Development of an Indirect ELISA Test Using Tachyzoite Crude Antigen for Sero-Diagnosis of Sheep *Toxoplasma gondii* Infection

Adriana TITILINCU, Viorica MIRCEAN, Anamaria IOVU, Vasile COZMA

University of Agricultural Science and Veterinary Medicine, Faculty of Veterinary Medicine, Department of Parasitology and Parasitic Diseases, 3-5 Manastur Street, Cluj Napoca, Romania, Fax: 0040264-593792; Phone: 0040264-596384 int. 165; E-mail: titilincu@yahoo.com

**Abstract.** The aim of this study was to use crude *T. gondii* tachyzoite antigen in an indirect ELISA and to compare it with indirect fluorescent antibody test (IFAT) considered as a “gold standard”. Sheep serum samples with known (49 negative and 45 positive) and unknown (94) status were tested. It was tried two types of microtiter plates and different antigen, sera and blocking dilution. The sensitivity, specificity positive and negative predictive value, accuracy and efficiency of test were evaluated. The chosen dilutions after CBT were 1:1000 for antigen and 1:400 for controls/sera samples on PolySorp 96-well microtiter plate (Nunc). 3% BSA had the best results in blocking phase of assay. The ELISA agreement with IFAT was relatively very good ($k=0.81$). The sensitivity and specificity of ELISA were 95.75%, respectively 85.11%. The accuracy of test was 81% and the efficiency 91.35%. ELISA using a crude tachyzoite antigen can be used for detecting anti-*T. gondii* antibodies in sheep sera, and consequently in the epidemiological studies and its uses instead of a commercial kit reduce the cost of the analysis.

**Keywords:** ELISA, crude antigen, sheep, *Toxoplasma gondii*

**INTRODUCTION**

*Toxoplasma gondii* is an apicomplexan parasite of warm-blooded animals, with cats as definitive and other animals and humans as intermediate hosts (Frenkel, 1970). The parasite has public health and zoonotic importance because it causes abortions, neonatal complication and chorioretinitis in humans (Montoya and Liesenfeld, 2004). Humans can be infected transplacentaly or by oral route with oocysts shed by cats and *T. gondii* cysts with raw meat.

The most important intermediate hosts among farm animals are sheep, goats and pigs. In sheep, infection may cause foetal death and abortion following a primary infection during pregnancy. Recently, it has been suggested that there is a possible recrudescence of infection during pregnancy (Duncanson et al., 2001). *T. gondii* infection in sheep is distributed worldwide, with seroprevalences of 20–91% in different countries (Dubey, 2009). In our country the seroprevalence is very high being between 36.3-64.3% in Transylvania and Banat regions (Cozma et al., 2008; Hotea et al., 2008; Iovu et al., 2008a; Titilincu et al., 2008a). Also, in 50% abortions were detected *T. gondii* DNA by PCR (Iovu et al., 2008b).

Control strategies of food borne diseases should be based on systematical serological investigations on farm level and the improvement or the development of more sensitive methods to detect the parasite in slaughtered animals that would allow an appropriate judgement during meat inspection and minimize the consumer exposure to this parasite (Lhafi et al., 2004; Pozio, 2008).

*T. gondii* infection in animals and humans can be diagnosed directly (detection of the parasite) by bioassay, histology, immunohistochemistry, Polymerase Chain Reaction (PCR)
and hybridisation, and indirectly (detection of antibodies) by serological methods as Dye test (DT), Modified Agglutination test (MAT), Immunofluorescence Assay test (IFAT) and Enzyme-Linked ImmunoSorbant Assay (ELISA).

ELISA is the most widely used test for screening toxoplasmosis in sheep and other animals. The test is both highly sensitive and specific (some false positives have been reported) when compared to the IFAT (used as a reference test). It is appropriate for routine serology and for epidemiological surveys of Toxoplasma infection. There were developed many ELISAs based on purified or recombinant antigens (Garcia et al., 2006; Hosseininejad et al., 2009), but ELISAs based on tachyzoite crude antigens or soluble extracts, have a higher sensitivity rates (Aduriz et al., 2007).

Therefore, the aim of this study was to develop an indirect ELISA for sero-diagnosis of *T. gondii* infection in sheep using tachyzoite crude antigen. ELISAs based on tachyzoite crude antigens or soluble extracts are highly specific and may be a cost-effective alternative of recombinant ELISAs, because the antigens can be relatively easy obtain.

**MATERIALS AND METHODS**

*Antigen.* The antigen used on ELISA was represented by a crude tachyzoite antigen. This was obtained by detergent lysis of *T. gondii* tachyzoites, RH strain maintained in cell culture. It was kindly given by Dr. Joke van der Giessen and her team from National Institute for Public Health and the Environment Bilthoven, Netherlands.

*Negative and positive controls.* The controls were represented by a pool of 10 sheep serum samples. The negative serum samples were obtained from 2-months-years-old lambs that tested negative in indirect fluorescent antibody test (IFAT) and in a commercial ELISA in two successive controls at 3 weeks interval. The positive serum samples were obtained from lambs infected with 3X10^3 *T. gondii* oocysts VEG strain (genotype II). The controls were divided into 50 µl volume and kept at -20°C till use.

*Antigen and controls dilution.* An optimal serum dilution for controls and antigen was established by checkerboard titration (CBT).

*Serum samples.* Sheep serum samples with known (49 negative and 45 positive) and unknown (94) status were tested to evaluate the analytical and diagnostic performance of developed ELISA. IFAT was considered the “gold standard”. The sera with known status were obtained from lambs negative in IFAT at two successive controls and from infected lambs with 3X10^3 *T. gondii* oocysts VEG strain (genotype II). The sera with unknown status were randomly chosen from a serosurvey accomplished in Transylvania in 2008.

*ELISA.* Nunc MaxiSorp and PolySorp 96-well microtiter plates were used for coating. Crude tachyzoite antigen was diluted starting from 1:800 to 1:2500 in phosphate buffer saline (PBS) as a coating buffer, and then incubated at 4°C overnight. Plate was then washed and incubated with blocking solution (1-3% bovine serum albumine in PBS) at 37°C for 1 h. All washing procedures were done three times, using PBS-T (PBS, pH 7.2, 0.05% Tween-20) in a washer machine (BioRad PW 40). Plate was then emptied, washed and controls (negative and positive) and serum samples were added. There were used serial dilutions of sera ranging from 1:100 to 1:500 in PBS. After serum incubation (37°C for 1 h) and washing, an anti-sheep IgG, whole molecule peroxidase conjugate (713-035-003, Jackson Immunoresearch Laboratories Inc.) diluted 1:100,000 in PBS was added to the wells and incubated at 37°C, 1 h. Plate was washed and bound antibodies were detected by incubation at room temperature with 3,30,5,50-tetramethylbenzidine liquid substrate 0.2%. After 10 min, 2N sulfuric acid was used to stop the reaction and optical density (OD) values were measured at 450 nm on a
The results were expressed as the S/P percentage (S/P%) according to the formula: (P-N/S-N)X100, where P is the OD_{450} of positive control, N is the OD_{450} of negative control and S is the OD_{450} of sample.

**IFAT.** Indirect fluorescent antibody test was considered as a “gold standard”. Antigen slides were incubated with sheep sera diluted 1:32 in PBS and then with a fluorescein isothiocyanate-labeled rabbit anti-sheep IgG (313-095-003, Jackson Immunoresearch Laboratories Inc.) diluted 1:100 in PBS. The incubation was made in a humidity chamber at 37°C for 30 minutes, and after each incubation the slides were washed 3 times with PBS. Positive and negative control sera were included in each slide. Slides were examined under fluorescence microscopy and only a bright, linear peripheral fluorescence of the T. gondii tachyzoites was considered positive.

**Test evaluation and statistical analysis.** The sensitivity and specificity of the ELISA were evaluated by Evaluation Test using Win Episcop e 2.0 program. Also, positive and negative predictive value and agreement level between ELISA and “gold standard” (IFAT), and a commercial ELISA (Toxotest, IDEXX Laboratories), respectively were assayed. The accuracy of test was evaluated by calculating Youden’s J index, efficiency (E) and positive and negative Likelihood Ratio (LR). Finally, repeatability (CVs) interplates was checked during the same day, and in different days.

**RESULTS AND DISCUSSION**

The chosen dilutions after CBT were 1:1000 for antigen and 1:400 for controls/sera samples on PolySorp 96-well microtiter plate (Nunc). 3% BSA had the best results in bloking phase of assay. In this condition the positive control had an OD of 1.083 and the negative control 0.122. The samples with S/P value higher then 30% were considered positive.

When sheep serum samples with known status were tested there was a perfect agreement between ELISA and “gold standard” (IFAT) (k=1), and sensitivity and specificity of ELISA were 100%.

From 94 sheep sera with unknown status tested for IgG antibodies to T. gondii, 47 (50%) were positive in IFAT, 52 (55.3%) in ELISA and 61 (64.9) in Toxotest. The ELISA agreement with “gold standard” (IFAT) and Toxotest was relatively very good (k=0.81; 0.80) (Tab. 1, 2). The sensitivity and specificity of ELISA were 95.75%, respectively 85.11%. The accuracy of test was 81% (Tab. 1) and the efficiency 91.35%. LR+ was 6.43 and LR- 0.05. Interplates CVs during the same day was between 5.26% and 12.63% for negative control and between 8.88% and 9.17% for positive control. Interplates CVs during 3 days was 4.3% for negative control and 8.07% for positive control (Tab. 1).

Advantages of ELISA over the other diagnostic methods are that it can be semi automated, the reading of results is objective and does not require skilled personnel. Relative to the IFAT as a reference test, we have developed an ELISA test that shows very good sensitivity (95.75%), good specificity (85.11%) and a relatively very good agreement (k=0.81). Hosseininejad et al. (2009) using a purified tachyzoite surface antigen SAG1 for sero-diagnosis of canine Toxoplasma gondii infection obtained a value of 94.52% for sensitivity and of 93.60% for specificity.

45 sera samples were positive in both tests and there were another 7 samples positive in ELISA but negative in IFAT. Similar to us Shapran et al. (2008) obtained a higher prevalence by ELISA (41.7%) comparing with IFAT (37%). We used as antigen, crude tachyzoite lysate of T. gondii tachyzoites. It is widely known that this antigen contains a large number of antigens including those from intracellular origin, whereas in IFAT the antibodies
recognize preferentially surface membrane antigens. IFAT has been considered a more species-specific test, but its sensitivity and specificity can be influenced by the established cut off values (Björkman and Uggla, 1999).

The specificity of 85.11% can be explained by a possible cross-reaction with other apicomplexan parasites as *Sarcocystis* spp. and *N. caninum*. Uggla *et al.* (1987) demonstrated in cow’s sera cross-reactivity with *Sarcocystis cruzi* when *T. gondii* soluble antigens were used in indirect ELISA. In Romania, the prevalence of *Sarcocystis* spp. infection in sheep is very high (81.6%) (Titilincu *et al.*, 2008b) and of *N. caninum* relatively low (2.5%) (unpublished data).

Otherwise 91.35% of tested subjects were correctly classified as positive or negative. The chance as a positive subject using the developed ELISA to be diagnose as a negative is very low (LR- 0.05), but there is a chance of 6.43X to diagnose a negative subject as a positive one.

<table>
<thead>
<tr>
<th>Evaluation of ELISA performance using IFAT as gold standard</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sensitivity (%)</strong></td>
</tr>
<tr>
<td><strong>Specificity (%)</strong></td>
</tr>
<tr>
<td><strong>True prevalence (%)</strong></td>
</tr>
<tr>
<td><strong>Apparent prevalence (%)</strong></td>
</tr>
<tr>
<td><strong>Positive predictive value (%)</strong></td>
</tr>
<tr>
<td><strong>Negative predictive value (%)</strong></td>
</tr>
<tr>
<td><strong>Youden’s J</strong></td>
</tr>
<tr>
<td><strong>Agreement (k)</strong></td>
</tr>
<tr>
<td><strong>Efficiency</strong></td>
</tr>
<tr>
<td><strong>Positive Likelihood Ratio</strong></td>
</tr>
<tr>
<td><strong>Negative Likelihood Ratio</strong></td>
</tr>
<tr>
<td><strong>CVs (same day) /+ (%)</strong></td>
</tr>
<tr>
<td><strong>CVs (3 days) /+ (%)</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Comparison of positive results obtained by IFAT, ELISA and Toxotest for <em>T. gondii</em> antibodies in sheep sera</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Positive n(%)</strong></td>
</tr>
<tr>
<td><strong>CI 95%</strong></td>
</tr>
<tr>
<td><strong>Negative n(%)</strong></td>
</tr>
<tr>
<td><strong>Total</strong></td>
</tr>
</tbody>
</table>

**CONCLUSIONS**

Our results suggest that ELISA using a crude tachyzoite antigen can be used for detecting anti-*T. gondii* antibodies in sheep sera, and consequently in the epidemiological studies. By improving the specificity of assay by testing cross-reactivity with *Sarcocystis* spp. it can be used for diagnosis purpose in correlation with other techniques. Using an “in house” ELISA based on a crude tachyzoite antigen instead of a commercial kit reduce the cost of the analysis.

**Acknowledgments.** This study was financially supported by the Ministry of Education, Research and Innovation from Romania (grant PNII PC 51-013/2007).
REFERENCES


