



A purified tachyzoite protein based ELISA for the sero-surveillance of *Toxoplasma gondii* specific antibodies in domestic ruminants*

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ABSTRACT

Toxoplasmosis, an immensely significant zoonotic disease, is caused by coccidian *Toxoplasma gondii*. Data on prevalence of the parasite in economically important livestock species from India is scanty. An antibody detection ELISA specific for *T. gondii* was laboratory standardized using purified tachyzoite lysate antigen of *T. gondii* (RH strain). Sera samples (168) from sheep (60), goat (63) and cattle (45) were procured from regions of Uttar Pradesh and West Bengal for assessing the sero-surveillance of *T. gondii* specific antibodies. Among the small ruminants, 31.66% and 17.46% of the total sera samples collected from sheep and goats, respectively, were detected positive for *T. gondii* specific antibodies. The sero-surveillance of toxoplasmosis among cattle was 28.88%. The study has relevance for developing control strategies against this zoonotic organism.

Key words: Cattle, ELISA, Goat, Sheep, Sero-Surveillance, *Toxoplasma gondii*

Toxoplasmosis, caused by *Toxoplasma gondii*, is a zoonotic disease with global prevalence causing abortions, stillbirth, and neonatal losses especially in sheep, goats and swine (Hill and Dubey 2002). The WTO guidelines on control strategies, especially of food-borne diseases, insist on mandatory systematic serological investigations of the causative agent (s) at the farm level and in slaughtered animals (Pozio 2008). Enzyme linked immunosorbent assay (ELISA) is one of the widely used tests for screening antibodies against toxoplasmosis in sheep and other animals. The sensitivity and specificity of the test is high when compared to the IFAT, the standard reference test (Titilincu *et al.* 2009). Although many protocols have been developed based on purified or recombinant antigens (Hosseinienejad *et al.* 2009), crude tachyzoite antigens or soluble tachyzoite extracts based ELISAs showed higher sensitivity rates

(Aduriz *et al.* 2007). Hence, the present study was undertaken with the primary objective of generating data on the sero-surveillance of toxoplasmosis in sheep, goats and cattle using a purified tachyzoite lysate protein based ELISA.

MATERIALS AND METHODS

In vivo propagation of *Toxoplasma gondii* in mice: Cryopreserved *T. gondii* tachyzoites, maintained in the Divisional Laboratory of Parasitology for over a decade, were revived and passaged through Swiss Albino mice by intra-peritoneal inoculation of 1×10^3 tachyzoites (Singh *et al.* 2009).

Separation of host cell-free tachyzoites: On development of peritonitis following experimental infection, the peritoneal lavage containing free tachyzoites as well as infected macrophages were collected in phosphate buffered saline (PBS, pH 7.4) and washed thrice by centrifugation at 5000 rpm for 10 min. The washed cells were re-suspended in 10 ml of PBS (pH 7.4). The intracellular tachyzoites were rendered free by rupturing the intact parasitized macrophages mechanically by passing the contents through a 27 G needle fitted in a 10 ml sterile syringe. The resultant tachyzoites suspension was washed again by re-suspending in 20 ml of PBS and the debris was allowed to settle down in the centrifuge tube for 10 min. The supernatant was collected carefully and passed slowly @ 1 ml/2–3 min through a pre-wetted 3 mm pore size polycarbonate membrane filter (Gross *et al.* 1991). The filtered tachyzoites were sedimented by

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centrifuging the suspension at 3000 rpm for 10 min. The supernatant was completely removed and the tachyzoites were re-suspended in 1 ml of PBS (pH 7.2).

Preparation of whole tachyzoite lysate antigen (TLA): The purified tachyzoites were lysed by subjecting the suspension to rapid freeze-thaw for 5 times in liquid nitrogen. Thereafter, the suspension was subjected to ultrasonication for 10 cycles of 15 amplitude for 30 sec on ice with an interval of 30 sec between the cycles. The disruption of tachyzoites was monitored microscopically at each step. Following complete disruption of the tachyzoites, the sonicated product was centrifuged at 13,000 rpm for 30 min at 4°C. The supernatant was carefully aspirated and the protein concentration was estimated as per Lowry *et al.* (1951).

Serum samples: Sera samples (168) were randomly collected from apparently healthy goats (63), sheep (60) and cattle (45) from parts of West Bengal, Makhdoom and Bareilly region of Uttar Pradesh, respectively. Approximately 5 ml of blood was drawn aseptically from the jugular vein of each animal and allowed to clot for isolation of serum. The sera samples were centrifuged at 2500 rpm for 10 min and stored at -20°C after addition of thiomersal (10 mg/ml) @ 5 ml/ml of serum till use.

Tachyzoite lysate antigen-based enzyme-linked immunosorbent assay (TLA-ELISA): The TLA-ELISA protocol was laboratory standardized for optimum reactivity against each species of animal studied by checker-board titrations using different concentrations of antigen, serum and conjugate dilutions. The individual wells of ELISA plates were coated with 100 µl of TLA (concentration 8 mg/ml) in carbonate-bicarbonate buffer (pH 9.6). The plates were incubated at 4°C overnight. Excess unbound antigen was removed from the wells by washing 3-times with PBS-Tween 20 (PBS-T). The unused free binding sites of the wells were blocked with 3% fat-free milk powder dissolved in PBS. Following incubation at 37°C for 2 h, the plates were washed 3-times with PBS-T and the sera samples diluted 1:100 with PBS and 1% non fat milk powder were loaded in duplicate wells and incubated for 1 h at 37°C. The plates were washed thrice with PBS-T to remove the unbound antibodies. Species specific IgG molecules conjugated to horseradish peroxidase enzyme were diluted 1:10000 with PBS containing 1% fat-free milk powder for detection of *T. gondii* specific antibodies in the sera of sheep and goats and a conjugate dilution of 1:15000 was used for screening the cattle sera. The wells were incubated for 1 h at 37°C before washing with PBS-T thrice. The wells were developed with 100 µl of o-phenylenediamine dihydrochloride (OPD, 0.4 mg/ml) substrate dissolved in phosphate-citrate buffer (0.05 M, pH 5.0) containing H₂O₂. The reaction was allowed to progress for 15 min at dark and subsequently stopped by addition of 50 ml of 3N HCl. The absorbance of the wells was read at 492 nm in an ELISA reader (Microscan) and the OD₄₉₂ values were recorded.

Reference sera: Known positive and negative reference sera samples for sheep, goat and cattle were provided by Dr J P Dubey, USDA, Beltsville, USA.

RESULTS AND DISCUSSION

Observations on infectivity of cryopreserved *T. gondii*: Peritonitis developed in all the experimental mice between days 4 to 7 post-inoculation (PI) with *T. gondii* tachyzoites. The clinical signs included raised hair coat, pendulous abdomen filled with ascitic fluid, dullness, tachypnoea marked by resting of forelegs either on walls of the cages or on the nozzle of water bottle or on other resting mice, etc. Numerous tachyzoites were detectable either as free in the peritoneal fluid or within the macrophages under high power of microscope.

Protein concentration of whole tachyzoite lysate antigen (TLA): The processing of the peritoneal fluid of infected mice resulted in concentrating adequate tachyzoites in purified form which were used for preparation of whole tachyzoite lysate soluble protein. The protein concentration of the TLA was determined as 3.6 mg/ml.

Tachyzoite lysate antigen-based enzyme-linked immunosorbent assay (TLA-ELISA): Sera samples collected from sheep, goats and cattle were screened for the presence of *T. gondii* specific antibodies by ELISA. The cut-off values for individual species of animals tested were determined by adding the 4 standard deviations (SD) value to the mean OD₄₉₂ value of 6 negative controls. The negative cut-off values for sheep, goat and cattle were worked out as 0.232, 0.126 and 0.219, respectively. Among the small ruminants, 19 out of 60 (31.66%) field sera samples collected from sheep were positive (Fig.1), whereas 11 sera samples out of 63 samples (17.46%) collected from goats were detected positive for *T. gondii* specific antibodies (Fig. 2). So far as seroprevalence of toxoplasmosis among cattle is concerned, 13 sera samples out of 45 (28.88%) were assayed positive (Fig.3).

Detection of the parasite in the intermediate hosts through parasitological techniques is difficult as none of the biological stages are demonstrable in the blood or natural excretions/secretions. Among the various diagnostic techniques

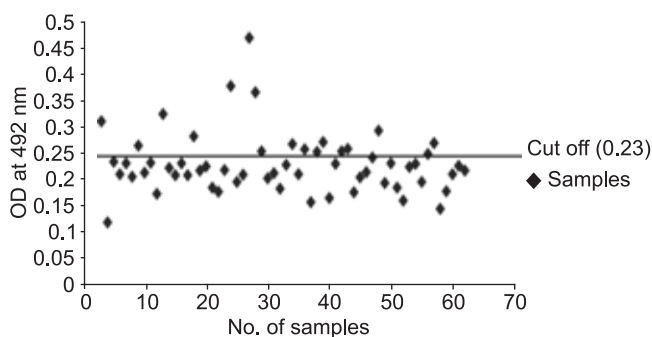


Fig. 1. Antibody response against *T. gondii* in field sera of sheep.

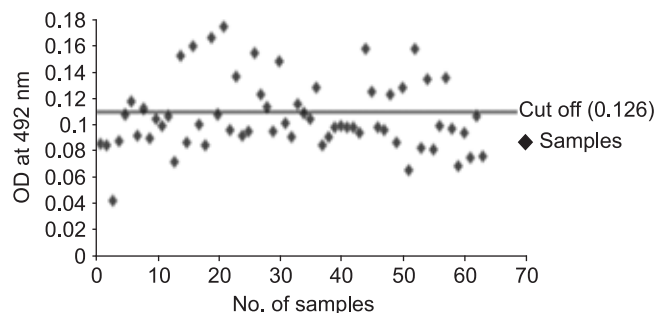


Fig. 2. Antibody response against *T. gondii* in field sera of goat.

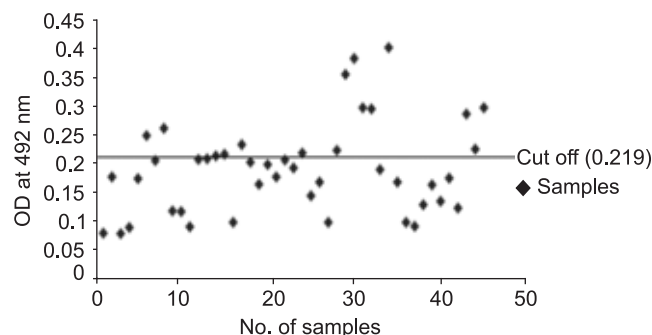


Fig. 3. Antibody response against *T. gondii* in field sera of cattle.

available, ELISA was chosen for standardization using a purified whole cell lysate tachyzoite antigen for the present study due to its superior sensitivity as well as high throughput. Since the tachyzoite contains virtually all of the antigenic moieties presented during the active infection of the host, it was an obvious choice for use as the detection antigen. The ELISA is well suited to laboratories required to analyze large numbers of samples and the results obtained are found to be in good agreement with that of IFAT in terms of sensitivity and specificity (Titilincu *et al.* 2009).

The ELISA test was standardized in the laboratory using the purified tachyzoite lysate antigen and further used for screening of *Toxoplasma* specific antibodies present in the sera samples procured from different species of animals from field conditions. The purified tachyzoite antigen could detect *T. gondii* specific antibodies in the serum of 31.66% sheep, 17.46% goat and 28.88% cattle. Comparatively lesser goats were found to be seropositive for *Toxoplasma* specific antibodies in comparison to higher seropositive values observed in sheep from an identical sample size. It is interesting to note that the seroprevalence of *T. gondii* among cattle (28.88%) is comparable to the same in sheep (31.66%). The positive correlation may be attributable to the fact that the sheep and cattle sera samples were procured from Mathura and Bareilly districts of Uttar Pradesh which are closely located in comparison to West Bengal from where the sera samples from goats were collected. From the analysis of moderate number of samples procured from 3 different species of animals from 2 different states of India, it is

deduced that seroprevalence of *T. gondii* is more in Uttar Pradesh than West Bengal.

Crude tachyzoite antigen based ELISA for seroprevalence study of *T. gondii* were reported by various workers from different parts of world (Shaapan *et al.* 2008, Titilincu *et al.* 2009) and is in good agreement with IFAT, the “gold standard” test in terms of sensitivity and specificity (Titilincu *et al.* 2009). On the basis of a significant positive correlation between the titers of *T. gondii* antibodies in goats obtained by ELISA, IHA and IFAT, these tests are considered appropriate for epidemiological surveys of *Toxoplasma* infection in goats (Figueiredo *et al.* 2001). So far as Indian scenario is concerned, reports on the serological surveys involving domesticated livestock are scanty (Dubey 1988, Dubey *et al.* 1993). Earlier also, 32.66% of goat sera samples were found positive for toxoplasmosis by a native protein based ELISA from slaughterhouses of Bareilly (Kumar 2004). The presence of *T. gondii* specific antibodies in different species of farm animals from 2 different states of India is suggestive of its wider prevalence that may be ascribed to the fact that all of the stages of the life cycle of *T. gondii* are potentially infective and the oocysts, voided through the faeces of cat, are quite resistant to environmental extremes. However, a wider study involving more number of samples from across the country is required for generation of conclusive data on prevalence of the causative agent of toxoplasmosis, an important zoonotic disease.

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