RESULTS
4.0 RESULTS

4.1 Immunodiagnosis of *Echinococcus granulosus* in dogs

4.1.1 Excretory/secretory antigen of *E. granulosus*

The excretory/secretory antigen of *E. granulosus* was obtained after culturing the worms for 24 hours when the worms were very active. The worms became immobile afterwards and the culturing was stopped at this point and the excretory/secretory products were pooled, concentrated and dialyzed. The protein concentration of the excretory/secretory antigen was found to be 540 μg/ml of antigen (Plate. 1 and 2).

4.1.2 Somatic antigen of *E. granulosus*

The somatic antigen of *E. granulosus* was prepared and utilized for the standardization of ELISA, SDS-PAGE and Western blotting (Enzyme immunotransfer blot). The protein concentration of the somatic antigen was found to be 940μg per ml of antigen (Plate. 3, 4 and 5).

The somatic antigens of *T. hydatigena* and *D. caninum* were also prepared and had 1250μg per ml and 1450μg protein per ml of antigen, respectively (Plate. 6 and 9).

4.1.3 Testing of hyper immune serum

Hyper immune serum raised in rabbits against both somatic and excretory/secretory antigen of *E. granulosus* tested by CIEP and AGPT gave
positive results to both somatic and excretory/secretory antigens, respectively by 90 minutes.

4.2 Counter immuno electrophoresis (CIEP)

The test was initially standardized with positive dog serum and E/S and somatic antigen and a clear band could be seen after running the system for 90 minutes. The test was then evaluated with hyper immune serum raised against the excretory/secretory antigen and somatic antigen and two clear bands could be seen after running for 90 minutes. Then the test was performed with field serum (1:2 dilutions).

Ten serum samples from dogs, which were found to be infected with E.granulosus worms during necropsy, gave positive results against E/S and somatic antigens. The CIEP slides showed one clear band after staining. The dogs, which were heavily infected with E. granulosus during necropsy, revealed three bands against hyper immune serum. The other positive samples resulted in one band against hyper immune serum. The other entire ninety field samples screened showed negative results. No cross-reaction was found when serum positive for T.hydatigena and D.caninum were used. The sharpness of the band was clearer with E/S antigen compared to somatic antigen (Plate. 11 and 12).

The sensitivity and specificity of CIEP with E/S and somatic antigen are depicted in Table 1 & 2. Among 100 serum samples screened, the sensitivity
was found to be 90 and 80 per cent and the specificity was 100 per cent with both E/S and somatic antigen, respectively (Fig. 9 and 10).

4.3. Indirect Enzyme Linked Immuno Sorbent Assay (ELISA)

4.3.1 Assay standardization by Checker board titration

The working dilutions of conjugate, excretory/secretory antigen and positive serum were found to be 1:10,000, 2μg/well and 1:100, respectively by checkerboard assay method. The working dilution for somatic antigen of conjugate was 1:10,000; antigen was 3 μg/well and 1:200 dilution for serum (Fig. 1, 2, 3 and 4).

4.3.2 Determination of cut off value

The results of the preliminary assays performed on sera from 10 dogs in which no helminth ova were detected yielded a mean background absorbance value (x) of 0.378 and 0.334 and a standard deviation of 0.036 and 0.031 for E/S and somatic antigen of *E. granulosus*, respectively. In the present study the cut off OD value for E/S antigen was 0.486 and for somatic antigen of *E. granulosus* was 0.427 (Mean + 3 SD) (Fig. 5).

4.3.3 Detection of *E. granulosus* antibodies in dogs

4.3.3.1 Detection of *E.granulosus* specific antibodies with E/S antigen by indirect ELISA

The results of indirect ELISA to detect specific antibodies against E/S antigen of *E.granulosus* are presented in Table. 4. *E. granulosus* E/S antigen
specific antibodies were detected in 81 (32.4 per cent) out of the 250 field serum samples examined (Plate. 14). The sensitivity and specificity of the test are detailed in Table. 1. The sensitivity and specificity were 100 and 79.20 per cent with E/S antigen respectively (Fig. 9). All negative serum controls were negative in the assay similar to the substrate and conjugate controls. The OD values of positive serum ranged from 0.486 to 0.850 and are depicted in the graph (Fig. 6).

4.3.3.2 Detection of *E.granulosus* specific antibodies with somatic antigen by indirect ELISA

Out of 250 serum samples examined for presence of anti-*E.granulosus* antibodies with somatic antigen in dogs, 62 samples (24.8) per cent were found to be positive (Table. 4; Plate. 13). The sensitivity of the test was found to be 100 per cent and specificity was 71.60 per cent (Table. 2 & Fig. 10). The OD values were in the range of 0.427 to 0.980 (Fig. 7). Statistical analysis by Chi-square test revealed significant difference between the two antigens (P≤0.05).

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

In the present study the protein profile of somatic and excretory/secretory antigens of *E.granulosus* were studied using 10 per cent resolving gel and 4.5 per cent stacking gel.

4.4.1 Protein profile of somatic and excretory/secretory antigens
The molecular weights of somatic and excretory/secretory antigens were calculated by comparing the results with standard curve. The standard curve was obtained by plotting the Rf values on y-axis and molecular weights on the x-axis using semi log graph paper (Fig. 8).

In somatic antigen a total of 11 polypeptides were found ranging between 114 kDa to 16 kDa. The Rf values of each polypeptide are presented in Table. 7. The protein profiles of somatic antigen were studied by SDS-PAGE at a protein concentration of 60μg. The major bands were 94, 66, 45, 34, 24 and 16 kDa. The minor bands included 114, 84, 74, 68 and 56 kDa and one band above 97.4 kDa was observed (Plate. 15).

A total of twelve polypeptides were identified in excretory/secretory Ag ranging from 110 kDa to 14 kDa. The protein concentration used was 40μg / lane to study the protein profile of E/S antigen of *E.granulosus*. The Rf values for all the peptides are presented in Table. 8. Three peptides were identified above 97.4 kDa. The major bands included 66, 45, 38 and 34 kDa. The minor bands were of 82, 78, 30, 24 and 14 kDa (Plate. 16).

4.4.2 Protein profile of *T. hydatigena* and *D. caninum* somatic antigen

Twenty-one polypeptide bands were found in the somatic antigen of *T.hydatigena* ranging from 104 kDa to 14 kDa (Table. 9). The major bands included 83, 66, 46, 34, 37, 28 and 23 kDa. Fourteen peptides were identified as minor bands (Plate. 17). Twenty-two polypeptide bands were
found in the somatic antigen of *D. caninum* ranging from 101 kDa to 12 kDa (Table. 10). The major bands included 64, 50, 43, 35, 33, 30, 27, 18 and 15 kDa and 13 polypeptides were considered as minor bands (Plate. 18).

### 4.4.3 Comparison of protein profile of somatic and excretory/secretory antigens of *E.granulosus* and *T.hydatigena* and *D.caninum* somatic antigen by SDS-PAGE

Out of 11 polypeptides identified in somatic and 12 polypeptides in excretory/secretory antigens, only four peptides of molecular weight 66, 45, 34 and 24 kDa were found to be common between somatic and excretory/secretory antigens of *E.granulosus*. *T.hydatigena* showed only two bands i.e. 66 and 34 which were common with somatic antigen of *E.granulosus* where as three bands viz., 66, 34 and 14 were common with excretory/secretory antigen of *E.granulosus*. However, somatic antigen of *D.caninum* shared two bands of 84 and 68 kDa with somatic antigen of *E.granulosus* and three bands of 78, 30 and 14 kDa with excretory/secretory antigen of *E.granulosus* (Table. 12).

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

The immunoreactive polypeptides in somatic and excretory/secretory antigens were identified using Dot-ELISA and Enzyme Immuno Transfer Blot (EITB) by probing against known positive sera.
4.5.1 Titration of known positive homologous serum by DOT-Enzyme linked immunosorbent assay (DOT-ELISA)

The different dilutions of homologous known positive sera of *E.granulosus* were probed against E/S and somatic antigen by DOT-ELISA with antigen concentration of 2µg and 5µg/disc in 2µl of coating buffer (appendix) respectively. The highest dilution of homologous known positive serum showing clearly visible brown dot was considered as end point titre of antigen-antibody reaction. The end point titre based on presence or absence of brown dot was found to be 1:200. Based on the results of DOT-ELISA all the serum samples were tested at 1:100 and 1:200 for E/S and somatic antigen (Plate. 20 and 21).

4.5.2 Detection of Immunoreactive peptides in somatic and excretory/secretory antigens by EITB

The results of EITB carried out with 1:200 and 1:100 dilutions of known positive sera against somatic and excretory/secretory antigens of *E.granulosus* are as follows.

4.5.3 Immunoreactive polypeptides in somatic antigen of *E.granulosus*

The polypeptides detected on western blots using homologous known positive serum of *E.granulosus* with anti dog IgG conjugate at 1:1000 dilution ranged between 84 to 16 kDa (Table. 13). A total of four polypeptides of size 84, 66, 45 and 16 kDa were identified on blots (Plate.
24). Two peptides having molecular weight of 66 and 16 kDa were seen as diffuse bands.

4.5.4 Immunoreactive polypeptides identified in excretory/secretory antigen of *E.granulosus*

The polypeptides detected on western blots using homologous positive serum of *E.granulosus* with anti dog IgG conjugate at 1:1000 dilution ranged between 98 to 24 kDa (Table. 14). A total of six polypeptides of size 98, 82, 66, 45, 34 and 24 kDa were identified on the blots (Plate. 23). Two peptides having molecular weight of 98 and 34 kDa were seen as diffuse bands.

4.5.5 Immunoreactive polypeptides identified in *T.hydatigena* and *D.caninum* somatic antigen with known positive serum of *E.granulosus*

*T.hydatigena* and *D.caninum* somatic Ag blot were treated with homologous known positive serum of *E.granulosus* with anti-dog IgG conjugate at 1:1000 dilution. Two polypeptides of size 69 and 42 kDa were detected with *T.hydatigena* (Plate. 29) and only one polypeptide of 35 kDa was detected in *D.caninum* somatic antigen (Plate. 30) when reacted with the serum of *E.granulosus*.

None of the polypeptides were identified in negative sera when probed against E/S, somatic and F/S antigens of *E.granulosus* (Plate. 31).
4.5.6 Serodiagnosis of somatic antigen of *E.granulosus* in dogs by EITB

The antibodies were detected at 1:200 serum dilution. The antidog IgG conjugate was used at 1:1000 dilution. The polypeptides of 84, 66 and 45 kDa were detected in all the positive serum samples (Plate. 26). Out of 250 blood samples examined for *E.granulosus* with somatic antigen in dogs by EITB, specific antibodies were detected in 41 samples (16.4 per cent) (Table. 5). The sensitivity and specificity was found to be 100 and 83.60 per cent, respectively (Table. 2; Fig. 10).

4.5.7 Serodiagnosis of E/S antigen of *E.granulosus* in dogs by EITB

The antibodies could be detected at 1:100 serum dilution. The antidog IgG conjugate was used at 1:1000 dilution. The polypeptides of 66, 45 and 34 kDa were detected in all the positive serum samples (Plate. 27). Out of 250 serum samples examined by EITB, specific antibodies were detected in 52 samples (20.80 per cent) (Table. 5). The sensitivity and specificity was found to be 100 and 88.40 per cent, respectively (Table. 1; Fig. 9).

4.5.8 Comparison of sensitivity and specificity of ELISA and EITB with somatic and E/S antigen

The sensitivity and specificity of ELISA and EITB with somatic and E/S antigens varied significantly (P≤0.05). The sensitivity and specificity
by ELISA with somatic antigen was found to be 100 and 71.60 per cent respectively. The sensitivity and specificity in EITB was found to be 100 and 83.60 per cent respectively. The sensitivity and specificity by ELISA with E/S antigen was found to be 100 and 79.20 per cent where as the sensitivity and specificity of EITB was found to be 100 and 88.40 per cent respectively.

4.6 Immunodiagnosis of *E.granulosus* with faecal supernatant antigen

The faecal supernatant antigen with protein concentration of 1150µg per ml was used to standardize the Latex agglutination test, Dot-ELISA, SDS-PAGE and in Western blotting (Enzyme immunotransfer blot).

4.6.1 Latex agglutination test

Latex agglutination test was conducted with polystyrene latex beads (0.81µ) sensitized with F/S antigen. Different dilutions of antigen including undiluted and diluted (1:2, 1:4 and 1:8) were used to sensitize the latex beads of 1:100 dilution and was tested for *E.granulosus* infection using different dilutions of serum (undiluted, 1:2, 1:4, 1:8, 1:16 and 1:32).

The optimum dilution of faecal supernatant (F/S) antigen to coat the latex beads was found to be 1:4 dilution with 1:100 concentration of latex beads. Similarly the end titre of the serum was found to be 1:4 dilution with the faecal supernatant (F/S) antigen. The optimum concentration of
the antigen to coat the latex beads and different dilutions of serum samples are depicted in the Table. 6.

The results of latex agglutination test were determined by the extent of visual agglutination reaction, which was scored as 1+, 2+, 3+ and 4+ reactions. A 3+ and 4+ agglutination reaction was considered as positive and 2+ and 1+ agglutination was negative. The negative reaction was indicated by uniform turbidity without agglutination (Plate. 33).

The latex agglutination test was evaluated with a total of 250 serum samples using E.granulosus F/S antigen. The test indicated 47 samples (18.8 per cent) samples to be positive. The sensitivity and specificity of latex agglutination was found to be 100 and 78.80 per cent respectively (Table. 3; Fig. 11).

4.6.2 Sodium do-decyl sulphate polyacrylamide gel electrophoresis

(SDS-PAGE) of faecal supernatant antigen

The protein profile of faecal supernatant antigen by SDS-PAGE was performed with 10 per cent resolving and 4.5 per cent stacking gel.

4.6.3 Protein profile of faecal supernatant antigen

The protein concentration of 25μg/lane was used to observe the protein profile of faecal supernatant antigen of E.granulosus. A total of eight polypeptides were noticed in the faecal supernatant antigen ranging from 76 kDa to 17 kDa. The Rf values for all the peptides are presented in
Table. 11. The major bands included 54, 45, 37 and 34 kDa and the minor bands were of 76, 66, 24 and 17 kDa (Plate. 19).

4.6.4 Identification of Immunoreactive polypeptides by Enzyme Immuno Transfer Blot (EITB)

The immunoreactive polypeptides in fecal supernatant antigen were identified using Dot-ELISA and Enzyme Immuno Transfer Blot (EITB) by probing against homologous sera.

4.6.5 Titration of known positive homologous serum by DOT-Enzyme linked immunosorbent assay (DOT-ELISA)

The different dilutions of positive sera of *E.granulosus* were probed against fecal supernatant antigen by DOT-ELISA. The antigen concentration used was 3µg/disc in 2µl of coating buffer (appendix). The highest dilution of homologous serum showing clearly visible brown dot was considered as end point titre of antigen-antibody reaction (Plate. 22). The end point titre based on presence or absence of brown dot was found to be 1:400.

EITB was carried out using 1:400 dilution of positive sera against faecal supernatant antigen of *E.granulosus*.

4.6.6 Immunoreactive polypeptides in faecal supernatant antigen of *E.granulosus*
The polypeptides detected on western blots using positive serum of *E.granulosus* with anti dog IgG conjugate at 1:1000 dilution ranged between 66 to 18 kDa (Table. 15). A total of four polypeptides of size 66, 45, 34 and 17 kDa were identified on blots (Plate. 25).

**4.6.7 Serodiagnosis of *E.granulosus* in dogs by EITB with faecal supernatant antigen**

The antibodies were detected at 1:400 serum dilution. The antidog IgG conjugate was used at 1:1000 dilution. The polypeptides of 45 kDa and 34 kDa were detected in all the positive serum samples (Plate. 28). Out of 250 serum samples examined for *E.granulosus* in dogs by EITB with faecal supernatant antigen, specific antibodies were detected in 47 samples (18.8) per cent (Table. 5). The sensitivity and specificity was found to be 100 and 86.80 per cent, respectively (Table. 3, Fig. 11).

**4.6.8 Serodiagnosis of *E.granulosus* in dogs by Dot-ELISA with faecal supernatant Ag**

The antibodies were detected at 1:400 dilution. The antidog IgG conjugate was used at 1:1000 dilution. Out of 250 serum samples examined for *E.granulosus* in dogs by Dot-ELISA with faecal supernatant antigen, specific antibodies were detected in 45 samples (18 per cent). The sensitivity and specificity was found to be 100 and 82.00 per cent, respectively (Table. 3; Plate. 32).
4.6.9 Comparison of sensitivity and specificity of LAT, Dot-ELISA and EITB

The sensitivity and specificity of LAT, Dot-ELISA and EITB in the diagnosis of *E.granulosus* of dogs (Fig. 11). The sensitivity by LAT and Dot-ELISA was found to be 100 and specificity was 78.80 and 82.00 per cent respectively. The sensitivity and specificity in EITB was found to be 100 and 86.80 per cent, respectively (Table. 3).

4.7 Copro-Polymerase Chain Reaction

In the present study, 100 faecal samples were examined by copro-microscopy and 19 samples were positive for *Taeniid* ova. Among the 19 positive samples, 10 faecal samples belonged to dogs which had *E.granulosus* worms at intestinal examination (Plate. 7 and 8) and no *Taenia* species of worms were found.

Copro-PCR was carried out using two sets of *E.granulosus* specific primers namely Eg 1f, Eg 1r and JB 3f, JB 4.5r as per Stefanic *et al.* (2004) and Boweles and McManus (1994).

Two sets of primers of nucleotide length 23bp and 24bp were used in copro-PCR assays where the optimal annealing temperature was 53°C and 50°C. The primers directed the amplification of at least a single DNA fragment using genomic DNA from *Taenia* eggs as the template.

19 samples were subjected to Copro-PCR by using the above said primers. In the present study Eg 1f, Eg 1r primer amplified 255 bp, which is
specific to *E.granulosus*, in ten samples found infected with *E.granulosus* worms at necropsy (Plate. 35) and no bands were observed in nine fecal samples which were positive for *Taenia* eggs by copromicroscopy (Plate. 10).

The other primers JB 3f, JB 4.5r which amplified 440 bp, also specific for *E.granulosus*, in the ten positive samples (Plate. 34) and no bands were observed in nine faecal samples which were positive for *Taenia* eggs by copromicroscopy.

The Copro-PCR was found to be 100 percent sensitive and specific in the detection of *E.granulosus* eggs in the faecal samples.