COMPARISON OF ELISA AND IFA FOR ESTIMATION OF ANTIBODY LEVELS OF CATTLE TO THEILERIA ANNULATA VACCINE

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ABSTRACT

Bovine tropical Theileriosis caused by Theileria annulata is an economically important disease of cattle. An enzyme linked immunosorbent assay (ELISA) was used to determine antibody levels in vaccinated and unvaccinated cattle, using cellular schizont as antigen and its results were compared with immunofluorescent assay (IFA). For this test 126 sera collected (105 vaccinated, 31 not vaccinated) from cows and assayed with ELISA which among them 104 sera were positive and 32 sera were negative. Same sample assayed with IFA in which 99 were positive sera and 37 were negative sera. Thereby the sensitivity and specificity of this ELISA on comparison with IFA were 95.5% and 66.6% respectively. This study revealed that ELISA could be successfully used for both differentiating vaccinated and not vaccinated cattle and obtaining the titer of vaccinated cattle.

Keywords: Theileria annulata, ELISA, IFA, vaccine.

INTRODUCTION

Tropical bovine Theileriosis, caused by Theileria annulata (T.annulata), is an economically important tick-borne protozoan disease in many parts of the world. This disease in its milder form is responsible for lowered milk production while in severe cases causes high mortality resulting in economic losses to the dairy farmers. An array of diverse immunological tests, including indirect fluorescent antibody technique, complement fixation, passive haemagglutination, agar gel precipitation, counter immune-electrophoresis and capillary tube agglutination tests, are employed for monitoring antibody responses following Theileria infection/vaccination. ELISA have been used extensively in recent years as aids in serological diagnosis of parasitic diseases and in vaccinated cattle (Bidwell et al 1978, Luckins and Mehlitz 1978, Pipano E. 1990, Beniwal et al 1997, Gubbelts et al 2000 and Manuja et al 2001). ELISA is the test of choice for the assay of antibody and offers an unprecedented combination of sensitivity, specificity, speed, economy, laboratory safety and automation. This paper describes an evaluation of ELISA for detection Theileria annulata vaccination and its comparison with IFA which the results clearly showed ELISA can be used successfully even with cellular schizont antigen.

MATERIALS AND METHODS

A) Schizont infected lymphoid cells of Theileria annulata

Schizont infected lymphoid cells which had been isolated from infected animal with Theileria annulata infection were grown in RPMI(Gibco), with 10/15% FCS(Gibco) and after some passages, the cells transferred to modified stoker(Gibco) with 10% FCS which from this, the cell culture containing 28,000,000 cells/ml were isolated.

B) Preparation of antigen from concentrated
Comparison of ELISA and IFA for Antibody Estimation

Schizont infected lymphoid cells

The cells were washed twice with PBS (0.01M, pH=7) then the cells treated with sonicator (28000 W) for 5 min. in ice and centrifuged 10 min, 100,000 g. Cells were precipitated and the supernatant was collected by washing the pellet three to four times with PBS (Kachani et al 1992). The collected supernatant as antigen was analysed in ELISA test and its protein concentration was determined by Lowry (1951).

C) Preparation of Rabbit Anti-Cow-\gamma G-HRP conjugate

Rabbit anti - cow immunoglobulin was conjugated to peroxidase by the method of Wilson and Nakane (1978) in which sodium periodate (12mM) were used and the amount of \gamma G was twice the peroxidase. This couple was passed through sephadex G-25 and sodium borohydride was used for the reduction and the sample was dialysed against PBS (0.01 M).

D) Optimising antigen concentration and conjugate dilution

By checkerboard analysis, different concentrations of antigen against different dilutions of conjugate were examined according to the method of ELISA in section E to obtain the best concentration and dilution of antigen and conjugate.

E) Enzyme linked immunosorbent assay (ELISA)

Theileria Schizont antigen diluted with 0.05 M carbonate / bicarbonate buffer (pH=9.6) were dispensed in 100\mu l into the wells of polystyrene micro titer plates (Nunc). The plates were left overnight at 4\degree C to coat with antigen and then washed three times with PBS/Tween 20 and shaken dry, then blocked with PBS/Tween 20, 0.5% gelatin and washed again three times with PBS/Tween 20. Pooled standard positive and negative sera diluted 1/100 with PBS/Tween 20/ 0.1% gelatin and added to each well in volumes of 100\mu l then the plates were incubated for 1.5 h at 37\degree C. The plates were washed and dried as before and 100\mu l of enzyme conjugate diluted 1/2000 was added to each well, followed by further incubation for 1.5h at 37\degree C. After washing and drying again, 100\mu l volumes of orthophenylenediamine (1mg/ml) in Phosphate/Citrate buffer pH 5.5 containing 0.01 % hydrogen peroxide was added to each well and incubated at room temperature for 10 min. The enzyme reaction was then stopped with

**Correlation 0.8869**

![Correlation graph](image)

**y = 0.339x - 0.0009, \ R^2 = 0.7866**

Fig. 1. Correlation curve between IFA and ELISA.

Table I. Results of schizont antigen concentration and Anti-Cow-HRP Conjugate dilution by presenting their optical density (OD).

<table>
<thead>
<tr>
<th>Schizont Ag (µg/mL)</th>
<th>Conjugate Ag (µg/mL)</th>
<th>80</th>
<th>80</th>
<th>40</th>
<th>40</th>
<th>20</th>
<th>20</th>
<th>10</th>
<th>10</th>
<th>Blank</th>
<th>No Coating</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/1000</td>
<td>1.27</td>
<td>0.93</td>
<td>1.26</td>
<td>0.86</td>
<td>1.23</td>
<td>0.83</td>
<td>1.21</td>
<td>0.81</td>
<td>0.42</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>1/2000</td>
<td>1.12</td>
<td>0.78</td>
<td>1.10</td>
<td>0.46</td>
<td>0.93</td>
<td>0.43</td>
<td>0.76</td>
<td>0.41</td>
<td>0.36</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>1/4000</td>
<td>0.84</td>
<td>0.62</td>
<td>0.71</td>
<td>0.40</td>
<td>0.65</td>
<td>0.36</td>
<td>0.52</td>
<td>0.33</td>
<td>0.32</td>
<td>0.31</td>
<td></td>
</tr>
</tbody>
</table>

Table II. The cut off points for standard positive and negative sera.

<table>
<thead>
<tr>
<th>Serum dilution</th>
<th>1/50</th>
<th>1/100</th>
<th>1/200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard sera</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>positive</td>
<td>1.224</td>
<td>1.121</td>
<td>1.028</td>
</tr>
<tr>
<td>positive</td>
<td>1.312</td>
<td>1.267</td>
<td>1.122</td>
</tr>
<tr>
<td>positive</td>
<td>1.232</td>
<td>1.211</td>
<td>1.110</td>
</tr>
<tr>
<td>positive</td>
<td>1.154</td>
<td>1.028</td>
<td>0.984</td>
</tr>
<tr>
<td>positive</td>
<td>1.289</td>
<td>1.278</td>
<td>1.124</td>
</tr>
<tr>
<td>positive</td>
<td>1.293</td>
<td>1.273</td>
<td>1.121</td>
</tr>
<tr>
<td>negative</td>
<td>0.998</td>
<td>0.685</td>
<td>0.770</td>
</tr>
<tr>
<td>negative</td>
<td>0.967</td>
<td>0.776</td>
<td>0.768</td>
</tr>
<tr>
<td>negative</td>
<td>0.865</td>
<td>0.654</td>
<td>0.652</td>
</tr>
</tbody>
</table>

CUTOFF = mean (OD positive sera) + 2 SD
CUTOFF = 1.257

Optimum sera for cut off = 1/100

2M Sulphuric acid and the intensity of development of
the yellow colour reaction was determined at 492nm .
The controls included in each assay were wells con-
taining PBS instead of antiserum and known positive
and negative reference sera. By this system 116 sera (95
vaccinated, 21 not vaccinated) collected from the field
were examined.

F) Immunofluorescent assay test (IFA)

IFA was performed as described by Burridge (1971)
with minor modifications. Theileria Schizont antigen
was fixed and incubated with the same serum samples
which used for ELISA then after washing, rabbit
antibody γG - FITC conjugate was added and the
reaction was observed under fluorescent microscope.

G) Comparison of IFA and ELISA

116 samples titre were compared by IFA and ELISA
and the correlation factor was estimated. (Watt.T.A.
1995)

RESULTS

Table III. Determination of Intra and Inter assays for showing the reproducibility of ELISA.

<table>
<thead>
<tr>
<th>OD</th>
<th>NO</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Mean</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra Assay</td>
<td>1.332</td>
<td>1.314</td>
<td>1.121</td>
<td>1.282</td>
<td>1.282</td>
<td>0.095</td>
<td>%7.25</td>
<td></td>
</tr>
<tr>
<td>Inter Assay</td>
<td>1.273</td>
<td>1.278</td>
<td>1.028</td>
<td>1.267</td>
<td>1.211</td>
<td>0.1</td>
<td>%8.25</td>
<td></td>
</tr>
</tbody>
</table>

SD = Standard Deviation
CV = Coefficient of Variation

Checker board assay for determination the dilution of
antigen and conjugate

Developing an ELISA system for assessment of hu-
moral immunity of vaccinated cows against Theileria, was
achieved using concentrated lymphoid Schizont antigen (2.2
mg/ml), Then coating 40µg/ml of this antigen on ELISA
plates. In this system 1/2000 dilution of rabbit anti-cow-
HRP conjugate was used (Table I).

Assessment of antibody levels by ELISA

The cut off points for standard positive and negative sera
were found to be 1.257 OD (Table II). In this
system 116 sera collected from cows were assayed with
ELISA which among them 94 sera were found positive
Comparison of ELISA and IFA for Antibody Estimation

Table IV. Comparison of IFA and ELISA for selected samples to show their differences. ELISA was found to be more sensitive than IFA, thus detecting antibody in higher dilutions of serum samples.

<table>
<thead>
<tr>
<th>NO</th>
<th>IFA Titer</th>
<th>ELISA Serum Dilution</th>
<th>ELISA OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/64</td>
<td>1/400</td>
<td>1.423</td>
</tr>
<tr>
<td>2</td>
<td>1/64</td>
<td>1/400</td>
<td>1.324</td>
</tr>
<tr>
<td>3</td>
<td>1/128</td>
<td>1/800</td>
<td>1.642</td>
</tr>
<tr>
<td>4</td>
<td>1/128</td>
<td>1/800</td>
<td>1.486</td>
</tr>
<tr>
<td>5</td>
<td>1/254</td>
<td>1/1600</td>
<td>1.621</td>
</tr>
<tr>
<td>6</td>
<td>1/254</td>
<td>1/1500</td>
<td>1.632</td>
</tr>
<tr>
<td>7</td>
<td>1/32</td>
<td>1/100</td>
<td>1.345</td>
</tr>
<tr>
<td>8</td>
<td>1/128</td>
<td>1/900</td>
<td>1.598</td>
</tr>
<tr>
<td>9</td>
<td>1/254</td>
<td>1/1400</td>
<td>1.604</td>
</tr>
<tr>
<td>10</td>
<td>1/64</td>
<td>1/200</td>
<td>1.574</td>
</tr>
<tr>
<td>11</td>
<td>1/32</td>
<td>1/200</td>
<td>1.436</td>
</tr>
<tr>
<td>12</td>
<td>1/254</td>
<td>1/1400</td>
<td>1.321</td>
</tr>
<tr>
<td>13</td>
<td>Negative</td>
<td>1/100</td>
<td>0.875</td>
</tr>
<tr>
<td>14</td>
<td>Negative</td>
<td>1/100</td>
<td>0.764</td>
</tr>
<tr>
<td>15</td>
<td>Negative</td>
<td>1/100</td>
<td>0.648</td>
</tr>
</tbody>
</table>

and 22 sera were negative.

Antibody levels by IFA and its comparison with ELISA

In testing the same sera with IFA, it was found that 89 sera were positive and 27 sera were negative. These two tests were compared and the results are presented in Table IV. The sensitivity and specificity of ELISA on comparison with IFA were found to be 95.5% and 66.6% respectively.

Intra and Inter assay

The intra and inter assays were also calculated and the results are presented in Table III in which their percentage of correlation variation were found to be 7.25% and 8.25% respectively. IFAT and ELISA results were compared for some alternate samples to show their differences (Table IV) and its correlation factor was shown in Fig.1.

Samples titer comparison between IFA and ELISA tests

The titers of randomly selected samples were compared by IFA and ELISA and are shown in Table IV in which the titers of positive and negative samples are shown in IFA as well as ELISA and their correlation curve is shown in Fig.1 in which their calculated correlation factor is indicated as 0.88 which is in acceptable range.

DISCUSSION

The availability and quality of suitable antigens, and technical reproducibility, are important features to consider in the development of a successful serodiagnostic test. The antigen used in ELISA in the present work was prepared from schizont of T. annulata. The antigenic material was stored at -70°C and used successfully in the test for periods up to one year. The serological methods which have been used previously to determine the antibody content of sera from animals infected with Theileria parasites include complement fixation, immune precipitation, capillary agglutination, indirect haemagglutination and indirect fluorescent antibody tests (Pipino 1977, Purnell 1977). Among these, the technique most widely used has been immunofluorescence. Pipino and Cahana (1969) used the IFA to monitor antibody formation in cattle following infection with T. annulata and other researchers showed also similar technique for vaccinated cattle. In the present work, ELISA was compared with IFA, these two tests did not show significant differences under conditions used in the present study regarding the results in antibody estimation. The analysis of schizont antibody response in vaccinated animals is justified since the vaccine contained schizonts of T. annulata and the seroconversion can be effectively moni-
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tored for the evaluation of vaccination campaign. The ELISA may prove useful in seroepidemiological studies as it detects antibodies to schizont which is found in carrier status in the population. This technique also provides automation for large scale seromonitoring program. In present study comparative evaluation of serum samples using ELISA and IFA showed that ELISA is more sensitive and specific than IFA, similar results have been reported by other researchers (Beniwal et al 1997, Kachani et al 1996, Kachani et al 1992, Katende et al 1990). This comparison warranted the possibility of using ELISA as a test of choice in seromonitoring of carrier and vaccinated populations. In same way Manuja et al (2001) showed that single ELISA dilution could be successfully used for studies of theileriosis by using cellular schizont. Gubbels et al (2000) showed also ELISA performed as well as IFAT and T. annulata remained positive in the ELISA for at least one year. They showed also limited cross-reaction was found only with T. Parva antiserum, but not with any other Theileria or Babesia species. The coefficient of variation (CV) of inter and intra assays in this study as mentioned in results were 8.25 and 7.25 respectively and the correlation factor between IFA and ELISA was estimated to be 0.88 which is acceptable.

REFERENCES


