4.0 RESULTS

4.1 Morphological identification of sarcocyst

The identification of the *Sarcocystis* species based on morphological characters are described below.

4.1.1 Microscopic examination of *Sarcocystis bovicanis*

The microsarcocysts of *S. bovicanis* were elongated with tapering ends. The cyst wall was very thin, smooth and lobulations were present (Plate 1 and 2). The microcyst measured 138 to 520µm in length and 25 to 150µm in width with an average of 350µm±15µm and 55µm±5µm. The cyst wall appeared smooth without any structures. The bradyzoites were crescent and banana shaped measuring 14.19µm x 5.10µm in fresh preparations (Plate-3) and 13.63µm x 4.80µm in stained smears (Plate-4). (Jain and Shah (1988); Pandit *et al.* (1994); Bhatia (2000)).

4.1.2 Macroscopic and microscopic examination of *Sarcocystis bovifelis*

The macro sarcocysts of *S. bovifelis* were few in number or sparsely distributed in the musculature of oesophagus (Plate-5). The macrocyst of *S. bovifelis* recovered from the oesophagus were elongated and tapered at both the ends (Plate-6). The cysts were creamy white in colour with thick cyst wall. The cysts measured 1.2mm to 6.5mm in length and 0.20mm to 0.80mm in width with an overall mean length x breadth of 4.5mm±0.65mm x 0.75mm±0.15mm respectively.
The microcysts of *S. bovifelis* were slender with rounded ends. The cyst wall was thick, transversely striated with hair like projections (Plate 7). Lobulations were absent and sarcocysts measured 620 to 850 \( \mu \)m in length and 45 to 90 \( \mu \)m in width with mean value of 715.65±8.5\( \mu \)m x 76.31±2.3\( \mu \)m respectively. Bradyzoites were 13.43\( \mu \)m x 5.0\( \mu \)m in fresh preparations and 13.27\( \mu \)m x 4.34\( \mu \)m in stained smears and were banana or crescent shaped (Plate-8).

4.2 Immunodiagnosis of Sarcocystosis in cattle

4.2.1 Soluble extracts of *S. bovicanis* and *S. bovifelis*

The soluble extracts used for the standardization of ELISA, SDS-PAGE and Western blotting (Enzyme immunotransfer blot) had a protein concentration of 550\( \mu \)g per ml and 920\( \mu \)g per ml of antigen in the case of *S. bovicanis* and *S. bovifelis* respectively (Fig. 1).

4.2.2 Host tissue antigen

Host tissue soluble extract prepared from the heart of non-affected cattle heart had a protein concentration of 2mg per ml.

4.3 Enzyme Linked Immuno Sorbent Assay (ELISA)

4.3.1 Assay standardization

The working dilutions of conjugate, antigen and serum was determined to be 1:5000, 5\( \mu \)g/well and 1:100 for *S. bovicanis* respectively by checkerboard assay method. The working dilution for *S. bovifelis* of
conjugate was 1:5000, antigen was 1µg/well and 1:200 dilution for serum (Fig.2, 3, 4).

4.3.2 Determination of cut off value

The results of the preliminary assays performed on sera from 25 cattle in which no microcyst or macrocyst of *S.bovicanis* and *S.bovifelis* were detected yielded a mean background absorbance value (x) of 0.151 and 0.149 and a standard deviation of 0.020 and 0.024 respectively. The cut off value for *S.bovicanis* was 0.210 and 0.221 for *S.bovifelis* (Fig. 5).

4.3.3 Detection of Sarcocystosis antibodies in cattle

4.3.3.1 Detection of *S.bovicanis* specific antibodies by ELISA

The results of ELISA to detect specific antibodies against *S.bovicanis* are presented in Table-1. *S.bovicanis* specific antibodies were detected in 146 (48.66%) out of the 300 serum samples examined. The sensitivity and specificity of the test are detailed in Table-2. The sensitivity and specificity were 76.4 and 66.6 per cent respectively. All negative serum controls were negative in the assay similar to the substrate and conjugate controls. The positive and negative results were read based on colour development and OD values (Plate-9). The OD values of positive serum ranged between 0.220 to 0.850 and are depicted in the graph (Fig. 6).

4.3.3.2 Detection of specific anti- *S.bovifelis* antibodies by ELISA
Out of three hundred serum samples examined for presence of anti-
*Sarcocystis bovifelis* antibodies in cattle, 15.33 per cent were found to be
positive (Table-1) (Plate-10). The sensitivity of the test was found to be 81.8
per cent and specificity was 71.4 per cent (Table-2). The OD values were in
the range of 0.239 to 0.880. Statistical analysis by Chi-square test revealed
significant difference between the two infections (P≤0.05).

**4.3.3.3 Cross reactivity between the species**

The cross reaction was observed between the *S.bovicantis* and
*S.bovifelis* of cattle (Plate-11). Cross reactivity was also tested between the
heterologous pathogenic species of *Sarcocystis* of goat. *S.bovifelis* showed
cross reaction with *Sarcocystis capracanis* whereas *S.bovicantis* did not
react with *S.capracanis*.

**4.4 Sodium do-decyl sulphate polyacrylamide gel electrophoresis
(SDS-PAGE)**

In the present study the protein profile of *S.bovicantis* and *S.bovifelis*
was studied by SDS-PAGE including host tissue antigen. The soluble
extracts of host tissue antigen was used to check the cross reactivity with
*Sarcocystis* species. Protein profile of the samples were studied using 10%
resolving gel and 4.5% stacking gel.

**4.4.1 Protein profile of soluble extracts of S.bovicantis and S.bovifelis**
The molecular weight of soluble extracts of *S. bovicanis* and *S. bovifelis* was calculated by comparing the results with standard curve. The standard curve was obtained by plotting the $R_f$ values on y-axis and molecular weights on the x-axis using semi log graph paper (Fig. 7).

In *S. bovicanis* soluble antigen a total of 10 polypeptides were found ranging between 170 kDa to 12 kDa. The $R_f$ values of each polypeptide are presented in Table-3. The protein profiles of *S. bovicanis* studied by SDS-PAGE at a protein concentration of 27 $\mu$g is depicted in Plate-12. The major bands were 170, 100, 82, 36, 34, 15 and 12 kDa. The minor bands included 62 and 36 kDa and two bands below 29 kDa were observed.

A total of eleven polypeptides were identified in *S. bovifelis* ranging from 100 kDa to 15 kDa. The protein concentration used was 18 $\mu$g/lane to study the protein profile of *S. bovifelis*. The $R_f$ values for all the peptides are presented in Table-4. Two peptides were identified below 29 kDa. The major bands included 100, 82, 60, 38 and 31 kDa. The minor bands were of 68, 54, 49, 45, 22 and 15 kDa (Plate-13).

### 4.4.2 Protein profile of host tissue antigen
Nine polypeptide bands were found in the host tissue ranging from 80 kDa to 11 kDa (Table-5). The major bands included 61, 50, 29 and 11 kDa. Five peptides were identified as minor bands (Plate-14).

4.4.3 Comparison of protein profile of *S.bovicanis*, *S.bovifelis* and host tissue antigen by SDS-PAGE

Out of 10, 11 and 9 polypeptides identified in *S.bovicanis*, *S.bovifelis* and host tissue antigen respectively only three peptides of molecular weight 100, 82 and 15 kDa were found to be common between *S.bovicanis* and *S.bovifelis*. Whereas host antigen showed only one band in common with *S.bovifelis*. However none of the bands were shared with *S.bovicanis* (Table-6).

4.5 Identification of Immunoreactive polypeptides by Enzyme Immuno Trasfer Blot (EITB)

The immunoreactive polypeptides in soluble extracts of *S.bovicanis*, *S.bovifelis* and host tissue antigen were identified using Dot-ELISA and Enzyme Immuno Transfer Blot (EITB) by probing against homologous and heterologous hyperimmune sera. The hyperimmune serum was raised in calves and presence of antibodies were checked by Agar gel Precipitation test (AGPT) and Counter Current Immunoelectrophoresis (CIEP) test.

4.5.1 Titration of hyperimmune sera raised against *S.bovicanis* and *S.bovifelis* by DOT-Enzyme linked immunosorbent assay (DOT-ELISA)
The different dilutions of hyperimmune sera of *S. bovicanis* was probed against homologous antigen by DOT-ELISA. The antigen concentration used was 7µg/disc in 2µl of coating buffer (appendix). The highest dilution of hyperimmune serum showing clearly visible brown dot was considered as end point titre of antigen-antibody reaction (Plate-15). The hyperimmune sera and normal cattle sera collected before inoculation were tested with conjugate control. The end point titre based on presence or absence of brown dot for *S. bovicanis* was found to be 1:3000. In normal sera visible dot was not found even at 1:500 dilution.

Similarly for *S. bovifelis* different dilutions of hyperimmune sera were tested against homologous soluble extract from cystozoites. The serum dilution upto 1:8000 showed clearly visible brown dot at antigen concentration of 5µg/disc (Plate-16).

### 4.5.2 Titration of known positive and negative sera by DOT-ELISA

The cut off value of positive and negative serum samples were determined by DOT-ELISA. The antibody titres for *S. bovicanis* ranged between 1:50 to 1:800 (Plate-17) and 1:50 to 1:3200 for *S. bovifelis* (Plate-18). Based on the results of DOT-ELISA all the serum samples were tested at 1:100 and 1:200 for *S. bovicanis* and *S. bovifelis*, respectively.

### 4.5.3 Detection of Immunoreactive peptides in soluble extracts of *S. bovicanis* and *S. bovifelis* by Enzyme Immuno Transfer Blot (EITB)
EITB was carried out using 1:3000 and 1:8000 dilutions of homologous and heterologous hyperimmune sera against *S.bovicanis* and *S.bovifelis* soluble antigen extracts.

### 4.5.4 Immunoreactive polypeptides in soluble extract of *S.bovicanis*

The polypeptides detected on western blots using HIS of *S.bovicanis* with anti bovine IgG conjugate at 1:1000 dilution ranged between 100 to 12 kDa (Table-7). A total of six polypeptides of size 100, 82, 52, 36, 15 and 12 kDa were identified on blots (Plate-19). Two peptides having molecular weight of 100 and 82 kDa were seen as diffuse bands.

*S.bovicanis* soluble extract separated by SDS-PAGE blotted on to the nitrocellulose membrane and probed against *S.bovifelis* HIS revealed only two polypeptides of size 48 and 15 kDa (Plate-21). However it did not react with any of the peptides from host tissue extracts.

### 4.5.5 Immunoreactive polypeptides identified in *S.bovifelis* soluble extract

*S.bovifelis* blots were treated with homologous hyperimmune serum and the polypeptides of size 100, 82, 49, 45, 43, 31, 22 and 14 kDa reacted with the serum (Plate-20) (Table-8). *S.bovifelis* was found to have 82, 49 and 14 kDa polypeptides as immunoreactive when probed by HIS raised against *S.bovicanis* (Plate-22). None of the polypeptides were recognized in host tissue antigen when probed against HIS of *S.bovifelis*. Polypeptides of
molecular weights of 100, 22 and 14 kDa were seen as diffuse bands. *S. bovicanis* and *S. bovifelis* blots did not react with normal sera from non-infected cattle.

**4.5.6 Immunoreactive peptides detected in *S. bovicanis* and *S. bovifelis* probed against known positive and negative serum**

A total of 30 known positive and 30 negative serum samples were tested against soluble extract of *S. bovicanis* and *S. bovifelis*. The probing of homologous sera with soluble extract of *S. bovicanis* resulted in reactivity with polypeptides of 170, 83, 82, 48, 36 and 15 kDa (Table-9). The 170 kDa and 36 kDa peptides were present in all the 30 samples with 100 percent sensitivity. However other immunodominant proteins viz., 83, 48, 36, 29 and 15 kDa were not recognized by all the positive sera (Plate-23, 24 & 25).

In *S. bovifelis* immunoreactive peptides of size 76, 68, 67, 45, 38 35, 32 and 31 kDa were identified (Plate-26) (Table-10). The polypeptides of molecular weight 76, 68 and 38 were identified in all the positive samples with 100 per cent sensitivity (Plate-27). None of the polypeptides were identified in negative sera (Plate- 28 and 29).

**4.5.7 Serodiagnosis of Sarcocystosis in cattle by EITB**

**4.5.7.1 Serodiagnosis of *S. bovicanis***
The antibodies were detected at 1:100 dilution. The antibovine IgG conjugate was used at 1:1000 dilution (Table-11). The polypeptides 170 kDa and 36 kDa was detected in all the positive serum samples. Out of 300 blood samples examined for *S.bovicanis* in cattle by EITB, specific antibodies were detected in 175 (58.3%). The sensitivity and specificity was found to be 87.5 and 81.8 per cent, respectively.

### 4.5.7.2 Serodiagnosis of *S.bovifelis*

The EITB was carried out at 1:200 serum dilution with 1:1000 antispecies conjugate. Specific antibodies were detected in 89 (29.6%) serum samples out of 300 serum samples examined (Table-11). The polypeptides of size 76 kDa and 38 kDa were commonly identified in all the serum samples which showed positive results. The sensitivity of 93.5 per cent and specificity of 87.5 per cent was recorded for *S.bovifelis*.

### 4.5.8 Comparison of sensitivity and specificity of ELISA and EITB

The sensitivity and specificity of ELISA and EITB varied significantly in diagnosis of *S.bovicanis* and *S.bovifelis* of cattle (Fig. 8 & 9). The sensitivity and specificity by ELISA was found to be 76.4 and 66.6 per cent and 81.8 and 71.4 per cent for *S.bovicanis* and *S.bovifelis*, respectively. The sensitivity and specificity in EITB was found to be 87.5 and 81.8 per cent and 93.5 and 87.5 per cent for *S.bovicanis* and *S.bovifelis* respectively.

### 4.6 Differentiation of *S.bovicanis* and *S.bovifelis* by Random
Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR)

RAPD-PCR was carried out using four arbitrarily designed primers in which primer 1 and primer 2 were chosen based on previously published reports. The primers 3 and 4 were newly designed and examined for the amplification of *S. bovicanis* and *S. bovifelis* genomic DNA and for use as a probe.

Four primers of nucleotide length 20, 18, 16 and 22 bp with GC content more than 50% were used in PCR assays where the optimal annealing temperature was 36°C. All the four primers directed the amplification of at least a single DNA fragment using both *S. bovicanis* and *S. bovifelis* genomic DNA as the template.

In this study the number of DNA fragments amplified by primers 1, 2, 3 and 4 for *S. bovicanis*, *S. bovifelis* and host DNA are presented in Table-14. Primer 1 amplified two fragments of size 1.3 and 1.2 kb for *S. bovicanis* where as for *S. bovifelis* four fragments 1.1, 1.0, 0.85 and 0.75 kb were amplified and none of the fragments were observed in host DNA (Plate-30). The intensity of the fragments amplified are presented in Table-15.

A second primer with 19 nucleotides in length produced DNA fragments of 0.4, 0.6 and 0.7 kb for *S. bovicanis* (Plate-31). The fragments of 1.2, 1.3 and 1.5 kb were amplified for *S. bovifelis*. No bands were visible in host DNA after gel electrophoresis.
The Primer 3 amplified two fragments of 0.8 and 0.5 kb were *S. bovicanis* (Plate-32). In the case of *S. bovifelis* two fragments of size 1.0 and 1.1 kb were obtained. There were no DNA fragments present in the host DNA.

In the present study, *S. bovicanis* DNA produced only single fragment of size 0.5 kb whereas *S. bovifelis* DNA amplified 0.7, 0.9 and 1.0 kb (Plate-33) for primer 4. In host DNA none of the fragments were visible. The similarity co-efficient between the two species by RAPD-PCR was found to 0.375(Table-16).

5.0 DISCUSSION

Sarcocystosis an occult protozoan disease is now recognized as an emerging coccidian disease in livestock particulary in cattle (Shah, 1983). The *Sarcocystis* species in cattle are known to produce morbidity and mortality in naturally occurring and experimentally induced infections.