

IMMUNOREACTIVE POLYPEPTIDE PROFILES OF *SARCOCYSTIS FUSIFORMIS* USING HYPERIMMUNE AND NATURALLY INFECTED SERUMAmit Singh<sup>1\*</sup>, Daya Shanker<sup>2</sup>, Amit Kumar Jaiswal<sup>2</sup> and Sanjeev Kumar<sup>2</sup>

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## ABSTRACT

Immunoreactive polypeptide profiles study was made in the whole cyst lysate antigen of *Sarcocystis fusiformis* which was collected from buffaloes slaughtered at local slaughterhouse, Mathura, U.P. When whole cyst lysate antigen was subjected to SDS-PAGE, twelve major polypeptides (78, 66, 53, 50, 42, 39, 32, 29, 27, 24, 19 and 15kDa) were observed. On western blotting, out of twelve, six (32, 39, 42, 53, 66, 78kDa) and two polypeptides (53kDa and 66kDa) were found to be immunoreactive when probed with homologous hyperimmune and pooled known positive natural serum. No immunoreactivity was seen with pooled known negative natural serum. These two polypeptides (53kDa and 66kDa) bands can be explored for the development of sensitive and specific diagnostic assays.

**Keywords:** *Bubalus bubalis*, buffaloes, *Sarcocystis fusiformis*, polypeptides, SDS-PAGE, western blot

## INTRODUCTION

Sarcocystosis caused by protozoan *Sarcocystis* species is one of the commonest and important entities affecting the livestock sector. The infection causes immense economic losses due to poor growth, decreased milk production, abortion, death and due to downgrading or condemnation of infected carcasses. (Dubey *et al.*, 1989a). Economic loss alone due to the condemnation or downgrading of meat containing sarcocysts has been estimated to be in terms of millions of dollars to the cattle industry (Fayer and Dubey, 1986).

Diagnosis of sarcocystosis is not possible since the clinical signs are non-specific and parasitaemia during the acute phase of the disease may be brief or too low for easy detection (Fayer and Dubey, 1986). It is an occult infection and cannot be diagnosed by using conventional parasitological techniques and is noticed only in slaughtered animals or during postmortem examination. The development of serological tests is therefore

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essential for the diagnosis of sarcocystosis. For the development of effective serological assays, study of immunogenic polypeptides is warranted. In the present study, sodium do-decyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting have been attempted to reveal the specific proteins in *Sarcocystis fusiformis* which may help in developing genus or the species-specific diagnostic tests.

## MATERIALS AND METHODS

### Collection of Sarcocysts and blood samples

Approximately 25 gm of representative tissues from oesophagus, tongue and heart were collected in clean plastic bags from each of the 160 buffaloes slaughtered at the local slaughterhouse of Mathura. Blood samples were also collected from the same buffaloes for harvesting of sera. Representative tissues collected from the slaughtered buffaloes were subjected to macroscopic and microscopic examination for the presence of sarcocysts. Sarcocysts were collected from positive samples and species identification was done as per the morphological features of sarcocysts described by Bhatia (2000). Sera from 20 animals in which cysts were detected were pooled hence considered as pooled known positive serum whereas sera from another 20 animals in which cysts were not seen were pooled and hence regarded as pooled known negative sera.

### Preparation of whole cyst lysate antigen

Whole cyst lysate antigen of *Sarcocystis fusiformis* recovered from tissues was prepared as per Gasbarre *et al.* (1984) with slight modifications. About 20 large size macrocyst separated from tissues were homogenized in

tissue homogenizer (Heidolph, silent crushers) in PBS (pH -7.2). This was then freeze-thawed four times, and ultrasonicated (Misonix- Ultrasonic liquid processor) three times for 20 seconds each at 100 W. The suspension was centrifuged at 15000 g for 30 minutes. The supernatant collected was used as antigen. The protease inhibitor phenyl methyl sulphonyl fluoride (PMSF) (100 mM) was added at a concentration of 1 µl/ml of antigen. The protein concentration of whole cyst lysate antigen of *Sarcocystis fusiformis* was estimated as per the method of Bradford *et al.* (1976). The antigen was aliquoted and stored at - 20°C till further use.

### Raising of hyperimmune sera

Two wistar Rats weighing around 125 to 150 g were used for raising the hyperimmune serum (HIS) against whole cyst lysate of *Sarcocystis fusiformis*. Permission to raise HIS in rats was obtained from CPCSEA.

Rats were inoculated intramuscularly with 75 µg of protein after emulsified with equal amount of Freund's complete adjuvant (Santa cruz). After primary immunization, booster doses were given intramuscularly on 14<sup>th</sup>, 21<sup>st</sup> and 28<sup>th</sup> day of 1<sup>st</sup> injection but with Freund's incomplete adjuvant (Santa cruz). The blood was collected after 7<sup>th</sup> day of last injection by puncturing the choroid plexus using fine smooth edge capillary tube. Sera were separated and presence of specific antibodies was checked by Agar Gel Precipitation test (AGPT). The serum samples were aliquoted and stored at -20°C till further use.

### Protein profiles

#### Sodium do-decyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted as per the description of Laemmli (1970) using discontinuous

gel and buffer system. Mididial slab gel electrophoresis (Bangalore Genei) was used to perform the electrophoresis. Electrophoresis was done using 12% resolving gel and 5% stacking gel. 50 µl of WCL antigen of *Sarcocystis fusiformis* containing 50 µg protein with 2X Laemmli buffer was loaded in the wells. Prestained coloured protein marker (genetix) was loaded in separate well as standard marker. Electrophoresis was carried out at a constant current of 10 mA (50v) for stacking gel and 16 mA (100v) for the resolving gel using power pack (Bangalore Genei). After completion, gel was removed and stained with Coomassie blue stain according to the descriptions of Laemmli (1970).

Molecular weight of each polypeptide was calculated by using 'Image analysis' software.

### Western blotting

The Western blotting was carried out as per the method of Towbin *et al.* (1979); Granstrom *et al.* (1990). Protein sample was loaded in triplicate, one each for hyperimmune serum, known positive serum and known negative serum. Prestained coloured protein marker (genetix) was also loaded with each protein samples. After electrophoresis, gel was incised into three parts and used separately for electro transfer blot and transferred on Polyvinylidene difluoride membrane (0.45 µm) using trans-blot semidry electrophoresis transfer cell (BioRad) for 45 minutes at a constant voltage (12V). Once the transfer was completed, membranes were immersed in blocking buffer for 1.30 h at 37°C. After blocking, membranes were then washed thrice with washing buffer and incubated separately with 1: 100 dilutions of HIS, 1: 50 dilutions of pooled known positive and 1: 50 dilutions of pooled known negative serum at 37°C for 1.30 h. All the membranes were again

washed with washing buffer and then incubated with corresponding antisppecies HRP-conjugate (1:5000) at 37°C for 1 h. After washing 5 times with washing buffer, membranes were incubated with substrate (DAB) at room temperature for 5 to 10 minutes. The results were read by comparing with prestained standard molecular weight protein marker.

## RESULTS AND DISCUSSIONS

Sarcocystosis is one of the commonest disease entities in bovines and is difficult to diagnose in live animals. Development of effective diagnostic assay based on serological tools will certainly help in ante-mortem diagnosis. However, development of serological based assay needs study of immunoreactive polypeptides present in the parasites. So, in this study, whole cyst lysate antigen of *Sarcocystis fusiformis* was prepared and subjected to protein profiling study to detect the immunoreactive polypeptides.

Protein profile of whole cell lysate antigen (WCL) of *Sarcocystis fusiformis* was studied by SDS-PAGE using 12% resolving gel and 5% stacking gel to obtain the polypeptides. The molecular weight of polypeptides observed in SDS-PAGE was calculated by using 'Image analysis' software. This software calculates the molecular weight by comparing the results with standard curve which was obtained by plotting the Rf values against molecular weight of protein marker.

Twelve major polypeptides in WCL antigen of *Sarcocystis fusiformis* ranging from 15kDa to 78kDa were observed in SDS-PAGE study. The major bands were 78, 66, 53, 50, 42, 39, 32, 29, 27, 24, 19 and 15kDa (Figure 1).

Almost similar number of polypeptides

has also been observed by Khulbe *et al.* (1989) who carried out electrophoretic separation of various fractions of *Sarcocystis fusiformis* (cyst wall, cyst fluid and zoites). Six components in cyst wall and four components each in cysts fluid and zoites i.e total 14 polypeptides with varied Rf values were obtained. Electrophoretic studies of soluble antigens of other species of *Sarcocystis* have also been studied by several workers. Abbas and Powell (1983) detected eight detectable polypeptides ranging in molecular weight from 10kDa to 220kDa in surface antigens of *Sarcocystis muris*. Granstorm *et al.* (1990) observed 20 polypeptides in the range of 22 to 215kDa in bradyzoites antigen. In another electrophoretic study by Mamatha *et al.* (2008), 10 and 11 polypeptides in whole cyst soluble antigens of *Sarcocystis cruzi* and *Sarcocystis bovifelis*, respectively were detected. More number of polypeptides reported by Granstorm *et al.* (1990) may be due to use of silver nitrate staining which is said to be more sensitive (Laemmli, 1970) in comparison to coomassie blue staining. The differences in the banding patterns compared to previous reports could be due to type of species or parasitic stage used for antigen or methodology used for preparing antigen. The percentage of resolving gel may also influence the separation of polypeptides based on molecular size.

Western blot analysis was carried out after the SDS PAGE to identify the immunoreactive polypeptides in *Sarcocystis fusiformis*. Protein bands resolved in SDS PAGE gel were transferred to PVDF membrane and probed with different types of sera. When probed with homologous hyperimmune serum raised in rats, six (32, 39, 42, 53, 66, 78kDa) out 12 polypeptides obtained in electrophoretic gel were found to be immunoreactive (Figure 2).

However, when probed with pooled positive known natural serum, only two bands

of 53kDa and 66kDa size were observed. No immunoreactivity was seen with pooled known negative natural serum (Figure 3).

Immunoreactive profiles of *Sarcocystis fusiformis* were also studied by Hamza (2004). On immunoblot, the author found several bands to be immunogenic (20- 27kD, 43kD, 90kD, 160kD, and 215kD). The author also studied the effect of treatment on the immunoreactive profile of *Sarcocystis fusiformis*. After heat treatment, the pattern of detected protein bands became slightly faint at 65°C, while at 70°C most bands disappeared. Mamatha *et al.* (2008) carried out polypeptides profile study of *Sarcocystis* species of cattle origin by probing with their respective homologous hyperimmune and homologous positive field sera. The probing of homologous sera with soluble extract of *Sarcocystis bovicanis* resulted in reactivity with polypeptides of 170, 83, 82, 48, 36 and 15kDa. The 170kDa and 36kDa peptides were present in all the natural sera but other immunodominant proteins viz., 83, 48, 36, 29 and 15kDa were not recognized by all the positive sera. In *Sarcocystis bovifelis*, immunoreactive peptides of size 76, 68, 67, 45, 38, 35, 32 and 31kDa were identified. The polypeptides of molecular weight 76kDa and 38kDa sera were identified in all the positive samples. In our study, pooled positive serum was used to probe the immunoreactive polypeptides and with this serum too, only two polypeptides of size 53kDa and 66kDa were observed but they differ in size. The reason for differing in size may be that antigen used by Mamatha *et al.* (2008) was of cattle origin and other reason could be that they used 10% resolving gel instead of 12% used in the present study. In another antigenic analysis of *Sarcocystis* species of cattle (Hettiarachchi and Rajapakse, 2008), probing with homologous hyperimmune serum revealed seven distinct antigenic bands, three of

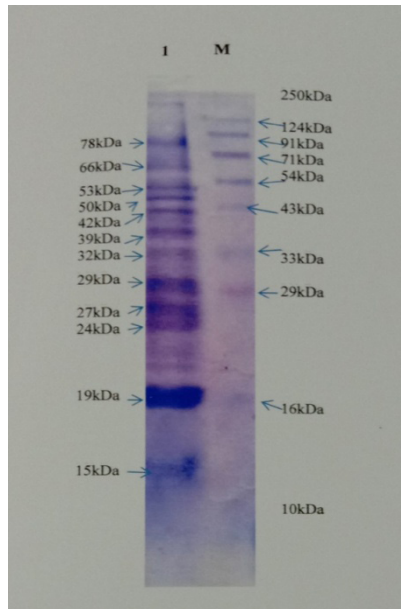


Figure 1. SDS PAGE analysis of WCL antigen of *S. fusiformis*.

Lane 1: WCL;

Lane M: Prestained protein molecular weight marker.

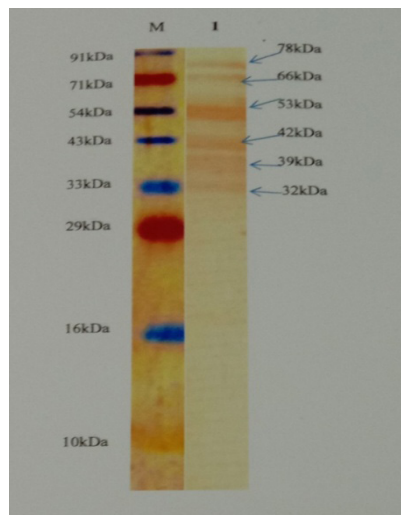


Figure 2. Western blot analysis of WCL of *S. fusiformis* with HIS.

Lane M: Prestained coloured protein marker;

Lane 1: Immunoreactive polypeptides when WCL antigen of *S. fusiformis* probed with homologous HIS.

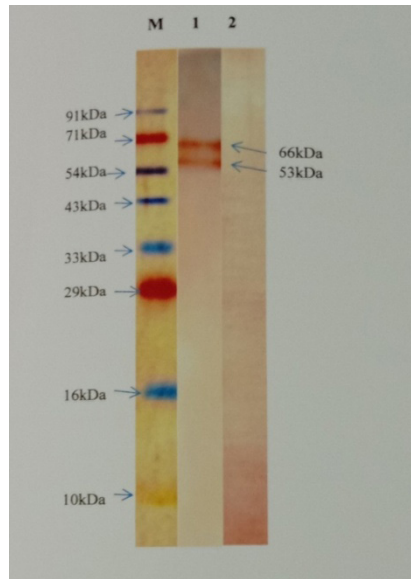


Figure 3. Western blot analysis of WCL antigen of *S. fusiformis* with natural sera.

Lane M: Prestained coloured protein marker;

Lane 1: Immunoreactive polypeptides when WCL antigen of *S. fusiformis* probed with pooled known positive sera;

Lane 2: Immunoreactive polypeptides when WCL antigen of *S. fusiformis* probed with pooled known negative sera.

which were prominent. Out of these three, two were complex bands of around 66kDa and above and the other one was of approximately 45kDa. The two protein bands (53kDa and 66kDa) observed with known positive serum in the present study can be isolated and purified and should be assessed for its diagnostic potential which may increase the sensitivity and specificity of the antibody based serological assay.

## REFERENCES

- Abbas, M.K. and E.C. Powell. 1983. Identification of surface antigens of *Sarcocystis muris* (Coccidia). *J. Protozool.*, **30**(2): 356-361.
- Bhatia, B.B. 2000. *Textbook of Veterinary Protozoology*. Indian Council of Agricultural Research - Krishi Anusandhan Bhavan, New Delhi, India. p. 236-255.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**(1-2): 248-252. DOI: 10.1016/0003-2697(76)90527-3
- Dubey, J.P., C.A. Speer and R. Fayer. 1989. *Sarcocystosis of Animals and Man*, CRC Press, Inc. Boca Raton, Florida, USA.
- Fayer, R. and J.P. Dubey. 1986. Bovine sarcocystosis. *Compendium on Continuing*

- Education for the Practising Veterinarians*, **8**: 130-142.
- Gasbarre, L.C., S. Pamela and R. Fayer. 1984. Humoral and cellular immune responses in cattle and sheep inoculated with *Sarcocystis*. *Am. J. Vet. Res.*, **45**(8): 1592-1596.
- Granstrom, D.E., R.K. Ridley, Y. Baoan and L.J. Gershwin. 1990. Immunodominant proteins of *Sarcocystis cruzi* bradyzoites isolated from cattle affected or nonaffected with eosinophilic myositis. *Am. J. Vet. Res.*, **51**(7): 1151-1155.
- Hamza. 2004. Identification and characterization of *Sarcocystis fusiformis* antigen before and after heat treatment. *El-Minia Medical Bulletin*, **15**: 253-260.
- Hettiarachchi, D.C. and R.P.V.J. Rajapakse. 2008. Antigenic analysis of bovine *Sarcocystis* spp. in Sri Lanka. *Journal of National Science Foundation*, Sri Lanka, **36**(3): 239-244. Available on: <http://dl.nsf.ac.lk/handle/1/6715>
- Khulbe, D.C., A. Kushwah, H.S. Kushwah and M.A. Quadri. 1989. Electrophoretic separation of the soluble proteins of different fractions of the sarcocyst of *Sarcocystis fusiformis* of buffalo (*Bubalus bubalis*). *Vet. Parasitol.*, **32**(3): 127-131. DOI: 10.1016/0304-4017(89)90113-1
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature*, **227**: 680-685. DOI: 10.1038/227680A0
- Mamatha, G.S., P.E. D'Souza and V.V.S. Suryanarayana. 2008. Serodiagnosis of bovine sarcocystosis by enzyme immune transfer blot (EITB) in naturally infected cattle. *J. Vet. Parasitol.*, **22**(2): 69-72.
- Towbin, H., T. Staehelin and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *P. Natl. Acad. Sci. USA.*, **76**(9): 4350-4354. DOI: 10.1073/pnas.76.9.4350