Use of antigen capture tube enzyme-linked immunosorbent assay for the diagnosis of Trypanosoma evansi infections in dromedary camels (Camelus dromedarius)

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Summary: Parasitological diagnosis of Trypanosoma evansi infection in camels is hampered by the small number of parasites in blood circulation, coupled with the tendency of this trypanosome to invade tissues. To overcome this, a more sensitive assay, an antigen enzyme-linked immunosorbent assay (ELISA), was developed, capable of detecting trypanosome antigens released into the bloodstream by dying parasites. To evaluate the usefulness of this assay in assisting chemotherapy, an experiment was designed to compare the ability of a Trypanozoon subgenus-specific monoclonal antibody (TR7) to capture antigens in whole blood and serum of camels in a T. evansi-endemic area of the Marsabit district in northern Kenya. The tests were performed in polystyrene tubes coated with TR7. Antigen ELISA using whole blood was performed in the field, while serum collected on the same day from the same animals was stored at -20°C and tested in the laboratory at a later date. A total of 100 camels were examined. Twelve per cent of the camels were found to be antigenaemic when whole blood was tested, compared to thirteen per cent detected using serum. Thus, the results obtained so far do not show a significant difference in the sensitivity of tube ELISA when detecting antigens either in whole blood or serum.


INTRODUCTION

Trypanosomiasis caused by Trypanosoma evansi, a mechanically-transmitted trypanosome, is the most important disease of dromedary camels (1, 13). T. evansi infection may be fatal, rapidly leading to death, or it may take a chronic course manifested by fever, emaciation, anaemia and oedema (1, 2, 3). Camels in the tsetse-infected areas are also affected to a lesser extent by the tsetse-transmitted trypanosome species.

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Previously, trypanosomiasis diagnosis in the field was based on clinical signs and the demonstration of trypanosomes in blood using standard trypanosome detection methods (5). However, these approaches are inadequate, as the clinical signs are not pathognomonic, while parasitological diagnosis is curtailed by the low level of parasitaemia, often too low to be detected (7, 11).

Recently, an antigen-trapping microplate enzyme-linked immunosorbent assay (ELISA) based on a monoclonal antibody specific for the *Trypanozoon* subgenus-specific invariant antigen was developed for diagnosis of *T. evansi* infections (9, 10, 11). This antigen capture ELISA was subsequently simplified and adapted for field use through the development of an antigen capture tube ELISA. The results obtained by tube ELISA were identical to those from the microplate ELISA (11). Tube ELISA using whole blood had been tested previously and had also yielded good results (V.M. Nantulya, unpublished findings).

Since the antigen ELISA using whole blood had not undergone field trials, the study of its application in the field was of great interest and importance. This paper describes data obtained from a field trial of this version of the test.

**MATERIALS AND METHODS**

**Animals**

One hundred camels in a *T. evansi*-endemic area of the Marsabit district in northern Kenya were tested. The animals belonged to a demonstration herd at the FARM (Food and Agriculture Research Management)-Africa Marsabit District Mobile Outreach Camp (MOC).

**Control sera**

Positive control serum was obtained from an incidental finding of trypanosomiasis in a camel admitted for surgical excision of an epulis at the Veterinary Clinic of the University of Nairobi. The condition of this camel deteriorated rapidly even after complete recovery from surgery. Serum from this animal was found to be strongly positive for trypanosome antigens by microplate antigen ELISA. Subsequently, blood taken from the animal revealed the presence of numerous trypanosome parasites by wet blood film examination.

Negative control serum was obtained from uninfected camels on a farm at Athi River, near Nairobi. The animals were confirmed to be negative by mouse inoculation, antigen ELISA and antibody ELISA.

**The field kit**

The field kit consisted of the following components:

- washing buffer (phosphate-buffered saline [PBS] with 0.05% Tween 20), pH 7.4
- bovine serum albumin (BSA)
- citrate buffer, pH 4.0
- 20% D-glucose
- 1% horseradish peroxidase (HRPO)
- 2,2'-azino-di-(3-ethylbenzthiazoline sulfate) (ABTS)
- control sera
- antibody-coated polystyrene tubes
- conjugate (TR7/47.34.16 monoclonal antibody labelled with glucose oxidase).

**Test blood and sera**

Whole blood and serum samples were collected from all camels. Both whole blood and serum were assayed for antigens, the former in the field and the latter in the laboratory. Whole blood was tested within a few hours of collection, while the serum was separated twelve hours after collection and transported to the laboratory within two days of separation where it was stored at -20°C until required.

**Tube ELISA technique**

Polystyrene tubes were coated overnight at 4°C with 1 ml (50 µg/ml) per tube of a partially purified immunoglobulin (Ig)M fraction of a monoclonal antibody TR7/47.34.16 in carbonate/bicarbonate buffer (pH 9.6). This antibody is *Trypanozoon* subgenus-specific (7). The tubes were rinsed once using washing buffer (50 mM PBS containing 0.5 M NaCl and 0.05% Tween 20 pH 7.4). Excess buffer was drained and 500 µl of whole blood or serum was added per tube. Each sample was tested in duplicate. The samples were diluted in an equal volume of washing buffer and incubated for 30 min at room temperature. The tubes were soaked and rinsed three times at 10 min intervals using washing buffer before adding the conjugate (glucose oxidase-labelled IgM fraction of the same monoclonal antibody), diluted 1:1,000 in washing buffer containing 1% BSA. The tubes containing conjugate were incubated at room temperature for 30 min, after which they were washed three times at 10 min intervals. Substrate and chromogen (250 µl) were then added. The chromogen and substrate used contained 40 mM of ABTS, 2% D-glucose and 0.01% HRPO. These tubes were incubated with gentle mixing at room temperature and colour changes were observed after 15 min.

**Microplate ELISA**

Flat bottomed micro-ELISA plates were coated overnight at 4°C with 100 µl (5 µg/ml) per well of the same partially-purified IgM fraction of monoclonal antibody TR7/47.34.16 as for tube ELISA.

The procedure followed was similar to the tube ELISA (described above) except that 50 µl of serum and 100 µl of conjugate and substrate were added per well.

The conjugate used was a HRPO-labelled IgM fraction of the same monoclonal antibody diluted 1:1,000 in washing buffer containing 1% BSA. The chromogen used was the same as that used in tube ELISA but the substrate contained 0.003% hydrogen peroxide in 50 mM citric acid buffer (pH 4.0). The optical densities (ODs) were read after 30 min at a wavelength of 414 nm using a micro ELISA reader.

**Other laboratory investigations**

Whole blood samples were also tested for trypanosomes using the buffy coat technique (6, 12).
Statistical analysis

A statistical analysis of the results was carried out using the chi-square ($\chi^2$) test of association (14). A $\chi^2$ value greater than or equal to 3.84 was regarded as significant ($P < 0.05$).

RESULTS

Of 100 camels, 21 were positive for one or more of the three tests performed, namely: tube ELISA using whole blood or serum, and microplate ELISA using serum. Thirteen of the positive camels were antigenaemic as determined by tube ELISA using serum, twelve by tube ELISA using whole blood and eight by microplate ELISA. Eight camels were detected by tube ELISA using both serum and whole blood, while only four of the eight had antigens detected by microplate ELISA.

One camel had parasites detected by microscopic examination of the buffy coat smear. In this case, no antigens were detected by tube ELISA using whole blood or serum. However, some antigenaemia was detected using microplate ELISA.

A visible green colour change of the substrate was regarded as positive for tube ELISA. For microplate ELISA, an OD greater than or equal to 0.045 was regarded as positive (Table I).

The statistical analysis showed no significant difference between the three tests at $P \leq 0.05$ (Tables II and III).

DISCUSSION

It has been suggested that demonstration of trypanosomal antigens in blood of an infected animal is synonymous with parasitological diagnosis of the disease (4, 15). This is true in the detection of T. brucei, as the antigen detected by the specific monoclonal antibody is released into circulation only after destruction of the trypanosomes (4).

In this study, trypanosome infection in one camel was diagnosed parasitologically using the dark ground phase contrast buffy coat technique (6, 12). This proven infection was not detected by tube ELISA using either whole blood or serum. However, it was detected by microplate ELISA performed with the serum sample (10). The ability of microplate ELISA to detect antigens in this case, and in four other cases which had been missed by tube ELISA, could be attributed to greater accuracy of the spectrophotometric assessment of the microplate ELISA results, compared with the less accurate visual assessment of the tube ELISA results. These results may suggest that microplate ELISA is more sensitive than tube ELISA. However, 13 of the 21 antigenaemic animals were detected by tube ELISA compared to 8 by microplate ELISA, when the two tests were performed on serum samples. Also, the tube ELISA detected 12 of the 21 cases when whole blood was used. This ability of tube ELISA to detect infections which are not detected by microplate ELISA could be due to the greater sensitivity of tube ELISA, which could be attributed to the larger volumes of test samples used in the latter assay.
# TABLE I

Results obtained from twenty-one antigenaemic camels tested for *T. evansi* antigens by tube enzyme-linked immunosorbent assay (ELISA) using whole blood and serum, and microplate ELISA

<table>
<thead>
<tr>
<th>Animals</th>
<th>Identification No.</th>
<th>Tube ELISA Whole blood</th>
<th>Serum</th>
<th>Microplate ELISA OD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>+</td>
<td>+</td>
<td>0.087</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>–</td>
<td>+</td>
<td>0.042</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>+</td>
<td>–</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>216</td>
<td>–</td>
<td>–</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td>326</td>
<td>–</td>
<td>+</td>
<td>0.017</td>
</tr>
<tr>
<td></td>
<td>332</td>
<td>–</td>
<td>+</td>
<td>0.021</td>
</tr>
<tr>
<td></td>
<td>504 **</td>
<td>–</td>
<td>–</td>
<td>0.085</td>
</tr>
<tr>
<td></td>
<td>703</td>
<td>–</td>
<td>–</td>
<td>0.047</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>–</td>
<td>–</td>
<td>0.055</td>
</tr>
<tr>
<td></td>
<td>504 **</td>
<td>–</td>
<td>–</td>
<td>0.085</td>
</tr>
<tr>
<td>Females</td>
<td>20</td>
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<td>23</td>
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<td>+</td>
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<td>30</td>
<td>–</td>
<td>+</td>
<td>0.018</td>
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<td></td>
<td>41</td>
<td>++</td>
<td>+</td>
<td>0.005</td>
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<td>+</td>
<td>–</td>
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<td>55</td>
<td>+</td>
<td>–</td>
<td>0.028</td>
</tr>
<tr>
<td></td>
<td>59</td>
<td>+</td>
<td>–</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>77</td>
<td>+</td>
<td>–</td>
<td>0.028</td>
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<tr>
<td></td>
<td>252</td>
<td>+++</td>
<td>+++</td>
<td>0.642</td>
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<tr>
<td></td>
<td>294</td>
<td>+</td>
<td>–</td>
<td>0.011</td>
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<tr>
<td></td>
<td>C20</td>
<td>+</td>
<td>+</td>
<td>0.064</td>
</tr>
<tr>
<td></td>
<td>C298</td>
<td>+++</td>
<td>+++</td>
<td>0.325</td>
</tr>
</tbody>
</table>

- + slight green colour change
- ++ obvious green colour change
- +++ deep green colour change
- - no colour change

* an optical density (OD) ≥ 0.045 was regarded as positive

** this camel had parasites detected by the dark ground buffy coat technique

# TABLE II

Comparison of the results obtained by tube enzyme-linked immunosorbent assay (ELISA) using whole blood and serum in twenty-one antigenaemic camels

<table>
<thead>
<tr>
<th>Whole blood Serum</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>8</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>Negative</td>
<td>4</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>12</td>
<td>9</td>
<td>21</td>
</tr>
</tbody>
</table>
TABLE III

Comparison of the results obtained by tube enzyme-linked immunosorbent assay (ELISA) using whole blood and by microplate ELISA using serum from twenty-one antigenaemic camels

<table>
<thead>
<tr>
<th>Microplate ELISA</th>
<th>Tube ELISA</th>
<th>Whole blood</th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>8</td>
<td>5</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>9</td>
<td>21</td>
<td></td>
</tr>
</tbody>
</table>

However, statistical analysis showed that the differences between the three ELISA tests were not significant. A similar observation was made in an earlier investigation (11).

The antigen trapping tube ELISA is a significant improvement in field diagnosis from the dark ground buffy coat technique used previously. The aim of this study was to develop a test which is easy to perform in the field, is sensitive, easy to read and yields immediate results. Antigen trapping tube ELISA would appear to be a useful test in the field diagnosis of trypanosomiasis. More importantly, tube ELISA using whole blood was the most suitable of the three tests examined, as results were obtained almost immediately after sampling, and treatment could be commenced without delay.

However, antigen detection assays are known to miss a few early infections, since parasites have to be lysed before the non-secretory antigens are released (4, 7, 16). Furthermore, antigens are also known to persist for up to 30 days in camels (16) and 55 days in cattle (9) after successful chemotherapy. Under field conditions, it would therefore be necessary to supplement tube ELISA using whole blood with one of the more sensitive, easily-applied parasitological methods, such as the buffy coat technique.

This trial was carried out on 100 camels. Further work on a larger scale is required to confirm these findings.

ACKNOWLEDGEMENTS

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Résumé : Le faible nombre de trypanosomes dans le sang et la tendance de ce parasite à envahir les tissus rendent le diagnostic de l'infection des dromadaires par Trypanosoma evansi difficile. On a donc mis au point un test
d'immunocapture enzymatique (enzyme-linked immunosorbent assay : ELISA), plus sensible, qui permet de détecter les antigènes libérés dans le sang circulant par les trypanosomes sur le point de mourir. Pour évaluer l'intérêt de ce test en cours de chimiothérapie, on a comparé expérimentalement l'aptitude d'un anticorps monoclonal spécifique du sous-genre Trypanozoon (TR7) à capturer des antigènes dans le sang complet et dans le sérum de dromadaires. L'expérience a été menée dans une zone d'infection endémique par T. evansi du district de Marsabit au nord du Kenya. Les tests ont été réalisés dans des tubes en polystyrène tapissés de TR7. Une épreuve d'immunocapture ELISA à partir du sang complet a été effectuée sur le terrain, tandis que le sérum prélevé le même jour sur les mêmes animaux a été conservé à −20 °C et analysé plus tard. Sur les cent dromadaires ainsi examinés, douze ont été reconnus porteurs de l'antigène par le test réalisé sur sang complet contre treize à partir du sérum. D'après les résultats obtenus jusqu'ici, l'épreuve ELISA en tubes ne présente donc pas une grande différence de sensibilité selon qu'elle est appliquée au sang complet ou au sérum.

MOTS-CLÉS : Détection de l'antigène - Diagnostic - Dromadaires - ELISA en tubes - Sang complet - Trypanosomose.

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Resumen: La poca cantidad de parásitos en la sangre y la tendencia de Trypanosoma evansi a invadir los tejidos dificultan el diagnóstico parasitológico de la infección de los dromedarios por este tripanosoma. Para paliar esta dificultad, se puso a punto una prueba de inmunocaptura enzimática (ELISA), más sensible, capaz de detectar los antígenos del tripanosoma liberados en la sangre por los parásitos que están a punto de morir. Para evaluar el interés de esta prueba en quimioterapia, se comparó experimentalmente la capacidad de un anticuerpo monoclonal específico del subgénero Trypanozoon (TR7) para capturar antígenos en la sangre entera y en el suero de dromedarios. La experiencia se llevó a cabo en una zona de infección endémica por T. evansi en el distrito de Marsabit, al norte de Kenya. Las pruebas se realizaron en tubos de poliestireno con TR7. Una prueba de inmunocaptura ELISA a partir de la sangre entera se efectuó en el terreno mientras que el suero recogido el mismo día en los mismos animales se conservó a −20° C y se chequé en laboratorio en una fecha posterior. De los cien dromedarios así examinados, doce fueron reconocidos portadores del antígeno por la prueba a partir de la sangre entera contra trece a partir del suero. Según los resultados así obtenidos, la prueba ELISA en tubos no presenta una gran diferencia de sensibilidad según se aplique a la sangre entera o al suero.

REFERENCES


