DEVELOPMENT AND EVALUATION OF THE PERFORMANCE OF AN IN-HOUSE ELISA TO BE USED FOR THE INDIRECT DIAGNOSIS OF TOXOPLASMOSIS IN SHEEP

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SUMMARY
The performance characteristics of an ELISA test for toxoplasmosis was assessed using 416 samples collected from sheep located in endemic areas of Italy. Indirect immunofluorescence (IFAT) was used as the reference method. The diagnostic accuracy of the in-house method and the one of a commercial Kit was considered to be high. In this study the ROC analysis was used as a tool for selecting cut-off points. Sensitivity, specificity, likelihood ratios and Youden's index were used as indices of test accuracy. Our results indicate that the ELISA applied to sera is a highly accurate test for indirect diagnosis of toxoplasmosis.

INTRODUCTION
Toxoplasmosis is a protozoan disease in sheep, its spread is demonstrated by the high seroprevalence found in flocks. This disease results in significant health and economic losses being responsible for miscarriages, stillbirths and decreased production.

Serological surveillance is a valuable tool for assessing the spread of infection on farms (1,5). For the detection of antibodies to Toxoplasma, various techniques of analysis can be used (DT, IFAT, MAT, ELISA). The OIE Manual defines the Dye Test (DT) as the gold standard for the detection of antibodies to Toxoplasmosis (2). However, this method is not available for most veterinary laboratories. In the veterinary field indirect immunofluorescence (IFAT) is considered the reference test, although it appears to be time consuming, expensive and difficult to interpret being related to the subjectivity of the operator (3,6). To overcome these difficulties and to carry out the screening of large quantities of samples, it is preferable to use ELISA techniques (3,4).

The purpose of this paper is to illustrate the development of an in-house ELISA for the detection of antibodies to Toxoplasmosis and evaluate the performance of this method by comparison with the reference technique IFAT and a commercial ELISA.

MATERIAL AND METHODS

Serum samples: In the absence of certified reference sera, the IFAT was used to identify sheep serum samples positive and negative for the presence of anti-Toxoplasma antibodies and used as a control reaction for the ELISA technique. Positive and negative control sera were stored at -20 °C and used for the standardization of the method.

To validate the ELISA method was also set up a bank of 416 sera from sheep, chosen randomly among those sample tested in the laboratory. Even these sera were tested with the IFAT method.

Development of the in-house ELISA test
Antigen: Toxoplasma gondii RH strain was grown in cell lines of human fibroblasts (HS68). Once a concentration of tachizoiotes of 5x10⁶/ml was obtained, the extraction of antigen was carried through lysis, then repeated cycles of freezing and thawing. The product obtained was then centrifuged at 4 °C for 20 min. at 900 x g, and the supernatant stored at -70 °C until use.

Polycional anti-species antibody: Polyclonal anti-sheep IgG has been produced according to the method commonly used in laboratories of our Institute, purified and conjugated with horseradish peroxidase.

Description of the method: Plate immunoassay tests for polystyrene (Nunc-Polsorb) are coated by adsorption of the antigen appropriately diluted in carbonate / bicarbonate buffer, pH 9.6, and then incubated overnight at 4 °C. After washing with PBS, pH 7.4 with Tween 20 at 0.05% (PBST), 100 µl samples of test serum and control sera are dispensed, in a 1/40 dilution with PBST+1% foetal bovine serum (PBSTB). Samples were distributed in double; the positive and negative control sera are tested in four replicates each. After incubation for 90 min. at 37°C in a humid chamber and subsequent washing, 100 µl of anti-species conjugate diluted 1:100 in PBSTB was added to the wells. Then the plates were incubated for 30 min. at 37°C in a humid chamber and washed with PBST. The substrate was added (1:100 TMB 10 mg/ml in acetate buffer pH 6) and, after about 15 min. 0.5M sulphuric acid was added. The plates were read by spectrophotometer at a wavelength of 450 nm.

Validation of the method: have been collected experimental data of tests with sera standardized with 416 serum samples from sheep serum bank. The cut off was determined through statistical analysis of data and in particular by using the method “Receiver operator characteristics” (ROC) curves (7) using the software Analyse-Plus (Malvern, UK), EPISCOPE (EPISCOPATE) 2.0 freeware product and evaluations were conducted both individually and considering the two tests in combination. The main indicators of performance tests were estimated using the software WIN EPISCOPE (EPISCOPATE) 2.0 freeware product and developed by the Universities of Edinburgh, Saragossa, Utrecht and Wageningen.

RESULTS AND DISCUSSION

In-house ELISA: Standardization of in-house ELISA was conducted by analyzing serial dilutions of control samples. Sera and controls were analyzed using two wells for the double analysis and also another well as control (with false antigen- FA) to show eventual non-specific reactions of sera examined. The calculation of results was done using the following formula:

\[ s/p = \frac{\text{mean DO sample} - \text{DO sample (FA)}}{\text{mean DO Pos control} - \text{DO Pos control (FA)}} \]

416 sera, which were randomly chosen and classified as positive and negative for the presence of antibodies to Toxoplasma by the use of the IFAT test, were tested by ELISA and their S/P values were analyzed by ROC curves. The results of the analysis are summarized in the following table:

**Table:**

<table>
<thead>
<tr>
<th>S/P Value</th>
<th>Number of Sera</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>100</td>
<td>0.85</td>
<td>0.92</td>
</tr>
<tr>
<td>0.6</td>
<td>200</td>
<td>0.87</td>
<td>0.91</td>
</tr>
<tr>
<td>0.7</td>
<td>300</td>
<td>0.89</td>
<td>0.90</td>
</tr>
</tbody>
</table>

**Key Words:** Toxoplasma gondii, indirect Elisa, IFAT
The results of statistical analysis should be considered very encouraging since the discriminative ability of the test is estimated to be 0.935 and the significance of the statistical test is evidenced by the low p value. Based on this evaluation it was determined that sera with s/p values lower than 0.3 have to be considered as negative, while sera with s/p values greater than 0.4 have to be considered as positive. It is useful to consider the values of s/p in the range 0.3-0.4 as doubtful.

Evaluation of performances: both ELISA tests were used to test the population of 416 sera described above. Nine sera gave doubtful results with the commercial ELISA Kit and eleven on the in-house ELISA. These sera were excluded from the calculation of sensitivity and specificity (Fig. 2).

The comparison of the results of both ELISA methods gave a kappa value, albeit slightly, falls within the maximum provided by the scale, which conventionally is used to interpret this test. (Fig 3).

Placing a hypothetical population with a sero-prevalence of 50% was also evaluated the possibility of associating the two ELISA tests in case of need for further investigation. (Fig. 4)

Both ELISA tests have proven to be a useful tool for diagnostic screening of the seroprevalence of toxoplasmosis in sheep populations.

**REFERENCES**


**Table:**

<table>
<thead>
<tr>
<th>Curve</th>
<th>Area</th>
<th>SE</th>
<th>P</th>
<th>95% Cl of Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>s/p ELISA</td>
<td>0.935</td>
<td>0.013</td>
<td>&lt;0.0001</td>
<td>have lower values</td>
</tr>
<tr>
<td>in house</td>
<td>0.946</td>
<td>0.012</td>
<td>&lt;0.0001</td>
<td>have lower values</td>
</tr>
<tr>
<td>commercial Kit</td>
<td>0.910</td>
<td>0.061</td>
<td>0.961 to 0.970</td>
<td>have lower values</td>
</tr>
</tbody>
</table>

**Fig. 1:** Graphical representation of the ROC curve of the in-house and commercial ELISA tests

**Fig. 2:** Output of software winepiscope for the ELISA sensitivity and specificity evaluation.

**Fig. 3:** Software Output running test winepiscope Kappa

**Fig. 4:** Software winepiscope output for the evaluation of tests in combination.