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Detection of African animal trypanosomes: The haematocrit centrifugation technique compared to PCR with samples stored on filter paper or in DNA protecting buffer

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\textbf{A B S T R A C T}

The present study aimed at comparing the trypanosome specific 18S-PCR-RFLP using samples stored either on Whatman filter papers (PCR-RFLP-fp) or in a commercial cell lysis and DNA protecting buffer (PCR-RFLP-pb) with the haematocrit centrifugation technique (HCT), a method widely used for the diagnosis of African Animal Trypanosomosis. Out of 411 head of cattle, 49 (11.92\%) (CI=8.95–15.45) scored positive for the presence of trypanosomes by HCT whereas 75 (18.25\%) (CI=14.63–22.33) and 124 (30.17\%) (CI=25.77–34.86) scored positive using PCR-RFLP-fp and PCR-RFLP-pb, respectively. Out of the 49 positives by HCT, 14 (28.57\%) (CI=16.58–43.26) and 28 (57.14\%) (CI=42.21–71.18) were concordant by PCR-RFLP-fp and PCR-RFLP-pb, respectively. None of the PCR techniques detected parasites from the Trypanozoon group. Although HCT detected more cases of Trypanosoma vivax (33), species identification using PCR-RFLP-fp and PCR-RFLP-pb were significantly different (p < 0.001) from the HCT technique. The use of DNA protective buffer is thus recommended as the output of the PCR-RFLP-pb is improved and the risk of contamination between samples is reduced.

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1. Introduction

African Animal Trypanosomosis (AAT) is a parasitic disease that causes serious economic losses in the livestock production system. Diverse clinical signs are observed from anaemia, loss of body condition and emaciation to death when untreated. In sub-Saharan Africa, the disease is caused by Trypanosoma congolense, Trypanosoma vivax and Trypanosoma brucei. Its distribution overlaps with the presence of the tsetse fly except for T. vivax and Trypanosoma evansi that can be transmitted mechanically by other hematophagous biting flies (CFSPh, 2009). AAT restricts agricultural production, limits the availability of food and contributes to poverty across rural sub-Saharan Africa (Grady et al., 2011).

Accurate diagnosis of trypanosome infections is required for a proper understanding of the epidemiology of the disease, which can then result in the implementation

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of adequate control strategies (FAO, 1992, 1998; Getachew, 2005). The diagnosis mainly involves examination of clinical signs and laboratory methods. A presumptive field diagnosis is often based on finding an anaemic animal with a poor body condition in an epidemic or endemic area. Even then, because of the various clinical manifestations, diagnosis of trypanosomosis cannot be based on these clinical signs only (Nantulya, 1990) as no pathognomonic clinical sign can confirm the disease. The presence of the parasite must thus be confirmed to ensure a proper diagnosis. Different techniques are therefore more or less routinely used by veterinarians: direct microscopy, concentration techniques, laboratory animal inoculation, detection of anti-trypanosomal antibodies and molecular assays (Moser et al., 1989; Murray et al., 1977; Nantulya, 1990). Each of these techniques presents some strong and weak points.

Serodiagnosis is a sensitive method but does not differentiate active infections from cured ones, nor does it distinguishes between different species of trypanosomes due to a poor specificity of the method (Desquesnes, 1997). However, Bossard et al. (2010) recently developed an inhibition ELISA based on the Heat Shock Protein 70 that is closely related to the mammalian Immunoglobulin Binding Protein. The specificity of this method has been reported as much better than previous tests.

Parasitological techniques such as the haematocrit centrifugation technique (HCT), Buffy Coat examination (BCE) and stained blood smear examination have been reported to be of low sensitivity but high specificity (Gardiner, 1989). These techniques are of limited significance when parasitaemias are low as often observed in endemic areas (Desquesnes, 1997).

The polymerase chain reaction (PCR) has been reported to be the most sensitive and specific technique to detect trypanosomal DNA in either the vector or the host (Dellespau et al., 2006; Gysen et al., 2003; Lefrancois et al., 1998; Majiwa et al., 1993; Reifenberg et al., 1997; Solano et al., 1999). There have been several advances in the development of PCR using different primer sets for the identification of trypanosomes at species and sub-species level. For instance, Masiga et al. (1992) designed oligonucleotide primers targeting the satellite DNA monomer to accurately identify Trypanosoma simiae, T. congolense, T. brucei and T. vivax. Gysen et al. (2003) developed a semi-nested PCR-Restriction Fragment Length Polymorphism (RFLP) based on the 18S ribosomal small subunit for the detection of all trypanosome species including the three subtypes of T. congolense using three primers. Desquesnes et al. (2001) identified the three T. congolense subtypes, the Trypanozoon group, T. vivax, T. simiae and Trypanosoma theleri by using a single PCR based on the Internal Transcribed Spacer 1 (ITS-1) of rDNA. However, PCR has been scarcely applied to assess the prevalence of trypanosomosis on field samples, due to its time-consuming and costly aspects as well as the requirement for technical expertise (Solano et al., 1999). For our study, the semi-nested PCR-RFLP developed by Gysen et al. (2003) was preferred to other molecular methods because of its high sensitivity (multicycopy gene) and its ability of differentiating the three subtypes of T. congolense. This PCR cannot differentiate species within the trypanozoon group but being not or weakly pathogen for cattle, this issue was not considered as a constraint.

Separating the sampling from the laboratory PCR activities involves the storage of the biological material for later processing. This might constitute a critical point as contamination between samples can easily occur in field conditions. Replacing the conventional filter papers for storage by blood aliquots preserved in a DNA protecting buffer decreases the time for the preparation of buffy-coats spotted on filter papers (e.g. impregnation, drying and storage), decreases also the potential contact between samples and thus the risk of contamination. Furthermore, the volume of blood that is collected is larger and subsequently allows the extraction of more target DNA. This should allow a higher number of detected cases by the PCR-RFLP. The objective of this study was to compare the detection of parasites by HCT, a technique routinely used in the field, with PCR-RFLP on samples that were stored either on Whatman® filter papers (PCR-RFLP-fp) or in a DNA protecting buffer (PCR-RFLP-pb). In the absence of gold standard, specificities and sensitivities were not calculated.

2. Materials and methods

2.1. Study animals and site

A heterogeneous group of 411 cattle of different ages and sexes were selected from different villages (kebeles) in and around the Ghibe valley, South-Western Ethiopia (Jimma and Gurge zones). Details on the epidemiology of trypanosomosis and trypanocidal drug resistance in the area have been mentioned elsewhere in Fikru et al. (2012) and Moti et al. (2012) respectively.

2.2. Protocol

2.2.1. Haematocrit centrifugation technique (HCT)

From each animal, whole blood was collected by puncture of the jugular vein into 5 ml heparinised Venosafe® tubes (Terumo Europe) for further analysis. The blood samples were then examined using the HCT as described by Woo (1970). The PCV values were recorded and a value below 24% was considered as anaemia (Marcotty et al., 2008).

2.2.2. Molecular assay

2.2.2.1. Preparation of the buffy coats spotted on filter paper and DNA extraction.

The buffy coat was prepared from about 50 μl of blood in plain capillary tubes and was extracted from each capillary tube after the HCT was completed by cutting the tube approximately 1 mm below the red cell/plasma interface. The buffy coat from each tube was spotted on filter papers (Whatman® n° 4, GE Healthcare Bio-Sciences AB, Sweden) and dried protected from direct sun light and flies. The filter papers were kept in individual envelopes and then in a plastic bag containing silica gel for storage at 2–8 °C (refrigerator) until further processing.

DNA extractions were done using the saponin-PBS method described by de Almeida et al. (1998). Briefly,
four confetti’s of 5 mm in diameter i.e. approximately 2/3 of the total surface of the dried buffy coat spot, were punched out for DNA extraction using 10% Chelex® (BIO-RAD, Belgium). To avoid contamination, two stainless steel punching tools were alternated after they were flame-sterilised and cooled.

2.2.2.2. Whole blood in protecting buffer and DNA extraction. Aliquots of whole blood (800 μl) were kept in an equal volume of AS1 buffer® (Qiagen, USA) for cell lysis and DNA preservation at ambient temperature for a period of less than 3 months. DNA extraction was done from 200 μl of blood/AS1 buffer® combination using QIAamp mini blood kit® according to the manufacturer’s instructions (Qiagen, USA). Extracted DNA was stored at −20 °C till further processing.

2.2.2.3. Trypanosome species identification. For species identification, DNA amplifications were done using three primers targeting the gene coding for the small ribosomal subunit 18S (semi-nested PCR) and followed by digestion of the amplicons using Msp I enzyme (New England BioLabs, USA) as described by Geysen et al. (2003). PCR conducted on DNA extracted from buffy coat spotted on filter paper was abbreviated PCR-RFLP-fp. When the DNA extraction was performed from whole blood in protection buffer PCR-RFLP-pb was used.

2.3. Data analysis

The percentage of positivity for each trypanosome species detected by the different methods together with their 95% exact (Clopper-Pearson) confidence intervals were calculated. Techniques (HCT and PCR-RFLP’s) were compared pairwise with respect to estimated prevalences by the Fisher exact test at the 5% significance level. The distribution of the positive cases over T. vivax and T. congolense was also compared pairwise between the methods by the Fisher exact test at the 5% significance level using SAS Version 9.3 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Percentage positivity using different sampling methods

 Compared to HCT, the percentage of positivity for the presence of trypanosomes was increased by more than 50% and 250% when using PCR-RFLP-fp and PCR-RFLP-pb respectively (see Table 1). The percentages of positivity for the three tests were statistically different from each other when compared two by two (p < 0.001).

The non-pathogenic trypanosome species T. theileri was excluded from the analysis to avoid exaggerating the differences between the tests during comparison.

Table 1

<table>
<thead>
<tr>
<th>Species</th>
<th>HCT positive N (% – LCL/UCL)</th>
<th>PCR-RFLP-fp positive N (% – LCL/UCL)</th>
<th>PCR-RFLP-pb positive N (% – LCL/UCL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tv</td>
<td>23 (5.60–3.58/8.28)</td>
<td>2 (0.49–0.06/1.75)</td>
<td>23 (5.56–3.58/8.28)</td>
</tr>
<tr>
<td>Tc + Tv</td>
<td>8 (1.95–0.84/3.80)</td>
<td>0 (0.00/0.089)</td>
<td>1 (0.2–0.01/1.35)</td>
</tr>
<tr>
<td>T + Tc</td>
<td>2 (0.49–0.06/1.18)</td>
<td>0 (0.00/0.089)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>49 (11.92–8.95/15.45)</td>
<td>75 (18.25–14.63/22.33)</td>
<td>124 (30.17–25.77/34.86)</td>
</tr>
</tbody>
</table>

With HCT: haematocrit centrifuge technique, PCR-RFLP-fp: 18S-PCR-RFLP using samples stored on Whatman filter papers, PCR-RFLP-pb: 18S-PCR-RFLP using samples stored in a commercial cell lysis and DNA protecting buffer, N: number, LCL: lower confidence level, UCL: upper confidence level, TV: T. vivax; Tc: T. congolense savannah; T: Trypanozoon.

3.2. Comparison of HCT and PCR-RFLP-fp

Out of 49 HCT positive samples, 14 (28.57%) (CI = 16.58–43.26) also scored positive when using PCR-RFLP-fp. In contrast, PCR-RFLP-fp detected more positive cases compared to HCT, but the difference was not statistically significant (Table 2).

In Table 1, are shown the results of species identification. Out of 411 samples, 24 (5.84%) (CI = 3.78–8.56) were detected as T. congolense savannah by HCT (16 single and 8 mixed) whereas PCR-RFLP-fp detected 73 (17.8%) positives (all single); 33 (8.0%) (CI = 5.59–11.09) were detected as T. vivax by HCT (23 single and 10 mixed) whereas PCR-RFLP-fp detected 2 (0.48%) (CI = 0.06–1.75) (all single). None were detected as Trypanozoon by PCR-RFLP-fp whereas HCT detected 2 (0.5%) (2 mixed). The difference in species identification using PCR-RFLP-fp compared to HCT was statistically significant (p < 0.001).

3.3. Comparison of HCT versus PCR-RFLP-pb

Out of 49 HCT positive samples, 28 (57.1%) also scored positive when using PCR-RFLP-pb. On the other hand, PCR-RFLP-pb detected more positives compared to HCT (Table 2) and the difference was statistically significant (p < 0.001).

For species identification, out of 411 samples, 24 (5.84%) (CI = 3.78–8.56) were detected as T. congolense savannah by HCT (16 single and 8 mixed) whereas PCR-RFLP-pb detected 101 (24.57%) (CI = 20.49–29.03) positives (100 single and 1 mixed); 33 (8.02%) (CI = 5.59–11.09) were detected as T. vivax by HCT (23 single and 10 mixed) whereas PCR-RFLP-pb detected 24 (5.8%) (CI = 3.78–8.56) (23 single and 1 mixed); 2 (0.48%) (CI = 0.06–1.75) were detected as Trypanozoon by HCT (2 mixed) whereas PCR-RFLP-pb detected none. Difference in species identification using PCR-RFLP-pb compared to HCT was statistically significant (p < 0.001).
Table 2
Comparison of the detection of trypanosome infection using HCT, PCR-RFLP-fp and PCR-RFLP-pb on cattle blood samples from Ethiopia.

<table>
<thead>
<tr>
<th></th>
<th>PCR-RFLP-pb negative</th>
<th>PCR-RFLP-pb positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT negative</td>
<td>PCR-RFLP-fp negative</td>
<td>243</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>PCR-RFLP-fp positive</td>
<td>23</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>266</td>
<td>96</td>
</tr>
<tr>
<td>HCT positive</td>
<td>PCR-RFLP-fp negative</td>
<td>11</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>PCR-RFLP-fp positive</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>21</td>
<td>28</td>
</tr>
</tbody>
</table>

3.4. Comparison of PCR-RFLP-pb and PCR-RFLP-fp

Out of 124 PCR-RFLP-pb positive samples, 42 (33.9%) (CI = 26.37–43.25) also scored positive when using PCR-RFLP-fp. On the other hand, PCR-RFLP-fp detected 33 positives that were not detected by PCR-RFLP-pb. The difference in detection of infection was statistically significant (p < 0.001).

For species identification, out of 411 samples, 101 (24.57%) (CI = 20.49–29.03) were detected as *T. congolense* savannah by PCR-RFLP-pb (100 single and 1 mixed) whereas PCR-RFLP-fp detected 73 (17.76%) (CI = 14.19–21.81) cases (all single); 24 (5.84%) (CI = 3.78–8.56) were detected as *T. vivax* by PCR-RFLP-pb (23 single and 1 mixed) whereas PCR-RFLP-fp detected 2 (0.49%) (CI = 0.06–1.75) (all single); *Trypanozoon* was detected by none of the PCR. Considering *T. congolense* and *T. vivax* detection, the percentage of positivity was higher with PCR-RFLP-pb (p < 0.001).

3.5. Relationship of PCV value with HCT and PCR-RFLP-fp or -pb

The mean PCV was 26.2 ± 5.0 and anaemic condition of the animal (PCV < 24) was found matching with diagnostic test positivity for trypanosomosis in 29 (59.2%) (CI = 44.23–73.01), 36 (48%) (CI = 36.35–60.07) and 53 (42.7%) (CI = 34.21–52.30) cases for HCT, PCR-RFLP-fp and PCR-RFLP-pb, respectively (Table 3).

4. Discussion

The aim of this study was to compare the HCT with the PCR-RFLP on samples from the same animal but stored under two different conditions, i.e. either buffy coat spots on filter paper or whole blood in lysis/stabilisation buffer. Filter papers are commonly used to store blood or buffy coat samples for epidemiological surveys. This method for field sample storage presents multiple advantages as a low cost, a low weight allowing easy transport and a long term preservation. However, two important drawbacks were observed from a long time practice in the field i.e. a potential contamination between samples by contact between the filters or unclean hands of the technician and the low quantity of blood that is effectively used for DNA extraction. To overcome these drawbacks, an alternative storage method is available using a commercial buffer (QIAGEN AS1® – QIAGEN, USA) that is lysing the cells and protecting the DNA present in the solution for several months at ambient temperature.

The main outcome of this study is clearly a better trypanosome detection with PCR performed on DNA extracted from a larger volume of whole blood (±three times the volume). The larger the volume of the blood sample used for DNA extraction, the higher the probability of detecting trypanosomes at low concentration. PCR-RFLP-fp method is performed routinely with 30 μl aliquots and PCR-RFLP-pb with 100 μl blood samples. The number of positives would have certainly increased if QIAGEN Midi-kit® (uses up to 2 ml blood) was used rather than the Mini-kit (uses up to 200 μl blood) for the DNA extraction. However, the price has to be considered with 0.44€ and 7€ for the mini and midi kits, respectively. In our study, the detection efficiency of the PCR was higher (124 against 75) when using PCR-RFLP-pb compared to PCR-RFLP-fp. The 65% gain in detection might however be less than expected when tripling the volume of the sample used for DNA extraction. An explanation could be a lower efficiency of the QIAGEN kit compared to the chelex-based extraction. However Hsiang et al. (2010) reported that the two methods were equally efficient.

Beside the considerations made here above, some results deserve more accurate observation such as (i) negative PCR’s that coincide with a positive HCT, (ii) two samples that were detected as *T. vivax* by PCR-RFLP-fp and as *T. theileri* by PCR-RFLP-pb.

Due to the sampling procedure i.e. a simultaneous processing of the microscopic examination and preparation of the buffy coats on filter paper or blood in the protective buffer, detection of trypanosome was done for each animal

Table 3
Relationship between PCV category and presence of trypanosomes infection with the three different tests used on cattle blood from Ethiopia.

<table>
<thead>
<tr>
<th>PCV category</th>
<th>HCT +N (%; LCL/UCL)</th>
<th>PCR-RFLP-fp +N (%; LCL/UCL)</th>
<th>PCR-RFLP-pb +N (%; LCL/UCL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCV &gt; 24 (n = 109)</td>
<td>29 (59.2–44.23/73.01)</td>
<td>36 (48–36.35/60.07)</td>
<td>53 (42.7–34.21/52.30)</td>
</tr>
<tr>
<td>PCV ≥ 24 (n = 302)</td>
<td>20 (40.82–28.06/54.94)</td>
<td>39 (52.00–40.78/63.02)</td>
<td>71 (57.26–48.42/65.66)</td>
</tr>
<tr>
<td>Total (n = 411)</td>
<td>49</td>
<td>75</td>
<td>124</td>
</tr>
</tbody>
</table>

With N: number; LCL: +: positive, lower confidence level; UCL: upper confidence level.
on three independent samples (obtained from the bleeding of the same animal, but being three distinct aliquots from the same 5 ml Vacutainer® tube). Parasitaemia observed in the field are commonly oscillating between the detection limits of the HCT and of the 18S-PCR being around 250–500 parasites/ml and 25 parasites/ml, respectively (Geyser et al., 2003; Paris et al., 1982). These low parasitaemia are likely to explain some differences between the aliquots that are tested with the different methods. Some capillary tubes filled from the same 5 ml Vacutainer® tube might contain no trypanosome or a cluster of trypanosomes or even different species of trypanosome. As stated above, the detection limit of the 18S-PCR is situated around 25 trypanosomes/ml meaning about 1 trypanosome/capillary tube. At this low level of parasitaemia, some of the capillaries may contain 1, 2 or more trypanosomes and some may be empty. This situation was previously reported by Junqueira et al. (1996) with negative PCR’s correlated to positive xenodiagnosis. This is likely the explanation of the positive HCT versus negative PCR and the two samples that were detected as T. vivax by PCR-RFLP-Fp and as T. theileri by PCR-RFLP-pb.

The number of T. vivax detected by HCT (33) was significantly higher than by PCR-RFLP-Fp and PCR-RFLP-pb that detected 2 and 24, respectively. A lower sensitivity for T. vivax detection using an 18S based PCR had already been reported elsewhere (Njiru et al., 2005). This might be explained by the limited sensitivity of the PCR for this particular species linked to a high genetic diversity of the 18S locus. Additionally, the microscopic observation of T. vivax is strongly facilitated by the high mobility of this species. On the other hand, the PCR-RFLP-Fp and PCR-RFLP-pb detected more T. congolense infections than the ITS1 PCR on the same samples (Fikru et al., 2012). The discrepancy in this case can be explained by the fact that the 18S-PCR is a semi-nested PCR with, in principle, a lower detection limit.

Finally, the question should be raised to know if the infection or the disease has to be diagnosed. This was already explored for trypanosomosis in goats in Burkina Faso where sick animals were always microscopically positive (Vitouley et al., 2012). Diagnosing the disease with a less sensitive method like the HCT keeps its full sense in the strategies aiming at reducing the economic impact of the disease at the herd level. In our study, the proportion of anaemic animals was higher in the HCT positive animals (59.2%) compared to 48% and 42.7% for PCR-RFLP-Fp and PCR-RFLP-pb, respectively. HCT remains a robust field method as it will be more efficient than PCR to detect clinical cases that have to be treated. Indeed, in routine veterinary practice diagnosing the disease remains the priority compared to detecting parasites. Alternatively, the PCR constitutes a valuable tool for epidemiological surveys and for drug efficacy testing where an evidence of cure is necessary. The parasitological technique combined with the clinical examination are ideal when the focus is on the disease. The molecular techniques are useful when the focus is on the presence of the parasite (presence/absence, geographic distribution, herd prevalences). The multi-species PCR present the advantage of decreasing the work load and the final cost of the operation. Only one PCR has to be done on each sample and the restriction step for species diagnosis applies only for the positives.

5. Conclusion

Major differences were observed during this study between the detection of trypanosome by the three methods. Therefore, it is of a high importance to consider carefully the protocol of any trypanosome prevalence survey before comparing any results. Prevalence’s might vary by a factors 2–3 depending on the method that is used.

Haematocrit values should also be systematically correlated with infection status as this is providing useful information on the impact of the disease in a specific area. Highly virulent parasites will strongly affect the PCV values of infected animals as opposed to more endemic situations where a better host/parasite balance is observed. This distinction is essential for the establishment of a coherent control strategy.

References


