INHIBITION OF THE DNA AMPLIFICATION OF TRYPANOSOMES PRESENT IN TSETSE FLIES MIDGUTS: IMPLICATIONS FOR THE IDENTIFICATION OF TRYPANOSOME SPECIES IN WILD TSETSE FLIES

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Summary:

The present study was carried out in order to investigate if there was really a failure of PCR in identifying parasitologically positive tsetse flies in the field. Tsetse flies (*Glossina palpalis gambiensis* and *Glossina morsitans morsitans*) were therefore experimentally infected with two different species of *Trypanosoma* (*Trypanosoma brucei gambiense* or *Trypanosoma congolense*). A total of 152 tsetse flies were dissected, and organs of each fly (midgut, proboscis or salivary glands) were examined. The positive organs were then analysed using PCR. Results showed that, regardless of the trypanosome species, PCR failed to amplify 40 % of the parasitologically positive midguts. This failure, which does not occur with diluted samples, is likely to be caused by an inhibition of the amplification reaction. This finding has important implications for the detection and the identification of trypanosome species in wild tsetse flies.

KEY WORDS : *Glossina palpalis gambiensis, Glossina morsitans morsitans, Trypanosoma brucei gambiense, Trypanosoma congolense,* PCR inhibition.

Résumé : INHIBITION DE L'AMPLIFICATION DE L'ADN DE TRYPANOSOMES PRÉSENTS DANS LE TUBE DIGESTIF DE MOUCHES TSÉTSÉ : IMPLICATION POUR L'IDENTIFICATION DES ESPÈCES DE TRYPANOSOMES CHEZ LES GLOSSINES SUR LE TERRAIN

*La présente étude a été conduite dans le but de vérifier si l'échec de la PCR pour identifier les mouches tsétsé porteuses de trypanosomes sur le terrain était du à une inhibition de la réaction d'amplification ou bien à l'absence d'amorces spécifiques de certaines souches de trypanosomes. Pour ce faire, des infections expérimentales de glossines (*Glossina palpalis gambiensis *et* Glossina morsitans morsitans*) avec deux espèces différentes de trypanosomes (*Trypanosoma brucei gambiense *ou* Trypanosoma congolense*) ont été réalisées. 152 glossines ont été disséquées et les organes de chaque mouche (intestin, proboscis ou glandes salivaires) ont été examinés. Les organes positifs ont ensuite été analysés par PCR. Les résultats montrent que, quelle que soit l'espèce de trypanosomes, 40 % des intestins positifs à l'observation ne sont pas amplifiés par la PCR. Ce problème, résolu en diluant les échantillons, est probablement dû à l'inhibition de la réaction d'amplification. Ceci a d'importantes implications pour la détection et l'identification des espèces de trypanosomes chez les glossines sur le terrain.*

MOTS CLÉS : Glossina palpalis gambiensisis, Glossina morsitans morsitans, Trypanosoma brucei gambiense, Trypanosoma congolense, *inhibition de la PCR.*

The various trypanosome species infecting tsetse flies must be accurately identified if a better understanding of the epidemiology of the disease is to be achieved. Due to its higher sensitivity, polymerase chain reaction (PCR) tends to complete or replace the traditional dissection and examination technique used to detect and identify trypanosome infections in wild tsetse flies (McNamara *et al.*, 1995; Solano *et al.*, 1995, 1996). Moreover, its high specificity, makes it possible to identify the trypanosomes below subgenus level and, importantly, to reveal mixed infections (Masiga *et al.*, 1996; Morlais *et al.*, 1998a).

However, PCR sometimes failed to identify parasitologically positive flies (Woolhouse *et al.*, 1996; Morlais *et al.*, 1998b). The question remains whether this could be due to inhibitors of the amplification reactions or to the absence of certain primers specific of existing trypanosome subgroups (McNamara *et al.*, 1994; Solano *et al.*, 1995). Another reason might be that divergence between trypanosome sequences could exist according to geographical location, thus preventing species-specific primers from recognizing all the trypanosomes of a same species (Morlais *et al.*, 1998b).

Previous observations (data not shown) led us to suspect PCR inhibition could exist. To test this hypothesis, experimental infections of tsetse flies were conducted with two *Trypanosoma*, *Trypanosoma brucei gambiense* or *Trypanosoma congolense* savannah type, for which specific primers are available. Tsetse organs were first observed by microscopy, then positive organs were subjected to PCR using the corresponding specific set of primers.

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MATERIALS AND METHODS

Tsetse and trypanosomes

The *Glossina palpalis gambiensis* and *Glossina morsitans morsitans* used here are bred in colonies kept at CIRAD-EMVT in Montpellier, France, and originally come from, respectively, Burkina Faso and Zimbabwe. *T. congolense* clone E325 (savannah type) was isolated from infected wild *Glossina pallidipes* caught in Uganda (Uilenberg *et al.*, 1973), whereas *T. b. gambiense* A005 was isolated in 1989 from humans, in Fontem, Cameroon (Dukes *et al.*, 1989).

Infection of tsetse flies

G. p. gambiensis were infected with procyclic culture forms of *T. b. gambiense* using the membrane feeding technique. Flies failing to feed were removed from the experiment and the remaining blood-filled flies were subsequently left to feed on an uninfected rabbit until the end of the experiment. *G. p. gambiensis* and *G. m. morsitans* were infected with *T. congolense* clone E325 by letting teneral flies feed on the belly of infected mice (when parasitaemia was between 3×10^7 and 10^8 trypanosomes/ml). Again, flies failing to feed were removed and the remaining blood-filled flies were subsequently maintained on two uninfected rabbits, one for each fly species, until the end of the experiment. Infection was assessed by dissecting each fly after four days starvation (five or six days for the most part) and 48 days post-infection, with examination of the midgut and salivary glands (when infected with *T. b. gambiense*) or midgut and proboscis (in the case of *T. congolense*) by phase-contrast microscopy.

20 *G. p. gambiensis* (numbered from 1 to 20) were given a feed infected with *T. b. gambiense* and subsequently checked for the presence of *T. b. gambiense* while 65 *G. p. gambiensis* (numbered from 21 to 85) and 67 *G. m. morsitans* (numbered from 86 to 152) were given a feed infected with *T. congolense* and subequently checked for the presence of *T. congolense* by microscopy.

PCR

Each parasitologically positive fly organ was recovered from slide in 30 µl of sterile water and incubated one hour at 56° C and 30 minutes at 95°C in 30 µl of a 5 % chelex 100 resin (Biorad, CA, USA). After centrifugation, 10 µl of the supernatant (referred as standard conditions), were used for subsequent DNA amplification. PCR was carried out using a DNA thermalcycler (MJ Research, Cambridge, UK) with final volumes of 50 µl containing 10 pmoles of each primer (TCS1 and TCS2 for *T. congolense,* or TBR1 and TBR2 for *T. b. gambiense,* described by Masiga *et al.*, 1992), 0.2 mM of each deoxyribonucleotide, 1 X incubation buffer with 1.5 mM MgCl2 and 0.5 units of Taq polymerase (QBIOgene, Ilkirch, France). Samples were initially denatured at 94°C for three minutes and then processed through 45 cycles, each consisting of a denaturation step at 94°C for 30 s, an annealing step at 55° C (TCS1/TCS2) or 56° C (TBR1/TBR2) for 30 s and an extension step at 72° C for one minute. The final elongation step was lengthened to five minutes. Amplification products were checked by electrophoresis in 2 % agarose gels and visualised by ethidium bromide staining under UV light.

RESULTS

Microscopic observation revealed *T. b. gambiense* in seven midguts and two salivary glands from *G. p. gambiensis*. *T. congolense* were observed in 17 midguts from *G. p. gambiensis*, 10 midguts from *G. m. morsitans*, one proboscis from *G. p. gambiensis* and eight probosces from *G. m. morsitans*.

When all these parasitologically positive organs were subjected to PCR with the appropriate sets of primers, five/seven midguts from *G. p. gambiensis* infected with *T. b. gambiense* and 12/27 midguts from the two tsetse fly species infected with *T. congolense*, came out negative using standard PCR conditions (see Materials and Methods), whereas all the probosces and salivary glands were positive. It had been previously checked that organs from uninfected flies, used as negative controls, could not be amplified with the two pairs of primers used in the study.

By reducing the volume of chelex supernatant used, i.e. by diluting the sample, all the 17 "standard PCR conditions" negative midguts were successfully amplified (Fig. 1). The dilution necessary to obtain successful amplification differed from one midgut sample to the next: whereas amplification of the parasite DNA present in the midgut of tsetse fly n° 27 succeeded with

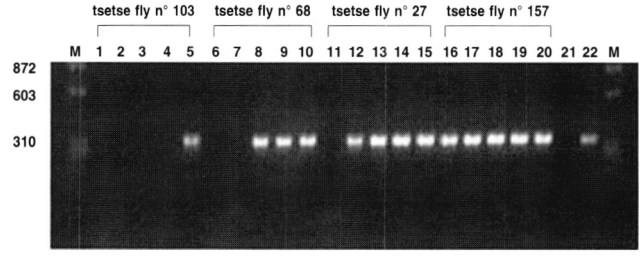

Fig. 1. – TCS1 and TCS2 PCR amplification of *Trypanosoma congolense* present into four different tsetse fly midguts using different volumes of chelex supernatant.

M: marker PhiX174/HaeIII; 21: PCR negative control; 22: PCR positive control.

1, 6, 11 and 16: PCR using 10 µl of chelex supernatant; 2, 7, 12 and 17: PCR using 5 µl of chelex supernatant; 3, 8, 13 and 18: PCR using 2 µl of chelex supernatant; 4, 9, 14 and 19: PCR using 1 µl of chelex supernatant; 5, 10, 15 and 20: PCR using 0.5 µl of chelex supernatant.

a volume of 5 µl (1/2 dilution), it needed a further reduction to 0.5 µl (1/20 dilution) in the case of tsetse fly n° 103. No amplification could be obtained by raising the volume of chelex supernatant above 10 µl. The "standard PCR conditions" positive midguts could also be amplified using a reduced volume of chelex supernatant (see tsetse fly n° 157, Fig. 1).

DISCUSSION

This study was designed to check whether PCR effectively fails to identify parasitologically positive tsetse flies in the field, as it has been previously hypothesized (Solano *et al.*, 1995; Morlais *et al.*, 1998b). PCR were carried out on tsetse organs that contained human (*T. b. gambiense*) or animal (*T. congolense*) trypanosomes, for which specific primers were available.

We observed that the amplification reaction only failed with the midgut samples. Such failure of PCR in identifying parasitologically positive midguts had already been reported (Masiga *et al.*, 1992; Masiga *et al.*, 1996; Woolhouse *et al.*, 1996; Reifenberg *et al.*, 1997; Lefrançois *et al.*, 1999). PCR lack of sensitivity cannot be incriminated since raising the quantity of parasite DNA used proved unsuccessful. On the other hand, since diluting the samples restored PCR performance, the likely cause of the failure lies in an inhibition of the parasite DNA amplification due to the cellular environment of the midgut. Although the optimal dilution varied, all the parasitologically positive midguts were successfully amplified using 0.5 µl of supernatant (1/20 dilution).

This has important implications in the field, where PCR is used to characterize trypanosome infections in wild tsetse flies (McNamara *et al.*, 1995; Lefrançois *et al.*, 1999). Even if species-specific primers did not cover all *Trypanosoma* possibly present in wild tsetse flies, our results suggest that PCR failure in the field may simply relate to an inhibition of the amplification process, leading to errors of estimation regarding trypanosomes of medical or veterinary importance.

In conclusion, great care must be taken when using PCR to detect and identify trypanosomes in the midguts of tsetse flies in the field. Several sample dilutions must be tested to avoid PCR failure, whether caused by inhibition or by the very small number of parasites sometimes present in tsetse midguts.

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