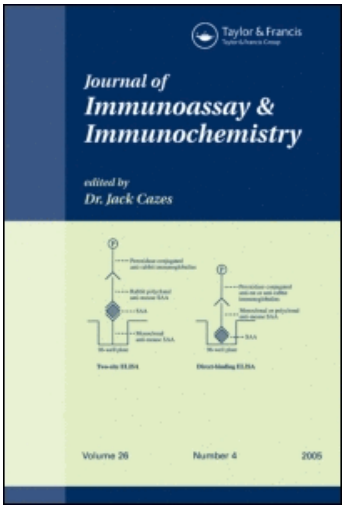

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Assessment of Chromogen Suitability in ELISA for the Detection of Anaplasmosis and Trypanosomosis

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Abstract: Two different ELISAs were routinely performed in our laboratory to detect bovine trypanosomosis and anaplasmosis. The ELISA test for trypanosomosis involved the adsorption of a soluble fraction of parasites as the antigen; and, the ELISA for anaplasmosis was performed with a purified recombinant protein MSP5r adsorbed to the plate. With the purpose of assessing the merit of ABTS and TMB, we compared the absorbance obtained from positive and negative control sera from both assays. The results obtained, suggest that TMB is more adequate for recombinant antigens and that ABTS is preferred when partially purified antigenic extracts are used in the ELISA test.

Keywords: Anaplasmosis, Tripanosomosis, TMB, ABTS, MSP5, ELISA

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INTRODUCTION

The indirect Enzyme Linked Immunosorbent Assay (ELISA), is based on the use of conjugated antibodies to detect the complex antigen-antibody. The antibodies, generally IgG, covalently bound to an enzyme (peroxidase or phosphatase) and are easily detected by the enzymatic reduction of a chromogen. Since the generation of oxidized chromogen is a first order reaction, affinity and specificity to the antigen can be easily determined.[1,2] Therefore, the choice of the chromogen is crucial in this assay.

Benzidine (a protons donator) has been shown to be an efficient chromogen for most ELISA assays; however, due to its high toxicity, some other derivatives have been developed. The 3,3′,5,5′ tetramethylbenzidine (TMB) is generally used in standard assays. The production of oxidized TMB is highly efficient and is one order of magnitude more sensitive than orthophenyl diamine (OPD), another chromogen currently used.[3] TMB has various advantages: it is stable, has low volatility, its toxicity is reduced to a minimum, it is as sensible as benzidine, and it's cost is low. TMB is soluble in an acidic pH, which partially limits its use in certain assays with antigen mixtures.[3] On the other hand, 2,2′-Azinobis(3- ethylbenzthiazoline −6- sulfonic acid (ABTS), which is also a widely used substrate in the immunoenzymatic assay technique; is stable, has low volatility, and is soluble in water, but its toxicity level is much higher.[4]

Bovine anaplasmosis and trypanosomosis are enzootic hemoparasites in most tropical and subtropical countries, including Venezuela, with a seroprevalence up to 30% for tripanosomosis and 50% for anaplasmosis.[5–9] It is known that both of them affect goats and sheep, but there is little information on the effects of these diseases in small ruminants.

Anaplasmosis is a tick borne disease caused by *Anaplasma marginale* (*A. marginale*). This obligatory intraerythrocytic rickettsia induces acute anemia, fever, weight loss, and possible death.[5,10] The causative agent of ruminant trypanosomosis in South America is *Trypanosoma vivax* (*T. vivax*). This hemoflagellate causes a disease characterised by a fever and anemia, similar to anaplasmosis.[11]

The aim of this study was to determine which of these two substrates (ABTS or TMB) may be efficiently used in the ELISA developed in our laboratory: one recombinant antigen of Mayor Surface Protein 5 (MSP5r) from *A. marginale* to detect bovine anaplasmosis, and the other to serodiagnosis of trypanosomosis, using soluble extract of *Trypanosoma evansi* (*T. evansi*) as the antigen.

EXPERIMENTAL

Reference Sera for *Anaplasma marginale*

Hyperimmune Positive Serum

A young bull[8,12] was inoculated subcutaneously with 100 μg of MSP5r contained in 2 mL of complete Freund's adjuvant, and 3 more times using

incomplete Freund's adjuvant in 15 day intervals under the same conditions. The hyperimmune serum was collected 10 days after the last inoculation. (Kindly donated by H. Caballero, Universidad Simón Bolívar).

Weak Positive Serum

The positive serum was collected from the experimentally infected calf[12] with *A. marginale* at the 32nd day after being infected.

Negative Serum

Serum Nos 5490 and 5644 came from France, where anaplasmosis is not endemic.

Reference Serums for *Trypanosoma vivax*

Positive Serum

Positive serum was obtained from the experimentally infected sheep with *Trypanosoma vivax* (S1+ from sheep OVTV 0203 and S2+ from sheep OVTV 0204) at the 25th day post-infection.

Negative Serums

Negative sera were collected from OVTV0203 (S1-) and OVTV0204 (S2-) before the experimental infection.

Purification of the MSP5r Antigen

MSP5r was previously purified[8] from the supernatant of *E. coli* transfected bacteria (pAR1903) using the Xpress System protein purification kit (TA cloning kit; Invitrogen, San Diego), according to the manufacturers instructions under denaturant conditions.

Soluble Antigenic Extract from *T. evansi*

Due to the cross reaction between some trypanosomes,[1,12] and specifically between *T. evansi* and *T. vivax*,[9] the ELISA was performed in order to detect bovine anti-*T. vivax* using *T. evansi* as the antigen. The soluble extract was purified according to Reyna-Bello et al.[9] Briefly, a cryopreserved sample of *T. evansi* was inoculated in a rat (*Spragüe Dawley*) and parasitemia was followed until it reached 10^9 parasites/mL. The parasites were purified using a DEAE-Cellulose column,[13] and then centrifuged at 16000 × g. The precipitate was resuspended in PBS at pH 8, and sonicated in five

cycles of 30 seconds. Then, the sample was centrifuged for 5 minutes at 16000 × g. The supernatant, containing the soluble antigenic extract was stored at −70°C. The protein concentration was determined using the MicroBCA kit with bovine albumin as the standard (Promega, Madison).

ELISA Using MSP5r as the Antigen (ELISA/MSP5r)

The 96 polyvinyle wells (Nunc, Polysop, Denmark) were sensitized with MSP5r diluted in 50 mM of carbonate bicarbonate buffer at pH 9.6, at a concentration of 2 μg/mL (100 μg/well). The plates were incubated over night in a humid chamber at 4°C. The plates were washed five times with 150 mM NaCl containing 0.1% Tween 20 (WS), blocked with 5% nonfat dry milk, diluted in phosphate buffer saline solution at 20 mM, 150 mM NaCl, pH 7.2 (PBS), and incubated for 1 hour at 37°C in a humid chamber. The plates were washed again under the same conditions as described above. The sera were diluted 1/200 in PBS containing 0.1% Tween 20 (PBS −T), and were added to a 100 μL/well for 1 hour at 37°C. The plates were washed again and the anti-bovine conjugate (Sigma, St. Louis) was added, diluted 1/2500, 1/5000, 1/10000, and 1/20000 in PBS-T, in order to attain optimal concentrations, for a time lapse of 1 hour at 37°C in a humid chamber. The plates were washed with WS and the chromogen TMB was added, diluted in a citrate phosphate buffer at 50 mM, pH 4, containing 10% dimethil sulfoxide and 1% H_2O_2 (50 μL/well) for 30 minutes at room temperature. The reaction was stopped with 2 M H_2SO_4 (50 μL/well) and the plates were read at 450–630 nm.

ELISA Using the Soluble Extract of *T. evansi* as the Antigen (ELISA/*T. evansi*)

The 96 polyvinyle wells (Nunc, Polysop, Denmark) were sensitized 100 μL/well with the antigenic extract isolated from *T. evansi*, diluted in 50 mM of carbonate bicarbonate buffer at pH 9.6 at a concentration of 32 and 8 μg/mL. The plates were incubated over night in a humid chamber at 4°C, and were washed five times using WS. The plates were then blocked with 5% nonfat dry milk diluted in PBS, and incubated for 1 hour at 37°C in a humid chamber. The plates were washed again under the same conditions as described above. The diluted serum (1/200 in PBS−T) were added to each well for 1 hour at 37°C, in order to attain optimal concentrations. The dilution of 1/200 was appropriate, as previously obtained by standarization.[9] The plates were washed again and the anti-bovine conjugate (Pierce, Rockford) was added at a dilution of 1/20000 (the conjugate was diluted in PBS-T), for a time lapse of 1 hour at 37°C in a humid chamber. The plates were washed with WS and the chromogen ABTS was added, diluted in

100 mM of citrate buffer pH 4 containing 0.5% H_2O_2 (100 μL/well) for 45 minutes, at room temperature. The plates were then read at 405 nm.

With the purpose of determining the optimal condition in both tests, we used the value of the optical density (OD) mean of the positive control sera, divided by the OD mean of the negative sera. The ELISA is a test to distinguish positive from negative sera, so the bigger the difference between positive and negative sera, the better will be the ELISA system.

RESULTS

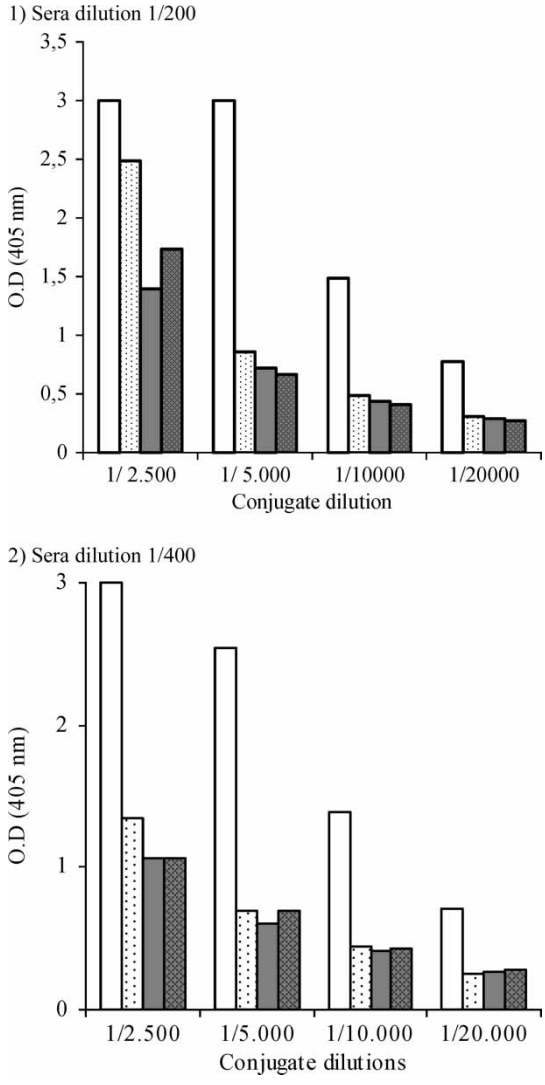
When the ELISA/MSP5r plate was revealed with ABTS (Figures 1 and 2), the ratio between the naturally infected serum and the mean of the two negatives, did not exceed 2 in either case. The major difference between the positive and negative serum was of 1.8, using dilutions of 1/200 for the serum and 1/2500 for the conjugate. In the remaining conditions that were observed, including figures 1 and 2, the hyperimmune serum was efficiently detected in the assay, indicating that the ELISA system worked. However, when we compared the naturally infected serum versus the two negatives ones with conjugate dilutions of 1/5000, 1/10000, and 1/20000 in either figure, it seemed like the system was not sensitive enough under these conjugate conditions, since there were no differences between positive and negative sera.

When TMB was used in the MSP5r assay, (Figures 3 and 4), the results differed from those of ABTS. On one hand, with a 1/2500 dilution of the conjugate, the system was completely saturated and all the sera, positive or negative, reached the maximum OD. Only the naturally infected serum did not reach the maximum OD; nevertheless, at the 1/400 dilution, it reached the maximum OD. Differences among the naturally infected serum versus the negative serum (dilutions of 1/200 and 1/400) were observed at conjugate dilutions of 1/10000 and 1/20000 in contrast to the results depicted in Figures 1 and 2. With the use of TMB, at a dilution of 1/200 of the serum and 1/10000 of the conjugate (Figure 3), the O.D ratio was 1.89, and at conjugate dilution 1/20000 the ratio was 2.8. These values showed that TMB works with higher conjugate dilutions as opposed to ABTS.

On the other hand, in Figures 5 and 6, we can observe that when we used trypanosome as the antigen, the difference between positive and negative sera becomes more evident when ABTS is used (Figure 5), resulting in a ratio that goes from 2.21 at an antigen concentration of 8 μg/L, to 2.69 at 32 μg/mL. On the contrary, with TMB this difference is 1.75 to 1.95 times, respectively (Figure 6).

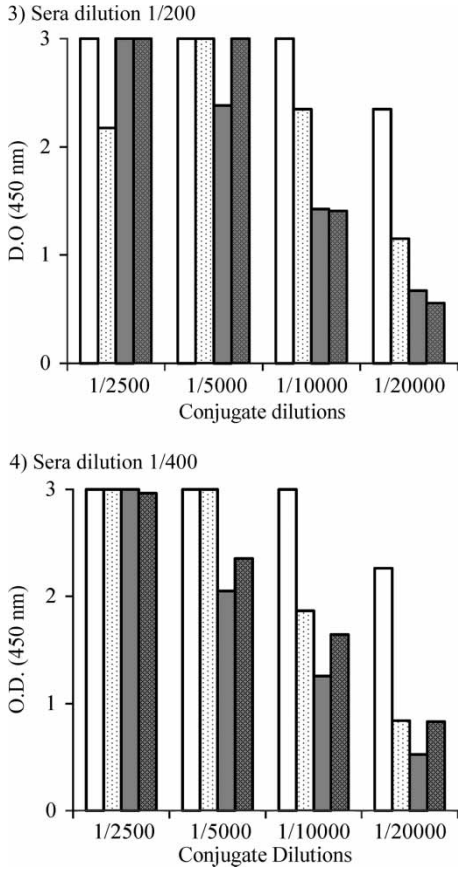
DISCUSSION

The proper standarization for the immunoenzymatic assay is the key for the development of a high precision test, the antigen being the most difficult



Figures 1 and 2. Evaluation of the chromogen TMB in an indirect ELISA using 1 μg/mL of MSP5r, different dilutions of the conjugate vs. two positive sera (hyper-immune serum ☐ and serum from experimental infected calf ⬚) and two negative control sera ▩, ▩. Figure 1 at dilutions of 1/200 of sera and Figure 2 at dilution 1/400, respectively.

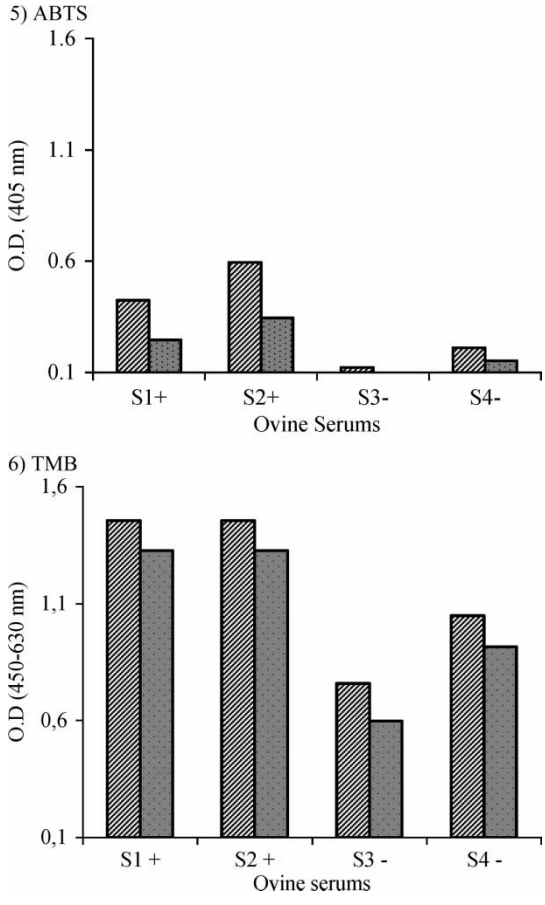
thing to standardize.[14] Once the antigen is standardized for the assay, all the elements that could influence the outcome of the assay, for instance the different solutions for the blocking procedure, dilutions of the sera, and the conjugate, must be controlled.[2] In this study, we have demonstrated that



Figures 3 and 4. Evaluation of the chromogen ABTS in an indirect ELISA using 1 μg/mL of MSP5r, different dilutions of the conjugate vs. two positive sera (hyperimmune serum ☐ and serum from experimental infected calf ⁙) and two negative control sera ▩, ▩, Figure 3 at dilutions of 1/200 of sera and Figure 4 at dilution 1/400, respectively.

the substrate used in the enzymatic assay is crucial for the proper functioning of this test.

In the study involving MSP5r, we can appreciate differences between positive and negative sera using both TMB and ABTS. However, these differences were more evident when the TMB substrate was used (Figures 3 and 4). This could be due to the fact that TMB is more sensitive,[15] and, thus, it is capable of detecting minimum concentrations of anti-MSP5 immunoglobulins that are present in the serum even at high serum (1/200) and conjugate (1/10000) dilutions, while ABTS is less sensitive. Another aspect that must be considered is that MSP5 is a purified protein, which diminishes unspecific



Figures 5 and 6. Evaluation of the conduct of the chromogens ABTS (5) and TMB (6) respectively in an indirect ELISA at different concentrations of the antigenic extract of *T. evansi* (32 μg/mL ▨ and 8 μg/mL ■) vs. two positive serums (S1+ and S2 +) and two negative ovine serums (S3- and S4-). The dilution of the ovine anti-IgG conjugate was of 1:20000.

adherence, lowering the risk of false positives in the presence of a highly sensitive substrate like TMB.

Curiously, when we used soluble antigenic extracts from *T. evansi* (Figures 5 and 6), as is the case with *T. evansi*, the best ratio between the positive and negative serum was achieved using ABTS substrate and not TMB. This effect could be due to unspecific binding of the immunoglobulins to certain surface glycoproteins that are inserted in the external layer of the plasmatic membrane, called variable surface glycoprotein (VSG),[16] that are present in the antigenic extract of *T. evansi*. These glycoproteins are partially responsible for the cross reaction between *T. vivax* and *T.*

evansi.[17–19] In our laboratory, we have seen how sera that come from non endemic zones for these parasites, are recognized in Western blots of VSGs from Venezuelan isolates (data not shown). Thus, a highly sensitive substrate like TMB gives a high background in the assay.

In other assays, employing the ELISA test and Western blots for the diagnosis of *Shistosoma mansoni* (*S. mansoni*), cross reactivity was described between this parasite and others like *Ancylostoma sp*. and *Ascaris lumbricoides*, which constitutes a limiting factor for the utilization of serologic tests in the diagnosis of this parasite.[20,21] In order to study this cross reactivity problem in serological tests to determine the presence of *S. mansoni*, Alarcón de Noya *et al.*,[22] demonstrated that when adding sodium metaperiodate to the antigen, the cross reaction of this parasite with other helminths like *Faciola hepática*, *Trichinilla sperillis*, *Toxocara canis*, etc; was inhibited. This demonstrated that in ELISA assays and Western blots the existent cross reaction between *S. mansoni* and other helminths was principally due to the glycosylated part of the glycoproteins, since the metaperiodate oxidizes the hydroxilic groups of the sugars, blocking the access of the antibodies to these epitopes.[22]

It is possible that the glycoproteins that are present in the antigenic extract of *T.* evansi[23] have played an important role in the appearance of false positives in the ELISA, because of the unspecific recognition of glycosydic epitopes by antibodies present in the negative serum of the studied animals. As we previously indicated, the TMB substrate is more sensitive than the ABTS substrate and this may be the reason for our observation, that when we used a soluble antigenic extract of *T. evansi*, the ABTS results are more optimal for the ELISA test. This could probably be the reason why when we used the TMB substrate, the negative samples presented elevated values of OD caused by the unspecific recognition of the sugars by the antibodies that come from negative serum, which reduces the differences between the positive and negative samples, making the standardization of the test very difficult.

Based on theses results, we can point out that the ELISA test is capable of differentiating positives and negatives, indifferently from the type of antigen or the used substrate. However, the differences between positive and negative sera were higher when we used TMB for the purified proteins; also, ABTS seemed to be a better substrate when using mixtures of antigens like that of *T. evansi*.

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