Comparison of different DNA preparation protocols for PCR diagnosis of Human African Trypanosomosis in Côte d’Ivoire

P. Solano a,*, V. Jamonneau b, P. N’Guessan a, L. N’Dri a, N.N. Dje c, T.W. Miezan c, V. Lejon d, P. Büscher d, A. Garcia a

a Institut Pierre Richet (IPR), IRD UR 035, BP 1500 Bouake, Ivory Coast
b Laboratoire Commun de Recherche sur les Trypanosomoses IRD/CIRAD, BP 5035 Baillarguet, France
c Projet de Recherches Cliniques sur la Trypanosomiase (PRCT), BP 1425 Daloa, Ivory Coast
d Department of Parasitology, Institute of Tropical Medicine, B-2000 Antwerpen, Belgium

Received 19 June 2001; received in revised form 20 September 2001; accepted 16 November 2001

Abstract

During a medical survey the sleeping sickness focus in Bonon, Ivory Coast, PCR with Trypanosoma brucei specific primers (TBR 1–2 from Parasitology 99 (1989) 57) was tested on DNA derived from blood samples. DNA purification using a chelating resin was performed either on whole blood or on the buffy coat prepared in two different ways. The preparation based on whole blood performed better than those using the buffy-coat. Using this first method, the sensitivity was 100% on parasitologically confirmed patients, and the specificity was 92%. However, problems of reproducibility of the technique were pointed out, particularly on samples from serologically positive but apparently aparasitemic individuals. It is suggested that the PCR could help in the diagnosis of Human African Trypanosomosis, but the use of other primers should be investigated. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Human African Trypanosomosis; Ivory Coast; PCR; Diagnosis; Trypanosoma brucei gambiense

1. Introduction

Human African Trypanosomosis (sleeping sickness) remains a significant public health problem in Sub-Saharan Africa. The impact of the disease is comparable to that of leishmanioses in terms of disability-adjusted life years lost (DALYS) (Molyneux, 1997). Since the disease is fatal if untreated and since some of the drugs used for treatment are very toxic, a reliable diagnosis is of paramount importance.

Mass screening of the population at risk is currently performed using serological tests in order to select individuals carrying trypanosome-specific antibodies, on which parasitological examinations are then carried out. The most commonly used serological tests in the field is the
Card Agglutination Test for Trypanosomiasis (CATT), developed for detection of antibody to *Trypanosoma brucei gambiense* (Magnus et al., 1978). Because of its simplicity and low price, the CATT is a very useful tool for field application. The parasitological diagnosis of sleeping sickness is based on the detection of trypanosomes in lymph node aspirates, blood, or cerebrospinal fluid, but currently available tests have low sensitivity and are hampered by fluctuating parasitaemia (Truc et al., 1994). The existence of seropositive but parasitologically unconfirmed subjects (CATT + on whole blood and plasma, but negative using parasitological examination) may however have epidemiological implications, since some of them might actually harbor the parasite and thus contribute to the persistence of the foci.

PCR has been introduced for detection of trypanosome DNA in biological samples. In animal trypanosomosis, PCR has been used as a tool allowing precise identification of the infecting trypanosome taxa (Majiwa et al., 1994), or to detect trypanosomes in the blood (Katakura et al., 1997; Clausen et al., 1998; Solano et al., 1999). The technique has also been used on humans as a more sensitive alternative to parasite detection for primary diagnosis of *T. b. gambiense* infection (Enyaru et al., 1998; Kabiri et al., 1999; Simo et al., 1999; Kyambadde et al., 2000; Penchenier et al., 2000), or for stage determination of sleeping sickness by amplifying trypanosome DNA from the cerebrospinal fluid (Truc et al., 1999; Kyambadde et al., 2000). However, in all these studies, few efforts have been made to standardise the procedures and to comparatively evaluate either the sample preparation methods or the primer sets.

The objective of the present study was to compare simple methods for DNA preparation requiring minimal sample manipulation, which would allow PCR amplification from blood without the need for organic extraction of DNA. In addition, it was intended to evaluate whether PCR can be considered as a reliable diagnostic tool for human sleeping sickness, taking into account sensitivity, specificity, reproducibility and feasibility in field conditions.

2. Material and methods

2.1. Origin of samples

A medical survey was carried out in April–May 2000, around the town of Bonon, in Central West Ivory Coast (6°55'N–6°W). A total of 13,900 people were screened using CATT on whole blood: among them 498 were positive (they were called ‘suspects’). Of these 498 individuals, 170 were confirmed serologically positive by CATT on plasma (i.e. ‘seropositive’) and 76 (i.e. ‘patients’) were found infected with trypanosomes, using either the mini-anion exchange column (mAECT) or lymph node aspirates (49 and 27, respectively). The patients subsequently went to the Projet de Recherches Cliniques sur la Trypanosomiase (PRCT) in Daloa to receive treatment.

2.2. Preparation of blood and buffy-coat samples

Blood was collected in heparinized vacutainers during the medical survey and processed following three different protocols.

For comparison of preparations, ‘true positives’ will refer to PCR positive results on patients with demonstrated trypanosomes in the blood by mAECT only. ‘False negatives’ are negative PCR results on similar subjects. ‘True negatives’ are PCR negative results on CATT negative individuals (on whole blood). ‘False positives’ are PCR positive on the same samples.

In the first protocol, 1 ml of whole blood was dispensed in a 1.5 ml eppendorf tube (preparation 1).

The two other protocols used the buffy-coat instead of whole blood, because it has been reported that trypanosomes are concentrated at this level after centrifugation (Woo, 1970; Murray et al., 1977).

In preparation 2, after centrifugation of the vacutainer containing 5 ml of blood at 2000 × g during 10 min, 50 μl of buffy-coat were put in a 0.5 ml eppendorf tube.

In preparation 3, 1 ml of blood was taken off from the vacutainer and put in a 1.5 ml eppendorf tube, whereafter the tube was centrifuged at
15,000 × g during 10 min; then 50 μl of buffy-coat were dispensed in a new 0.5 ml eppendorf tube.

The difference between preparations 2 and 3 relies on the assumption that in preparation 3, the buffy-coat would be easier to collect from a narrower tube than in preparation 2 thus reducing the risk of loss of trypanosome DNA.

After centrifugation and buffy-coat collection on the field, all these samples were kept in a coolbox during the day of collection, and put at −20 °C when back in the laboratory.

On the 50 first patients who went to Daloa for treatment, we also wanted to assess which anticoagulant, i.e. EDTA or heparin, was the best for the PCR. One milliliter of blood was taken in an EDTA-coated tube and 1 ml in a heparinized tube, which were frozen immediately. On these samples, DNA extraction and PCR were performed according to the preparation 1 protocol described below.

2.3. DNA extraction procedures and PCR conditions

DNA extraction from preparation 1 was based on the protocol of Penchenier et al. (1996) and was modified by using a Chelex resin (Biorad) instead of the READYAMP (Promega), because the Chelex resin is much cheaper.

Preparations 2 and 3 were processed in the same way following a modified procedure of Walsh et al. (1991). Briefly, 100 μl of a 1% suspension of Chelex in pure water were added to the buffy-coat in the eppendorf tube, which was incubated at 56 °C for 1 h and at 95 °C for 30 min, whereafter it was centrifuged at 15,000 × g during 2 min. The supernatant constituted the DNA sample for PCR amplification.

The primers used were TBR 1–2 of Moser et al. (1989), which were reported to specifically amplify DNA of the subgenus Trypanozoon which comprises the species Trypanosoma brucei, and which have already been used to detect T. b. gambiense in humans (de Almeida, 1998; Enyaru et al., 1998; Truc et al., 1999).

A positive control (DNA of T. brucei), a first negative control (all the components of the PCR mix with milliQ water instead of DNA) and a second negative control (1% suspension of Chelex without any other product and processed in the same way as the samples) were run in each PCR reaction.

For PCR amplification, 5 μl of the Chelex-extracted supernatant was added to 25 μl of final reaction mixture containing 10 mM Tris–HCl, 50 mM KCl, 1.5 mM MgCl₂, 200 μM dNTPs, 20 pmol primers and 0.5 U of Taq polymerase (Appligene): The DNA was first denatured at 95 °C for 1 min, then it was subjected to 35 cycles consisting in 1 min at 95 °C, 1 min at 55 °C, 1 min at 72 °C, followed by a terminal elongation for 10 min at 72 °C. PCR products were visualized on an ultraviolet trans-illuminator after electrophoresis in a 2% agarose gel.

2.4. Strategy of analysis

First, the effect of heparin and EDTA was assessed by comparing the sensitivities obtained by PCR on paired samples from 50 of the 76 patients.

To determine the best method for DNA preparation, the sensitivities and specificities of PCR obtained through the three different preparations were compared on sets of samples from confirmed patients and from randomly chosen CATT negative individuals (Fig. 1). The sensitivity was classically calculated as the number of true positives divided by the number of patients detected by mAEC. The specificity was the number of true negatives divided by the number of CATT negative individuals (on whole blood), assuming that the latter were not infected. As sample sizes were relatively small, both sensitivity and specificity values are to be considered as indicative and should be confirmed on a larger population. Taking this point into account, confidence interval values were computed under the exact theoretical binomial distribution, allowing a more precise interpretation of these values (Dixon and Massey, 1969).

To measure the reproducibility of the PCR within each of these preparations (intra-preparation reproducibility), some samples were amplified by blind test a second time by the same person, a few days after the first amplification and k values
Fig. 1. Sample analysis for comparisons of DNA preparations. T+: patient found infected with trypanosome using mAECI; CATT: individual negative using CATT on whole blood; ND: not done; suspect: individual positive using CATT on whole blood, negative using CATT on plasma (see also Section 2); seropositive: individual positive on both CATT on whole blood and plasma, but negative using mAECI.

for agreement were calculated. As proposed by Fleiss (1981), \( \kappa \) values lower than 0.40 reflect poor agreement, values between 0.40 and 0.75 reflect fair to good agreement and values above 0.75 indicate strong agreement.

The McNemar \( \chi^2 \)-test was used to compare the results obtained through different protocols performed (at blind) on the same individuals.

### 3. Results

#### 3.1. Heparin versus EDTA

From 50 patients who were included in the protocol, 47 and 25 tested positive with PCR on blood taken, respectively into heparin and EDTA.

#### 3.2. Sensitivity and specificity

In a first trial to compare the three preparations, the 14 first people who had been diagnosed as sleeping sickness patients, were tested. PCR gave 14 positive results with preparation 1, 13 positive results with preparation 2 and 11 positive results with preparation 3. We decided to continue the sensitivity and specificity tests only with preparations 1 and 2, because of the apparent low sensitivity of preparation 3.

<table>
<thead>
<tr>
<th>PCR</th>
<th>Disease status</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infected</td>
<td>Uninfected</td>
</tr>
<tr>
<td><strong>(a) Preparation 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>26</td>
<td>4</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>Total</td>
<td>26</td>
<td>49</td>
</tr>
<tr>
<td><strong>(b) Preparation 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>26</td>
<td>2</td>
</tr>
<tr>
<td>Negative</td>
<td>4</td>
<td>26</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>28</td>
</tr>
</tbody>
</table>

‘Infected’ refers to a sample where trypanosomes were found, ‘uninfected’ is CATT negative on whole blood.

\( ^a \) Sensitivity: \( 26/26 = 100\% \); specificity: \( 45/(45+4) = 92\% \) with CI\(_{95}\) = (0.84; 0.99).

\( ^b \) Sensitivity: \( 26/(26+4) = 87\% \) with CI\(_{95}\) = (0.70; 0.97); Specificity: \( 26/(26+2) = 93\% \) with CI\(_{95}\) = (0.74; 0.98).
3.4. Inter-preparation comparison

We compared the PCR results obtained through preparations 1 and 2 on 59 samples (Table 3). No significant differences were obtained when both preparations were performed on the same subjects (\(P > 0.20\)) indicating that none of these preparations led systematically to significantly more positive (or negative) results than the others. However, non-symmetric results appeared on 28.8% of the samples.

Moreover, when we checked the origin of the discordant results, we saw that, from 17 discordant results, again 11 originated from suspect or seropositive samples, four from CATT negative, and two from trypanosome infected samples.

4. Discussion

The presence in blood of components that inhibit PCR amplification has been a major problem associated with direct amplification of DNA in cell lysates (Higuchi, 1989). Therefore, many PCR methods involve organic extraction and ethanol precipitation to purify target DNA before amplification. While this yields DNA ready for amplification, the numerous manipulations required make organic extraction highly impractical for field use, and at the same time increase the risk of sample contamination with exogenous target DNA. To allow diagnosis of tropical diseases close to the field, PCR protocols should be simplified as much as possible without loss of reproducibility.

Table 2
Within preparation reproducibility of the PCR, for two amplifications

<table>
<thead>
<tr>
<th></th>
<th>Preparation 1 (1st)</th>
<th>Preparation 1 (2nd)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>23</td>
<td>13</td>
<td>36</td>
</tr>
<tr>
<td>−</td>
<td>6</td>
<td>51</td>
<td>57</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>29</strong></td>
<td><strong>64</strong></td>
<td><strong>93</strong></td>
</tr>
</tbody>
</table>

With preparation 1, from the 19 non-concordant results, 14 were suspect or seropositive individuals (see Fig. 1 for definition). On trypanosome-infected samples, 10 out of 12 gave concordant results, and on CATT negative samples, 44 out of 47 gave concordant results.

With preparation 2, from 16 non-concordant results, 14 were suspect or seropositive individuals. On infected samples, 20 out of 21 were concordant, and on CATT negative samples, 28 out of 29 were concordant.

### Table 3
Comparison of PCR results between preparations 1 and 2

<table>
<thead>
<tr>
<th></th>
<th>Preparation 1</th>
<th>Preparation 2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>14</td>
<td>11</td>
<td>25</td>
</tr>
<tr>
<td>−</td>
<td>6</td>
<td>28</td>
<td>34</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20</strong></td>
<td><strong>39</strong></td>
<td><strong>59</strong></td>
</tr>
</tbody>
</table>

Between preparations 1 and 2, McNemar \(\chi^2 = 1.47\) ns.
In a first attempt to identify an appropriate combination of blood sampling and PCR protocol for diagnosis of human sleeping sickness, we compared three different sample preparation procedures followed by the same PCR protocol, using the more often used TBR primer set of Moser et al. (1989). For DNA purification, we selected the Chelex procedure (based on Walsh et al., 1991) because of its simplicity and reported use (de Almeida et al., 1997; de Almeida, 1998; Solano et al., 1999; Truc et al., 1999).

In a first experiment, we looked at the effect of anticoagulants used for blood sampling, i.e. heparin and EDTA. It appeared clearly that heparin is more appropriate than EDTA when it is intended to perform PCR on the blood sample. Release of EDTA could provoke inhibition of the PCR. This confirms observations made by others who evaluated heparin, EDTA and citrate (Burckhardt, 1994).

On heparinized blood samples, we compared three DNA preparation protocols, one starting from whole blood and two starting from the buffy-coat, all being treated with a 1% aqueous Chelex suspension. Preparation 3 was only evaluated briefly, because it showed poor sensitivity on the first samples tested. Furthermore, it was the most time, labour and material consuming protocol, due to the transfer of the blood from the vacutainer to the eppendorf tube prior to centrifugation. The best compromise between sensitivity, specificity and simplicity was obtained with preparation 1, which showed 100% sensitivity on trypanosome infected patients detected during a medical survey and 92% specificity. It should be noticed here that, as we wanted the true positives to be positive with 100% certainty, we limited the sensitivity evaluation to samples that were taken from patients found infected with trypanosomes using the mAECT (26 for preparation 1, 30 for preparation 2). Further, we also tested the two preparations on patients who were found infected with trypanosomes by lymph node puncture (data not shown). Combining the two parasitological techniques (mAECT and lymph node puncture), the PCR results using the two preparations were the following: 100% sensitivity (43/43) with preparation 1, and 85.2% (46/54) with preparation 2, confirming the previous result.

At first instance, it seems surprising that with whole blood, the sensitivity appeared better than with the buffy-coat. Because of the fact that trypanosomes concentrate in a thin layer in the latter sample, we would have expected more trypanosome DNA to be present in the buffy-coat. An explanation for the lower sensitivity with the buffy-coat could be either the difficulty of properly taking the buffy-coat from the vacutainer tube, or the small volume taken (50 μl) taken for analysis, which would not allow enough DNA to be present for PCR amplification.

The 92% specificity of preparation 1 was due to four CATT-negative cases which appeared positive in PCR. We do not think that any mislabeling could account for this result. In addition, particular care was applied to avoid contamination by using two negative controls in each PCR. An infection with *T. b. brucei* might have occurred explaining a positive PCR result, but seems quite improbable. Although it has been shown that CATT may be negative in parasitologically confirmed patients (Dukes et al., 1992; Enyaru et al., 1998), this feature, to our knowledge has not yet been described in Ivory Coast. However, it cannot be excluded that some of these CATT false negatives could represent recently infected persons. It should be pointed out that PCR-positive results on CATT-negative samples have also been reported elsewhere using TBR primers (de Almeida, 1998; Penchenier et al., 2000). In the absence of more information, we consider these samples as inherent PCR false positives.

If the study had been stopped at this stage, the conclusion would have been that the technique used, i.e. PCR using TBR primers on whole blood treated with Chelex, is at least as satisfactory (100% sensitivity, 92% specificity) as other PCRs for sleeping sickness diagnosis (see Kabiri et al., 1999).

However, reproducibility was tested for each preparation and showed important discrepancies. On patients either ‘suspect’ or ‘seropositive’, the intra- or inter-preparation reproducibility appeared poor for both preparations. The CATT positive, but aparasitaemic subjects could repre-
sent either false CATT positives (but this cannot account for poor reproducibility), or false parasitological negatives with low parasitaemia. In this latter case, an explanation could be that very low amounts of DNA would induce a random possibility of taking sequences to be amplified. However, on patients on one hand, and on non-infected samples (i.e. CATT negative) on the other hand, the two preparations gave satisfactory, although not excellent, results (the number of concordant results on this type of samples was respectively 54 out of 59 for preparation 1 and 48 out of 50 for preparation 2).

In summary, the PCR using preparation 1 was the most sensitive, and gave satisfactory results on both trypanosome-infected and CATT-negative samples. On suspect samples (CATT positive on whole blood and/or plasma, but aparasaemic) the results were completely inconsistent. Aiming at the use of PCR as a diagnostic tool for human sleeping sickness, it appears from the present study that a PCR positive result cannot be interpreted as a definite trypanosome infection. Taking into account the number of studies in which the PCR revealed new epidemiological features in trypanosomoses and appeared to be a promising diagnostic tool for several diseases (see Tang et al., 1998), we think that the unexplained results obtained in this study could be linked to the primers used. Other authors reported some unexplained results with these primers (de Almeida, 1998). Garcia et al. (2000), using these TBR primers in a longitudinal survey of seropositive individuals, even showed that in a cross-sectional study, positive PCR results might appear randomly in a population living in an endemic area. Our present results confirm the extreme complexity of seropositivity. In the near future, the samples used in the present work should be analyzed with other primers specific for T. brucei s.l. or for T. b. gambiense. It will be of particular interest to interpret a PCR positive result on suspects or seropositive samples.

Acknowledgements

This investigation received financial support from the Belgian General Direction of International Co-operation, and from the International Atomic Energy Agency, Vienna.

References


