DISCUSSION
5.0 DISCUSSION

Echinococcosis is a cosmopolitan parasitic zoonosis caused by the dwarf dog tapeworm *Echinococcus granulosus*. The domestic life cycle is maintained through dogs and ungulates, mainly sheep and cattle. The disease has a worldwide distribution, with a considerable impact to both human and animal health and with important socio-economic consequences in endemic areas. Echinococcosis, the focus of exhaustive taxonomic and epidemiological studies, has a global economic impact through morbidity and mortality in man and production losses in the livestock industry.

The parasite *E.granulosus* is found worldwide especially in areas where hygienic conditions and education are poor. Another reason is the uncontrolled stray dog population in developing countries with a scavenger function. There is some evidence that the disease is spreading because of a lack of meat control, dog management and appropriate legislation (Schwabe, 1986).

In India, there are many reports of cystic Echinococcosis in man and livestock. The socio-economic, cultural and religious factors have frequently played an important role in the transmission of infection to human beings. *E.granulosus* tapeworms have also been reported in dogs.

This disease is one of the major health problems in India (D’Souza, *et al.*, 2001). Due to lack of awareness, raw meat is fed to domestic and stray dogs or they have a free access to offal. The slaughtering of domestic animals for meat is yet to be regulated. The intestinal contents and waste meat is not
properly disposed. Stray dogs roam freely and eagles, kites and other wild birds perch in the trees, waiting to feed from bits of carcasses. These are the major sources of infection of this disease. The main cause of this disease is due to a low level of awareness, lack of legislation, lack of meat inspection, water pollution and also the uncontrolled stray dog population.

The definitive host range for the *Echinococcus granulosus* is more restricted than the intermediate host range. According to the control perspective the diagnosis of the adult stages of these parasites is more important than that of the cystic stage (Allan and Craig, 2006).

Diagnosis of echinococcosis in live dogs is difficult as clinical signs are non-specific and not indicative in infected dogs. However, the correct diagnosis of infection and specific identification of the worm species is essential to reduce the dissemination and transmission, zoonotic impact and economic losses in terms of decreased growth rate and condemnation of infected organs etc.

The gravid proglottids and or eggs are shed in the faeces and the eggs are brown in colour and are morphologically indistinguishable from tapeworm eggs of the *Taenia* species (Eckert *et al.*, 2001a). The eggs can remain viable in the environment up to one year under varying conditions of temperature and moisture (Thompson, 1995). The adult worm passes out gravid proglottids containing eggs, or free eggs are passed out with the faeces. These gravid proglottids, or eggs, are dispersed and contaminate the environment, feed,
grass, water etc, which are sources of infection to intermediate hosts including humans over a wide area (Thompson, 1995).

Echinococcosis in dogs is prevalent throughout the year and many workers in India have reported its prevalence from different regions (Acharya, 1939; Maplestone and Bhaduri, 1940; Reddy et al., 1968; Sahai, 1969; Sahasrabudhe et al., 1969; Khuddus and Krishna Rao, 1971; Hedge et al., 1974; Sharma and Venkataratnam, 1974; Reddy and Reddy, 1988; Singh and Dhar, 1988; Chowdhury and Tada, 2001 and Prathiush, 2007) with different prevalence rates.

The development of sensitive and specific ante-mortem diagnostic methods for the detection of canine echinococcosis is important for epidemiological baseline data and for surveillance of hydatid control programmes. Screening of dogs for *E. granulosus* has traditionally been done by arecoline purgation followed by examination of the purge. Although the specificity of purgation can be 100 per cent, it is time-consuming, biohazardous, has variable sensitivity and requires trained personnel (WHO/OIE, 2001).

Furthermore, the eggs of Taeniid cestodes are morphologically indistinguishable by light microscopy due to extreme morphologic similarity and identification by microscopic examination of the feces is risky and non-specific. Two major diagnostic methods have been extensively used in dogs namely purgation with arecoline compounds and necropsy of the small
intestine. Necropsy is the method of choice and is considered as the gold standard (Eckert, 2003).

Clinical diagnosis of echinococcosis has always been problematic because of the variety and non-specificity of symptoms. A rapid and simple diagnostic method is needed in order to conduct epidemiological surveys successfully. For these reasons, immunodiagnostic tests play an important role in the diagnosis and epidemiology of the disease. The immunological methods vary in their design and adaptability and are useful for the detection and quantification of antigens and antibodies.

ELISA has emerged as a very useful immunological tool because of which it became one of the most widely used techniques in measuring antibody, antigen and protein (McLaren et al., 1979)

Different antigenic preparations of the adult worms and copro-antigen were used as source of antigen in immunoassays. They included either the crude homogenates of whole worm or partially purified antigens. Different sensitivities and specificities were reported with these antigens and the same antigenic preparations in different groups exhibited varied results.

Echinococcosis in canids cannot be diagnosed by microscopic egg detection in faecal samples, because these eggs are morphologically indistinguishable from those of *Taenia* species and egg excretion is often irregular (Dinkel *et al.*, 1998).
Direct examination method has disadvantages as small numbers of worms may be overlooked and parasites consisting of only one or two segments may escape detection (Eckert et al., 2001). Sedimentation and counting techniques (SCT) were found to have sensitivity and specificity nearing 100% and was considered as the “gold standard” (Eckert, 2003; Deplazes et al., 2004). But both techniques are time consuming and labor intensive and further cannot be applied to live animals and alternative techniques are necessary.

Hence a study was undertaken to characterize different antigens from the adult worms and the copro-antigen. The antigens were subjected to SDS-PAGE to know the protein profile and EITB to assess the immunodominant and immunodiagnostic antigens. The Copro-PCR was carried out for confirmatory diagnosis and differentiation from other *Taenia* species.

### 5.1 Counter immunoelectrophoresis (CIEP)

The counter immunoelectrophoresis (CIEP) was evaluated for the diagnosis of *Echinococcus granulosus* in dogs in the present study with E/S antigen of *Echinococcus granulosus* for the first time. The E/S antigen from heavily positive dog had given a clear band when positive dog serum was used. False negative reaction was obtained in the other moderate positive cases. This can be due to the low levels of antibody/antigen used in the assay. Rabbit anti-*Echinococcus granulosus* E/S hyperimmune serum with E/S antigen gave positive results. Two bands could be visualized indicating two
major antigenic components in the heavily positive dog sample. This was comparable with the results obtained by Ahmad and Nizami (1998). They obtained a maximum of four precipitin arcs when faecal supernatants of experimentally infected dogs were used against positive dog serum. The somatic antigen of *E.granulosus* also gave promising results with both positive sera and hyperimmune serum. This was in accordance with Katoch and Singh (1994) who used adult worm antigen for detection of echinococcus antibodies in experimentally infected pups by CIEP.

Katoch and Singh (1994) used standard CIEP for detection of echinococcus antibodies in sera of experimentally infected pups on day 20, 30 and 50 postinfection using protoscoleces and adult worm antigen. They detected antibodies on day 30 and 50 postinfection with adult worm antigen and 20, 30 and 50 days postinfection with protoscoleces antigen. Ahmad and Nizami (1998) used CIEP for the detection of coproantigens of *Echinococcus granulosus* in dogs on an experimental basis. They used positive dog serum against positive faecal supernatant in the assay. Konapur *et al.* (1999) used CIEP for diagnosis of hydatidosis in cattle and buffaloes using concentrated crude and partially purified antigens of hydatid cyst fluid, germinal membrane and protoscoleces. The sensitivity and specificity was 90.7 and 88.1 per cent in cattle and 69.2 and 87.8 per cent in buffaloes, respectively.
The specificity of CIEP was found to be 100 per cent in the present study with both E/S and somatic antigen. Sensitivity was found to be 90 and 80 with E/S and somatic antigen, respectively. The high sensitivity and specificity could have been due to the low prevalence of *Echinococcus granulosus* by necropsy method, which was taken as the standard for CIEP. Interestingly no cross reaction was found when somatic antigen of *Taenia hydatigena* and *Dipylidium caninum* were used. Tris-borate buffer was used in the present study whereas barbitone buffer was used by Ahmad and Nizami (1998). This was considered essential since sodium barbitone was not available.

It can be concluded that CIEP is a simple, inexpensive and rapid test. Results can be obtained within 90 min and has a potential for field application since it shows high specificity and sensitivity for both E/S and somatic antigen with positive sera and hyperimmune serum.

5.2 Detection of anti-*echinococcus* antibodies by ELISA

Indirect ELISA was conducted in the present study for detection of serum antibodies in dogs, specific to *Echinococcus granulosus* with E/S and somatic antigen. Out of 250 serum samples examined for anti-*Echinococcus* antibodies, 81 (32.4 per cent) and 62 (24.8 per cent) were found to be positive for E/S and somatic antigen of *E.granulosus*, respectively by indirect ELISA. Gasser *et al.*, (1993) used ELISA for the diagnosis of *E. granulosus* infection in dogs with worm excretory/secretory antigen and compared with protoscolex
somatic antigen. They tested 224 sera from dogs and found significant linear relationship between absorbance values of the sera tested against the two antigens.

ELISA based serum antibody detection is useful in the diagnosis of naturally acquired *Echinococcus granulosus* infection in dogs (Gasser *et al.*, 1988). Ersfeld *et al.* (1997) analyzed adult worm extracts of *Echinococcus granulosus* by ELISA and showed a sensitivity of 83 per cent for cystic echinococcosis. They showed that *Echinococcus granulosus* adult worms could provide an alternative source to metacestode antigens for the serodiagnosis of cystic echinococcosis. Zhang *et al.*, (2003) found poor sensitivity and specificity in ELISA based methods for detection of circulating antibodies in canines and found no correlation of ELISA results with worm burden. Carmena *et al.* (2005 a) found that excretory-secretory products of *Echinococcus granulosus* contain potential diagnostic antigens that can be used in the immunodiagnosis of canine echinococcosis.

In the present study cross-reactions were noticed in *Taenia hydatigena* and *Dipylidium caninum* positive samples. The OD values in *Echinococcus granulosus* positive dogs were in the range of 0.852 to 1.022. In the case of dogs naturally infected with *Taenia* the OD values were 0.336 to 0.754 and for *Dipylidium caninum* infected dogs it was 0.348 to 0.689. Since the cut off value was 0.486, the above results with *Taenia hydatigena* and *Dipylidium caninum* were considered as cross reactions. This could be possibly due to
sharing of common epitopes/antigenic determinants between the closely related species. In addition, the antigens used were only partially purified. Studies conducted by Gasser et al. (1988) demonstrated that 25 –60 per cent of the sera from dogs infected with *Echinococcus granulosus* did not show significant levels of specific antibody and revealed cross-reactivity with other parasite species. Similarly, Jenkins et al. (1990) evaluated somatic antigen of *Echinococcus granulosus* for serodiagnostic purpose and found variable diagnostic sensitivity and high cross-reactivity with antigens from other parasite species.

In the present study, the sensitivity and specificity of ELISA was found to be 100 and 79.20 per cent for E/S antigen and 100 and 71.60 per cent for somatic antigen, respectively. Out of 250 dogs serum samples examined, the false positive reaction was observed in 52 and 71 dogs with E/S and somatic antigen, respectively. On the other hand false negative results were not found against E/S and somatic antigens of *E.granulosus* infected dogs. The high sensitivity was observed due to the heavy worm burden in both the positive samples. The specificity of the ELISA in the present study was found to be lower which might have been due to more number of false positive cases. The reduced specificity may also be due to increased background reactivity with sera from dogs infected with *T.hydatigena*. There was a difference in the cut off values of 0.486 and 0.427 for E/S and somatic antigen contrary to 0.330 for worm excretory secretory-ELISA and 0.382 for protoscolex somatic-ELISA reported by Gasser et al. (1992). However, Gasser et al. (1992) had found a
higher specificity of 93.7 per cent with worm E/S antigen and 97.9 per cent with protoscolex somatic antigen. This may be attributed to the differences in antigen preparation and type of antigen used.

This is the first report where the comparison was made in detection of antibodies by ELISA using E/S and somatic antigens of *E.granulosus*. The present findings showed that the seroprevalence with E/S antigen was high compared to somatic antigen of *E.granulosus* in dogs.

5.3 Protein profile of somatic antigen and E/S antigen of *E.granulosus* and somatic antigen of *T.hydatigena* and *D.caninum*

Echinococcosis is considered to be an important helminthic disease but the antigenic profiles of the *E.granulosus* adult worms is not properly understood and there is scanty information on the antigenic nature of *E.granulosus* adult worms of dogs. Therefore, Sodium do-decyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was employed to study the protein profile.

In the present study the protein profile of the samples was observed by using 10% resolving gel and 4.5 per cent stacking gel. In the study of Cecilia Casaravilla *et al.* (2005) the protein profile of somatic extract and E/S products of adult *E.granulosus* was determined by SDS-PAGE in 10 per cent linear gel under reducing conditions. However, Gasser *et al.* (1992) characterized *Echinococcus granulosus* adult worm excretory/secretory
antigen by SDS-PAGE under reducing conditions, but did not indicate their molecular weights.

In the present study, the SDS-PAGE analysis of *E. granulosus* somatic antigen revealed a total of 11 polypeptide bands ranging from 114 kDa to 16 kDa and 12 with E/S antigen they ranged from 110 kDa to 14 kDa. Twenty-one polypeptide bands were found in the somatic Ag of *T. hydatigena* and Twenty-two with *D. caninum* ranging from 104 kDa to 14 kDa and 101 kDa to 12 kDa, respectively. Four peptides with molecular weights of 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 of 66 and 34 kDa, which were common with somatic antigen of *E. granulosus* where as three bands viz., 66, 34 and 14 kDa were common with excretory/secretory antigen of *E. granulosus*. The somatic antigen of *D. caninum* had two bands of 84 and 68 kDa were common with somatic antigen of *E. granulosus* and three bands of 78, 30 and 14 kDa as observed with excretory/secretory antigen of *E. granulosus*.

In the present study, the presence of common bands between somatic and E/S antigen of *E. granulosus*, somatic antigen of *T. hydatigena* and *D. caninum* indicated that the species are closely related.

The differences in the banding patterns compared to previous reports could be due to the type of antigen used. The percentage of resolving gel may also influence the separation of polypeptides based on molecular size.
This is the first report of comparative protein profile study of somatic and E/S antigen of *E. granulosus* and the somatic antigen of *T. hydatigena* and *D. caninum*.

### 5.4 Enzyme Immuno Transfer Blot (EITB) with somatic and excretory/secretory antigens of *E. granulosus*

Enzyme Immuno Transfer Blot was considered to be a valuable method for immunological diagnosis of echinococcosis, because of its efficacy in detection and requirement of very small amounts of antigen (Towbin *et al.*, 1979). The presence of small amounts of antibodies in a serum of low titre and even initial infections could be detected by this method. It also recognizes proteins, which are necessarily surface exposed (Blasser *et al.*, 1984). The EITB was conducted with partially purified antigens obtained by SDS-PAGE where in the proteins were separated based on their molecular weight.

The usefulness of serological tests in the diagnosis of *Echinococcus* infections has been limited due to the high antigenic cross reactivity among different *Taenia* species. A strategy to overcome the limitations of immunological tests is based on the identification of species-specific immunoreactive peptides. Hence EITB was carried out to identify the species-specific immunoreactive polypeptides in somatic and E/S antigen of *E. granulosus*.

The immunoreactive polypeptides detected on western blots in the present study in the somatic antigen of *E. granulosus* when probed with
known positive serum from dogs included those of four polypeptides of 84, 66, 45 and 16 kDa that could be identified on blots. A total of six polypeptides of size 98, 82, 66, 45, 34 and 24 kDa were identified in E/S antigen of *E.granulosus*. Only two polypeptides of 66 and 45 kDa were identified in both the antigens when probed with known positive serum.

Carmena *et al.*, (2005a) had also used somatic and E/S antigen of protoscoleces of *E.granulosus* for diagnosis of intestinal echinococcosis in dogs. The immunoblotting assay was performed by using sera from dogs infected with *E.granulosus* and other helminths. The assay showed four cross reacting proteins of 65, 61, 54 and 45-46 kDa with E/S-Ag. The antigens with polypeptides of 89 and 50 kDa in ES-Ag and 130 and 67 kDa in S-Ag were identified by sera of dogs infected with *E.granulosus* only, whereas protein of 41-43 KDa was recognized by the majority of the sera from dogs with other infections.

Casaravilla *et al.* (2005) produced two IgM murine monoclonal antibodies EgC1 and EgC3 against the excretory/secretory products of *Echinococcus granulosus* adult worms. Immunoblotting of somatic extract and E/S products of *E.granulosus* adult worm antigens were predominantly recognized as 50 kDa and 85 kDa, respectively. However western blot analysis of worm excretory secretory antigen and protoscolex somatic antigen of *E.granulosus* was studied by Gasser *et al.* (1992) using sera from infected and uninfected dogs. The results revealed that the relative molecular mass of antigenic components of worm excretory secretory antigen ranged between 94
to 39 kDa and these were specific for *Echinococcus granulosus* and were not identified in protoscolex somatic antigen. Carmena *et al.* (2005b) used hydatid cyst fluid (HCF), somatic antigen and E/S antigen of *Echinococcus granulosus* protoscoleces for immunodiagnosis of canine echinococcosis by immunoblotting. The immunoblot assay revealed the shared antigenic components of HCF, somatic antigen and E/S antigen with molecular masses of 4-6, 20-24, 52-80 and 100-104 kDa, including doublets of 41/45, 54/57 and 65/68 kDa. The non-shared polypeptides of each antigenic extract of *E.granulosus* were identified as 108 and 78 kDa for HCF, 124, 94, 83 and 75 kDa for somatic antigen and 89, 42, 39, 37 and 35 kDa for E/S antigen.

This cross-reactivity could be possibly due to sharing of antigenic determinants and also the use of polyclonal anti-sera during this study.

This is an original attempt in the detection of immunoreactive peptides of *E.granulosus* in dogs using partially purified somatic and E/S antigen with polyclonal antisera.

**5.4.1 Serodiagnosis of echinococcosis in dogs by EITB**

Gasser *et al.* (1992) conducted western blot analysis with *E.granulosus* worm excretory secretory antigen to identify immunodominant protein using sera from dogs infected with *E.granulosus*. They found major antigenic bands of relative molecular weight of 94-68 and 43-39 kDa in all the positive serum. In the present
study out of the 250 serum samples examined for *E.granulosus* with E/S antigen in dogs by EITB, specific antibodies were detected in 52 (20.80 per cent). The polypeptides viz. 66 kDa and 45 kDa were detected in all the positive serum samples. The sensitivity and specificity was found to be 100 and 88.40 per cent, respectively. The differences in this study could be possibly due to the type of antigen used.

Similarly in the 250 serum samples examined for *E.granulosus* infection with somatic antigen in dogs by EITB, specific antibodies were detected in 41 (16.40 per cent). The sensitivity and specificity was found to be 100 and 83.60 per cent, respectively. The polypeptides of 84, 66 kDa and 45 kDa were detected in all the positive serum samples.

Since no reports were found in literature with regard to serodiagnosis of *E.granulosus* with somatic antigen by EITB in dogs, it is considered to be the first attempt wherein partially purified somatic antigen of *E.granulosus* and polyclonal antisera were evaluated by EITB. The results indicated that EITB could be effectively used for detection of anti-*E.granulosus* antibodies in field serum samples. EITB can be employed for specific antibody detection in live animals and confirmatory diagnosis of echinococcosis can be made in definitive host and effective treatment can be undertaken.

### 5.4.2 Immunoreactive polypeptides identified in *T.hydatigena* and *D.caninum* somatic antigen with known positive serum of *E.granulosus*
Carmena et al. (2005b) found cross reactivity for somatic and excretory secretory antigen of protoscoleces with serum from dogs without Echinococcus infection. They identified 41 and 43 kDa, which were recognized by majority of the sera from dogs without Echinococcus infection. Similarly, in the present study cross-reaction was observed with somatic antigens of T. hydatigena and D. caninum with E. granulosus positive serum. Two polypeptides of size 69 and 42 kDa were detected with T. hydatigena and only one polypeptide of 35 kDa was detected in D. caninum somatic Ag when reacted with the positive serum of E. granulosus.

5.4.3 Comparison of sensitivity and specificity of ELISA and EITB

Gasser et al. (1992) used ELISA for the diagnosis of E. granulosus in dogs with worm E/S and protoscolex somatic antigen and found the sensitivity of 80.8 and 75.6 per cent and the specificity of 93.7 and 97.9 per cent with worm-ELISA and protoscolex-ELISA respectively.

Pathak et al. (1994) evaluated ELISA and EITB in the diagnosis of Taenia solium cysticercosis in pigs. The sensitivity and specificity of ELISA and EITB was 70 and 73 percent and 90 and 100 per cent, respectively. Sreenivasa Murthy et al. (1999) evaluated ELISA and EITB for the diagnosis of Taenia solium cysticercosis in pigs and found the sensitivity and specificity of 90 and 97.5 per cent and 50 and 100 per cent. Dhanalakshmi (2003) also used EITB for the sero-diagnosis of Taenia solium cysticercosis in pigs and
found that the sensitivity varied from 40-53, 57.4-91.4 per cent and 85-100 per cent with whole cyst antigen, somatic and excretory secretory antigen, respectively whereas their specificity was 100 per cent.

In the present study the sensitivity and specificity of ELISA and EITB with somatic and E/S antigens varied significantly in diagnosis of *E.granulosus* infection in dogs. The sensitivity and specificity of ELISA with somatic and E/S antigen was found to be 100 and 72.17 per cent and 100 and 79.03 per cent, respectively. However the sensitivity and specificity of EITB with somatic and E/S antigen was found to be 100 and 83.87 per cent, 100 and 88.21 per cent, respectively.

Similarly Gasser *et al.* (1993) reported that overall specificities ranged between 97.3 and 100 percent and sensitivities ranged between 73 and 84 percent for IgG, IgA and IgE ELISA. However Ersfeld *et al.*, (1997) analysed adult worm extracts of *Echinococcus granulosus* by ELISA and found the sensitivity of 83% for diagnosis of cystic echinococcosis. Zhang *et al.*, (2003) also reported poor sensitivity and specificity in ELISA based methods for detection of circulating antibodies in canines.

The sensitivity and specificity in the present study was found to be higher with E/S antigen compared to somatic antigen. The differences might be due to complex nature of somatic antigen compared to E/S antigen and methods employed in the preparation of antigen.
5.5 Immunodiagnosis of *E.granulosus* by using F/S antigen

5.5.1 Latex agglutination test (LAT)

The latex agglutination test is a simple and inexpensive diagnostic screening method for various parasitic and bacterial infections. The test was found to be rapid as the agglutination reaction could be visualized within 3-5 minutes and the test did not need any specific equipment as per Szyfers and Kagan (1963). This test has been evaluated in parasitic diseases like cystic echinococcosis and *Taenia solium* cysticercosis but there are scanty reports of this test on echinococcosis in dogs.

In the present study an attempt was made to evaluate the faecal supernatant antigen (FS) of *E.granulosus* and also to know the efficacy of LAT as a diagnostic method for *E.granulosus* in dogs. The test was initially standardized with naturally infected dogs and then the test was used to screen the field serum of dogs.

The test was found to be rapid, sensitive and easy to perform and the results could be visualized within 3-5 minutes. This is in accordance with Shimizu (2000) who had evaluated latex agglutination test for the detection of *Echinococcus* coproantigens in the definitive host and reported that LAT was simple and rapid test can be used for the detection of *E.granulosus* infection in dogs at field level.
Szyfers and Kagan (1963) evaluated a two-minute slide agglutination test for diagnosis of hydatid disease in man and found 100 percent sensitivity and 97.0 per cent specificity. In the present study the sensitivity and specificity of latex agglutination test was found to be 100 and 79.43 per cent respectively with faecal supernatant antigen. Similarly Shimizu (2000) used latex agglutination test for the detection of *E.multilocularis* coproantigens in the definitive host and found 47 and 61 per cent sensitivity and 94 and 86 per cent specificity with non-heated and heated faecal samples of wild foxes, respectively. Prasanna *et al.* (2001) used latex agglutination test to detect *Taenia solium* cysticercosis in pigs using whole cyst antigen (WCA) and partially purified antigen (antigen B) and reported sensitivity of 92.3 per cent with antigen B and 73.0 per cent with whole cyst antigen.

The latex agglutination test is a very simple and easy to perform test owing to one step antigen-antibody reaction at room temperature and required only 3-5 minutes for completion. Large number of serum samples can be screened and hence it was considered as a very useful on-site test for the detection of echinococcosis in dogs in naturally infected conditions at field level.

**5.5.2 Protein profile of F/S antigen of *E.granulosus***

Guarnera *et al.*, (2000) prepared three types of antigen viz, coproantigen from dry faeces, fresh faeces and adult worm antigen. They separated proteins of all the three antigens by SDS-PAGE but did not indicate the molecular
weights of the separated proteins. In the present study the protein profile of the F/S antigen was studied using 10% resolving gel and 4.5% stacking gel. During this study, the SDS-PAGE analysis of *E.granulosus* faecal supernatant antigen revealed a total of 8 polypeptides ranging from 76 kDa to 17 kDa. The major bands included 54, 45, 37 and 34 kDa and the minor bands were of 76, 66, 24 and 17 kDa. This found to be the first report where the molecular weight of the separated proteins from faecal supernatant antigen of *E.granulosus* were observed.

5.5.3 Serodiagnosis of Echinococcosis in dogs by Dot-ELISA

Dot-ELISA was widely used in serodiagnosis of parasitic diseases with good level sensitivity and specificity. It is desirable to develop a more convenient, less expensive immunoassay of high sensitivity and specificity for detection of infections. Dot-ELISA was therefore evaluated in the present study to detect echinococcus infection in dogs. It was widely used to detect antigen or antibody to various parasitic infections (Pappas *et al.*, 1988).

In this technique minute amounts of antigen are dotted on the nitrocellulose paper, which reacts with precipitable chromogenic substrate. This visually read enzyme immuno assay is rapid, portable, reagent conservative, equivalent in sensitivity and specificity. Therefore Dot-ELISA was used in the present study for detection of antibodies
against Echinococcus infection in dogs. Fecal supernatant antigen of 
*E. granulosus* was used in the present study for serodiagnosis.

Furusawa (1997) developed a Dot-ELISA, in which a nitrocellulose 
membrane coated with biotinylated capture antibody as primary antibody and 
streptavidin biotinylated horseradish peroxidase (HRP) complex were used to 
visualize the presence of the coproantigens for 'on the spot' diagnosis of 
*Echinococcus multilocularis* infection in definitive hosts, suitable for field 
surveys.

In the present study 250 serum samples were examined for *E. granulosus* 
infection in dogs by Dot-ELISA with faecal supernatant antigen 
and were positive in 45 (18 per cent) serum samples screened. The sensitivity 
and specificity was found to be 100 and 82.66 per cent, respectively.

This was an original attempt on serodiagnosis in the detection of 
*E. granulosus* infection by Dot-ELISA with F/S antigen of *E. granulosus*. The 
Dot-ELISA test is very simple to perform and large number of serum samples 
can be screened as such, it was considered as a very useful test for the 
detection of echinococcosis in dogs in naturally infected conditions at field 
level.

### 5.5.4 Enzyme Immuno Transfer Blot (EITB) with F/S antigen

Elayoubi and Craig (2004) reported that the immunoblotting assay of 
fractionated faecal supernatant of *E. granulosus* revealed that the molecular
weight was more than 670 kDa and ranged from 146 to 440 kDa. Similarly Guarnera et al. (2000) conducted copro-western blot and found only two bands of molecular weight of 40 and 45 kDa with faecal supernatant antigen prepared from the dry faeces collected from the environment. But in the present study immunoreactive polypeptides detected on western blot with faecal supernatant antigen of *E.granulosus* when probed with known positive serum from dogs included those of four polypeptides of size 66, 45, 34 and 17 kDa. The variation in the expression of immunodominant peptides in the present study might be due to the change in the preparation of antigen and the type of antigen used for detection of immunoreactive peptides.

5.5.5 Serodiagnosis of Echinococcosis in dogs by EITB

The polypeptides 45 kDa and 34 kDa were detected in all the positive serum samples. Out of 250 serum samples examined for *E.granulosus* in dogs by EITB with faecal supernatant antigen, specific antibodies were detected in 47 (18.80 per cent). The sensitivity and specificity was found to be 100 and 86.80 per cent, respectively. This is similar to the observation made by Guarnera et al. (2000) who found the sensitivity and specificity of copro-western blot with F/S antigen was 70 and 100 per cent respectively.

5.6 Copro-Polymerase chain reaction

The accurate identification of *Echinococcus* species and its detection by conventional techniques is time consuming, labour intensive and is difficult in live animals. Although *Echinococcus* species are known to be very specific to
definitive host, there are reports to indicate that *Echinococcus granulosus* and *Echinococcus multilocularis* may infect the same host in experimentally induced infections (Thompson and Eckert, 1983; Stefanic et al. 2004). Since microscopic or macroscopic examination of *Echinococcus* is possible only at post-mortem and serological tests are not species-specific, a diagnostic probe for the detection and differentiation of *Echinococcus* species was considered essential.

Molecular biological techniques have enabled technical innovations with potential applications to diagnostic Parasitology. The identification of parasite species or even stage-specific nucleic acid sequences has resulted in the development of DNA probes useful for hybridization of DNA from diagnostic samples. This technology has limited value in that its application focuses mainly on the characterization of *Echinococcus* isolates of strains thus providing epidemiological rather than clinical information.

A variety of DNA probes have been developed and used by several groups to characterize and identify different *E.granulosus* strains or isolates (Lymbery et al., 1989; Yap et al., 1988). Apart from the restricted availability of specific diagnostic probes, one major problem is the limited sensitivity of hybridization and labelling techniques. Current hybridization techniques do not allow identification of single taeniid eggs. The possibility of differentiating single cestode eggs at species level represents an important goal in parasite diagnosis as per McManus (1990). These technical limitations can now be
essentially eliminated by an extraordinary new tool, the PCR (Saiki et al., 1985). Diagnostic PCR depends on the availability of appropriate target nucleic acid sequences that flank regions of interest, which help in the design of synthetic oligonucleotide primers.

Currently, the classic and most reliable methods for diagnosis of *E.granulosus* infection rely on parasitological detection of adult worms at necropsy or after arecoline purgation. These methods are difficult to use in large-scale epidemiological studies because they are laborious, bio-hazardous and lack sensitivity (Abbasi et al., 2003). Copro-antigen detection assays developed recently are generally more suitable and practical for this purpose, eventhough they have lowered sensitivity and are more specific in areas where both *E.granulosus* and *E.multilocularis* are co-endemic, (Zhang et al., 2006). Copro-PCR was found to be valuable in the identification of the parasite to the species level and also to differentiate from *Taenia* spp. (Abbasi et al., 2003) and highly sensitive and specific copro-PCR assays have been developed for the detection of *E.granulosus* (Stefanic et al., 2004).

Copro-PCR assays have been used for screening of dogs in epidemiological surveys for detection of *E.granulosus* infection. The diagnosis of *E.granulosus* infection in canids has been largely used for the identification of target sequences suitable for sensitive and specific amplification (Zhang et al., 2006).
In the present study, Copro-PCR was carried out by using two sets of *E.granulosus* specific primers viz. Eg 1f, Eg 1r and JB 3f, JB 4.5r. The primers directed the amplification of a single DNA fragment using genomic DNA from *Taenia* eggs as the template. Totally 19 samples were subjected to Copro-PCR by using the above said primers. Ten dogs which were positive for *E. granulosus* worms at necropsy were positive by copro-PCR. The other 9 dogs which were negative at necropsy were also negative by copro-PCR.

Eg 1f, Eg 1r primer amplified 255 bp which is specific to *E.granulosus* in ten samples which were positive for *E.granulosus* at necropsy and no bands were observed in nine faecal samples which were positive for *Taenia* eggs by copromicroscopy. This is in accordance with Zhang *et al.* (2006) who examined 30 dogs by copro-PCR with Eg 1f, Eg 1r primer in Hejing County, and found that 17 dogs were infected with *E.granulosus* which were confirmed to belong to the sheep strain or the G1 genotype by sequencing. They suggested that the corresponding primer used in PCR could easily detect a single egg with no cross amplification of DNA from closely related cestodes, including *E.multilocularis* and *Taenia* spp. Similarly, Mathis and Deplazes, (2006) used Eg 1f and Eg 1r set of primer for diagnosis of *E.granulosus* infection in dogs from fecal samples and found upon amplification yielded a single band of 255 bp upon analysis in positive cases.

Shaikenov *et al.* (2004) had used the same set of primers for the detection of *E.granulosus* DNA from *Taenia* eggs, which were isolated from soil samples by copro-PCR. Of the 21 samples contaminated with taeniid eggs five
samples were found positive for *E.granulosus* yields an amplicon of 255 bp upon amplification, which was specific to *E.granulosus*.

In the present study the other primer JB 3f, JB 4.5r (mitochondrial CO1 primers) which amplified a single band of 440 bp was specific to *E.granulosus* in ten samples which were positive for *E.granulosus* at necropsy and no bands were observed in nine fecal samples which were positive for *Taenia* eggs by copromicroscopy. In the observations of Salina Manandhar (2005) similar mitochondrial CO1 primers were used for copro-PCR assay. Out of 47, 11 dog fecal samples were positive and the rest of the *Taenia* egg positive samples were PCR negative.

### 5.7 Conclusions

Based on the studies on immunological and molecular diagnosis of *E.granulosus* in dogs – the following conclusions were drawn:

- The CIEP was found to be a simple test, which can be used for the diagnosis of *E.granulosus* infection in dogs. The sensitivity and specificity of CIEP with somatic and E/S antigen of *E.granulosus* in dogs was recorded for the first time.

- Serodiagnosis of *E.granulosus* infection of dogs with somatic antigen of adult worm was attempted for the first time.

- Latex agglutination test was evaluated for the detection of echinococcosis in naturally infected dogs. The sensitivity and specificity of LAT in the detection of *E.granulosus* in dogs with F/S antigen was
considerably good.

- The protein profiles of all the three antigenic preparations viz. somatic, E/S, F/S antigen of *E.granulosus* and the somatic antigen of *T.hydatigena* and *D.caninum* were different but they shared some common bands among them. The comparative protein profile study of somatic, E/S, F/S antigen of *E.granulosus* and somatic antigen of *T.hydatigena* and *D.caninum* by SDS-PAGE was an original attempt.
- Detection of immunoreactive peptides by EITB was helpful in conjunction with SDS-PAGE for serodiagnosis.
- Serodiagnosis of *E.granulosus* in dogs by EITB and Dot-ELISA was standardised for the first time.
- The sensitivity and specificity of EITB was higher when compared with indirect ELISA and Dot-ELISA.
- Among the three antigens used, the E/S and F/S antigens had higher sensitivity and specificity when compared to somatic antigen.
- Copro-PCR technique was found to be highly sensitive technique for the detection of *E.granulosus* in dogs and no cross reactions were observed with other *Taenia* species.
- The two pairs of primers which are specific to sheep strain used in the present study are equally good in the detection of *E.granulosus* in dogs.