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Detection of trypanosome DNA in serologically positive but aparasitaemic horses suspected of dourine in Ethiopia

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A field study of horses was conducted in the province of Bale, Ethiopian highlands. A rapid questionnaire analysis indicated that dourine, known as "Dirressa", is a major health problem of equines in this area. A total of 121 horses suspected of dourine were examined by use of clinical, parasitological, serological and DNA based techniques. Incoordination of hindlegs (76 %), swelling of external genitalia (48.8 %) and emaciation (39.7 %) were the most common clinical signs observed. Using the haematocrit centrifugation technique (HCT), no trypanosomes were detected in blood, genital washes or tissue fluids. By contrast, trypanosome specific DNA products were amplified by PCR and subsequently detected by DNA probe hybridization in blood samples of 29 horses (29/104), all serologically positive by CFT and/or ELISA. Positive PCR results were significantly associated with swelling of external genitalia (*P*< 0.05). There is strong evidence, although there was no direct detection of *T. equiperdum*, that dourine is highly prevalent in the area, a finding which is in accordance with earlier reports. It is concluded, that this PCR assay provides a very sensitive tool in the diagnosis of active infections of dourine in endemic areas where trypanocidal drug use is common.

Keywords : Dourine, *Trypanosoma equiperdum*, Diagnosis, PCR, Ethiopia

INTRODUCTION

Dourine, caused by *Trypanosoma* (*Trypanozoon*) *equiperdum* Doflein, 1901, has been recognised as a disease of breeding solipeds for many centuries. Diagnosis of *T. equiperdum* in chronically infected horses or donkeys is difficult, due to the low number of parasites present in peripheral circulation or tissue fluids [1,4,8,13]. Serological tests relying on antibody detection are more sensitive. However, they fail to distinguish between an active infection and a cured one.

The objective of this study was to evaluate whether the polymerase chain reaction (PCR) might provide a more sensitive technique in the diagnosis of an active infection.

MATERIALS AND METHODS

Study area: The field study was conducted in the administrative region of Bale, 470 km south of Addis Ababa. The Bale people, like many others in remote areas of the Ethiopian highlands where communication is poor, use horses as pack animals as well as for farm work [1]. Dourine has been reported in the area in the past [1,7].

Study population: A total of 87 female and 34 male horses suspected of dourine by the veterinary authorities were examined by use of clinical, parasitological, serological and DNA based techniques.

Questionnaire analysis: The farmers were interviewed about breeding and management conditions, as well as predominant health problems among their horses. Veterinary personnel was also interviewed about control measures against dourine.

Clinical investigations: By giving special attention to the reproductive organs, each

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horse was physically examined. The external genitalia was observed for any abnormal conditions such as swelling and discharge. To detect any abnormal gait, each horse was observed while it was standing and trotting.

Parasitological investigations: a) Blood samples: A heparinized microhaematocrit capillary was filled with approximately 60μl of blood and centrifuged for 5 minutes at 10,000× g as done during measurements of PCV. The buffy coat-plasma junction was examined under a microscope using ×10 and ×40 objectives and ×10 eyepieces. In a positive diagnosis, trypanosomes are found at the junction of the plasma and buffy layer in the centrifuged blood [16]. b) Genital washes: Vaginal- and preputial washes were collected with normal saline and centrifuged for 5 min at 1,000× g. A drop of sediment was put onto a slide, covered with a cover slip and examined under a microscope using ×10 and ×40 objectives and ×10 eyepieces. c) Mice inoculation: Fresh blood (0.5 ml) and the sediment of genital washes of 35 horses were inoculated into 70 mice (Swiss white). The mice were monitored every other day for the presence of trypanosomes for a period of 60 days. A drop of tail blood was examined as a wet film under a microscope of 400× magnification.

Polymerase chain reaction (PCR) : a) DNA extraction: EDTA blood (250μl) was mixed with equal volume of lysis buffer (0.32 M sucrose; 0.01 M Tris; 0.005 M $MgCl_2$; 1% Triton X-100; pH 7.5), centrifuged at 12,000× g for 25 s, and the pellet which was formed was washed 3 times with lysis buffer. The final pellet was resuspended into a 250μl PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100; pH 8.3) containing 50μg proteinase K/ml, incubated at 56℃ for 1 h and at 95℃ for 10 min.
b) DNA amplification: Standard PCR amplifications were carried out in 25μl reaction mixtures containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.1% Triton X-100, 1.25 mM $MgCl_2$, 200μM from each of the four dNTP's, 0.5 units of Taq DNA polymerase (Appligene, Illkirch, France) and 1μM from each of the oligonucleotide primers. The template DNA added to the reaction mixture was either 10 ng of purified trypanosome DNA or, in the assays to detect trypanosome DNA in horses, 2% of the DNA extracted from 250μl blood. Oligonucleotide primers (nuclear repeat primers, NRP 1 and 2) specific for trypanosomes of the subgenus *Trypanozoon* were used [3]. The reaction mixtures were covered with 25μl of paraffin oil and cycled in a programmable thermocycler (Trio-Thermoblock®, Biometra, Goettingen, Germany). During each cycle the samples were incubated at 94℃ for 90 sec (to denature the template) followed by annealing the primers at 55℃ for 90 sec and extension of the annealed primers at 72℃ for 120 sec. This was repeated for 34 cycles. Fifteen μl of each sample were electrophoresed in a 3% agarose gel containing 0.5μg/ml ethidium bromide.

DNA hybridization: After DNA amplification of the test samples, the PCR products were immobilized onto nylon membranes by slot-blotting as described earlier [14]. The DNA probe was prepared as follows: A clone of *T. brucei* ILTat 1.4 [9] was expanded in mice, separated from infected blood by DEAE-cellulose [11] and submitted to DNA extraction and PCR as mentioned earlier. The generated PCR product was purified by use of the QIAquick PCR-purification kit® (Qiagen, Hilden, Germany) and the DNA yield was determined as described before. Probe labelling and hybridization was based on enhanced chemiluminescence using the ECL direct nucleic acid labelling and detection kit® (Amersham, UK) according to the manufacturer's recommendations.

Detection of trypanosomal antibodies: The complement fixation test (CFT) was performed in accordance with the procedure of Alton et al. [2] for the detection of brucella antibodies. The enzyme-linked immunosorbent assay (ELISA) was carried out according to O.I.E. standard procedures [13]. Antigen was isolated from highly parasitaemic rats infected with *T. equiperdum* (strain Alfort, BgVV). Trypanosomes were separated from blood cells by passing blood from infected rats through a column of anion exchanger, DEAE-cellulose [11]. For antigen preparation, the trypanosomes were washed and disintegrated by three times freezing/thawing or ultrasonification. The working dilution of the antigen was assessed by checkerboard titration against a positive serum [15].

RESULTS

Questionnaire analysis: According to the people interviewed, dourine is known locally

as 'Diressa' . The farmers reported that the first sign of the disease in affected horses is incoordination, especially of the hind quarters, and swelling of the external genitalia. The farmers claimed that the disease is a major health problem of horses causing high mortality. The disease has a seasonal character which coincides with the breeding season (autumn). Horses are treated against dourine only irregularly, when trypanocidal drugs are available. The drug used in the area is quinapyramine sulphate (Triquin-S®, Wockhardt Veterinary Ltd., India).

Clinical signs: The most dominant clinical signs observed were incoordination of hindlegs (76%), oedematous swelling of external genitalia (48.8%) and emaciation (39.7%). These signs were observed in both sexes with different frequencies in male and female horses. The frequency of oedematous swelling of external genitalia in female horses (48 out of 87 = 55.2%) was higher than in males (11 out of 34 = 32.4%), whereas the observation of incoordination of hindlegs in male horses (29/34 = 85.3%) was more common as in females (63/87 = 72.4%).

Parasitological examinations: No trypanosomes could be detected in blood samples by HCT. Mice inoculated with blood samples or genital washes remained parasitologically negative.

DNA amplification and probe hybridization: Using the nuclear repeat primer pair NRP 1/2, specific PCR products of 177 bp could be amplified in blood samples of 23 (23/104 = 22.1%) horses (Fig. 1). Subsequent slot-blot hybridization with a DNA probe specific for *Trypanosoma* spp. of the subgenus *Trypanozoon* produced strong signals with PCR products generated from blood samples of 29 horses (Fig. 2).

Detection of trypanosomal antibodies:

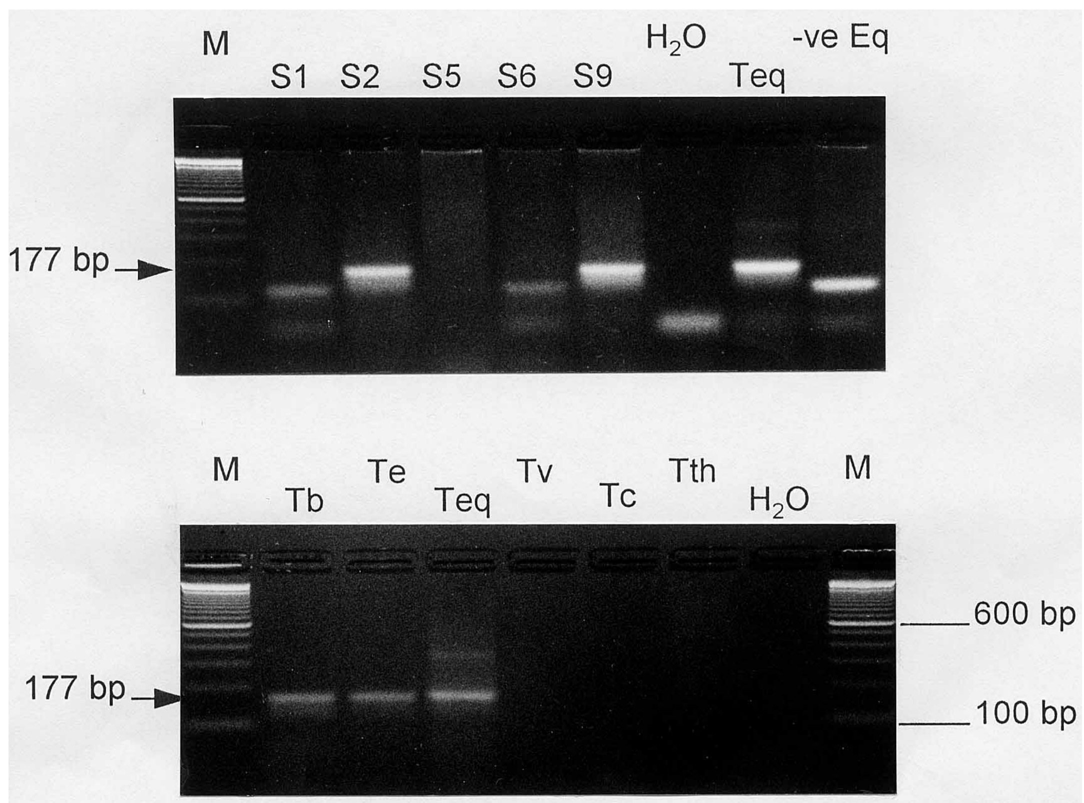

Fig. 1 Ethidium bromide stained agarose gel showing the PCR amplification products (177 bp) of the nuclear repeat primers (3) from blood samples of horses (S) suspected of dourine and DNA of *Trypanosoma* (*Trypanozoon*) *equiperdum* (Teq), *T.* (*T.*) *brucei* (Tb), *T.* (*T*) *evansi* (Te), *T. vivax* (Tv), *T. congolense* (Tc) and *T. theileri* (Tth). Sample S2 and S9 are PCR positive. (M: 100 bp ladder; -ve Eq: total DNA extracted from blood of an uninfected horse; H_2O: no DNA)

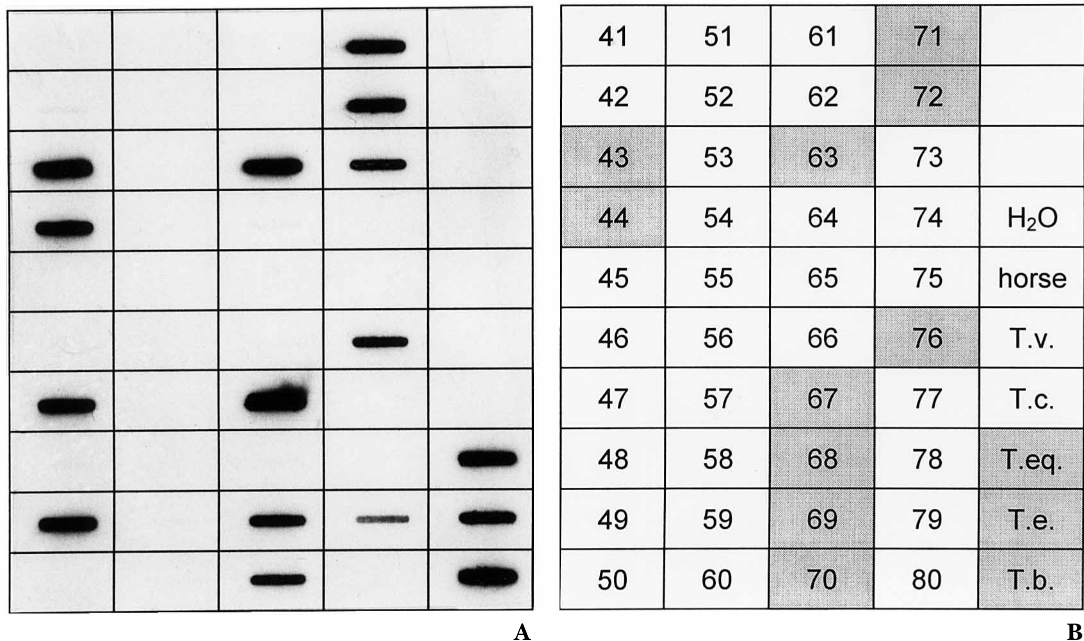

Fig. 2 **A.** Slot-blots of PCR amplified DNA products generated from 40 blood samples (41 - 80) of aparasitaemic horses suspected of dourine showing the hybridization with the *Trypanozoon* specific DNA probe. **B.** The designation of the slot-blotted samples on the hybridized membrane. Samples which tested already positive by PCR are indicated by a shadowed background. [*Trypanosoma* (*Trypanozoon*) *equiperdum* (T.eq.), *T.* (*T.*) *brucei* (T.b.), *T.* (*T.*) *evansi* (T.e.), *T. vivax* (T.v.), *T. congolense* (T.c.), DNA extracted from blood of uninfected horse (horse), no DNA (H_2O)]

Among 120 sera tested, 34 (28.3%) were positve by CFT (sera showing more than 50% fixation at a dilution of 1/5 or higher) and 51 (42.5%) had increasing ELISA readings. Of the 23 PCR positive horses, 22 were serologically positive by CFT. All horses which tested positive by PCR/DNA probe hybridization were serologically positive by CFT and/or ELISA. The detailed serological results will be published elsewhere.

DISCUSSION

The clinical signs common to dourine, such as incoordination of hindlegs and oedematous swelling of external genitalia [1,4, 8, 13,15], were observed in horses of this study. However, attempts to detect the causative agent of dourine by microscopy in blood samples and genital washes failed. By contrast, specific DNA products could be amplified by PCR and subsequently detected by DNA probe hybridization in blood samples of 29 horses (29/104), all serologically positive for trypanosomal antibodies by CFT and/or ELISA. This observation is in agreement with a report given by Kanmogne and co-workers [10] who were able to amplify trypanosome DNA in serologically positive but aparasitaemic Gambian sleeping-sickness suspects in Cameroon.

A higher sensitivity of the PCR assay as compared to the direct parasitological techniques has been reported in the past. Wuyts and colleagues [17] observed in a cow experimentally infected with *T. evansi,* that PCR signals appeared as soon as two days post-infection, whereas positive detection by microscopy followed two days later. Masake and co-workers [12] experimentally infected three cows with isolates of *T. vivax.* The PCR revealed *T. vivax* DNA in the blood samples as early as 5 to 7 days post infection. The parasites were detected by microscopy 4 to 5 days later. Clausen et al. [6] conducted a PCR assay on blood samples from Ankole cattle experimentally infected with *T. brucei.* Specific PCR products could be amplified as soon as one day post infection, whereas pos-

itive detection by microscopy followed 4 days later. PCR signals remained positive during infection, although aparasitaemic phases occurred.

Due to the uncertainty of detecting *T. equiperdum* in the tissues and in the bloodstream of equines suffering from dourine [1,4,8,13], in practice this disease is diagnosed indirectly on the basis of clinical signs, and serologically by the complement fixation test [13]. Serological tests relying on antibody detection are more sensitive, however, they fail to distinguish between an active infection and a cured one. Clausen and co-workers monitored the trypanocidal activity of diminazene aceturate (Berenil®, Hoechst AG) in experimentally with *T. brucei* infected cattle by DNA amplification [5,6]. PCR signals disappeared two to three days after treatment in cattle which responded clinically well to treatment. In cattle, however, which showed clinical signs of CNS involvement and which died before the end of the experiment, although aparasitaemic after treatment, specific signals could be amplified on several occasions post treatment. The authors concluded, that PCR provides a sensitive tool to assess the therapy effectiveness and disease progression in trypanosomosis, especially when trypanosomes are sequestered in cryptic sites. There is evidence that the occurrence of nervous symptoms in dourine infected horses is associated with the presence of *Trypansoma equiperdum* parasites in cerebrospinal fluid [4].

As *Trypanosoma equiperdum* is the only trypanosome to affect horses in temperate climates, the confirmation of the parasite - or the detection of trypanosomal antibodies - is sufficient for a positive diagnosis. However, in countries of Africa where overlap of *T. equiperdum* and *T. brucei* spp. exists, it is difficult to distinguish *T. equiperdum* microscopically — or serologically — from other members of the subgenus *Trypanozoon* [13]. The nuclear repeat primer set used in this study can neither distinguish between *T. equiperdum* and *T. brucei* spp. in PCR-based assays (3, Fig. 1). Until DNA sequence differences between these species are known, the diagnosis of dourine will rely on circumstantial evidence.

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