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 trypanosom species present in wild animal captured by hunters in the southern forest belt of Cameroon (Bipindi). Trypanosom species 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. I was 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 (Nandinia binotata and Genetta servalina) and in rodents (Cricetomys gambianus and Atherurus africanus). 13 species (54%) were carrying T. brucei s.l. identified as nongambiense group 1.

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

D espite 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 persistence 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 rhodesiensis 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

Résumé : Identification par PCR des trypanosomes chez les animaux sauvages du Sud-Cameroun


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

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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 enabled 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 MgCl2, 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).

RESULTS

64 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
The prevalences observed were as follows:

- T. b. gambiense (54%) and eight (33.3%) of the 24 animal species - 4.3% for “savannah type”
- 11% for “forest type”
- 0.6% for “forest type” T. congolense

Parasite was confirmed by PCR: one T. b. gambiense group 1, T. b. gam­biense (TBG1), T. congolense “forest type” (TCF), T. congolense “savannah type” (TCS), TV, and TSM.

Seven of the 35 KIVI were positive, but the parasites did not grow when transferred into Cunningham culture medium. For these seven positive samples, three were confirmed by PCR: one T. b. gambiense group 1 and T. congolense “forest type”, T. congolense “savannah type”, TV and TSM.

The PCR results obtained with 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. 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.

Two species of rodents (Antherurus africanus and Cricetomys gambianus), in two species of ungulates (Cephalophus dorsalis and C. monticola) and in two species of monkeys (Cercocetes 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. b. gambiense group 1 and TV (one M. talapoin, one A. africanus, one N. binitata and one P. potto),
- three with TBG1 and TV (one C. gambianus, one C. nictitans and one C. dorsalis),
- one with T. b. gambiense group 1 and TCF (A. calabarensis),
- one with T. b. 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).

Table 1. - PCR results on animal blood using different specific primers. TB: T. brucei non-gambiense group 1; TBG1: T. b. gambiense group 1; TCF: T. congolense “forest type”; TCS: T. congolense “savannah type”; TV: T. vivax; TSM: T. simiae.

<table>
<thead>
<tr>
<th>Common name</th>
<th>Species name</th>
<th>Total tested</th>
<th>Species of trypanosome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TB</td>
<td>TBG1</td>
</tr>
<tr>
<td>Brush-tailed porcupine</td>
<td><em>Atherurus africanus</em></td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>Giant rat</td>
<td><em>Cricetomys gambianus</em></td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>Sun squirrel (red-legged)</td>
<td><em>Heliosciurus rufobrachium</em></td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><strong>Rodents Sub-Total</strong></td>
<td></td>
<td>39</td>
<td>8</td>
</tr>
<tr>
<td>Duiker (blackstiped)</td>
<td><em>Cephalophus dorsalis</em></td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Blue Duiker</td>
<td><em>Cephalophus monticolana</em></td>
<td>30</td>
<td>3</td>
</tr>
<tr>
<td>Duiker (ogilby’s)</td>
<td><em>Cephalophus ogilby</em></td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>Duiker (yellow-backed)</td>
<td><em>Cephalophus silviculter</em></td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>Duiker (peter’s)</td>
<td><em>Cephalophus callipygus</em></td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>Sitatunga</td>
<td><em>Tragelaphus speki</em></td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>Royal antelope</td>
<td><em>Neotragus pygmaeus</em></td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td><strong>Ungulates Sub-Total</strong></td>
<td></td>
<td>45</td>
<td>5</td>
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<tr>
<td>Mangabey (white-eylid)</td>
<td><em>Cercocebus torquatus</em></td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>Moustached monkey</td>
<td><em>Cercopithecus cephus</em></td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>Mona monkey</td>
<td><em>Cercopithecus mona</em></td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>Greater white-nosed monkey</td>
<td><em>Cercopithecus nictitans</em></td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>Mandrill</td>
<td><em>Mandrillus sphinx</em></td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td>Dwarf guenon</td>
<td><em>Miopithecus talapoin</em></td>
<td>12</td>
<td>2</td>
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<tr>
<td>Galago</td>
<td><em>Perodicticus potto</em></td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Golden Potto</td>
<td><em>Arctocebus calabarensis</em></td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td><strong>Primates Sub-Total</strong></td>
<td></td>
<td>54</td>
<td>7</td>
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<tr>
<td>Long-tailed pangolin</td>
<td><em>Manis tetradactyla</em></td>
<td>5</td>
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</tr>
<tr>
<td>Tree pangolin</td>
<td><em>Manis tricuspis</em></td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td><strong>Pangolins Sub-Total</strong></td>
<td></td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Palm civet (two spotted)</td>
<td><em>Nandinia binitata</em></td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Small-spotted genet</td>
<td><em>Genetta servalina</em></td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>African civet</td>
<td><em>Vivera citetta</em></td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td><strong>Carnivores Sub-Total</strong></td>
<td></td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>Monitor lizard</td>
<td><em>Varanus niloticus</em></td>
<td>5</td>
<td>–</td>
</tr>
<tr>
<td><strong>Reptiles Sub-Total</strong></td>
<td></td>
<td>5</td>
<td>–</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>164</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>% 8</td>
</tr>
</tbody>
</table>

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Note de recherche
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 africanaus 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 (Lariviére, 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, Cercocbus, Erythrocebus). Important transmission indices have been observed with the Mangabey (Cercocbus 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 comprehension of epidemiology of sleeping sickness, on the phenomenon of cyclical resurgence, the maintenance and spread of the disease.

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