

SUITABILITY OF A RAPID DNA ISOLATION AND AMPLIFICATION FOR DETECTION OF *TRYPANOSOMA CRUZI* IN *TRITOMA INFESTANS* DRY FECAL SPOTS COLLECTED ON FILTER PAPER

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Summary:

A rapid DNA extraction was used for *T. cruzi* detection in triatomines dry fecal spots collected on filter paper and analyzed by PCR. Fifty *T. infestans* were fed on experimentally infected Balb/C mice with high *T. cruzi* parasitemia and divided into five groups of ten triatomines, and 100 triatomines were infected with lower parasitemia and divided into five groups of 20 triatomines. One dry fecal spot was analyzed per group on days 1, 2, 3, 4 and 5 post feeding. Amplification targeted *T. cruzi* TCZ sequence and resulted positive from day 4 after bugs feeding in the two models (high and lower parasitemia). The rapid DNA isolation and PCR proposed are suitable for detection of *T. cruzi* DNA in filter paper and should be considered in field research.

KEY WORDS : *Trypanosoma cruzi*, DNA, *Triatoma infestans*, filter paper, PCR, Chagas' disease.

Résumé : ADÉQUATION D'UNE TECHNIQUE RAPIDE D'EXTRACTION D'ADN ET AMPLIFICATION POUR LA DÉTECTION DE *TRYPANOSOMA CRUZI* DANS DES ÉCHANTILLONS DE FÈCES SECS DE *TRITOMA INFESTANS* RÉCOLTÉS SUR PAPIER FILTRE

Une technique d'extraction rapide d'ADN a été utilisée pour la détection de *T. cruzi* dans des excréments secs d'insectes obtenus sur papier filtre et analysés par PCR. Cinquante *T. infestans* ont été nourris dans des souris Balb/C infectées expérimentalement par *T. cruzi* (haute parasitémie), divisés dans cinq groupes de dix triatomines et 100 triatomines ont été infectées (faible parasitémie) et divisés dans cinq groupes de 20 triatomines. Un petit rond de papier filtre a été analysé par groupe aux premier, second, troisième, quatrième et cinquième jours après l'infection. L'amplification de la séquence TCZ de *T. cruzi* a été positive dès le quatrième jour dans les deux modèles (haute et faible parasitémie). La technique d'extraction d'ADN proposée suivie par PCR est appropriée pour la détection de *T. cruzi* sur papier filtre et pourrait être utilisée dans des travaux de terrain.

MOTS CLÉS : *Trypanosoma cruzi*, ADN, *Triatoma infestans*, papier filtre, PCR, maladie de Chagas.

Natural transmission of Chagas' disease occurs by skin contamination with feces of vectors (triatomines) containing infectant forms of *Trypanosoma cruzi* (Silveira & Vinhaes, 1998). Recently, an outbreak of acute Chagas' disease caused by infected sugar broth in southern Brazil reopened discussion on the use of molecular techniques in field research, and the need to reduce costs in order to make it feasible (Pérez-Gutierrez *et al.*, 2006). The aim of the present work was to determine whether a rapid and cost effective DNA isolation followed by PCR might be used for *T. cruzi* DNA detection in triatomines dry fecal spots collected on filter paper.

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MATERIALS AND METHODS

T. cruzi DNA was used to confirm analytical sensitivity of PCR and *Leishmania amazonensis*, *L. braziliensis* and *L. chagasi* DNA were tested to confirm specificity of our amplification system, prior to the present study.

Third- and fourth-instar *T. infestans* were fed on Balb/C mice infected with 10^4 parasites/ml of *T. cruzi* Y strain. Although it is well known that the Y strain is highly virulent, we decided to perform experimental infection on days 7 and 4 after mice inoculation attempting to attain approximately 3.2×10^5 parasites/ml and 10^3 parasites/ml, that we have called high and lower parasitemia, respectively. During preliminary studies we found that each insect sucked around 25 μ l of blood, although it is possible for one specific bug to fail to suck, or, on the contrary, to ingest more than 25 μ l. In the first part of the study, the mice presenting with high parasitemia (approximately 3.2×10^5 parasites/ml) were separated into five groups of ten triatomines each. As controls, 50 triatomines organized in five groups of ten insects each, were fed on non infected mice.

In the second part of the study, one hundred third- and fourth-instar *T. infestans* were fed on Balb/C mice with lower parasitemia or approximately 10^3 parasites/ml. Triatomines were organized in five groups of 20 triatomines each. As controls, 25 triatomines separated into five groups of five insects each were fed on non infected mice. Each group of triatomines was placed in a plastic vial internally recovered by a circular piece of filter paper (6.5 mm in diameter – FRAMA – 1064). Dry fecal spots of filter paper was obtained from each group by a puncher (ABC Instr. Cirurgicos-00426) and eluted in 200 μ l of sterile distilled water, in a 1.5 ml polypropylene microtube for the DNA extraction. Then, samples were boiled at 100° C for 20 min., re-centrifuged and supernatants containing DNA were stored at – 20° C until amplification (Breniere *et al.*, 1995). After thawing, samples were spun in a refrigerated micro centrifuge (Centrifuge 5417R, Eppendorf-2233, Hamburg, Germany) at 15,300 xg, for 5 min. and supernatants were submitted to quantification by UV spectrophotometry at 260 nm (DU-70 Spectrophotometer, Beckman-92634 California – USA) to roughly estimate DNA concentration. Amplifications were carried out with primers already described (Braz *et al.*, 2007) chosen within the *T. cruzi* TCZ repetitive sequence (Moser *et al.*, 1989) which generated a 144 bp amplification product. For the PCR, each 50 μ l reaction mixture contained approximately 100 ng of triatomines fecal DNA, 200 μ M dNTP, 0.4 μ M of each primer (sense F2: 5' – TGCACCTCGGCTGATCGTTTTTCGAG – 3' and anti-sense B3: 5'– AGGGTTGTTTG GTGTCCAGTGTGTG – 3'), synthesized by IDT (Integrated DNA Technologies, Belo Horizonte, Brazil), 1.5 mM MgCl₂, 2.5 U of Taq DNA polymerase (Invitrogen, Paisley, U.K.) and 25 mM (NH₄)₂SO₄, in a buffer containing 100 mM Tris-HCl and 500 mM KCl, at pH 8.4. PCR conditions were as follows: an initial denaturation step at 95° for 5 min, followed by 40 cycles of 95° C/1 min, 55° C/1 min, 72° C/1 min and a final extension of 72° C/5 min. One run with a positive control (*T. cruzi* DNA) and two with negative controls (sterile water instead of DNA) were included in each amplification experiment. Amplicons

were separated by electrophoresis in 3 % agarose gels (Sigma, USA), stained with ethidium bromide (0.5 ng/ml), and visualized by UV trans-illumination (Vilmar Lourmat, France). After the first amplification was performed in monoplicate, all negative samples had three other aliquots of the original extracted DNA retested by PCR. Finally, 200 fg of *T. cruzi* DNA (or the equivalent of one parasite genome) was added to all PCR-negative samples, in all groups, either from infected or non infected triatomines in order to certify the absence of amplification inhibitors.

RESULTS

Previous sensitivity tests demonstrated that the TCZ sequence is able to detect fractions (approximately 2 fg) of a single parasite (around 200 fg). When DNA from *Leishmania amazonensis*, *L. braziliensis* and *L. chagasi* were tested to verify specificity there was no amplification.

PCR was negative in all study times and all filter papers from non infected triatomines (controls), irrespective of the parasitemia considered (high or lower). All PCR negative samples revealed the 144 bp DNA fragment after addition of *T. cruzi* DNA.

Detection of parasite DNA occurred on day 5 post feeding in both parasite loads after the first amplification (monoplicate), whereas re-analyses of the primary DNA samples in triplicate have enabled us to detect the parasite one day earlier, *i.e.*, on day 4, also in the two situations, high and lower parasitemia (Table I).

DISCUSSION

PCR was negative in all non infected triatomines (controls), as expected, of either the high or the lower parasitemia studies. These results, together with those run with DNA from *Leishmania amazonensis*, *L. braziliensis* and *L. chagasi*, corroborated the specificity of our amplification system. Nevertheless, all negative filter paper samples revealed the 144 bp DNA fragment after addition of *T. cruzi* DNA thus confirming the inexistence of amplification inhibitor factors what was crucial to rule out the presence of inhibitors, that have already been reported by Breniere *et al.* (1995) who used the same boiling procedure of the present study, and Araújo *et al.* (2002) who used a more complex extraction procedure with phenol-chloroform extractions. Dorn *et al.* (1999; 2001) have associated amplification inhibition to the type of biological sample, suggesting that digestive tract samples, especially stomach contents are more prone to inhibit PCR.

We decided to increase the number of insects in the second study group (lower parasitemia model) with a

Days post-feeding

High parasitemia (n = 10 triatomines)	PCR	PCR-triplicate
Lower parasitemia (n = 20 triatomines)	high/lower parasitemia	high/lower parasitemia
1	negative/negative	negative/negative
2	negative/negative	negative/negative
3	negative/negative	negative/negative
4	negative/negative	positive/positive
5	positive/positive	positive/positive

Table I. – PCR results of filter papers contaminated with dry feces of *T. infestans* fed on mice infected by *T. cruzi* (high and lower parasitemia).

proportional reduction of the number of insects in the control group due to the loss of some insects during the first experiment (high parasitemia), and also because all negative triatomines did not generate any amplification during the first part of the study, taken into account that our capacity to breed and maintain triatomines was limited by the laboratory infrastructure. Another point that is worth mentioning was the decision to investigate one filter paper per group of insects instead of one filter paper per insect. When bugs are fed with animals not all of them get infected. This procedure was adopted in an attempt to homogenize the groups with respect to the number of infected insects and the total blood volume ingested considering the group as a whole.

Our DNA extraction method proved to be fast and cost-effective and succeed in amplifying *T. cruzi* DNA from day 4 post insects feeding what was more satisfactory than results obtained by others such as Machado *et al.* (2000) who have also used a boiling procedure to extract DNA and detected *T. cruzi* DNA on filter paper containing triatomines feces only on day 15 after the infective meal. Conversely, in the only report in which *T. cruzi* DNA was detected as soon as day 2 post feeding (Russomando *et al.*, 1996), the authors have studied digestive tract samples of insects fed on experimentally infected monkeys presenting with low parasitemia. It is noteworthy that they used the same PCR target (TCZ sequence), however with a distinct pair of primers, in a more complex and expensive proteinase K and phenol-chloroform DNA extraction protocol, and a different animal model (monkey). Shikanai-Yasuda *et al.* (1996) have compared a boiling procedure with a phenol-chloroform DNA extraction protocol and concluded that *T. cruzi* DNA detection was superior with the second procedure (28 % and 59 % of detection, respectively).

In a recent report (Braz *et al.*, 2007), DNA samples were obtained directly from digestive tract of infected triatomines, using the same murine model (high and lower parasitemia), and the same DNA isolation method, although directly from feces obtained from each insect. The authors detected positivity as soon as the first day after insects feeding, but digestive tract samples were obtained by extrusion during the first three days after blood meal, indicating that detection of *T. cruzi* DNA during the first 72 hours are more likely to be associated to parasites ingested during blood meal rather than to parasites derived from multiplication of epimastigotes within the insects intestinal tract.

After the first amplification was performed in triplicate, all negative samples had three other aliquots of the original DNA retested by PCR. This repetition was performed in an attempt to improve sensitivity by testing a more representative fraction of DNA samples. Re-amplification of initially PCR-negative samples, ana-

lyzed in triplicate, enabled us to detect the parasite one day earlier, *i.e.*, on day 4 after insects blood feeding, also in the two situations (high or lower parasitemia), even though we expected to detect *T. cruzi* DNA in the high parasitemia model before the lower parasitemia one. Simultaneous detection irrespective of the model seems to corroborate the idea that metacyclic tripomastigotes elimination time in the environment does not depend on the parasite load, and in our experiments has occurred from day four after blood meal. The decision to use PCR as a diagnostic tool depends on a number of parameters such as costs, laboratory infrastructure availability, trained personnel and the establishment of an adequate analytical sensitivity and specificity of amplification. The present work has confirmed the suitability of a rapid DNA isolation and amplification for detection of *T. cruzi* DNA on filter paper, although one should always consider the use of a more complex DNA extraction protocol whenever possible.

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