**
	Total	164	23	13	1	7	18	2
			14 %	8 %	0.6 %	4.3 %	11 %	12 %

Table 1. – PCR results on animal blood using different specific primers. TB: *T. brucei* non *gambiense* group 1; TBG1: *T. b. gambiense* group 1; TCRF: *T. congolense* "forest type"; TCS: *T. congolense* "savannah type"; TV: *T. vivax*; TSM: *T. simiae*.

but only one was confirmed positive by PCR and identified as *T. vivax*.

Seven of the 35 KIVI were positive, but the parasites did not grow when transfered into Cunnigham culture medium. For these seven positive samples, three were confirmed by PCR: one *T. brucei* s.l./*T. vivax* mixed infection and two *T. vivax* single infections.

The PCR results obtained with *T. brucei* s.l. (TB), *T. b. gambiense* group 1 (TBG1), *T. congolense* "forest" (TCF), *T. congolense* "savannah" (TCS), *T. vivax* (TV) and *T. simiae* (TSM) specific primers are given in Table I. The prevalences observed were as follows:

- 22 % for *T. brucei* s.l. of which 8 % were *T. b. gambiense* group 1,
- 11 % for *T. vivax*,
- 4.3 % for "savannah type" *T. congolense*,
- 1.2 % for *T. simiae*,
- 0.6 % for "forest type" *T. congolense*.

T. brucei s.l. (non-*gambiense* group 1) and *T. b. gambiense* group 1 DNA were respectivelly detected in 13 (54 %) and eight (33.3 %) of the 24 animal species studied. Trypanosomes pathogenic to Man (TBG1) were found in two species of rodents (*Atherurus africanus* and *Cricetomys gambianus*), in two species of ungulates (*Cephalophus dorsalis* and *C. monticola*), in two species of carnivores (*Nandinia binotata* and *Genetta servalina*) and in two species of monkeys (*Cercocebus torquatus* and *Cercopithecus nictitans*). *T. vivax* is fairly represented as 11 % of the animals were found positive: it was present in all groups of animals examined. 12 mixed infections were identified:

- four with *T. brucei* non-*gambiense* group 1 and TV (one *M. talapoin*, one *A. africanus*, one *N. binotata* and one *P. potto*),
- three with TBG1 and TV (one *C. gambianus*, one *C. nictitans* and one *C. dorsalis*),
- one with *T. brucei* non-*gambiense* group 1 and TCF (*A. calabarensis*),
- one with *T. brucei* non-*gambiense* group 1 and TCS (*A. africanus*),
- one with TBG1 and TCS (*C. gambianus*),
- one with TV and TSM (*A. africanus*),
- and one with TBG1, TCS and TV (*G. servalina*).

DISCUSSION

The detection of trypanosomes in the blood is often made difficult because of the relatively poor sensitivity of the parasitological techniques and parasitaemia that is often low and fluctuating. These was also the case in this study where the QBC method detected trypanosomes in only 6 % of our samples (24.5 % by PCR). Only three out of seven positive KIVI were confirmed by PCR suggesting that trypanosome species other than those detected by the specific primers used in this study could be present in our samples.

Because of its high sensitivity and specificity, PCR allows detection and identification of trypanosome species within naturally infected hosts thus avoiding selection bias introduced when parasites are grown in culture for example. This technique shows the important number of trypanosome species harboured by wild animals in the forest belt of southern Cameroon. Nevertheless, a PCR positive result indicates the presence of the corresponding parasite DNA and not necessarily an active infection.

The fact that 8 % of the animals examined were positive for *T. b. gambiense* group 1, the parasite responsible in 80 % of the cases for the chronic form of the disease in West and Central Africa (Gibson, 1986), is surprising as previous authors rarely reported the presence of this parasite in wild game (Truc *et al.*, 1997a).

Five of the 13 animals positive for TBG1 by PCR were rodents (*Atherurus africanus* and *Cricetomys gambianus*). This last rodent is an excellent laboratory host for *T. b. gambiense* and tse-tse flies feed avidly on this animal in experimental conditions (Larivière, 1957; Van den Berghes *et al.*, 1963). It has been demonstrated for *C. gambianus* that tse-tse flies are able to penetrate an experimentally constructed burrow to feed on them (Molyneux, 1971). These flies are probably attracted by the more favorable humidity and temperature conditions in the burrow and possibly odours. Moreover, it is possible for *C. gambianus* to be bitten by *G. palpalis* and *G. caliginea* because they are found together in peridomestic habitats in Cameroon (Nash, 1970; Molyneux, 1973).

Three monkeys (*Cercocebus torquatus* and *Cercopithecus nictitans*) among 54 non-human primates tested were positive for TBG1 by PCR. Several experimental transmissions have been carried out on different species of monkeys (*Cercopithecus*, *Cercocebus*, *Erythrocebus*). Important transmission indices have been observed with the Mangabey (*Cercocebus galeritus agilis*) though infection under experimental conditions have been difficult (Van Hoof, 1947). However, blood meal analysis of tsetse flies show that very few of them were taken from primates other than man (Jordan *et al.*, 1961).

Despite the low number of small carnivores, two *Nandinia* and one *Genetta* were found positive for *T. b. gambiense* group 1. As far as we know, it is the first time that *T. b. gambiense* group 1 was identified in such wild animals.

Several ungulates species (*Kobus kob*, *Alcelaphus bubalus*) were found positive for *T. b. gambiense*-like trypanosomes in West Africa (Mehlitz, 1986; Guedegbe *et al.*, 1992; Truc *et al.*, 1997a). Nevertheless, our study is the first report suggesting the presence of gambian trypanosomes in wild ungulates (duiker and blue duiker) in the central African forest belt.

Nevertheless, the presence of *T. b. gambiense* strains in a particular animal does not mean that such an animal is an important reservoir host.

There is an urgent need to confirm this presence of *T. b. gambiense* in wild animals by a large scale study of specimens sampled during various seasons of the year and a systematic use of KIVI in order to increase the chances of isolating parasite stocks for mass culture and isoenzyme characterization. Complementary studies remain necessary given the low number of specimens for some animal species included in our study. There is also need to study the trophic preferences of vectors and quantify the man/wild animal reservoir contacts within a given locality. These studies should enable a better comprenhension of epidemiology of sleeping sickness, on the phenomenon of cyclical resurgence, the maintenance and spread of the disease.

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