5. CHAPTER-II

EVALUATION OF *E. GRANULOSUS* RECOMBINANT ANTIGENS IN IMMUNODIAGNOSIS OF CYSTIC ECHINOCOCCOSIS IN HUMANS
Early diagnosis and treatment of CE is most important for management of the CE (Ammann et al., 1990). The diagnosis of CE based on clinical grounds is frequently difficult. Diagnosis of CE is primarily based on imaging techniques and serological methods (Pawlowski et al., 2001). The diagnosis of CE by imaging methods is too expensive and inaccessible in most endemic areas. The interpretation of results by imaging methods also found to be difficult in case of small lesions and/or atypical images (Pawlowski et al., 2001). Therefore, reliable and economical immunological assays are most useful for diagnosis of CE (Parija, 1994).

The serodiagnosis of CE based on serum antibody or antigen detection is of immense value to confirm clinical findings or render diagnostic help in radiologically unclear cases (Gottstein, 1992). Earlier, several immunological methods have been developed and evaluated for the detection of hydatid antibodies in serum with varying sensitivity and specificity for the diagnosis of CE. These serodiagnostic methods include; compliment fixation test (CFT) (Mahajan et al., 1976), agar-gel-precipitation test (Mahajan et al., 1976), bentonite flocculation test (Mahajan et al., 1973; Botros et al., 1975), immunoelectrodiffusion (IED) (Richard-Lenoble et al., 1978), indirect immunofluorescent test (IIFT) (Mahajan et al., 1976) and radioimmunoassay (RIA) (Richard-Lenoble et al., 1978), indirect haemagglutination (IHA) test (Mahajan et al., 1973; Picardo and Guisantes, 1981; Parija and Ananthakrishnan, 1985; Parija et al., 1986; Parija and Rao, 1987; Todorov et al., 2003), rapid IHA (Parija and Rao, 1986; Parija et al., 1987), latex agglutination test (LAT) (Picardo and Guisantes, 1981) and countercurrent immuno electrophoresis (CIEP) (Mahajan et al., 1976; Picardo and Guisantes, 1981; Parija, 1991).

The enzyme immunoassays evaluated for detection of hydatid antibodies in serum include, IgG-enzyme-linked immunosorbent assay (ELISA) (Kanwar and Vinayak, 1992; Kaur et al., 1999), IgM-ELISA (Wattal et al., 1986), IgE-ELISA (Wattal et al., 1988) and enzyme linked immuno-transfer blotting (EITB) (Maddison et al., 1989; Siracusano et al., 1991; Leggatt et al., 1992; Craig, 1993; Poretti et al., 1999).

ELISA has been used more frequently than other serological tests for diagnosis of CE because of its high sensitivity in CE (Force et al., 1992; Kaddah et al., 1992; Ramzy et al., 1999; Kaur et al., 1999). However, ELISA is a technically cumbersome procedure, needs
technically skilled personnel and could be carried out only in well equipped laboratories. Therefore, a rapid Dot-ELISA was developed for detection of hydatid antibodies in serum for diagnosis of CE with a sensitivity of 94% and a specificity of 90.3% (Rogan et al., 1991). The Dot-ELISA was found to be rapid, inexpensive and simple to perform and considered to be a useful supplement to the US scanning (Rogan et al., 1991).

Recently, EITB has been increasingly used in the diagnosis of CE (Maddison et al., 1989; Siracusano et al., 1991; Leggatt et al., 1992; Craig, 1993; Poretti et al., 1999). The EITB using native antigens has been reported to show higher sensitivity and specificity than the ELISA for detection of antibody in serum (Leggatt et al., 1992; Craig, 1993; Poretti et al., 1999). The EITB using hydatid cyst fluid antigen, showed a sensitivity of 90% and a specificity of 100% for diagnosis of hepatic hydatidosis, but it was less sensitive for detection of uncomplicated cysts located in the lung and brain (Maddison et al., 1989). In another study, the EITB using E. granulosus antigens showed a sensitivity of 90%, as compared to 78% with the immunoelectrophoresis (Siracusano et al., 1991). However, the low sensitivity and low specificity have been the major problems associated with the serodiagnostic tests employed for detecting hydatid antibodies for diagnosis of CE (Shepherd and McManus, 1987).

Detection of hydatid antigens in serum, urine, hydatid cyst fluid and other body fluids is another approach for diagnosis of CE. These include, rapid and simple immunoassays for detection of hydatid antigen in serum by CIEP (Shariff and Parija, 1991), co-agglutination (Co-A) test (Shariff and Parija, 1993), LAT (Sheela Devi and Parija, 2003); and Ag-ELISA (Craig et al., 1986; Kanwar et al., 1994) and Ag-Dot-ELISA (Wang et al., 2002). These also include antigen detection in urine by CIEP (Parija et al., 1997) and Co-A test (Ravinder et al., 2000), and antigen in the hydatid cyst fluid aspirates by Co-A test (Parija et al., 1996), LAT (Sheela Devi and Parija, 2003), CIEP (Ravinder and parija, 1997) and reverse latex agglutination (RLA) test (Mahmoud and Ezzat, 1999).

Despite the development of different techniques with variable sensitivities and specificities the immunodiagnosis of CE in clinical practice still remains a diagnostic challenge. This is mainly due to the use of native hydatid antigens in various serological tests (Babba et al., 1994). The false-positive (non-specific) reactions with sera from other parasitic diseases, malignancies (Dar et al., 1984), and liver cirrhosis (Iacona et al., 1980) is a noted problem associated with these serological tests. The analysis of E. granulosus native antigens by using one-dimensional/two-dimensional electrophoresis and micro-sequencing by Zhang and McManus, (1996) have also suggested that both antigen B (AgB) and antigen 5 (Ag5)
components of HCF are composed of a family of proteins, but not a single protein. This indicates the possibility of cross-reactions in the diagnosis of CE using native antigens.

Therefore, *E. granulosus* recombinant antigens (Helbig et al., 1993; Fernandez et al., 1996; Gonzalez-Sapienza et al., 2000; Rott et al., 2000; Kittelberger et al., 2002; Virginio et al., 2003; Li et al., 2003; Lorenzo et al., 2005a) and synthetic peptides (Facon et al., 1991; Chamekh et al., 1992; Gonzalez-Sapienza et al., 2000) have been evaluated in many immunodiagnostic tests to enhance specificity as well as sensitivity of these tests.

More attention has been focused on the development and evaluation of immunodiagnostic assays using *E. granulosus* recombinant AgB fractions for diagnosis of CE both in humans and animals. The *E. granulosus* recombinant AgB fractions were found to be highly immunogenic and specific in diagnosis of CE (Lorenzo et al., 2005a). The recombinant AgB fractions, EG55 and EM10, of the *E. granulosus* and *E. multilocularis* metacestodes, respectively, were found to be species specific for serodiagnosis and serological differentiation of CE and alveolar echinococcosis (AE) (Helbig et al., 1993). However, in few cases, the *E. granulosus* recombinant AgB fractions also appeared to be genus specific, but not species specific (Li et al., 2003). Only few studies evaluated the immunodiagnostic assays using *E. granulosus* recombinant Ag5 (Facon et al., 1991; Gonzalez-Sapienza et al., 2000), probably because of its low specificity (Facon et al., 1991).

Recently, two *E. granulosus* recombinant antigen B fractions including, Rec Eg-AgB8/1 and Rec Eg-AgB8/2 were found to be the most efficient and relevant diagnostic antigens for diagnosis of CE (Virginio et al., 2003). The ELISA using Rec Eg-AgB8/1 and Rec Eg-AgB8/2 demonstrated that Rec Eg-AgB8/2 provides better performance in serodiagnosis of human CE than does the Rec Eg-AgB8/1 (Rott et al., 2000; Virginio et al., 2003). Subsequently, Li et al. (2004) identified and evaluated a recombinant antigen, EpC1, for diagnosis of CE. The EpC1 recombinant antigen showed a sensitivity of 92.2% and a specificity of 95.6%.

In the first phase of the present study, two diagnostic relevant recombinant antigens, Eg-rCW12 and Eg-rCW24, were identified by immunoscreening of the ZAP Express cDNA library obtained from *E. granulosus* metacestode germinal membrane mRNA. Both recombinant antigens, Eg-rCW12, Eg-rCW24, showed 100% sensitivity and specificity in initial screening by ELISA and EITB as described earlier in the chapter 1. In the first phase of the present study, Rec Eg-AgB8/2 was also produced by cloning and expression of
corresponding genomic DNA fragment. Both ELISA and EITB using Rec Eg-AgB8/2 also demonstrated 100 % sensitivity and specificity in initial screening.

Therefore, in the second phase of the present study two simple, rapid and reliable serodiagnostic assays such as ELISA and Dot-ELISA were evaluated by using the \textit{E. granulosus} recombinant antigens, Eg-rCW24, Eg-rCW12 and Rec Eg-AgB8/2, for detection of hydatid antibodies in serum for diagnosis of CE. The polyclonal antibodies raised against \textit{E. granulosus} recombinant antigens, Eg-rCW24, Eg-rCW12 and Rec Eg-AgB8/2, were also evaluated for use in ELISA and Dot-ELISA for detection of hydatid antigen in the serum.
The objectives of this phase of the study are:

1. To develop and evaluate enzyme-linked immunosorbent assay (ELISA) and Dot-enzyme linked immunosorbent assay (Dot-ELISA) using *E. granulosus* recombinant antigens (Eg-rCW12, Eg-rCW24 and Rec Eg-AgB8/2) to detect hydatid antibodies in serum for diagnosis of cystic echinococcosis.

2. To develop and evaluate ELISA and Dot-ELISA using affinity purified anti-Eg-rCW12, anti-Eg-rCW24 and anti-Rec Eg-AgB8/2 antibodies to detect hydatid antigens in serum for diagnosis of cystic echinococcosis.
Chapter II

Study Groups

**Group-I: Surgically confirmed and ultrasound diagnosed cystic echinococcosis (n=100):**
This group included 45 cases of surgically confirmed cases of CE and 55 cases of radiological confirmed cases of CE. The cysts removed during surgery were confirmed to be of hydatid cyst etiology by histopathological evidence of germinal layer and by demonstration of scolices and hooklets in the aspirated HCF. The radiologically-diagnosed CE cases included un-operated cases of CE but diagnosed by ultrasonography (US)/computed tomography (CT)/magnetic resonance imaging (MRI).

**Group-II: Presumptive or suspected cases of cystic echinococcosis (n=60):** This group included clinically diagnosed (presumptive) cases of CE. These patients presented with the clinical signs and symptoms of CE but they are proved neither surgically nor by radio diagnosis.

**Group-III: Controls with other parasitic diseases (n=60):** This group included patients with other parasitic diseases like amoebiasis (n=8), filariasis (n=10), neurocysticercosis (n=30), toxoplasmosis (n=9) and schistosomiasis (n=3).

**Group-IV: Healthy controls (n=30):** This group included healthy adults (blood donors and students) who have not suffered from CE or any other disease in the recent past.

Specimens

**Serum**

Five milliliters of venous blood was collected from all patients (n=220) and healthy individuals (n=30) under aseptic precautions and was allowed to clot. The serum was separated and stored in aliquots at -20°C till use.

Preparation of *E. granulosus* recombinant antigens

The *E. granulosus* recombinant antigens Eg-rCW12, Eg-rCW24 and Rec Eg-AgB8/2, were synthesized and purified as described earlier (Chapter-I).
Chapter II

Preparation of polyclonal antibodies against *E. granulosus* recombinant antigens in rabbit

Polyclonal antibodies were raised in rabbits against each *E. granulosus* recombinant antigen (Eg-rCW12 and Eg-rCW24 and Rec Eg-AgB8/2) as described earlier (Chapter-I).

Chromatographic purification of total IgG antibodies from rabbit sera

The anti-Eg-rCW12, anti-Eg-rCW24 and anti-Rec Eg-AgB8/2 IgG antibodies were purified by using commercially available protein A-Sepharose CL 4B column (*Genei, India*) as per the method described by Choromansky et al. (1990).

Briefly, the pH of the each serum was adjusted to 8.0 by adding 1.0 M Tris drop by drop. The antibody solution was passed through the pre-packed protein A-Sepharose CL 4B column (*Genei, India*). The column was washed sequentially with approximately 500 µl of 100 mM glycine (pH 3.0) per sample in stepwise. The IgG fraction was analyzed by reading at 280 nm in a spectrophotometer (*Systronics, India*). Analysis of IgG fraction was done in the elute, wash and flow-through. Finally, the elute was subjected to SDS-PAGE in order to estimate the purification process. Similarly the wash and the flow-through were also subjected to SDS-PAGE. The affinity purified IgG antibodies were concentrated by ultra membrane filtration using Centricon concentrator tubes of 3 kDa cut-off (*Amicon, USA*).

Detection of hydatid antibodies in serum

**ELISA for detection of hydatid antibodies in serum**

The ELISA was evaluated by using *E. granulosus* recombinant antigens Eg-rCW12, Eg-rCW24 and Rec Eg-AgB8/2 separately for detection of IgG antibodies in serum from cases of CE and control subjects. All tests were performed three times to exclude variations from day to day.

**Standardization of ELISA:** The optimum antigen concentration, for coating microtiter wells, and serum dilution was standardized by checkerboard titration. The concentration of antigen and the dilution of serum, which gave the maximum ratio of OD values with known positive serum and that with negative serum, were considered to be the optimum conditions for the test. All the test sera were analyzed at 1:200 dilutions in PBST.
ELISA using *E. granulosus* recombinant antigens for detection of IgG antibodies in serum consisted of the following steps:

1. **Antigen coating:** Polyvinyl high binding microtiter plates (*NUNC, New Zealand*) were coated with 100 µl/well of appropriate dilution of the *E. granulosus* recombinant antigen (0.5 µg/well) in carbonate bicarbonate buffer (pH 9.6) and incubated overnight at 4°C undisturbed.

2. **Washing:** The un-adsorbed antigen was removed by washing the plates with PBST (sterile PBS (pH 7.2) containing 0.1% Tween-20).

3. **Blocking:** The uncoated reactive sites in the wells were blocked using PBS (pH 7.2) containing 2% BSA by incubating for 3 h at 37°C.

4. **Washing:** Plates were washed three times with PBST as described before.

5. **Sample serum dilution and incubation:** Appropriate dilution of the patient sera (1:200) was prepared in PBST and 100 µl of each diluted serum was added in duplicate and incubated for 1.5 h at 37°C.

6. **Washing:** The plates were washed three times with PBST as before to remove unbound antibodies in serum.

7. **Second antibody (conjugate) incubation:** Rabbit anti-human-IgG-HRP conjugated second antibody (*Genei, India*) was used as per the manufacturers instruction (1:2000) with PBS (pH 7.2) containing Tween-20 (0.05%) and 100 µl volume was dispensed to all wells and incubated for 0.5 h at 37°C in dark.

8. **Washing:** Plates were washed three times with PBST as before to remove unbound conjugate.

9. **Colour development:** Substrate solution was prepared freshly by adding 6 mg of OPD (*S.D. Fine Chemicals, India*) in 10 ml of PBS (pH 7.2) containing 0.05% Tween-20 and 10 of µl H₂O₂ was added just before adding to the wells. Finally, 100 µl/well of the substrate solution was dispensed to all the wells and incubated for 15-20 min at 37°C in dark.

10. **Stop reaction:** The reaction was stopped by adding 50 µl of 2N H₂SO₄ per well. The absorbance value was recorded at 492 nm using ELISA reader (*Bio-Rad, USA*).

The same ELISA procedure was performed by using Eg-rCW12, Eg-rCW24 and Rec Eg-AgB8/2 for detection of hydatid IgG antibodies in the same batch of serum samples from cases of CE and control groups as mentioned earlier. Various controls such as antigen blank, first antibody blank, conjugate blank, and substrate blank, healthy normal serum
blank were used during the standardization of the ELISA. Antibody blank, positive control serum and negative control serum were used in every run for the validation of the test.

Dot-ELISA for detection of hydatid antibodies in serum

The Dot-ELISA was evaluated by using *E. granulosus* recombinant antigens Eg-rCW12, Eg-rCW24 and Rec Eg-AgB8/2 separately for detection of hydatid antibodies in in the same batch of sera from cases of CE and control subjects. The Dot-ELISA was optimized to estimate the concentration of antigen to be dotted and the dilution of serum to be used by checkerboard titration. All tests were performed three times to exclude variations from day to day.

Dot-ELISA using *E. granulosus* recombinant antigens for detection of hydatid antibody consisted of the following steps:

1. **Antigen spotting**: A nitrocellulose membrane (NCM) (*Hybond ECL, Amersham bioscience, Germany*) of size 0.5 x 0.5 cm was cut and mounted onto a plastic strip (0.5 cm x 5cm). A volume of 2µl/strip (0.2 µg/strip) of antigen was dotted on to individual strip and air dried for 30 min.
2. **Washing**: The un-adsorbed antigen was removed by washing the strips with PBST (sterile PBS (pH 7.2) containing 0.1% Tween-20).
3. **Blocking**: The uncoated reactive sites on the strips were blocked with PBS (pH 7.2) containing 2% BSA by incubating for 3 h at 37°C with constant shaking.
4. **Washing**: Strips were washed three times with PBST as described before.
5. **Sample serum dilution and incubation**: A 1:1000 dilution of the patient sera was prepared in PBST and the strips were incubated for 1.5 h at RT with constant shaking.
6. **Washing**: The strips were washed three times with PBST as before to remove unbound antibodies in the sample serum.
7. **Second antibody (conjugate) incubation**: A 1:2000 dilution of rabbit anti-human-IgG-HRP conjugated second antibody (*Genie, India*) was used as per the manufacturers’ instructions with PBS (pH 7.2) containing Tween-20 (0.1%) and incubated for 30 min at RT in the dark with constant shaking.
8. **Washing**: Strips were washed three times with PBST as before to remove unbound conjugate.
9. **Colour development**: Substrate solution was prepared freshly by adding 3 mg of 3,3’ dianinobenzidine (DAB) (*Sigma, USA*) in 5 ml of PBS (pH 7.2) containing 0.1%
Tween-20 and 5 μl H₂O₂ was added just before adding to the plastic tray. The strips were dispensed and incubated for 15-20 min at 37°C in dark under constant shaking.

10. **Stop reaction:** The reaction was stopped by washing the strips with double distilled water.

The positive reaction was indicated by the development of brown coloured spot in 2–5 min. When the reaction was complete, the strips were washed with distilled water and allowed to dry. Once dried, the strips were stored in the dark.

**Detection of hydatid antigens in serum**

**ELISA for detection of hydatid antigen in serum (Ag-ELISA)**

**Standardization of Ag-ELISA:** The optimum concentration of capturing antibody (affinity purified polyclonal antibodies raised against *E. granulosus* recombinant cyst wall antigens) and optimum dilution of the detecting antibody (hyperimmune polyclonal antiserum raised against *E. granulosus* recombinant antigens) were initially standardized by checkerboard titration.

The Ag-ELISA for detection of hydatid antigen in serum consisted of the following steps:

1. **Capturing antibody coating:** Polyvinyl high binding microtiter plates (*NUNC, New Zealand*) were coated with 100 μl/well (4 concentrations of 18 μg, 28 μg, 48 μg and 8 μg/well) of the affinity purified antibody (anti-Eg-rCW12, anti-Eg-rCW24 and anti-Rec Eg-AgB8/2 antibodies) in carbonate bicarbonate buffer (pH 9.6) and incubated overnight at 4°C undisturbed.

2. **Washing:** The un-adsorbed antibodies were removed by washing the plates with PBST (sterile PBS (pH 7.2) containing 0.1% Tween-20).

3. **Blocking:** The uncoated reactive sites in the wells were blocked by PBS (pH 7.2) containing 2% BSA by incubating for 3 h at 37°C.

4. **Washing:** Plates were washed three times with PBST as before.

5. **Dilution and incubation of *E. granulosus* recombinant antigens:** Known quantities of *E. granulosus* recombinant antigens (Eg-rCW12, Eg-rCW24 and Rec Eg-AgB8/2) were prepared in PBST. A concentration of 400 ng/100 μl of each diluted antigen was added in duplicate and incubated for 1.5 h at 37°C.

6. **Washing:** The plates were washed three times with PBST to remove unbound antigen molecules.
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7. **Detecting antibody:** Polyclonal hyperimmune serum was diluted by serial dilution (1:25 to 1:3200) in PBS (pH 7.2) and 100 μl volume was added to each well.

8. **Washing:** The plates were washed three times with PBST to remove unbound detecting antibodies.

9. **Second antibody (conjugate):** Goat anti-rabbit-IgG-HRP conjugated second antibody (Genei, India) was used as per the manufacturers instruction (1:2000) with PBS (pH 7.2) containing Tween-20 (0.05%) and 100 μl volume was dispensed to all wells and incubated for 30 min at 37°C in dark.

10. **Washing:** Plates were washed three times with PBST to remove the unbound conjugate.

11. **Colour development:** Substrate solution was prepared freshly by adding 6 mg of OPD (S.D. Fine Chemicals, India) in 10 ml of PBS containing 0.05% Tween-20 and 10 μl H₂O₂ was added just before adding to the wells. 100μl volume of the substrate solution per well was dispensed to all wells and incubated for 15-20 min at 37°C in dark for the development of optimum colour.

12. **Stop reaction:** The reaction was stopped by adding 50μl of 2N H₂SO₄ per well.

13. The absorbance values were recorded at 492 nm using ELISA reader (Bio-rad, USA).

The same Ag-ELISA procedure was performed by using anti-Eg-rCW12, anti-Eg-rCW24 and anti-Rec Eg-AgB8/2 monospecific antibodies for detection of hydatid antigens in the same batch of serum samples from cases of CE and control groups as mentioned earlier. The microtiter plates were coated with 4 μg/well of the capturing antibody (anti-Eg-rCW12, anti-Eg-rCW24 and anti-Rec Eg-AgB8/2 antibodies). The test sera and detecting antibody were used at dilutions of 1:100 and 1:200 respectively. Blank, positive control serum and negative control serum were used in every run for the validation of the test. All tests were performed three times to exclude variations from day to day.

**Estimation of antigen detection limit of Ag-ELISA**

The Ag-ELISA using anti-Eg-rCW12, anti-Eg-rCW24 and anti-Rec Eg-AgB8/2 antibodies for verification of the antigen detection limit consisted of the following steps:

In Step-1 of the Ag-ELISA protocol as described above, the polyvinyl high binding microtiter ELISA plates were coated with 100 μl (containing 4 μg) of the affinity purified antibody (anti-Eg-rCW12, anti-Eg-rCW24 and anti-Rec Eg-AgB/2 antibodies) in carbonate bicarbonate buffer (pH 9.6). Step-2 to Step-4 was same as described in Ag-ELISA.
In the Step-5 of the above Ag-ELISA protocol, different concentrations (2 µg, 1 µg, 800 ng, 400 ng, 200 ng, 100 ng, 50 ng, 25 ng and 12.5 ng/ml) of each antigen preparation were added. The plates were incubated for 1.5 h at 37°C. The plates were washed for removing unbound antigens (Step-6). In Step-7 of the above Ag-ELISA protocol, the polyclonal sera (detecting antibody) at appropriate dilution (standardized by checkerboard titration) estimated in the previous step were added. The plates were developed using anti-rabbit-IgG–HRP conjugate and substrate solution as described before (Step-8 to Step-12 of the Ag-ELISA protocol).

**Dot-ELISA for detection of hydatid antigen in serum**

The Dot-ELISA using anti-Eg-rCW12, anti-Eg-rCW24 and anti-Rec Eg-AgBBR monospecific antibodies, was evaluated to detect circulating *E. granulosus* antigens in serum of the cases with CE and controls. The Dot-ELISA was optimized to estimate the concentration of coating antibody to be dotted and the dilution of test serum to be used by checkerboard titration. All tests were performed three times to exclude variations from day to day.

Dot-ELISA procedure consisted of the following steps:

1. **Capturing antibody spotting:** A NCM (*Hybond ECL, Amersham bioscience, Germany*) of size 0.5 x 0.5 cm was cut and mounted onto a plastic strip (0.5 cm x 5cm). A volume of 2µl (2 µg/strip) of affinity purified IgG fraction of anti-Eg-rCW12 IgG, anti-Eg-rCW24 IgG and anti-Rec Eg-AgBBR antibody was dotted on to individual strips and air dried for 30 min.
2. **Washing:** The un-adsorbed antibodies were removed by washing the strips with PBST (sterile PBS 7.2 containing 0.1% Tween-20).
3. **Blocking:** The uncoated reactive sites on the strips were blocked using PBS (pH 7.2) containing 2% BSA by incubating for 3 h at RT on rocker shaker.
4. **Washing:** Strips were washed three times with PBST as described before.
5. **Sample serum dilution and incubation:** One ml of 1:10 dilution of the patient sera was prepared in PBST and the strips were incubated for 1.5 h at RT with constant shaking.
6. **Washing:** The strips were washed three times with PBST as before to remove unbound antibodies in sample serum.
7. **Second antibody (conjugate) incubation:** Rabbit anti-Eg-rCW12 IgG, anti-Eg-rCW24 IgG and anti-Rec Eg-AgBBR antibody coupled to HRP was used at a dilution of
1:400 in PBS (pH 7.2) containing Tween-20 (0.1%) and the strips were incubated for 1 h at RT in the dark with constant shaking.

8. **Washing**: Strips were washed three times with PBST as before to remove unbound conjugate.

9. **Colour development**: Substrate solution was prepared freshly by adding 3 mg of 3, 3’ diaminobenzidine (DAB) (Sigma, USA) in 5 ml of PBS (pH 7.2) containing 0.1% Tween-20 and 5 µl H2O2. The strips were dispensed and incubated for 15-20 min at RT in dark under constant shaking.

10. **Stop reaction**: The reaction was stopped by washing the strips with double distilled water.

The positive reaction was indicated by the appearance of brown coloured spot in 2–5 minutes. When the reaction was complete, strips were washed with distilled water and allowed to dry. Once dried, the strips were stored in the dark.

**Statistical analysis**

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and efficiency of the diagnostic methods were calculated as per the standard method (Park and Park, 2001). Statistical analysis for test of significance (chi-square test) for comparing the subgroups was done using the software Epi-info 2001 and Graph Pad (Online version) softwares. “P-value” <0.05 was considered as significant. The mean, median, standard deviation calculations were performed using Windows Microsoft Excel, 2003 software.
Clinical profile of the patients

The CE was more frequently observed in the age group of 40-60 years (46.87%) followed by the age group 20-40 years (38.75%). The age group ranged from 41-60 years (middle age people) appeared to involve highest by CE infection in males (51.38%) and followed by 20-40 age group (29.16%). However, the age group 21-40 appeared to involve highest (46.59%), followed by the age group 40-60 years (43.18%) respectively, in females. The sex and age distributions among confirmed and suspected cases of CE were summarized in Table-5-1.

Table-5-1: Age and sex distribution of Group-I and Group-II cases of CE

<table>
<thead>
<tr>
<th>Age groups (years)</th>
<th>Group-I</th>
<th>Group-II</th>
<th>Total No of male cases (%)</th>
<th>Group-I</th>
<th>Group-II</th>
<th>Total No of female cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 20</td>
<td>3</td>
<td>3</td>
<td>6 (8.33)</td>
<td>2</td>
<td>1</td>
<td>3 (3.40)</td>
</tr>
<tr>
<td>21-40</td>
<td>9</td>
<td>12</td>
<td>21 (29.16)</td>
<td>28</td>
<td>13</td>
<td>41 (46.59)</td>
</tr>
<tr>
<td>41-60</td>
<td>26</td>
<td>11</td>
<td>37 (51.38)</td>
<td>23</td>
<td>15</td>
<td>38 (43.18)</td>
</tr>
<tr>
<td>&gt; 60</td>
<td>6</td>
<td>2</td>
<td>8 (11.11)</td>
<td>3</td>
<td>3</td>
<td>6 (6.81)</td>
</tr>
<tr>
<td>Total No of cases</td>
<td>44</td>
<td>28</td>
<td>72 (45.00)</td>
<td>56</td>
<td>32</td>
<td>88 (55.00)</td>
</tr>
</tbody>
</table>

[Group-I: Cases of CE with confirmed diagnosis; Group-II: Cases of CE with presumptive diagnosis]

In the present study, 83.00% confirmed CE cases and 75.00% suspected CE cases presented with pain, 48.00% and 48.33% of cases presented with swelling, 24.00% and 21.65% of cases with vomiting, 24.00% and 33.33% of cases with weight loss, 19.00% and 31.66% of cases presented with cough, 10.00% and 3.33% cases with hemoptysis out of 100 confirmed CE cases and 60 suspected CE cases respectively. A total 25 of 100 confirmed CE cases showed other complications such as cyst rupture in 5 (5.00%) cases, 7 (7.00%) cases with calcified cyst, 5 (5.00%) cases with infected cyst, one cases each of renal calculus, carcinoma cervix, diabetes, tuberculosis, gallbladder calculus, hemiplegia, pleural effusion, pregnancy termination and one (1.00%) cases with T. versicolor. A total 5 of 60 (8.33%) suspected cases of CE showed other complications such as tuberculosis, ascites, neck swelling, pleural effusion and renal calculus.

The predilection of hydatid cysts towards different body parts in confirmed CE cases and in suspected CE cases was summarized in Table-5-2. The predilection of hydatid cysts among the South Indian patients involved in this study, were found more frequently in
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76% of confirmed and 41% of suspected CE cases in liver, followed by lungs (9% and 16%) respectively. Multi organ location of hydatid cysts was found in 7% of confirmed and 1.66% of suspected CE cases.

Table-5-2: Predilection of hydatid cysts towards different body parts in patients with CE

<table>
<thead>
<tr>
<th>Site of the hydatid cyst</th>
<th>Group-I No (%) (n=100)</th>
<th>Group-II No (%) (n=60)</th>
<th>Total number of cases No (%) (n=160)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>76 (76)</td>
<td>41 (68.33)</td>
<td>117 (73.12)</td>
</tr>
<tr>
<td>Lung</td>
<td>9 (9)</td>
<td>16 (26.66)</td>
<td>25 (15.62)</td>
</tr>
<tr>
<td>Peritonium</td>
<td>2 (2)</td>
<td>2 (3.33)</td>
<td>4 (2.5)</td>
</tr>
<tr>
<td>Spleen</td>
<td>2 (2)</td>
<td>0 (0)</td>
<td>2 (1.25)</td>
</tr>
<tr>
<td>Pelvis</td>
<td>2 (2)</td>
<td>0 (0)</td>
<td>2 (1.25)</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>2 (2)</td>
<td>0 (0)</td>
<td>2 (1.25)</td>
</tr>
<tr>
<td>Liver + Lung</td>
<td>3 (3)</td>
<td>1 (1.66)</td>
<td>4 (2.5)</td>
</tr>
<tr>
<td>Liver + Peritonium</td>
<td>2 (2)</td>
<td>0 (0)</td>
<td>2 (1.25)</td>
</tr>
<tr>
<td>Liver + Spleen + Peritonium</td>
<td>1 (1)</td>
<td>0 (0)</td>
<td>1 (0.62)</td>
</tr>
<tr>
<td>Lung + Liver + Spleen + Peritonium</td>
<td>1 (1)</td>
<td>0 (0)</td>
<td>1 (0.62)</td>
</tr>
</tbody>
</table>

[Group-I: Cases of CE with confirmed diagnosis; Group-II: Cases of CE with presumptive diagnosis]

The occupational profile of the study cases were summarized in Table-5-3. The CE was prevalent more frequent among the agricultural workers (about 41.41% and 43.33% of confirmed and suspected cases of CE respectively), followed by house wives (with a frequency of 26.26% and 28.33% of confirmed and suspected CE cases respectively) and public service undertaking/corporate sector employees (22.22% and 16.66% of confirmed and suspected CE cases respectively).

Table-5-3: Occupational profile of patients with CE

<table>
<thead>
<tr>
<th>Occupation</th>
<th>Group-I Number (%) (n=99*)</th>
<th>Group-II Number (%) (n=60)</th>
<th>Total number of cases No (%) (n=159)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agriculture</td>
<td>41 (41.41)</td>
<td>26 (43.33)</td>
<td>67 (42.13)</td>
</tr>
<tr>
<td>PSU/corporate sector</td>
<td>22 (22.22)</td>
<td>10 (16.66)</td>
<td>32 (20.12)</td>
</tr>
<tr>
<td>House wife</td>
<td>26 (26.26)</td>
<td>17 (28.33)</td>
<td>43 (27.04)</td>
</tr>
<tr>
<td>Mechanic/maid</td>
<td>2 (2.02)</td>
<td>2 (3.33)</td>
<td>4 (2.51)</td>
</tr>
<tr>
<td>Butcher</td>
<td>1 (1.01)</td>
<td>0 (0)</td>
<td>1 (0.62)</td>
</tr>
<tr>
<td>Student</td>
<td>7 (7.07)</td>
<td>5 (8.33)</td>
<td>12 (7.54)</td>
</tr>
</tbody>
</table>

[Group-I: Cases of CE with confirmed diagnosis; Group-II: Cases of CE with presumptive diagnosis; PSU- Public service undertaking; * One case with age less than 5 years]
Chapter II

Detection of *E. granulosus* IgG antibodies in serum by ELISA

**Standardization of ELISA**

The optimum concentration of the purified recombinant antigens, Eg-rCW12, Eg-rCW24 and Rec Eg-AgB8/2, for coating microtiter wells were estimated by checkerboard titration to be 0.5 μg per well. The optimum dilution of serum for the ELISA using each of the recombinant antigen preparations for detection of IgG antibodies was standardized by checkerboard titration to be 1:200.

**ELISA for detection of *E. granulosus* IgG antibodies in serum**

The cut-off OD\textsubscript{492} values of the ELISA using *E. granulosus* recombinant antigens (Eg-rCW12, Eg-rCW24 and Rec Eg-AgB8/2) for detection of IgG antibodies in serum were summarized in Table-5-4.

Table-5-4: Estimation of the cut-off OD\textsubscript{492} values for the ELISA for detection of *E. granulosus* IgG antibodies in serum

<table>
<thead>
<tr>
<th>OD\textsubscript{492} of normal sera (n = 30)</th>
<th>Detection of anti-<em>E. granulosus</em> antibody in serum using</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eg-rCW12</td>
</tr>
<tr>
<td>Absorbance range</td>
<td>0.052-0.129</td>
</tr>
<tr>
<td>Mean average OD</td>
<td>0.101533</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.019237</td>
</tr>
<tr>
<td>Cut-off</td>
<td>0.140007</td>
</tr>
</tbody>
</table>

The results of ELISA using *E. granulosus* recombinant antigens Eg-rCW12, Eg-rCW24 and Rec Eg-AgB8/2 for detection of IgG antibodies in serum from the cases of CE and controls were summarized in Table-5-5.

ELISA using Eg-rCW12 demonstrated diagnostic level of antibodies in 92 of 100 (92.00 %) sera of confirmed CE cases (Group-I) (Table-5-5). The assay also demonstrated a diagnostic level of antibodies in 31 of 60 (51.66 %) sera of Group-II cases. The test detected antibodies in 5.00 % sera from controls with other parasitic diseases (Group-III), but did not show any diagnostic antibodies in the sera of healthy controls (Group-IV).
ELISA using Eg-rCW24 demonstrated diagnostic level of antibodies in 95 of 100 (95.00 \%) sera of confirmed CE cases (Group-I) (Table-5-5). The assay also demonstrated a diagnostic level of antibodies in 36 of 60 (60.00 \%) sera of Group-II cases. The test detected antibodies in 6.66 \% sera from controls with other parasitic diseases (Group-III), but did not show any diagnostic antibodies in the sera of healthy controls (Group-IV).

Table-5-5: ELISA using \textit{E. granulosus} recombinant antigens for detection of \textit{E. granulosus} IgG antibodies in serum from cases of CE and controls

<table>
<thead>
<tr>
<th>Study groups</th>
<th>No of subjects</th>
<th>Number (%) of sera positive for \textit{E. granulosus} IgG antibody by ELISA using</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Eg-rCW12</td>
</tr>
<tr>
<td>Group-I</td>
<td>100</td>
<td>92 (92.00)</td>
</tr>
<tr>
<td>Group-II</td>
<td>60</td>
<td>31 (51.66)</td>
</tr>
<tr>
<td>Group-III</td>
<td>60</td>
<td>3 (5.00)</td>
</tr>
<tr>
<td>Group-IV</td>
<td>30</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity (%)</th>
<th>92.00</th>
<th>95.00</th>
<th>94.00</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specificity (%)</td>
<td>96.66</td>
<td>95.55</td>
<td>93.33</td>
</tr>
<tr>
<td>Positive Predictive Value (%)</td>
<td>96.84</td>
<td>95.95</td>
<td>94.00</td>
<td></td>
</tr>
<tr>
<td>Negative Predictive Value (%)</td>
<td>91.57</td>
<td>94.50</td>
<td>93.33</td>
<td></td>
</tr>
<tr>
<td>Efficiency (%)</td>
<td>94.21</td>
<td>95.26</td>
<td>93.68</td>
<td></td>
</tr>
</tbody>
</table>

[Group-I: Cases of CE with confirmed diagnosis; Group-II: Cases of CE with presumptive diagnosis; Group-III: Controls with other parasitic diseases; Group-IV: Healthy controls]

ELISA using Rec Eg-AgB8/2 demonstrated diagnostic level of antibodies in 94 of 100 (94.00\%) sera of confirmed CE cases (Group-I) (Table-5-5). The assay also demonstrated a diagnostic level of antibodies in 39 of 60 (65.00\%) sera of Group-II cases. The test detected antibodies in 8.33\% sera from controls with other parasitic diseases (Group-III), and 3.33\% sera from healthy controls (Group-IV).

The sensitivity, specificity, PPV, NPV, and efficiency of the ELISA using \textit{E. granulosus} recombinant antigens Eg-rCW12, Eg-rCW24 and RecEg-AgB8/2 for detection of \textit{E. granulosus} IgG antibodies in serum were presented in Table-5-5. The scatter plots showing relative distribution of OD\textsubscript{492} values in the ELISA using Eg-rCW12, Eg-rCW24 and RecEg-AgB8/2 for detection of \textit{E. granulosus} IgG antibodies in serum from cases with CE and controls were presented in Fig 5-1 to 5-3. The difference in the mean OD\textsubscript{492} values between groups (Group-I and IV; Group-II and IV) was analyzed by Mann-Whitney test.
Fig 5-1: Scatter plots showing relative distribution of OD₄₂ values in the ELISA using *E. granulosus* recombinant antigen Eg-rCW12 for detection of antibodies in serum from cases with CE and controls. The mean OD₄₂ values of the ELISA in Group-I cases (p=0.0001) and Group-II (p=0.0001) cases was significantly different compared to healthy controls. The p values were estimated using Graph Pad software (Online version).

Fig 5-2: Scatter plots showing relative distribution of OD₄₂ values in the ELISA using *E. granulosus* recombinant antigen Eg-rCW24 for detection of antibodies in serum from cases with CE and controls. The mean OD₄₂ values of the ELISA in Group-I cases (p=0.0001) and Group-II (p=0.0001) cases was significantly different compared to healthy controls. The p values were estimated using Graph Pad software (Online version).
Antibody-ELISA using Rec Eg-AgB8/2 antigen

Fig 5-3: Scatter plots showing relative distribution of OD values in the ELISA using *E. granulosus* recombinant antigen B fraction Rec Eg-AgB8/2 for detection of antibodies in serum from cases with CE and controls. The mean OD values of the ELISA in Group-I cases (p=0.0001) and Group-II (p=0.0001) cases was significantly different compared to healthy controls. The p values were estimated using GraphPad software (Online version).

**Dot-ELISA for detection of *E. granulosus* IgG antibodies in serum**

Dot-ELISA using Eg-rCW12 demonstrated diagnostic level of antibodies in 88 of 100 (88.00 %) sera of confirmed CE cases (Group-I) (Table-5-6). The assay also demonstrated a diagnostic level of antibodies in 34 of 60 (56.66 %) sera of Group-II cases. The test detected antibodies in 6.66 % sera from controls with other parasitic diseases (Group-III), and 3.33 % sera from healthy controls (Group-IV).

Dot-ELISA using Eg-rCW24 demonstrated diagnostic level of antibodies in 91 of 100 (91.00 %) sera of confirmed CE cases (Group-I) (Table-5-6). The assay also demonstrated a diagnostic level of antibodies in 36 of 60 (60.00 %) sera of Group-II cases. The test detected antibodies in 6.66 % sera from controls with other parasitic diseases (Group-III), and 3.33 % sera from healthy controls (Group-IV).

Dot-ELISA using Rec Eg-AgB8/2 demonstrated diagnostic level of antibodies in 90 of 100 (90.00 %) sera of confirmed CE cases (Group-I) (Table-5-6). The assay also demonstrated a diagnostic level of antibodies in 38 of 60 (63.33 %) sera of Group-II cases. The test detected antibodies in 8.33 % sera from controls with other parasitic diseases (Group-III), and 3.33 % sera from healthy controls (Group-IV).
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The sensitivity, specificity, PPV, NPV, and efficiency of the Dot-ELISA using Eg-rCW12 and Eg-rCW24 in comparison to RecEg-AgB8/2 for detection of *E. granulosus* IgG antibodies in serum were presented in Table-5-6.

Table-5-6: Dot-ELISA using *E. granulosus* recombinant antigens for detection of IgG antibodies in sera from cases of CE and controls

<table>
<thead>
<tr>
<th>Study groups</th>
<th>No of subjects</th>
<th>Number (%) of sera positive for <em>E. granulosus</em> IgG antibody by Dot-ELISA using</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Eg-rCW12</td>
</tr>
<tr>
<td>Group-I</td>
<td>100</td>
<td>88 (88.00)</td>
</tr>
<tr>
<td>Group-II</td>
<td>60</td>
<td>34 (56.66)</td>
</tr>
<tr>
<td>Group-III</td>
<td>60</td>
<td>4 (6.66)</td>
</tr>
<tr>
<td>Group-IV</td>
<td>30</td>
<td>1 (3.33)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity (%)</th>
<th>88.00</th>
<th>91.00</th>
<th>90.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity (%)</td>
<td>94.44</td>
<td>94.44</td>
<td>93.33</td>
<td></td>
</tr>
<tr>
<td>Positive Predictive Value (%)</td>
<td>94.62</td>
<td>94.79</td>
<td>93.75</td>
<td></td>
</tr>
<tr>
<td>Negative Predictive Value (%)</td>
<td>87.62</td>
<td>90.42</td>
<td>89.36</td>
<td></td>
</tr>
<tr>
<td>Efficiency (%)</td>
<td>91.05</td>
<td>92.63</td>
<td>91.57</td>
<td></td>
</tr>
</tbody>
</table>

[Group-I: Cases of CE with confirmed diagnosis; Group-II: Cases of CE with presumptive diagnosis; Group-III: Controls with other parasitic diseases; Group-IV: Healthy controls]

Detection of *E. granulosus* antigen in serum by Ag-ELISA

*Standardization of Ag-ELISA*

The optimum concentration of each of the affinity purified anti-Eg-rCW12, anti-Eg-rCW24 and anti-Rec Eg-AgB8/2 monospecific IgG antibodies (coating antibody) was found to be 4 µg/well. The optimum dilution of each of the affinity purified anti-Eg-rCW12, anti-Eg-rCW24 and anti-Rec Eg-AgB8/2 monospecific IgG antibodies (detecting antibody) was found to be 1:100. The detection limits of the *E. granulosus* antigens were 50 ng/ml to 100 ng/ml for the Ag-ELISA using all three anti-Eg-rCW12 (Fig 5-5a), anti-Eg-rCW24 (Fig 5-5b) and anti-Rec Eg-AgB8/2 (Fig 5-5c) antibodies.
Fig 5-4a: Chromatogram showing elution of IgG fraction from rabbit immune sera raised against Eg-rCW12 antigen.

Fig 5-4b: Chromatogram showing elution of IgG fraction from rabbit immune sera raised against Eg-rCW24 antigen.

Fig 5-4c: Chromatogram showing elution of IgG fraction from rabbit immune sera raised against Rec Eg-AgB8/2 antigen.
Fig 5-5a: Estimation of antigen detection limit for the Ag-ELISA using anti-Eg-rCW12 antibody. HS: hyperimmune serum raised against Eg-rCW12 antigen; NS: Normal rabbit serum as control

Fig 5-5b: Estimation of antigen detection limit for the Ag-ELISA using anti-Eg-rCW24 antibody. HS: hyperimmune serum raised against Eg-rCW24 antigen; NS: Normal rabbit serum as control

Fig 5-5c: Estimation of antigen detection limit for the Ag-ELISA using anti-Rec Eg-AgB8/2 antibody. Series 1: hyperimmune serum raised against Rec Eg-AgB8/2 antigen; Series 2: Normal rabbit serum as control
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Ag-ELISA for detection of *E. granulosus* antigens in sera from cases of CE and controls

The cut-off OD$_{402}$ values of the Ag-ELISA using anti-Eg-rCW12, anti-Eg-rCW24 and anti-Rec Eg-AgB8/2 antibodies for detection of antigen in serum were estimated to be 0.118201, 0.118969 and 0.117355 respectively (Table-5-7).

**Table-5-7: Estimation of the cut-off OD$_{402}$ values for the Ag-ELISA for detection of *E. granulosus* antigens in serum**

<table>
<thead>
<tr>
<th>OD$_{402}$ of normal sera (n = 30)</th>
<th>Detection of <em>E. granulosus</em> antigen in serum by Ag-ELISA using</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eg-rCW12 antibody</td>
<td>Eg-rCW24 antibody</td>
<td>Rec Eg-AgB8/2 antibody</td>
<td></td>
</tr>
<tr>
<td>Absorbance range</td>
<td>0.075-0.117</td>
<td>0.085-0.114</td>
<td>0.075-0.116</td>
<td></td>
</tr>
<tr>
<td>Mean average OD$_{402}$</td>
<td>0.097367</td>
<td>0.101267</td>
<td>0.095833</td>
<td></td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.010417</td>
<td>0.008851</td>
<td>0.010761</td>
<td></td>
</tr>
<tr>
<td>Cut-off</td>
<td>0.118201</td>
<td>0.118969</td>
<td>0.117355</td>
<td></td>
</tr>
</tbody>
</table>

The results of Ag-ELISA using *E. granulosus* Eg-rCW12 antibody, Eg-rCW24 antibody and Rec Eg-AgB8/2 antibody for detection of *E. granulosus* antigens in serum from the cases of CE and controls were summarized in Table-5-8.

The Ag-ELISA using anti-Eg-rCW12 monospecific antibody demonstrated *E. granulosus* antigen in 80 of 100 (80.00 %) sera of confirmed CE cases (Group-I) (Table-5-8). The assay also demonstrated the antigen in 23 of 60 (38.33 %) sera of Group-II cases. The test detected antigens in 2 (3.33 %) sera from controls with other parasitic diseases (Group-III), but did not show any *E. granulosus* antigens in the sera of healthy controls (Group-IV).

The Ag-ELISA using anti-Eg-rCW24 monospecific antibody demonstrated *E. granulosus* antigen in 83 of 100 (83.00 %) sera of confirmed CE cases (Group-I) (Table-5-8). The assay also demonstrated the antigen in 23 of 60 (38.33 %) sera of Group-II cases. The test detected antigens in 2 (3.33 %) sera from controls with other parasitic diseases (Group-III), but did not show any *E. granulosus* antigens in the sera of healthy controls (Group-IV).

The Ag-ELISA using anti-Rec Eg-AgB8/2 monospecific antibody demonstrated *E. granulosus* antigen in 83 of 100 (83.00 %) sera of confirmed CE cases (Group-I) (Table-5-
The assay also demonstrated the antigen in 23 of 60 (38.33 %) sera of Group-II cases. The test detected antigens in 1 (1.66 %) serum specimen from controls with other parasitic diseases (Group-III), but did not show any *E. granulosus* antigens in the sera of healthy controls (Group-IV).

The sensitivity, specificity, PPV, NPV, and efficiency of the antigen-ELISA using *anti-Eg-rCW12* antibody, *anti-Eg-rCW24* antibody and anti-RecEg-AgB8/2 antibody for detection of *E. granulosus* antigens in serum were presented in Table-5-8. The scatter plots showing relative distribution of OD_{492} values in the Ag-ELISA using *anti-Eg-rCW12*, *anti-Eg-rCW24* and anti-RecEg-AgB8/2 antibodies for detection of *E. granulosus* antigen in serum from cases with CE and controls were presented in Fig 5-6, Fig 5-7 and Fig 5-8. The difference in the mean OD_{492} values between groups (Group-I and IV; Group-II and IV) was analyzed by Mann-Whitney test.

Table-5-8: Ag-ELISA in serum using *anti-Eg-rCW12*, *anti-Eg-rCW24* and anti-RecEg-AgB8/2 antibodies for detection of *E. granulosus* antigen in sera from cases with CE and controls

<table>
<thead>
<tr>
<th>Study groups</th>
<th>No of subjects</th>
<th>No (%) of sera positive for <em>E. granulosus</em> antigen by Ag-ELISA using</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Anti-rec Eg-AgB8/2 Ab</td>
</tr>
<tr>
<td>Group-I</td>
<td>100</td>
<td>83 (83)</td>
</tr>
<tr>
<td>Group-II</td>
<td>60</td>
<td>23 (38.33)</td>
</tr>
<tr>
<td>Group-III</td>
<td>60</td>
<td>1 (1.66)</td>
</tr>
<tr>
<td>Group-IV</td>
<td>30</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>83.00</td>
<td>80.00</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>98.88</td>
<td>97.77</td>
</tr>
<tr>
<td>Positive Predictive Value (%)</td>
<td>98.80</td>
<td>97.56</td>
</tr>
<tr>
<td>Negative Predictive Value (%)</td>
<td>83.96</td>
<td>81.48</td>
</tr>
<tr>
<td>Efficiency (%)</td>
<td>90.52</td>
<td>88.42</td>
</tr>
</tbody>
</table>

[Group-I: Cases of CE with confirmed diagnosis; Group-II: Cases of CE with presumptive diagnosis; Group-III: Controls with other parasitic diseases; Group-IV: Healthy controls]
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Fig 5-6: Scatter plots showing relative distribution of OD_{492} values in the Ag-ELISA using anti-Eg-rCW12 antibody for detection of antigens in sera from cases with CE and controls. The difference in mean OD_{492} values of the Ag-ELISA between Group-I cases and healthy individuals (p=0.0001) as well as between Group-II and healthy individuals (p=0.0001) were found to be statistically significant. The p values were estimated using Graph Pad software (Online version).

Fig 5-7: Scatter plots showing relative distribution of OD_{492} values in the Ag-ELISA using anti-Eg-rCW24 antibody for detection of antigens in sera from cases with CE and controls. The difference in mean OD_{492} values of the Ag-ELISA between Group-I cases and healthy individuals (p=0.0001) as well as between Group-II and healthy individuals (p=0.0002) were found to be statistically significant. The p values were estimated using Graph Pad software (Online version).
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Ag-ELISA using anti-RecEg-AgB8/2 antibody

![Graph showing OD values for different groups](image)

Fig 5-8: Scatter plots showing relative distribution of OD_{405} values in the Ag-ELISA using anti-Rec Eg-AgB8/2 antibody for detection of antigens in sera from cases with CE and controls. The difference in mean OD_{405} values of the Ag-ELISA between Group-I cases and healthy individuals (p=0.0001) as well as between Group-II and healthy individuals (p=0.0001) were found to be statistically significant. The p values were estimated using Graph Pad software (Online version).

Dot-ELISA for detection of *E. granulosus* antigens in serum

The Dot-ELISA using anti-Eg-rCW12 monospecific antibody demonstrated *E. granulosus* antigen in 76 of 100 (76.00 %) serum samples of the cases with confirmed CE (Group-I) (Table-5-9). The assay also demonstrated the antigen in 22 of 60 (36.66 %) sera of Group-II cases. The test detected antigens in 3 (5.00 %) serum specimens from controls with other parasitic diseases (Group-III), but did not show any *E. granulosus* antigens in the sera of healthy controls (Group-IV).

The Dot-ELISA using anti-Eg-rCW24 monospecific antibody demonstrated *E. granulosus* antigen in 83 of 100 (83.00 %) serum samples of the cases with confirmed CE (Group-I) (Table-5-9). The assay also demonstrated the antigen in 23 of 60 (38.33 %) sera of Group-II cases. The test detected antigens in 1 (1.66 %) serum specimen from controls with other parasitic diseases (Group-III), but did not show any *E. granulosus* antigens in the sera of healthy controls (Group-IV).

The Dot-ELISA using anti-Rec Eg-AgB8/2 monospecific antibody demonstrated *E. granulosus* antigen in 82 of 100 (82.00 %) serum samples of the cases with confirmed CE (Group-I) (Table-5-9). The assay also demonstrated the antigen in 21 of 60 (35.00 %) sera of Group-II cases. The test detected antigens in 3 (5.00 %) serum specimens from controls...
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with other parasitic diseases (Group-III), but did not show any *E. granulosus* antigens in the sera of healthy controls (Group-IV).

The sensitivity, specificity, PPV, NPV, and efficiency of the Dot-ELISA using anti-Eg-rCW12 antibody, anti-Eg-rCW24 antibody and anti-Rec Eg-AgBI2 antibody for detection of *E. granulosus* antigens in serum were presented in Table-5-9.

**Table-5-9: Ag-Dot-ELISA using anti-Eg-rCW12, anti-Eg-rCW24 and anti-Rec Eg-AgB8/2 antibodies for detection of *E. granulosus* antigen in sera of cases with CE and controls**

<table>
<thead>
<tr>
<th>Study groups</th>
<th>No of subjects</th>
<th>No (%) of sera positive for <em>E. granulosus</em> antigen by Dot-ELISA using</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Anti-rec Eg-AgB8/2 Ab</td>
</tr>
<tr>
<td>Group-I</td>
<td>100</td>
<td>82 (82)</td>
</tr>
<tr>
<td>Group-II</td>
<td>60</td>
<td>21 (35)</td>
</tr>
<tr>
<td>Group-III</td>
<td>60</td>
<td>3 (5)</td>
</tr>
<tr>
<td>Group-IV</td>
<td>30</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>82.00</td>
<td>76.00</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>96.66</td>
<td>96.66</td>
</tr>
<tr>
<td>Positive Predictive Value (%)</td>
<td>96.47</td>
<td>96.20</td>
</tr>
<tr>
<td>Negative Predictive Value (%)</td>
<td>91.57</td>
<td>78.37</td>
</tr>
<tr>
<td>Efficiency (%)</td>
<td>88.94</td>
<td>85.78</td>
</tr>
</tbody>
</table>

[Group-I: Cases of CE with confirmed diagnosis; Group-II: Cases of CE with presumptive diagnosis; Group-III: Controls with other parasitic diseases; Group-IV: Healthy controls]
Clinical manifestations in patients with CE

Cystic echinococcosis is an important public health problem in many regions of the world, both in areas where the disease is endemic, and in regions where the disease is emerging or re-emerging (Craig et al., 1996). Though CE is preventable by efficient and sustained control campaigns (Torgerson and Heath, 2003), numerous reports indicated that its incidence has been increasing in various regions of the world (Eckert et al., 2000) including India (Parija, 1994; Khurana et al., 2007). The accurate assessment of the prevalence of CE is therefore a major element to expose the magnitude of the problem and evaluate the success of the control strategy. This involves clinical diagnosis of the disease and epidemiological surveillance of high-risk populations (Parija, 1994).

CE is a chronic zoonosis, usually characterized by slow growing fluid-filled cysts in the liver, lungs or other organs (Pawlowski, 1993). The clinical diagnosis of CE is invariably made when the disease is relatively advanced. The signs and symptoms of CE are also dependant on the location, number, size, topographic relationships of the cysts in the human body (Thompson and Lymbery, 1988; Eckert and Thompson, 1988; Thompson et al., 1995; Eckert and Thompson, 1997; Rosenzvit et al., 1999; Kamenetzky et al., 2000; Kamenetzky et al., 2002).

The infection generally occurs between 2-5 years of age (mean: 3 years), when children begin to go upon all fours and become friendly with dogs (Thompson et al., 1995; Pawlowski, 1997; Eckert and Thompson, 1997). But the clinical symptoms have been evident after many years of infection, generally in middle aged patients (Gottstein et al., 1987; Ammann et al., 1990; Pawlowski, 1997). Paul and Stefaniak (1997) observed that females (82.71%) were more frequently infected with CE compared to males (17.28%).

In the present study, CE was more frequently observed in the age group of 40-60 years (46.87%) followed by the age group 20-40 years (38.75%)(Table-5-1). The age group ranged from 41-60 years (middle age people) appeared to involve highest by CE infection in males (51.38%) followed by 20-40 age group (29.16%) (Table-5-1). The age group 21-40 affected highest (46.59%), followed by the age group 40-60 years (43.18%) in females. The CE was more frequently observed in females (55.00%) compared to males (45.00%)
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(Table-5-1). However, the difference was narrow in contrast to the observation made by Paul and Stefaniak (1997).

In CE of humans, primary hydatid cysts have been found most frequently in liver (approximately 65% of the cases), but also in lungs (25%) and other organs such as kidney, spleen, brain, heart, and bone (Schantz and Gottstein, 1986; Thompson et al., 1995; Eckert and Thompson, 1997; Rosenzvit et al., 1999; Kamenetzky et al., 2000). In other study, primary hydatid cysts were found most frequently in the liver (58.6 %), followed by lungs (29.2 %) and spleen (2.4 %) and the multi organ location of hydatid cysts were found in 9.8 % of CE cases (Guarnera et al., 2004).

In the present study, the predilection of hydatid cysts among the South Indian patients were found more frequently in 76 % of confirmed and 41 % of suspected CE cases in liver, followed by lungs (9 % and 16 %) respectively (Table-5-2). Multi organ location of hydatid cysts were found in 7 % of confirmed and 1.66 % of suspected patients with CE, which were similar to that of previous studies (Table-5-2).

The clinical symptoms in patients with liver cyst include right hipochondrial pain (48.27 %), vomica (37.93 %), fever (17.24 %), palpable tumor (17.24 %) and very subjective symptoms like astenia, anorexia and postprandial fullness sensation (Guarnera et al., 2004). The clinical symptoms in patients with non complicated lung cysts include, thoracic pain (50 %), cough (37.5 %), vomits (37.5 %), astenia (25 %) and anorexia (12.5 %)(Guarnera et al., 2004). These symptoms were found to be similar in CE patients with any strain of *E. granulosus* (Schantz and Gottstein, 1986; Pawlowski, 1997; Ortona et al., 2003).

In the present study, 83.00% confirmed CE cases and 75.00% suspected CE cases presented with pain, 48.00% and 48.33% of cases presented with swelling, 24.00% and 21.66% of cases with vomiting, 24.00% and 33.33% of cases with weight loss, 19.00% and 31.66% of cases presented with cough, 10.00% and 3.33% cases with hemoptysis out of 100 confirmed CE cases and 60 suspected CE cases respectively.

The present study also demonstrated that, CE was prevalent more frequently among the agricultural workers (about 41.41% and 43.33% of confirmed and suspected cases of CE respectively), followed by house wives (with a frequency of 26.26% and 28.33% of confirmed and suspected CE cases respectively) and public service undertaking/corporate
sector employees (22.22 % and 16.66 % of confirmed and suspected CE cases respectively) (Table-5-3).

Detection of *E. granulosus* antibodies in serum

**ELISA for detection of *E. granulosus* antibodies in serum**

Detection of antibodies in serum is found to be the most convenient and commonly used method for the diagnosis of CE (Siracusano et al., 1991; Parija, 1994). Antibody-based serological tests are useful not only for confirming the diagnosis of CE in an individual patient, but are also helpful in epidemiologic studies. Several antibody detection assays, such as IED, ELISA, Dot-ELISA and EITB, have been used to detect hydatid antibodies in serum for diagnosis of CE (Varela-Diaz, et al., 1978; Coltorti and Varela-Diaz, 1978; Coltorti, 1986; Maddison et al., 1989; Siracusano et al., 1991).

Both ELISA and Dot-ELISA were found to be more reliable tests, cheap and rapid, hence most frequently used in recent times (Kanwar et al, 1992; Li et al., 2004). The ELISA has been evaluated for diagnosis of CE by detecting *E. granulosus* IgG antibodies in serum (Facon et al., 1991; Chamekh et al., 1992; Kanwar et al, 1992; Liu et al., 1993; Heibig et al., 1993; Leggatt and, McManus, 1994; McVie et al., 1997; Kaur et al., 1999), IgM antibodies (IgM-ELISA) (Wattal et al., 1986) and IgE (IgE-ELISA) (Wattal et al., 1988). Therefore, the present study was designed to develop and evaluate ELISA using *E. granulosus* recombinant antigens, Eg-rCW12 and Eg-rCW24 and Rec Eg-AgB8/2, to detect hydatid antibodies in serum for diagnosis of CE.

The recombinant AgB fractions were found to be the most relevant diagnostic antigens for diagnosis of CE (Matossian et al., 1972; Maddison et al., 1989; Kanwar et al., 1992; Rott et al., 2000; Virginio et al., 2003; Li et al., 2003; Li et al., 2004). The recombinant antigens identified in the first phase of the present study also appears to be AgB fractions.

Therefore, in the present study, the ELISA was developed and evaluated by using *E. granulosus* recombinant antigens, Eg-rCW12, Eg-rCW24 and Rec Eg-AgB8/2 for the diagnosis of CE. For interpretation of seropositivity, a threshold (cutoff) value was calculated for each antigen using 30 serum samples collected from healthy individuals (Table-5-4). The mean OD$_{492}$ plus two standard deviations was taken as the cutoff.
The ELISA using *E. granulosus* recombinant antigens, Eg-rCW12, Eg-rCW24, for detection of antibodies in serum, showed a sensitivity of 92.00% and 95.00% respectively (Table-5-5). The specificity of the ELISA using Eg-rCW12, Eg-rCW24 antigens for detection of antibodies in serum was 96.66% and 95.55% respectively (Table-5-5). The sensitivity and specificity of the ELISA using *E. granulosus* recombinant antigen-B/2 (Rec Eg-AgB/2) for detection of antibodies in serum was 94.00% and 93.33% respectively (Table-5-5). In comparison, the efficiency of the ELISA using Eg-rCW24 (95.26%) for detection of IgG antibodies in serum was found to be higher than the ELISA using both Eg-rCW12 (94.21%) and Rec Eg-AgB/2 (93.68%) (Table-5-5).

In the present study, the sensitivity (92.00%, 95.00%) (Table-5-5) of the ELISA using Eg-rCW12 (12 kDa fraction) and Eg-rCW24 (24 kDa fraction) appeared to be similar to that of rEpC1-GST (92.20%) reported by Li et al. (2003), 8 kDa subunit (94%) reported by Shambesh et al. (1995), an antigen (92%) reported by Ito et al. (1999) and 12-kD subunit (90%) described by Leggatt et al. (1992), but higher than the 12-kD subunit (80%) reported by Ioppolo et al. (1996). However, the specificity of the ELISA using Eg-rCW12 and Eg-rCW24 was in accordance with that of these previous studies.

The ELISA using Rec Eg-AgB8/2, in the present study, showed a sensitivity of 94.00% (Table-5-5) which was in accordance with previous studies (Rott et al., 2000; Virginio et al., 2003). But, the specificity of the ELISA using Rec Eg-AgB8/2 (93.33%) was found to be lesser than the previous studies (99.50%) (Rott et al., 2000; Virginio et al., 2003).

False positive reactions have been the frequently noted problem in serological tests in patients infested by tapeworm and other helminths (Guisantes et al., 1981; Sjolander et al., 1989; Maddison et al., 1989; Force et al., 1992; Wen and Craig, 1994; Taherkhani et al., 2007). The serodiagnostic tests using *E. granulosus* antigens also showed cross reactions with sera from patients with AE, schistosomiasis and trichinosis (Sjolander et al., 1989; Force et al., 1992), sera from onchocerciasis and ascariasis (Sjolander et al., 1989) and sera from cysticercosis (Maddison et al., 1989; Wen and Craig, 1994). The ELISA using purified *E. granulosus* antigen also showed cross reactions with the sera collected from *Taenia saginata*, *Enterobius vermicularis* and *Fasciola hepatica* (Guisantes et al., 1981).

In the present study, the ELISA using Eg-rCW12, Eg-rCW24 and Rec Eg-AgB8/2 demonstrated a cross reactivity of 5%, 6.66% and 8.33% respectively (Table-5-5), with sera from neurocysticercosis cases, which was similar to those of other studies reported elsewhere (Maddison et al., 1989; Leggatt et al., 1992; Wen and Craig, 1994; Shambesh et
al., 1995). However, no cross-reactions were observed with sera from neurocysticercosis cases as reported by Ito et al. (1999). The ELISA using Eg-rCW12, Eg-rCW24 and Rec Eg-AgB8/2, in the present study, also did not show cross reactions with sera from patients with other parasitic diseases such as filariasis, amoebiasis, schistosomiasis and malaria (Table-5-5).

The GST-fusion proteins, even after affinity purification, are known to show false-positive reactions with the *E. coli*-specific antibodies in patient sera (Gasser et al., 1990; Facon et al., 1991; Helbig et al., 1993). The use of GST-fusion proteins in serodiagnosis of CE have shown false-positive reactions with sera from cases of schistosomiasis (Smith and Johnson, 1988; Gasser et al., 1990; Facon et al., 1991), and 47% of those patients exhibited IgG antibodies against GST (Auriault et al., 1990). In the present study, the recombinant antigens that were cleaved from their GST moiety using Factor X did not show cross reactions with sera from schistosomiasis cases, as previously observed by Helbig et al. (1993) and Li et al. (2004).

The antigen B (8 kDa) subunit of *E. granulosus* is known to produce cross-reactions with sera from AE cases (Leggatt et al., 1992; Shambesh et al., 1995; Ito et al., 1999). This would limit its usefulness for serologic differentiation of CE from AE. In the present study, the recombinant antigens were not evaluated using sera from AE and polycystic echinococcosis (PE) cases. Since, *E. multilocularis, E oligarthrus* and *E. vogeli* infections have not been prevalent in India, these recombinant antigens could be efficiently used as the diagnostic antigens for diagnosis of CE in this part of the world and other places, where these parasitic diseases are not commonly prevalent.

In the present study, ELISA was not evaluated using sera from patients with liver cirrhosis (Lacona et al., 1980), anti-P1 antibodies (Ben Ismail et al., 1980), and malignancies (Varela-Diaz et al., 1978), which could also produce false positive reactions. Although further tests with a significantly greater number of heterologous sera are required, the results of the present study (Table-5-5), suggested that any of the three recombinant antigens (Eg-rCW24, Eg-rCW12 and EG-AgB8/2) evaluated in the present study could be used for the diagnosis of CE.

In the present study, all sera were tested thrice, including the sera used for determination of the cutoff values, to exclude variations from day to day. However, all independently performed ELISAs showed the same results. This high level of reproducibility of these ELISAs is most important and especially useful when there are low serum antibody titers.
In the present study, the efficiency of ELISA using Eg-rCW12 and Eg-rCW24 (Fig 5-1 and Fig 5-2) was similar to the previous studies where variations in the antibody response to CE were associated with the location, size, number and/or condition of the hydatid cysts (Verastegui et al., 1992; Craig, 1997). These results were also similar to the previous studies where seroconversion was found to be correlated with increased immune stimulation with respect to the cyst growth and degeneration (Shambesh et al., 1999).

Given the diagnostic properties of the *E. granulosus* recombinant antigens (Eg-rCW12 and Eg-rCW24) (Table-5-5) evaluated in the present study and the fact that large quantities of the protein in pure form could be produced, these antigens were found to be of diagnostic value to develop various simple, cheap and rapid immunodiagnostic assays for diagnosis of CE in both humans as well as livestock.

The Dot-ELISA is a rapid, inexpensive and simple test to perform (Rogan et al., 1991). Pappas et al. (1986) also observed that the Dot-ELISA was a rapid and economical enzyme immunoassay which was antigen-conservative and serum-conservative. Such simpler assays have been developed for the detection of antibodies against a range of infectious agents including *Schistosoma* (Zhu et al., 2002) and *Leishmania* (Schalling et al., 2004). Therefore, the present study was further extended to evaluate Dot-ELISA using these *E. granulosus* recombinant antigens (Eg-rCW12 and Eg-rCW24), which were found to be diagnostic.

**Dot-ELISA for detection of *E. granulosus* antibodies in serum**

A rapid, simple, cheap and relevant antibody-based test using recombinant *E. granulosus* antigen is a necessity for diagnosis of CE in humans and animals (Muller et al., 1989; Frosch et al., 1993; Ferreira and Zaha, 1994; Li et al., 2004).

Dot-ELISA is a highly versatile solid-phase immunoassay for detection of antibody in the diagnosis of a number of parasitic diseases including, leishmaniasis (Pappas et al., 1983), toxoplasmosis (Pappas, 1988), malaria (Pappas, 1988), trypanosomiasis (Araujo, 1985), cysticercosis (Tellez-Giron et al., 1987) and CE (Schantz and Kagan, 1980). The test is found to be suitable for routine application in the clinic, laboratory and in the field for community surveillance surveys (Rogan et al., 1991). The assay uses minute amounts of reagents dotted onto solid surfaces such as nitrocellulose or cellulose acetate membranes.
which avidly bind proteins (Pappas, 1988). Hence the present study was undertaken to develop and evaluate Dot-ELISA using Eg-rCW12 and Eg-rCW24 for diagnosis of CE.

In the present study, the Dot-ELISA using *E. granulosus* recombinant antigens, Eg-rCW12, Eg-rCW24, for detection of antibodies in serum, showed a sensitivity of 88.00% and 91.00% respectively (Table-5-6). The specificity of the Dot-ELISA using either Eg-rCW12 or Eg-rCW24 antigens was observed to be 94.44%. The sensitivity and specificity of the Dot-ELISA using Rec Eg-AgB/2 for detection of antibodies in serum was observed to be 90.00% and 93.33% respectively (Table-5-6). In comparison, the efficiency of the Dot-ELISA using Eg-rCW24 (92.63%) for detection of IgG antibodies in serum was observed to be higher than the Dot-ELISA using both Eg-rCW12 (91.05%) and Rec Eg-AgB/2 (91.57%) (Table-5-6).

In the present study, the sensitivity and specificity of Dot-ELISA using Eg-rCW12 (88.00%, 94.44%), Eg-rCW24 (91.00%, 94.44%) and Rec Eg-AgB/2 (90.00%, 93.33%) (Table-5-6) and is in agreement with the studies of Romia et al. (1992) who demonstrated a sensitivity of 88.9% and a specificity of 96.9% by Dot-ELISA for diagnosis of CE. The sensitivity of Dot-ELISA obtained in the present study, however, was slightly lesser than the Dot-ELISA using sheep HCF antigen, which showed a sensitivity of 96% and specificity of 98% (Table-5-6) (Pappas et al., 1983; Mistrello et al., 1995).

Rogan et al. (1991) reported lower specificity (52%) and higher sensitivity (97%) by using crude sheep HCF as diagnostic antigen. It was suggested that the use of more purified antigen preparation, greatly improve the assay reliability by reducing false-positive reactions (Schantz and Kagan, 1980; Rogan et al., 1991; Wen and Craig, 1994). In the present study, the Dot-ELISA using recombinant antigens (Eg-rCW12 and Eg-rCW24) demonstrated high specificity (94.44%) (Table-5-6).

In the present study, the Dot-ELISA showed some problems associated with differentiating weak positive reactions from negative ones. In some cases the repetition of the tests using freshly collected serum samples produced clear results. Similar results were also observed in previous studies, where they suggested that the use of adequate and freshly collected negative and positive control sera could minimize these problems (Pappas et al., 1984; Rogan et al., 1991).

The antibody detection assays (ELISA and Dot-ELISA), as demonstrated in the present study, were found to be useful for serodiagnosis of CE. However, the inability to
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discriminate between recent and old infections and false-negativity were the major disadvantages of these immunassays. Some of the reasons for the occurrence of such false negative reactions were due to (i) secretion/ excretion of high levels of circulating antigen by the parasite which generate circulating immune complexes after reacting with antibodies (Craig and Nelson, 1984; Craig et al., 1986), (ii) the parasite with dormant or calcified cyst that may cease to produce antibodies (Biffin et al., 1993), (iii) antibody mopping by circulating antigen (Craig and Nelson, 1984), and iv) some of the cysts of lung associated with low serum antibodies and hydatid cysts of the eye and brain produce no or very low serum antibodies (Maddison et al., 1989; Siracusano et al., 1991).

Detection of *E. granulosus* antigens in serum

**Ag-ELISA for detection of *E. granulosus* antigens in serum**

The antibody detection assays in CE were neither sensitive nor very specific and there was no correlation between the levels of anti-hydatid antibodies and the burden of the disease (Rickard et al., 1984). Moreover, the persistence of anti-hydatid antibodies for years after surgical removal of the cyst (Rickard et al., 1984) and the cross-reaction with other parasitic infections (Larralde et al., 1989) further question their suitability for diagnosis of CE.

The hydatid antigens are generally excreted into serum during the active infection and hence detection of serum hydatid antigen always indicates active or recent infection (Shariff and Parija, 1991). Some studies have also shown the usefulness of the serum antigen detection for monitoring the post-treatment evaluation of the CE cases (Candolfi et al., 1985; Craig et al., 1986; Ravinder et al., 1997).

Several serodiagnostic tests have been evaluated for detection of hydatid antigen in the serum and other body fluids for diagnosis of CE. These tests include CIEP (Shariff and Parija, 1991), Co-A (Shariff and Parija, 1993), LAT (Sheela Devi and Parija, 2003), ELISA (Gottstein, 1984; Candolfi et al., 1985; Craig et al., 1986; Kanwar and Vinayak, 1992) and Dot-ELISA (Romia et al., 1992). These tests demonstrated varied sensitivities and specificities, which were unsatisfactory. Therefore, the ELISA was developed and evaluated by using affinity purified anti-Eg-αCW12, anti-Eg-αCW24 and anti-Rec Eg-AgB8/2 IgG antibody for detection of hydatid antigen in serum for the diagnosis of CE.
Antigen-ELISA is a specific test which enables qualitative or quantitative determination of specific antigen through chromogenic reaction (Gottstein, 1984). Ag-ELISA for quantification of antigen is especially valuable when the concentration of antigen is low and/or they are contained in high concentration of contaminating protein (Gottstein, 1984; Candolfi et al., 1985; Craig et al., 1986; Kanwar and Vinayak, 1992).

In the present study, the Ag-ELISA using anti-Eg-rCW12 and anti-Eg-rCW24 antibodies for detection of hydatid antigens in serum, demonstrated a sensitivity of 80.00% and 83.00% respectively, with a specificity of 97.77% (Table-5-8). The Ag-ELISA using affinity purified anti-Rec Eg-AgB/2 antibodies for detection of hydatid antigens in serum, showed a sensitivity of 83.00% and a specificity of 98.88% (Table-5-8). The cut-off point was calculated from the mean OD_{492} of the Ag-ELISA using 30 healthy individuals (Table-5-7). The mean OD_{492} plus two standard deviations was taken as the cutoff.

The hydatid antigen in the serum was also demonstrated, in our laboratory, by other simple assays using polyclonal antibodies. The CIEP test for detection of serum antigen, showed a moderate sensitivity of 55.55% in surgically proved and high sensitivity of 100% in ultrasound proved CE cases for the diagnosis of CE (Shariff and Parija, 1991). The Co-A test showed a sensitivity of 95% and specificity of 89% for the diagnosis of CE by detection of serum antigen. False positive rate of 18.5% was also observed with control sera from patients with various other parasitic diseases by the Co-A (Shariff and Parija, 1993). The LAT showed a sensitivity of 72% and a specificity of 98% for the diagnosis of CE (Sheela Devi and Parija, 2003). In comparison, in the present study, the efficiency of the Ag-ELISA using anti-Eg-rCW24 (90.00%) and anti-Rec Eg-AgB/2 (90.52%) antibodies for detection of hydatid antigen in serum was observed to be higher than the ELISA using anti-Eg-rCW12 antibody (88.42%) (Table-5-8).

In the present study, the sensitivity and specificity of Ag-ELISA using anti-Eg-rCW24 and anti-Eg-rCW12 antibodies (Table-5-8) were similar to the studies reported by Kanwar and Vinayak, (1992). In their study the ELISA using monospecific antibodies against specific hydatid antigens (8 kDa antigen and 116 kDa antigen) showed a sensitivity of 85% and 90% respectively for detection of hydatid antigen in serum for diagnosis of CE (Kanwar and Vinayak, 1992).

Gottstein (1984) demonstrated circulating antigen (concentration of 355-580 ng/ml) in only 40% of CE cases before surgery by employing double-antibody sandwich ELISA using polyclonal antibodies. Candolfi et al. (1985) demonstrated circulating antigens in
only 21% of sera form patients with CE. The low sensitivity might be due to either low level of protein excretion or due to the formation of immune complexes (d'Amelio et al., 1983; Gottstein, 1984; Candolfi et al., 1985). Craig (1986) observed that the sensitivity of ELISA was 90% for Turkana patients but as low as 50% for UK patients. The Ag-ELISA using anti-Eg-rCW24 antibody demonstrated a sensitivity of 83.00% in the present study.

In the present study, the antigen detection limit of Ag-ELISA using all three monospecific antibodies was 100 ng/ml to 50 ng/ml (Fig 5-5a, 5-5b, 5-5c). This was much lower than the antigen detection limit of 270 ng/ml reported by Gottstein (Gottstein, 1984). The possible reason for difference in the detection limit of antigen might be due to qualitative and quantitative difference in the concentration of coating antibody and the quality of the serum sample (Craig, 1986).

The host antigens are also found to interference with the antigen detection assays producing false positive reactions (Gottstein, 1984; Craig, 1986). However, in the present study, the use of monospecific antibodies against the *E. granulosus* recombinant antigens eliminated the interference of host components. The Ag-ELISA using anti-Eg-rCW24 showed false positive reactions in 2 (6.66%) of 30 patients with cysticercosis. The Ag-ELISA using anti-Eg-rCW12 showed false positive reactions in only one (3.33%) of 30 patients with cysticercosis (Table-5-8). The Ag-ELISA did not show cross reactions with sera from patients with other parasitic diseases and healthy controls (Table-5-8).

**Dot-ELISA for detection of *E. granulosus* antigens in serum**

Although, the Ag-ELISA is sensitive and specific, it is expensive, required technical expertise, perishable reagents and is difficult to adopt in small poorly equipped laboratories. Therefore, the present study was undertaken to develop and evaluate Dot-ELISA using affinity purified monospecific antibodies for the detection of hydatid antigen in the serum.

In bancroftian filariasis, Dot-ELISA was compared with sandwich ELISA for the detection of antigen in serum (Zheng et al., 1990). The result showed that in Dot-ELISA, 67 of 70 serum samples from microfilaremic patients were positive at a dilution of 1:50. In sandwich ELISA, 64 of the 70 serum samples were positive at a dilution of 1:10. The specificity of both assays was over 91%, but their sensitivity was markedly different. Dot-ELISA could detect as little as 0.055 ng/ml microfilarial antigens added to normal human
serum, whereas the lower limit of detection by sandwich-ELISA was 10 ng/ml parasite antigens (Zheng et al., 1990).

In the present study, the Dot-ELISA using affinity purified anti-Eg-rCW12 and anti-Eg-rCW24 monospecific antibodies for detection of hydatid antigens in serum, showed a sensitivity of 76.00% and 83.00% respectively, and a specificity of 96.66% and 98.88% respectively (Table-5-9). The Dot-ELISA using affinity purified anti-Rec Eg-AgB/2 monospecific antibodies for detection of hydatid antigens in serum, showed a sensitivity of 82.00% and a specificity of 96.66% (Table-5-9). In comparison, the efficiency of the Dot-ELISA using anti-Eg-rCW24 antibodies (90.52%) for detection of hydatid antigens in serum was observed to be higher than the Dot-ELISA using both anti-Eg-rCW12 (85.78%) and anti-Rec Eg-AgB/2 (88.94%) antibodies (Table-5-9).

The results of the present study were in contrast to the study reported by Romia et al. (1992). In their study the Dot-ELISA using anti-HCF hyperimmune rabbit serum demonstrated less sensitivity (55.6%). Low sensitivity is attributed to the small amounts of circulating antigens and/or immune complexes formation (Romia et al., 1992).

In conclusion, in this study, both Ag-ELISA and Dot-ELISA using affinity purified anti-Eg-rCW24 and anti-Eg-rCW12 monospecific antibodies for detection of hydatid antigens in serum showed high sensitivity and high specificity (Table-5-8 and Table-5-9). Hence both the techniques could be used for diagnosis of CE. The Dot-ELISA, has the added advantage of being more convenient, simple, rapid, cheap and reliable, does not require costly equipment and requires only lesser amounts of specimens and reagents.
Evaluation of ELISA and Dot-ELISA using *E. granulosus* recombinant antigens to detect hydatid antibodies in serum for diagnosis of cystic echinococcosis

The ELISA using *E. granulosus* recombinant antigens, Eg-rCW12, Eg-rCW24, for detection of antibodies in serum, showed a sensitivity of 92.00% and 95.00% respectively. The specificity of the ELISA using Eg-rCW12, Eg-rCW24 antigens for detection of antibodies in serum was observed to be 96.66% and 95.55% respectively. The sensitivity and specificity of the ELISA using *E. granulosus* recombinant antigen-B/2 (Rec Eg-AgB8/2) for detection of antibodies in serum was observed to be 94.00% and 93.33% respectively. In comparison, the efficiency of the ELISA using Eg-rCW24 (95.26%) for detection of IgG antibodies in serum was observed to be higher than the ELISA using both Eg-rCW12 (94.21%) and Rec Eg-AgB8/2 (93.68%).

The Dot-ELISA using *E. granulosus* recombinant antigens, Eg-rCW12, Eg-rCW24, for detection of antibodies in serum, showed a sensitivity of 88.00% and 91.00% respectively. The specificity of the Dot-ELISA using either Eg-rCW12 or Eg-rCW24 antigens was observed to be 94.44%. The sensitivity and specificity of the Dot-ELISA using *E. granulosus* recombinant antigen-B/2 (Rec Eg-AgB8/2) for detection of antibodies in serum was observed to be 90.00% and 93.33% respectively. In comparison, the efficiency of the Dot-ELISA using Eg-rCW24 (92.63%) for detection of IgG antibodies in serum was observed to be higher than the Dot-ELISA using both Eg-rCW12 (91.05%) and Rec Eg-AgB8/2 (91.57%).

The Dot-ELISA using Eg-rCW24 showed the highest efficiency in comparison to other two antigens (Eg-rCW12 and Rec Eg-AgB8/2) for demonstration of hydatid antibodies in serum. The ELISA using Eg-rCW24 however, showed a sensitivity similar to that of the ELISA using other antigens (Eg-rCW12 and Rec Eg-AgB8/2). The Dot-ELISA, therefore, using Eg-rCW24 showed to be a simple and rapid diagnostic test for diagnosis of CE.
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Evaluation of ELISA and Dot-ELISA to detect hydatid antigens in serum by using affinity purified antibodies raised against *E. granulosus* recombinant antigens for diagnosis of cystic echinococcosis

The Ag-ELISA using affinity purified anti-Eg-rCW12 and anti-Eg-rCW24 monospecific antibodies for detection of hydatid antigens in serum, showed a sensitivity of 80.00% and 83.00% respectively with a specificity of 97.77%. The Ag-ELISA using affinity purified anti-Rec Eg-AgB8/2 monospecific antibodies for detection of hydatid antigens in serum, showed a sensitivity of 83.00% and a specificity of 98.88%. In comparison, the efficiency of the Ag-ELISA using anti-Eg-rCW24 (90.00%) and anti-Rec Eg-AgB8/2 (90.52%) antibodies for detection of hydatid antigen in serum was observed to be higher than the ELISA using anti-Eg-rCW12 antibody (88.42%).

The Dot-ELISA using affinity purified anti-Eg-rCW12 and anti-Eg-rCW24 monospecific antibodies for detection of hydatid antigens in serum, showed a sensitivity of 76.00% and 83.00% respectively and a specificity of 96.66% and 98.88% respectively. The Dot-ELISA using affinity purified anti-Rec Eg-AgB8/2 monospecific antibodies for detection of hydatid antigens in serum, showed a sensitivity of 82.00% and a specificity of 96.66%. In comparison, the efficiency of the Dot-ELISA using anti-Eg-rCW24 antibodies (90.52%) for detection of hydatid antigens in serum was observed to be higher than the Dot-ELISA using both anti-Eg-rCW12 (85.78%) and anti-Rec Eg-AgB8/2 (88.94%) antibodies.

The Dot-ELISA using affinity purified anti-Eg-rCW24 antibody showed the highest efficiency in comparison to other two antibodies (anti-Eg-rCW12 and anti-Rec Eg-AgB8/2) for detection of hydatid antigens in serum. The Ag-ELISA using anti-Eg-rCW24 antibody showed the efficiency similar to that of the Ag-ELISA using other antibodies (anti-Eg-rCW12 and anti-Rec Eg-AgB8/2). The Dot-ELISA, therefore, using anti-Eg-rCW24 antibody showed to be a simple and rapid diagnostic test for diagnosis of CE.

In conclusion, in the present study, both Ag-ELISA and Dot-ELISA using affinity purified anti-Eg-rCW24 and anti-Eg-rCW12 monospecific antibodies for detection of hydatid antigens in serum showed high sensitivity and high specificity. Hence both the techniques could be used for diagnosis of CE. The Dot-ELISA, has the added advantage of being more convenient, simple, rapid, cheap and reliable, does not require costly equipment and requires only lesser amounts of specimens and reagents.