Enzyme-linked immunosorbent assay (ELISA) for detection of anti-*Trypanosoma evansi* equine antibodies

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Abstract

The standardization of ELISA for the detection of anti-*Trypanosoma evansi* antibodies in naturally and experimentally infected horses is described. Bayesian analysis was used to establish the cutoff between positive and negative sera. In order to determine the assessment of the ELISA test, the results obtained were compared with those from an IFA. A relative sensibility of 98.39%, a specificity of 95.12% and a predictive value of 96.83% were determined. The standardized technique was used to evaluate the antibody production against trypanosome in an experimentally infected equine, in which the sera converted 15 days after infection. The test was also used for a study of sera prevalence in a non-random sample from two different populations. A prevalence of 81.7% in workhorse and 57.14% in stable horses was found. © 1998 Elsevier Science B.V. All rights reserved.

**Keywords:** *Trypanosoma evansi*; Horse; ELISA; IFA; Bayesian analysis

1. Introduction

The *Trypanosoma evansi* is largely distributed throughout the world, but it is principally found in areas with hot weather as North Africa, South-east Africa, Central and South America (Hoare, 1972).
The clinical manifestations of the trypanosomiasis by *T. evansi* can be acute or chronic. The acute symptoms are fever, emaciation, anemia and sudden death of the animal in some cases. The chronic cases are presented with progressive weakness, emaciation, a notable decrease in the productive capacity and possible death (Luckins, 1988).

In 1939 Kubes (1939) reported that the equine infectious anemia and the Trypanosomiasis or ‘derrengadera’ are the two principal causes of death in horses in Venezuela. Fifty years later, García et al. (1989) showed a 69.3% prevalence of *T. evansi* through an indirect fluorescent antibody test (IFA) in a sample of 140 horses from the Venezuelan savanna (Estado Apure).

The diagnosis of trypanosomiasis by direct parasitological techniques is feasible in the acute state of the illness, when the blood is colonized by a large number of parasites. In the chronic state of the illness, which is characterized by low parasitemia, a good parasitological diagnosis is rather difficult (Luckins et al., 1978; Rae and Luckins, 1984). Thus, the serological techniques, which detect the anti-Trypanosoma circulating antibodies, are more effective for the diagnosis of the disease (Molyneux, 1975; Voller et al., 1979).

The objective of the present work was to develop an enzyme immunoassay (ELISA) in order to detect anti-*T. evansi* antibodies in equine serum and to examine its value as a diagnostic tool to study equine trypanosomiasis.

### 2. Materials and methods

#### 2.1. Antigen preparation

*T. evansi* was obtained from naturally infected horses from the venezuelan savanna (Estado Apure). The parasites were expanded by inoculating 0.5 ml of horse infected blood into two adult albino rats (Sprague Dawley). When the parasitemia reached 10^6–10^8 Trypanosomas ml\(^{-1}\), the blood was extracted by cardiac puncture using EDTA as anticoagulant. The *T. evansi* were purified by anion exchange chromatography using DEAE-cellulose as described Lanham and Godfrey (1970). The eluted parasites were washed three times by centrifugation at 1475 \( \times \) g for 20 min, in 20 mM phosphate buffer pH 7.2, containing 1% glucose. The parasites were resuspended in 2 ml of 20 mM phosphate buffer saline pH 7.2, (PBS) and sonicated four times for 1 min at a time. The resulting homogenate was centrifuged at 7000 \( \times \) g for 15 min. and the supernatant was stored at \(-20^\circ\)C and used as antigen in the ELISA, as described by Voller et al. (1975) and Luckins (1977). The concentration of protein in the supernatant was determined as 2.7 mg ml\(^{-1}\) by the Lowry et al. (1951) method.

#### 2.2. Preparation of secondary antibody conjugated to peroxidase

The secondary antibody was prepared in goat by inoculating purified horse IgG. Goat anti-horse IgG were purified by DEAE-cellulose according to standard methodology. The purity of the IgG was determined by immunodiffusion and immunoelectrophoretical
techniques. The goat anti-horse IgG was coupled to peroxidase as described by Wilson and Nakane (1978).

2.3. Equine sera and reference sera

In order to evaluate the developed ELISA for studies of trypanosomosis seroprevalence, samples from three Venezuelan populations were tested, 93 sera from the savanna (Hato el Frío, Estado Apure), 21 from the central region (Estado Miranda) and 27 sera from the stable in La Rinconada racetrack in Caracas, Venezuela.

From these equine populations, all the 27 sera from racehorses living in a trypanosomosis-free area at La Rinconada Racetrack and determined to be negative to trypanosomosis by IFA were taken as negative reference sera. Sixty-one sera from horses living in a protozoan endemic area at the venezuelan savanna and determined to be positive by IFA were used as positive reference sera.

2.4. The ELISA test standardization

The 96-well polyvinyl plate (Coster, Cambridge, MA) was sensitized with antigen (100 μl/well) diluted in buffer 50 mM carbonate-bicarbonate, pH 9.6 at the optimal dilution for 18 h at 4°C in a humid chamber. Three washes were performed for 5 min each, using 200 μl of 20 mM PBS pH 7.2 with 0.1% Tween 20 (PBS-Tween) per well. PBS-tween containing 0.5% gelatin (200 μl/well) was used as blocking buffer and applied for one hour at 37°C. The equine serum (100 μl/well) was diluted 1 : 100 in a blocking buffer and incubated for 1 h at 37°C in a humid chamber. The plates were washed under the same conditions. The conjugate (100 μl/well) was diluted 1 : 100 in blocking buffer and incubated for 1 h at 37°C in a humid chamber. The plate was washed with PBS-tween as described before and the substrate solution (100 μl H2O2 at 0.5%) and the chromogens (500 μl of 2,2-azino-bis-3-ethylbenz-thiazoline-6- sulfonic acid at 2%) (ABTS, Sigma) were added in 10 ml of 100 mM citrate buffer, pH 4, for 45 min in the darkness at ambient temperature. Finally the OD was read in the ELISA reader (Titertek Uniskan II) at 405 nm.

The optimal dilution of the conjugate was obtained using four different concentrations of equine IgG (200; 20; 2 and 0.2 ng), against four different dilutions of the conjugate (1 : 250, 1 : 500, 1 : 1000 and 1 : 2000) by triplicate. In order to establish the optimal dilution of the antigen, four dilution of the antigen were tested (1 : 10; 1 : 20; 1 : 50 and 1 : 100), two different dilution of conjugate (1 : 1000 and 1 : 1500) and two concentrations of equine reference sera (1 : 100 and 1 : 500). All of them were performed by duplicate. The determination of the cutoff was done through variance, therefore, the 27 reference negative equine serum from La Rinconada racetrack were analyzed by ELISA, in order to establish a confidence interval on the basis of the normal distribution of the OD as described De Savigny and Voller (1980). The cutoff point was also established using a Bayesian classification technique (Geissel, 1964), the method implied that the absorbimetric values of an ELISA of equine negative and positive referential sera were logarithmicaly transformed obtaining a normal distribution according to the graphical and Kolmogorov–Smirnov test.
High and low positive sera and negative ones were included in each plate as controls. For blanking, three wells without sera were included in each plates.

2.5. *Indirect fluorescent antibody test (IFA)*

The IFA was developed by the standard methodology described by García (1988), using the dilution 1 : 80 as cutoff to differentiate between the negative and positive sera.

2.6. *Determination of the assessment*

In order to determine the assessment of the test, 76 sera from venezuelan savanna and 27 sera from La Rinconada racetrack, were analyzed by ELISA and IFA, to allow a comparison of sensitivity, specificity, predictive value and the correlation between both techniques as described Tizard (1982) and Monfort and Miller (1990). The accuracy of the assay was determined by repeating the analysis of one serum 68 times in the same plate, to estimate the variation coefficient according to Tizard (1982).

2.7. *Kinetic of anti-*T.evansi* specific antibodies during experimental infection in a horse*

To study the immune-serological response to *T. evansi*, sera were obtained for 53 days from an equine that was experimentally infected with a *T. evansi* isolated from a rat. Through ELISA, the specific IgG antibody production against the parasite was measured.

3. Results

3.1. *Standardization of assay*

The optimal dilution for the goat anti-horse IgG peroxidase-conjugate was 1 : 1000. The optimal incubation period for the ABTS-H$_2$O$_2$ substrates was 45 min. The optimal antigen dilution was determined as 1 : 20 (13.5 µg/well), which resulted in a 4.72 ratio between the absorbance values for the positive and negative sera (data not shown).

In the estimation of the cutoff for variance methods, a reference horse population negative to trypanosomosis was used, and its OD mean ($\mu$) was 0.135 and its variance ($\sigma^2$) was determined as 0.035, therefore $\mu + 3(\sigma) = 0.135 + 3(0.035) = 0.240$. This value, was used as cutoff point for the other assays.

The cutoff point was established within a Bayesian framework which used the referential positive and negative population as a priori knowledge. The cutoff can be obtained from Fig. 1 corresponding to the OD at which the plotted curves intercept. Each of the curves corresponds, respectively, to $P_2(H_i/Y_i, X)$. $Y$ is the logarithms of the absorbance in the ELISA of a given animal. $X$ corresponds to the data of the referential populations, $H_i$ is the hypothesis that the animal is sero-positive and $H_1$ is the hypothesis that the animal is sero-negative. This $P_2(H_i/Y_i, X)$ is the possibility of hypothesis ‘$i$’ is true for a specific OD after observing the whole population. The cutoff point 0.243 corresponds to a serum that has an equal chance of being positive or negative.
Note that the two curves are complementary and that even for values that are close to 0.243 they provide very clear-cut evidence in favor of one of the two hypothesis.

3.2. Criteria for assessment of assay

The comparison between ELISA and IFA (Table 1) showed an ELISA relative sensitivity of 98.39%, a relative specificity of 95.12% and a relative predictive value of 96.83%. A correlation value of 0.93 between the ELISA and IFA test was obtained. The variation coefficient was calculated as 18.7% (data not shown).

3.3. Kinetic of the specific anti-*T. evansi* antibody during experimental infection

The result of the OD and the parasitemia obtained during experimental infection of the horse C1 is presented in Fig. 2. This figure (Fig. 2(A)) shows an increase in the OD during the first 10 days post-infection, and afterwards OD presents variation but it is never as low as the pre-infection values. During this period the parasitemia oscillated from zero to $10^6$ trypanosomes ml$^{-1}$ (Fig. 2(B)).

3.4. Sero-prevalence in different local horse populations

The sample of 93 horses (located in Estado Apure) resulted in serum-positive prevalence of 81.7%, while 21 horses from a stable (Estado Miranda) resulted in 42.9%

<table>
<thead>
<tr>
<th>ELISA/IFA</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>61</td>
<td>2</td>
<td>63</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>39</td>
<td>40</td>
</tr>
<tr>
<td>Total</td>
<td>62</td>
<td>41</td>
<td>103</td>
</tr>
</tbody>
</table>

Seventy-six equine sera from work horses of venezuelan savanna and 27 from La Rinconada racetrack.

Specificity: $36/41 = 95.12\%$.
Predictive value: $61/63 = 96.83\%$. 

![Equine serum probability of being serologically positive or negative in a ELISA to detect anti-*T. evansi* antibodies, according to its optical density.](image-url)
serum-positive prevalence and in the sample of 27 racehorses (Distrito Federal), no sera-positive animals were found.

4. Discussion

This paper presents the standardization of an immunoenzymatic assay, able to detect anti-T. evansi equine antibodies and the establishment of a cutoff point of 0.24. This OD permits to differentiate between positive and negative equine sera. The optimal antigen dilution was 1:20 (13.5 μg/well) and for the sera was 1:100.

Since there is no information about the variable antigen-type (VAT) from venezuelan strains of T. evansi, in this work, we did not study the influence of VAT in ELISA/T. evansi. Nevertheless, it is possible that after sonication and centrifugation of the antigen, most of the parasite membranes and the variant-specific glycoproteins responsible for VAT in the parasites (Vickerman, 1989), remain in the pellet and not in the supernatant. Also, since we did not use a cloned strain of T. evansi for antigen preparation, we may have a pool of VAT from T. evansi, making our antigen a heterogeneous one.
The Bayesian test establishes in a statistical way the probability that a serum can be positive or negative to trypanosomosis according to its OD. The determination of the cutoff through the variance values in the ELISA only constitutes a limit, therefore, those sera which are closer to this point are expressed as suspicious animals (Voller et al., 1979; De Savigny and Voller, 1980). On the other hand, the discrimination method permits a fixation of the probability for positive or negative sera that are close to the cutoff (Fig. 1), granting the clinician more precise and easy information about the result of the ELISA.

The relative assessment of the immunoenzymatic assay against IFA (Table 1) was demonstrated in a sensibility of 98.39%, specificity of 95.12% and predictive value of 96.83%. Similar values were found by other authors (World Health Organization, 1976). The agreement of the ELISA and the IFA results demonstrate that these tests can identify animals infected with trypanosomosis, recognize those that are healthy and predict the prevalence of the disease in a particular moment.

In Fig. 2, the variation of the antibody response during the course of the experimental infection is shown. Demonstrating that the level of antibodies remained higher (more than DO ≈ 0.5) throughout the experimental infection and the sero-conversion began 13 days after infection. These results are in agreement with those observed in Trypanosoma equiperdum by Hagebock et al. (1993). These results explain the effectiveness of the serological technique in the diagnosis of horse trypanosomosis.

Due to the cross reaction between some trypanosomes (Voller et al., 1975; Luckins, 1977; Rae and Luckins, 1984) and specifically between T. evansi and T. vivax (Toro et al., 1980) a preliminary ELISA was performed in order to detect anti-T. vivax bovine antibodies using the T. evansi antigen. A clear differentiation between pre-infected and post-infection serum of a experimentally infected bovine was found (data not shown).

We use this test on the venezuelan equine populations from different geographic origin. In the first sample, which consisted of 93 sera from horses from the venezuelan savanna (Estado Apure), 81.7% were sero-positive to T. evansi. In another population of 21 equines originally from the venezuelan central region, 42.9% were sero-positive. In the 27 equines from La Rinconada racetrack (Caracas), no positives were found.

The high prevalence of trypanosomosis detected in the venezuelan savanna population could be explained by the large size of the vector population and the close proximity between susceptible and infected animals. These two factors have been cited by Luckins (1988) in a study of T. evansi in Asia. In addition, the large rodent-like capybara (Hydrochaeris hydrochaeris) infected with T. evansi in the venezuelan savanna (Arias et al., 1997), could act as reservoirs for the infection of horses. On the other hand, the absence of trypanosomosis in the horse population from La Rinconada racetrack may be due to the strict supervision and the periodic fumigation for fly control.

These results show the advantage of using the ELISA/T. evansi test in epidemiological studies for trypanosomosis equine in the same manner as those reported by Rae et al. (1989) or by Olaho-Mukani et al. (1993) with ELISA/T. evansi for Camelus dromedaries.

The development of an ELISA for the diagnosis of equine trypanosomosis, constitutes a first step in performing a complete study of sero-epidemiology distribution of the disease, and its modification by environmental factors. It also provides a better tool for the study of immunological response caused by the trypanosomiasis in horses.
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References