J.Natn.Sci.Foundation Sri Lanka 2008 **36** *(3):239-244*

SHORT COMMUNICATION

Antigenic analysis of bovine *Sarcocystis* spp. in Sri Lanka

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Revised: 04 April 2008 ; Accepted: 20 June 2008

Abstract: *Sarcocystis* is an intracellular parasite, known to cause acute and chronic diseases in a variety of animal hosts, including cattle. *Sarcocystis* has also been reported as a zoonotic form and causes cysts in meat resulting in economic losses. This study was designed to characterize *Sarcocystis* antigens by molecular weight and to investigate the possibility of cross-reaction with related Apicomplexan *Toxoplasma gondii*.

Sarcocystis antigen was prepared by chemical lysis (10% Triton) of bradyzoites/cystozoites contained in tissue cysts. Healthy rabbits were inoculated with *Sarcocystis* (antigen and whole parasite) and *Toxoplasma* and antibodies were harvested after a month. A higher antibody titre was elicited by the inoculation of *Sarcocystis* cystozoites as opposed to the antigen. The latex agglutination test confirmed the presence of anti *Toxoplasma* antibodies in test serum and negated cross-reactivity between *Sarcocystis* and *T. gondii* at 1:100 serum dilution. Immunoblot results also did not support cross-reactivity. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent immunoblot analysis at 1:100 serum dilution revealed twelve antigenic bands, three of which were prominent. Out of these three, two were complex bands of ~ 66 kMW and above. The other was a single band of ~ 45 kMW. Monoclonal antibodies can be developed against these potent antigens for diagnostic purposes in the future.

Keywords: Antigens, cross-reactivity, protozoan parasite, *Sarcocystis*, Sri Lanka, *Toxoplasma*

INTRODUCTION

Sarcocystis is a protozoan parasite causing acute and chronic diseases in its intermediate hosts, commonly cattle and sheep. It is responsible for abortion, acute illness, growth retardation and ultimately death in livestock, generating significant economic losses. The presence of sarcocysts in meat can lead to a parasitic hazard in humans[1]. The beef industry in Sri Lanka is affected by condemnation of the carcasses of infected cattle at the time of inspection (*personal communication* with the meat inspectors at the Colombo Municipal Abattoir). Infected female cattle undergo abortion and weight reduction[2]. The low milk production negatively affects the milk industry.

Previous studies have portrayed *Sarcocystis* as a zoonotic parasite. The dissemination of *Sarcocystis* to animals largely depends on human factors and the human is also an essential part of the life cycle[3] for the two species *S. hominis* and *S. suihominis*. In a developing country such as Sri Lanka, poor hygienic conditions and traditional farm practices may facilitate contact between human and parasite, thereby increasing the risk of infection of humans. Three species of *Sarcocystis*, namely *S.cruzi (S.bovicanis), S.hirsute (S.bovifelis)* and *S. hominis (S.bovihominis)* were found to occur in cattle of Sri Lanka, by a study based on cyst morphology [4]. The definitive diagnosis of *Sarcocystis* has traditionally been accomplished through inspection of cattle carcasses at the abattoir. Infected animals are not detected due to repressed pathological symptoms. Currently, there is no effective prophylaxis or therapy for the disease caused by *S.cruzi*, which is generally referred to as sarcocystosis[5]. Livestock should be diagnosed routinely and the infected animals quarantined to prevent the spread of this parasite.

The primary objective of this study was to characterize antigens of *Sarcocystis* by molecular weight in order to facilitate development of a precise diagnostic tool. The SDS-Polyacrylamide Electrophoresis (SDS-PAGE) technique was employed for this study, as this approach allows separate visualization of each antigenic

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polypeptide band in a complex protein mixture, unlike the enzyme linked immunosorbent assay (ELISA). Analysis and characterization of parasite antigen can be achieved by the Western blot. This technique is justified by the fact that many proteins retain their antigenicity when transferred from Polyacrylamide gels onto Nitrocellulose filters [5]. The Western blot has been the preferred method for testing seroprevalence of *Sarcocystis, Toxoplasma* and *Neospora* antigens [6,7].

The possibility of cross-reaction with similar protozoa must be explored for correct interpretation of the results of a serological test. Hence, this study was expanded to investigate the possibility of cross-reaction with related Apicomplexan, *Toxoplasma gondii*, in light of the inconsistent results observed in the past (mentioned in detail in the Discussion)[5-9]. Both these parasites produce cysts in host tissue and it may be difficult to differentiate each symptom of sarcocystosis and toxoplasmosis[10]. Although infection with *Sarcocystis* is very common in cattle throughout the world, *T.gondii* has rarely been identified from bovine tissues. The frequent demonstration of antibodies in serum suggests however, that unapparent *Toxoplasma* infection may be common[5].

METHODS AND MATERIALS

Macroscopically visible sarcocysts were obtained from the thigh muscle of heavily infected cattle carcasses at the Colombo Municipal Abattoir by identifying the gross morphology. These cysts were narrow, elongated and cream/white in colour (Figure 1). They were teased out of the muscle with fine forceps, collected into phosphate buffered saline (PBS) and transported in an icebox. In the laboratory the specimens were stored at –20 °C.

Toxoplasma was obtained in the trachyzoite stage from a previously established and cryopreserved vero cell culture of the Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Peradeniya. The vial of trachyzoites was kept in a -20 °C freezer and thawed immediately prior to use.

Ten sarcocysts were collected into an eppendorf tube and washed with saline thrice to remove attached bovine tissue. Using fine forceps the cysts were macerated in 100 µL of saline at room temperature until a cream-coloured suspension formed. A drop of the suspension was examined under the light microscope (x 40) to confirm presence of banana-shaped cystozoites. These cystozoites were centrifuged, resuspended in PBS, recentrifuged and the supernatant discarded. The pellet of cystozoites was mixed with about 10 mL of 50% isotonic Percoll solution in a conical tube and centrifuged at 400 g for 10 min[11]. Extraction buffer consisting of 180 µL PBS and 20 µL 10% Triton (TritonX-100, BDH laboratory, BDH Chemical Australia Pvt. Ltd., Victoria 3284, Australia) in PBS was then added to the eppendorf. The sample was mixed well by a whirly mixer for 5 min, while alternatively placing it in ice to avoid heating. The sample was then centrifuged for 10 min at 14000 rpm in 4 °C. The resulting supernatant (antigen) was collected as aliquots into eppendorfs and stored at –20 °C.

The *Toxoplasma* culture was washed thrice with saline in an eppendorf and subjected to the lysis buffer (0.5 M Tris HCl, 10% SDS, DiThioThreitol, distilled water) for 10 min. The resulting antigen suspension was stored in –20 °C.

Cattle tissue was macerated in 100 µL of saline at room temperature using fine forceps. This sample was then centrifuged for 10 min at 10000 rpm in 4 °C. The resulting supernatant (antigen) was collected as aliquots into eppendorfs and stored in –20 °C.

The dot blot-ELISA [12] was used to check the antigen sample for host protein contamination. Two dots were impregnated with 1µL of *Sarcocystis* antigen and 1 µL host tissue preparation separately. They were incubated with peroxide conjugated anti-bovine IgG (Sigma Chemicals, St.Luise, USA) at a 1:200 dilution. No colour development in the antigen-impregnated dot suggested that host protein contamination in the prepared antigen was negligible, if any, and that it can be used for further analysis.

For estimation of protein quantity in the antigen preparation the Bicinchoninic acid protein assay kit No. BCA 1 (Sigma Chemicals, St. Luise, USA) and Sigma procedure no. TPRO 562 were used. A standard curve was prepared by plotting the net absorbance at 562 nm versus the concentration of known protein standards. This curve was used to determine the amount of protein in the *Sarcocystis* antigen sample. Using this value the true concentration of proteins in the antigen preparation was calculated as follows:

$$\frac{\text{mg antigen per assay from standard curve x dilution factor}}{\text{Volume (mL) of antigen sample used for assay}} = \text{protein mg/mL}$$

Four laboratory bred (Medical Research Institute, Borella) New Zealand white rabbits of ~ 3months of

age were labelled as R1, R2, R3 and R4. They were fed on Broiler Finisher and guinea grass throughout the experiment. After a week of orientation, the rabbits were bled and pre-immunization serum samples collected. As these samples tested negative for *Sarcocystis* and *Toxoplasma* infection by a dot blot assay (as previously described), the four rabbits were inoculated intramuscularly (IM) on the 21st of February and subcutaneously (SC) on the 2nd and 9th March (Table 1). Freund's incomplete adjuvant (Sigma Chemicals, St. Luise, USA) was mixed with the respective antigens [13] within 2.5 mL syringes, just before inoculation.

Blood (~ 4 mL) was drawn from the central artery of the ear [14] of each rabbit on three days (15th, 23rd and 30th of March) in order to select a serum sample with the highest concentration of antibodies. The serum for each animal, for each of the three collection dates, was stored in an eppendorf tube at – 20 °C.

The dot blot assay was used to detect presence of anti *Sarcocystis* and anti *Toxoplasma* antibodies in the collected serum. The *Sarcocystis* antigen imbibed dots were incubated with anti *Sarcocystis* serum of each animal (R2, R4), for each collection date. The three *Toxoplasma* antigen imbibed dots were incubated with anti *Toxoplasma* serum of R1 rabbit for each collection date. Out of the six PBS imbibed dots, three were incubated with anti *Sarcocystis* serum (of R2, R4) and the other three with anti *Toxoplasma* serum (of R1) for each collection date. Serum was thus tested at 1:100 dilution. Incubation with Peroxidase conjugated anti rabbit IgG (1:4000, Sigma Chemicals, St. Luise, USA) was followed by the chromogen solution (Amino Ethyl Carbazole 20 mg; Dimethyl formamide 2.5 mL; Acetate buffer 47.5 mL; 30 % H_2O_2 25 µL) . The rabbit serum which gave a colour reaction was used as positive serum for further analysis.

As a relatively low colour reaction was observed for anti *Toxoplasma* serum in the dot blot, the latex agglutination test [15] was performed to confirm the presence of anti *Toxoplasma* antibodies. Previous studies have shown that latex agglutination test is high in sensitivity [16] and is an effective method for routine serological screening for *T. gondii* antibodies .

Anti *Toxoplasma* serum sample was diluted with serum diluting buffer in a microtitre plate, starting at 1:25 up to1: 400. Anti *Sarcocystis* serum sample was diluted with serum diluting buffer (0.01 M phosphate buffered saline or PBS) in the same plate, starting at 1:25, upto 1: 100. Diluted (1:200) *Toxoplasma* positive human serum and *Toxoplasma* negative human serum were the positive and negative controls, respectively. The antigen mixture was freshly prepared with 2.5 mL antigen diluting buffer (0.4 g bovine serum albumin in 100 mL borate buffer), 35 µL 2-Mercaptoethanol, 50 µL Evans Blue dye solution (2 mg/mL water) and 0.15 mL of antigen (formalin fixed parasite). Agglutination was done in the microtitre plate. Twenty five µL of antigen mixture were pipetted out to each well immediately after mixing followed by 25 µL of appropriate serum dilutions and mixed gently by repeated pipetting action. Then, plates were covered with sealing tape and incubated at 37 °C overnight.

The Mini gel apparatus (Bio-Rad, Hercules, CA, USA) with a 12% Polyacrylamide gel was used to carry out electrophoresis for 50 min (stacking gel:100 V, 15 min and separating gel:200 V, 35 min). The immunoblot [17] was used to analyze the antigenic properties of the electrophoretically separated proteins.

Table 1: Inoculation of rabbits with *Sarcocystis* & *Toxoplasma* antigens

Date and type of inoculation	Rabbits inoculated			
	R1	R2	R3	R4
21st February (IM)	*Toxoplasma* antigen + incomplete adjuvant(1:1) ~ 0.5 mL	*Sarcocystis* bradyzoites + Incomplete adjuvant(1:1) ~ 0.5 mL	PBS + incomplete adjuvant(1:1) as control ~ 0.5 mL	*Sarcocystis* antigen + Incomplete adjuvant(1:1) ~ 0.5 mL
2nd March (SC)	*Toxoplasma* antigen ~ 0.2 mL	*Sarcocystis* bradyzoites ~ 0.2 mL	PBS ~ 0.2 mL	*Sarcocystis* antigen ~ 0.2 mL
4th March (SC)	*Toxoplasma* antigen ~ 0.2 mL	*Sarcocystis* bradyzoites ~ 0.2 mL	PBS ~ 0.2 mL	*Sarcocystis* antigen ~ 0.2 mL

IM= intramuscular ; SC= subcutaneous

In the SDS-Page, the positive control (serum from R3) was run in two lanes, in order to compare the banding patterns between each sample more accurately.

RESULTS

The concentration of protein in the cystozoite antigen (Ag) preparation, as determined by the Bicinchoninic acid test, was 3.02 mg/mL. Serum samples collected from rabbits on the 15th of March showed negative results in the dot blot assay. However, serum collected on the 23rd and the 30th of March tested positive for antigens in the immunoblot, with a stronger result for the samples collected on the 30th of March. Therefore, serum samples collected on the 30th of March were selected for the antigen analysis.

The results of the dot blot indicated that the serum sample collected from rabbit R1 was positive for *Toxoplasma* and that those collected from R2 and R4 were positive for *Sarcocystis*. A comparatively strong colour development was observed in the serum sample from R2 suggesting a high antibody titre and hence, this sample was selected to represent *Sarcocystis* antigens.

The latex agglutination test yielded positive results for all the serial dilutions of anti *Toxoplasma* serum by expressing a clear solution in the appropriate wells (A1-A5) of the microtitre plate. (Figure 2) The wells containing anti *Sarcocystis* serum and *Toxoplasma* antigen together showed a blue dot at the bottom, a negative result.

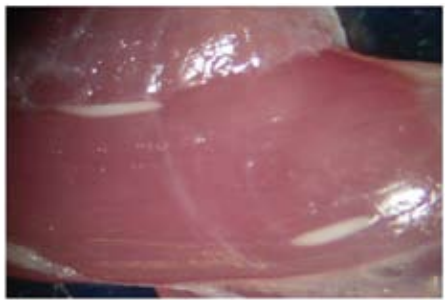

Figure 1: Sarcocysts in muscle tissue of cattle (10 x 40)

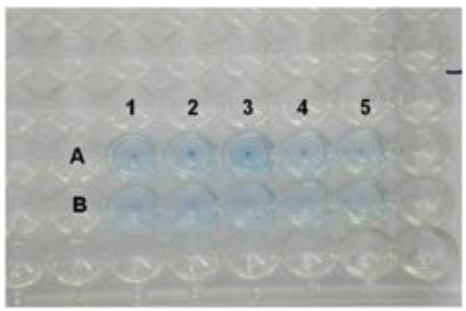

Figure 2: Latex agglutination test

A_1-A_4= anti *Sarcocystis* positive serum, A_5= negative control, B_1-B_4 = anti *Toxoplasma* positive serum, B_5= positive control

In the immunoblot and serum incubation, Ponceau S staining of the nitrocellulose blot revealed a significant protein-banding pattern for the *Sarcocystis* antigen preparation (Figure 3). Seven bands were distinct. Three bands were visualized between the 66-45 kMW markers and one band was approximately 45 kMW. Yet another band appeared to be slightly over 29 kMW. Two bands between 20 and 29 kMW were also visualized.

The immunoblot incubated with anti *Sarcocystis* serum showed a clear and distinct banding pattern (Figure 4). Three prominent bands were identified, two complex, one single. One of the 2 complex bands was approximately 66 kMW while the other had a higher molecular weight. The single band was approximately 45 kMW. The rabbit antiserum also reacted with nine other antigens. Seven of these bands were low in intensity and the other two faintly visible. No bands appeared for the fetal calf serum and *Toxoplasma* samples. The rabbit serum (positive control) yielded one conspicuously thick band. Three bands, one prominent and two of low intensity, appeared for the *Toxoplasma* sample lane of the blot incubated against anti *Toxoplasma* serum (Figure 5). No banding was seen for the *Sarcocystis* sample lane.

DISCUSSION

The antigens of *Sarcocystis* spp. infecting cattle, without discriminating between the different species, were investigated in this study. Cystozoites were chosen as the antigen source due to their high availability in abattoirs. A sufficient antibody titre was elicited against the cystozoites (in R2). Inoculation of the rabbit with the whole parasite (cystozoite) yielded better results in the dot blot than inoculation with the prepared antigen.

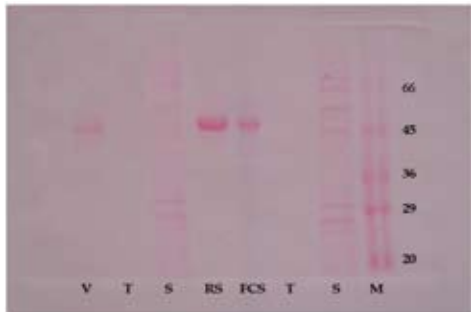

Figure 3: Western blot with Ponceau S stain

R=rabbit serum (from R3), T=*Toxoplasma* Ag (from R1), S=*Sarcocystis* Ag (from R2), F=fetal calf serum, V=vero cells, M=molecular marker /kMW

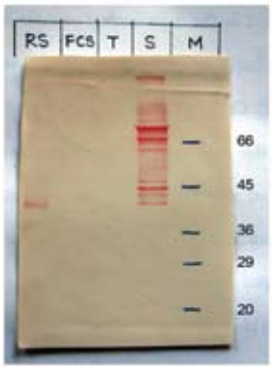

Figure 4: Immunoblot, incubated with anti *Sarcocystis* serum

RS=rabbit serum (from R3), FCS=fetal calf serum, T=*Toxoplasma* Ag (from R1), S=*Sarcocystis* Ag (from R2), M=molecular marker/kMW

Figure 5: Immunoblot, incubated with anti *Toxoplasma* serum

V=vero cells, T=*Toxoplasma* Ag (from R1), S=*Sarcocystis* Ag (from R2)

Infection of *Sarcocystis* (as studied in *S.cruzi*) normally leads to serological patterns similar to other parasitic diseases. Initial production of specific IgM antibody is subsequently followed by production of IgG antibodies, and the latter remain at high levels for a longer period. A persistent and often slowly rising IgG response seems to appear approximately five weeks post infection (pi) in sheep and cattle[18]. In the present study, the IgG response in rabbits was first detected 31 days pi (dpi). This response elevated by 38 dpi and the corresponding sera were thus used for the immunoblot.

The results of the immunoblot are consistent with previous serological studies that have used monoclonal anitibodies (Mabs)[11]. Two prominent cystozoite antigens of 45.5 and 64 kMW were recognized in the above study whereas the present study identified two bands of ~ 45 and ~66 kMW respectively. Another strong band was also observed above the 66 kMW marker in this study. It cannot be said at this point which, if any, of the molecules of *Sarcocystis* identified by rabbit hyper-immune sera may be surface molecules.

Sarcocystis bands did not appear below the 36 kMW marker although protein bands were seen at that same level in the Ponceau S stained blot. A plausible reason for this absence may be the denaturation of epitopes during the SDS-PAGE procedure. Extreme sensitivity to endogenous proteases could have also caused destruction of epitopes on surface antigens[11].

Cross-reactivity between *Sarcocystis* spp. and *T. gondii* has been investigated in the past, as the possibility cannot be overlooked during routine diagnosis. In 1971, the indirect florescence antibody test and Sabin-Feldman test delivered a negative verdict for cross-reactivity as only specific reactions had occurred[10] .In 1977, antigens of *Sarcocystis* cystozoites from cattle did not cross-react with serum of humans naturally infected with *T. gondii* [19]. In 1984, sera from 2 calves inoculated 28-45 days previously with $5x10^4$ *T. gondii* oocysts showed no reaction with *S.cruzi* antigen by means of ELISA. However a study[9] in 1987 described a very contrasting outcome in the cross reactivity tests. Savini [5] was also able to demonstrate by the ELISA that cross-reactions occur between *T. gondii* and *S. cruzi.* In the present study, no cross reactivity was evident either in the immunoblot or the latex agglutination test. *Toxoplasma* antigen did not react with antibodies to *Sarcocystis*, or *vice versa*. Due to this inconsistency in results, it is necessary to investigate possible cross-reaction, when conducting diagnostic procedures for *Sarcocystic* and *Toxoplasma* infection. A sensitive test should be developed to ascertain cross reactivity.

Acknowledgement

The technical support extended by the staff of the Department of Veterinary Pathobiology, Faculty of Veterinary Medicine & Animal Science, University of Peradeniya and the financial support offered by the Asian Development Bank are gratefully acknowledged.

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