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Evaluation of diagnostic potential of whole cyst lysate of *Sarcocystis fusiformis* for bubaline sarcocystosis

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Sarcocystis spp. has been observed to parasitize a wide variety of vertebrates including humans. Economic damage to livestock sector due to sarcocystosis is enormous. Not only is the financial loss associated with the abortion, reduced milk yield, neurological signs, loss of weight and death caused by it, but also from condemnation or downgrading of sarcocysts infected meat. Meat infected with sarcocysts is also not fit for human consumption as it likely to cause diarrhoea, bloating, dyspnoea, tachycardia, nausea and loss of appetite (Fayer 2004). Diagnosis of sarcocystosis in live intermediate hosts is difficult, since no stages of these intracellular protozoa come out of the infected animal or in blood and also clinical signs in sarcocystosis are non-specific. Conventional diagnostic methods such as muscle squash, rapid isolation technique, muscle digestion and histopathological examination (Dubey *et al*. 2015) are time consuming and not practical for mass screening and ante-mortem diagnosis of sarcocystosis. Immunodiagnostic assays are useful in detecting sarcocystosis in live animals which can be helpful in creating epidemiological map of the disease. Buffaloes play a vital role in Indian economy as it contributes maximum in terms of milk and meat production. Epidemiological studies based on slaughterhouse survey have revealed that sarcocystosis is very common in buffaloes. So, the present investigation was undertaken to evaluate the diagnostic potential of whole cyst lysate antigen of *Sarcocystis fusiformis* for the diagnosis of sarcocystosis in buffaloes by using indirect antibody detection enzyme immunoassay.

Tissue (oesophagus, tongue, heart, and diaphragm) and blood samples were collected from each of the 160 buffaloes slaughtered at abattoir of semi-arid India and then brought to Parasitology Laboratory for processing. Tissue samples were washed thoroughly under running water, superficial fascia and fat removed and after rinsing them with normal saline, they were then stored at 4ºC for further examination. Serum was harvested from blood samples which then were stored at -20˚C till further use. Tissue samples were subjected to macroscopic and microscopic examination for the presence of sarcocysts. Macroscopic size sarcocysts were carefully isolated and kept in phosphate buffered saline, *p*H 7.2). After removal of superficial sarcocysts, the whole organs were then thoroughly searched for the presence of visible sarcocysts and they were removed carefully without rupturing. Tissue samples were also subjected to pepsin digestion and then examined microscopically for the presence of sarcocysts.

For antibody detection ELISA, whole cyst lysate antigen of *Sarcocystis fusiformis* was prepared as per Mamatha *et al*. (2008). After protein estimation, antigen was stored in aliquots at -20ºC till further use.

For standardization of antibody detection ELISA, optimum dilutions of antigen and test sera were determined by checker board titrations in 96 wells ELISA plate (Greiner). Different concentrations of antigens (10 µg, 5 µg, 1 µg, 500 ng per 100 µl per well), different dilutions of pooled positive test serum, pooled positive negative serum and foetal calf serum (1:10, 1:20, 1:50, 1:100, 1:200, 1:400) were used, keeping the antiglobulin conjugate (Sigma) dilution as per manufacturer recommendation (1:5000). The optimum concentrations of antigen and dilution of natural serum was found to be 1 µg/100 µl and 1:200, respectively. The cut-off value was determined using the 20-each of known negative and known positive natural sera. Foetal calf serum was also used as additional negative control. Serum samples from 20 animals which did not reveal sarcocysts in tissues by macroscopic and microscopic examination were regarded as known negative serum samples and another 20 sera in which sarcocysts were detected were regarded as known positive sera samples. Mean absorbance value of known negative serum samples was 0.347 with standard deviation (S.D.) of 0.021. Mean absorbance value of 20 known positive sera were 0.954 with standard deviation of 0.018. Mean OD value of single foetal calf serum (10 times in duplicate) was also

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taken as additional negative control which was 0.322 with a standard deviation of 0.017. Cut–off absorbance value was determined by adding three standard deviation to the mean absorbance value of known negative sera. Hence, the cut-off OD value was taken at 0.410 and animals having OD value higher than this threshold value were regarded as positive. In the standardization process, blocking was done with 100 µl of 3% skimmed milk powder in PBS, enzyme substrate was OPD (o-phenylenediamine dihydrochloride). The colour reaction was monitored in dark place. The reaction was stopped by adding 50 µl of 1M H_2S0_4. The absorbance values were recorded at 450 nm.

After standardization, test sera were screened using reference positive and negative sera as controls. Diagnostic potential of WCL based antibody detection ELISA was evaluated by estimating the relative sensitivity and specificity, keeping tissue examination as standard by following formulae:

$$\text{Sensitivity} = a/(a + c) \times 100$$

Where a, number of animals positive by ELISA and tissue examination, i.e. true positive; c, number of animals positive by tissue examination but negative by ELISA, i.e. false negative.

$$\text{Specificity} = d/(b + d) \times 100$$

Where d, number of animals negative by ELISA and tissue examination, i.e. true negative; b, number of animals negative by tissue examination but positive by ELISA, i.e. false positive.

Kappa value of ELISA was calculated using online software QuickCalcs (https://www.graphpad.com/quickcalcs).

Immunodiagnostic approach has given the hope of diagnosing sarcocystic infection in live animals which is not feasible by conventional methods of examination. In the present investigation, whole cyst lysate antigen (WCL) based antibody ELISA could detect more animals with *Sarcocystis* infection than tissue examination. Out of total 160 buffaloes, tissue examination showed the presence of *Sarcocystis* infection in 90 animals (56.25%) whereas indirect ELISA, antibodies against sarcocystosis were detected in 102 animals (63.75%). Absorbance (OD) values of positive animals ranged from 0.703 to as high as 1.38 (Fig. 1). Earlier also, antibody ELISA using *S. fusiformis* antigen detected more number of positive animals in comparison to tissue examination. In buffaloes, Metwally *et al.* (2014) reported 98% infection by ELISA, 27.7% by microscopic examination and 25.5% by macroscopic studies.

Keeping tissue examination as a gold standard for detecting number of confirmed positive cases, sensitivity and specificity of whole cyst lysate antigen based antibody detection ELISA in the present study was found to be 88.88% and 68.57, respectively. Kappa value was found to be 0.586, and the strength of agreement was moderate. False positive results were seen in 22 animals in which no sarcocyst was seen. On the other hand, false negative result

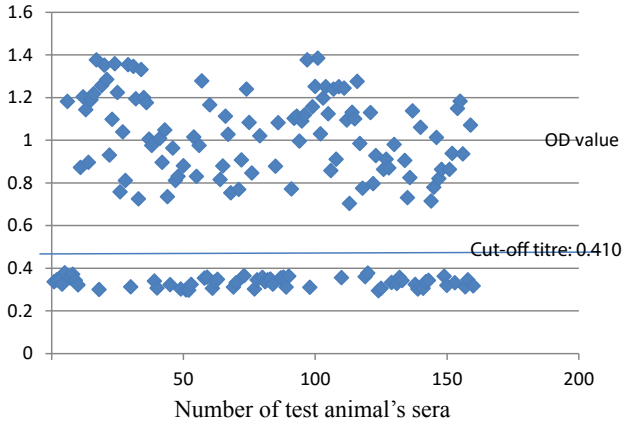

Fig. 1. O.D. values of test serum samples of buffaloes.

was observed in 10 animals in which microscopic and macroscopic size cysts were found. Kappa value was found to be 0.586, and the strength of agreement was found to be moderate. False positive results observed in the present study may be explained if either microscopic examinations employed in the assay failed to demonstrate the cyst and cystozoites in 22 animals or alternatively, antibodies were present, but cysts were not formed as the infections may have been at an early stage. On the other hand, the false negative results may be explained if the infections had been at the late stage where cysts were present, but antibodies had declined below the detectable level.

Type of antigen affects the sensitivity and specificity of the test. Savini *et al.* (1994) studied the sensitivity and specificity of ELISA, one using merozoite antigen grown *in vitro* and the other using antigen from cystozoites. Though sensitivity of both, merozoites based assay (98%) and cystozoittes based assay (95%) was comparable but the specificity was significantly higher in former (97%) than the later (84%). Savini *et al*. (1994) also reported that the antigens derived from merozoites are more suitable for the diagnosis of early stage acute infection whereas antigens derived from cystozoites are suitable only for diagnosis of chronic infections since cysts or cystozoites in the cysts in muscles are restricted to the chronic infection stage. Since present investigation was based on antigens derived from cysts, the test is more justifiable for the detection of chronic infections.

Presence of cross-reacting antigens between organisms of same or different genera also affects the specificity of sero-diagnostic tests. The cross reactions between the *Sarcocystis* species have been reported by several authors (Savini *et al*. 1994, Mamatha 2008, Kalita *et al*. 2015). However, development of genus specific assay can be helpful in epidemiological survey of sarcocystosis. Cross-reaction between *Sarcocystis* species and the closely related genera *Toxoplasma gondii* has also been studied by some authors. Uggla and Buxton (1990) found cross reactivity between *S. tenella* and *S. cruzi* antibody positive serum and *T. gondii* antigen. Savini *et al.* (1994) also observed cross reactions between *S. cruzi* antibodies and *Toxoplasma gondii* antigen and indicated that possibility

of cross reaction is more if cut-off threshold is determined by calculating the mean absorbance of negative sera and adding only two standard deviations. Contrary to these reports, no cross reaction was reported between *S. cruzi* antigen and *T. gondii* antibodies in ELISA assay (Opsteegh *et al*. 2011). Using modified agglutination test, Kalita *et al.* (2015) did not observe cross-reaction between *S. hirsuta* and *T. gondii*. By latex agglutination test and immunoblot analysis, no cross reaction was observed between *Sarcocystis* species of cattle origin and *T. gondii* at 1:100 serum dilution (Hettiarachchi and Rajapakse 2008). In the current investigation, buffalo sera was used at 1:200 dilution and the cut-off titre was at mean absorbance value of negative sera added by three standard deviation, so possibility of cross reaction between *S. fusiformis* and *T. gondii* is negligible. However, in the wake of inconsistency in the results of various investigators, it is necessary to make more intensive cross-reactivity study so as to develop effective immunodiagnostic assay.

In conclusion, the present study reported considerable number of animals to be infected with *Sarcocystis* in semi-arid region of India. It seems that epidemiological factors in the region are conducive for disease transmission, propagation and maintenance of the sarcocystosis. Performance of the WCL based ELISA was found to be moderate, though it detected more number of cases than the macroscopic and microscopic tissue examinations. Identification of dominant immunoreactive polypeptides shared by all life cycle stages of *Sarcocystis* will help in development of effective immunodiagnostic tests. This not only helps in epidemiological mapping of the disease but diagnosis of sarcocystosis during acute infection will help in curing the infection by timely intervention using therapeutic drugs.

SUMMARY

Antibody detection enzyme immunoasssay using whole cyst lysate antigen of *Sarcocystis fusiformis* was standardized and evaluated for the diagnosis of bubaline sarcocystosis. For the study, tissue (oesophagus, tongue, heart and diaphragm) and blood samples from each of the 160 buffaloes slaughtered at local abattoir of Mathura, Uttar Pradesh, India, were collected. Tissue samples were subjected to macroscopic and microscopic examinations for the detection of sarcocysts whereas blood samples were used for sera extraction for detection of antibodies by ELISA. *Sarcocystis fusiformis* isolated from tissues were used for preparation of whole cyst lysate antigen and subsequently used for development of antibody-detection ELISA. Ab-ELISA was standardized using pooled known negative, pooled known positive, hyper immune and foetal calf serum and was then compared with tissue examination. Antibody-ELISA could detect antibodies against *Sarcocystis* in 63.75% serum samples with 88.88% sensitivity and 68.57% specificity. Kappa value showed moderate efficacy of the WCL based antibody ELISA.

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