Chapter IV

Detection of hydatid antigen in the serum
Over the past many decades, serodiagnosis of cystic echinococcosis (CE) was based on the demonstration of circulating antibodies to *E. granulosus* in the serum. The major disadvantage of antibody detection in the serum is that it could not differentiate between past or current infection due to persistence of antibodies in the serum for a longer time even after surgical removal of the cyst or after chemotherapy. Moreover, the frequent occurrence of false negatives in surgically confirmed cases of CE is an inherent disadvantage of antibody detection assays (141). The occurrence of such false negatives has prompted to search for an alternative to antibody-based serology. This resulted in detection of cellular components and metabolites of the parasites that are present in blood stream during the acute phase of the infection.

Antigen detection in serum has been reported in many of the parasitic diseases such as amoebiasis (372, 373), malaria (374), leishmaniasis (375), filariasis (376), schistosomiasis (377) and also CE (129, 137, 170).

The detection of specific circulating antigen in serum is suggested to be more efficacious than antibody detection as it helps in the diagnosis of active infection and also in assessing post-chemotherapeutic treatment of CE (378). Therefore, attempts have been made in several studies to devise tests to detect circulating antigen in serum for the diagnosis of CE.

In CE, circulating hydatid antigen was first described in experimental *E. granulosus* infection of animals and subsequently in human infection (379). Immunodiffusion-in-gel was the first qualitative test to be employed for detection of circulating antigen in the diagnosis of six cases of CE (380). Subsequently, a double
antibody sandwich ELISA was developed and evaluated for the detection of circulating hydatid antigen (168).

The countercurrent immunoelectrophoresis (CIEP) (137), bacterial Co-agglutination (Co-A) test (129) and latex agglutination test (LAT) (170) have also been evaluated as simple and rapid diagnostic tests for detection of hydatid antigen in serum for the diagnosis of CE in the field or in the poorly equipped laboratories.

The circulating antigen in serum may be either free or bound with antibodies forming immune complexes. In CE, studies have demonstrated free antigen in serum by various assays such as CIEP (137), Co-A (129), LAT (170) and ELISA (168). Few studies have also demonstrated the presence of antigen in circulating immune complexes (CIC) in the serum for the diagnosis of CE (272, 273).

In this laboratory, the CIEP was standardized and evaluated for the first time to demonstrate circulating hydatid antigen in sera for the diagnosis of CE. The test showed a moderate sensitivity (55.55%) to detect antigen in surgically proved and a higher sensitivity (100%) in ultrasound proved CE cases. It was highly specific and no false positive reactions were observed with sera from diseased or healthy controls (137).

Another test, namely the Co-A test employing Staphylococcus aureus (Cowan I strain) bearing protein A coated with specific hydatid antibodies, was also used to demonstrate circulating hydatid antigen in the serum for the diagnosis of CE. The test had a sensitivity of 95% and specificity of 89% in detecting hydatid antigen in serum. False positive reaction was observed with 18.5% of control sera from patients with various other parasitic diseases (129).

The LAT using polyclonal hydatid antibody sensitized latex particles was also evaluated in this laboratory for detection of hydatid antigens in serum. The test could detect the circulating hydatid antigens in 13 of 18 (72%) patients with surgically confirmed CE, 17 of 26 (65%) patients with ultrasound proven CE and 19
of 47 (40%) presumptive cases of CE. The test detected antigen in 1 of 25 (4%) controls with other parasitic disease and no antigen was detected in the serum of 25 healthy controls. The LAT showed a sensitivity of 72%, a specificity of 98%, a positive predictive value of 93% and a negative predictive value of 91% (170).

**Sandwich ELISA**

Gottstein (168) employed double antibody sandwich ELISA to detect circulating antigens in patients with CE. He examined sera of 21 patients with CE and detected circulating antigens in seven of the patients sera in the concentration ranging between 310ng and 680ng protein per ml. The results showed 4 of 9 sera were positive for circulating antigen before and 3 of 9 sera were positive after operation for CE.

Craig (276) demonstrated circulating antigen, specific immune complexes (IgG and IgM) and specific antibodies (IgG, IgM, IgE and IgA) in the sera of CE patients from Turkana (Kenya) and the UK by ELISA. Specific IgG and IgM antibodies predominated in UK patients with CE, while all classes of specific antibodies were lower in the Turkana patients. Circulating antigen detected in 3% polyethylene glycol (PEG) precipitated complexes using peroxidase conjugated hyper immune human hydatid IgG was more specific in ELISA than either antibody or immune complex assays where peroxidase conjugated anti-human IgG was used (276). The sensitivity of circulating antigen was more with Turkana patients (90%) compared to UK patients (50%). In another study, Craig and Nelson (272) reported that sera from CE patients which were serologically negative in antibody tests often contained measurable circulating immune complexes and circulating antigens by sandwich ELISA.

In the present study the sandwich ELISA is evaluated for the detection of serum antigen in the diagnosis of CE.
CHAPTER IV

Dot-ELISA

The sandwich ELISA although highly sensitive and specific, is expensive and needs highly skilled personnel to perform the assay. The test also needs highly expensive reagents and sophisticated instruments which may not be available in many of the endemic areas with poorly equipped laboratories.

The search for relatively simple enzyme based immunoassay which does not require highly sophisticated equipment or skilled man power and the test which can be used in the field has led to evaluate the Dot-ELISA for serodiagnosis of many parasitic diseases (381-383).

The Dot-ELISA has been evaluated widely for detection of antibodies in the serum for the diagnosis of leishmaniasis (331), malaria (332), trypanosomiasis (332), fascioliasis (332), schistosomiasis (332) and CE (173). Subsequently, the Dot-ELISA has also been used for the detection of serum antigen in the diagnosis of malaria (381), bancroftian filariasis (382) and visceral leishmaniasis (383).

Reports are scanty on the use of Dot-ELISA for detection of serum antigen for diagnosis of CE (176). Therefore, in the present study, an attempt has been made to evaluate Dot-ELISA for detection of circulating hydatid antigen in the serum for the diagnosis of CE.

EITB

EITB, a highly sensitive and specific test, is increasingly used, for demonstration of specific antibodies in the serum, for the diagnosis of many parasitic diseases including the CE (151, 220, 268, 270). EITB for detection of specific and cross reactive antibodies in serum has been evaluated by many authors for the diagnosis of CE (151, 154, 156, 177, 178, 220, 229, 267, 269, 271, 325, 336, 356).
However, there are very few reports available on the use of EITB for detection of antigen in serum for diagnosis of parasitic diseases. The test has been evaluated for demonstration of serum antigen in the diagnosis of only toxoplasmosis (341). In that study, 79 serum specimens from pregnant women and 29 from immunocompromised patients were classified into three groups according to their serologic status to T. gondii as determined by immunofluorescence and ELISA: no antibodies (group 1), acute acquired infection (group 2) and reactivation (group 3). The samples were tested for the presence of circulating antigens of T. gondii by capture ELISA and EITB. The presence of circulating antigen was detected by at least one of the two techniques in 6 of 31 subjects in group 1, 51 of 68 subjects in group 2 and 7 of 9 subjects in group 3. Of the total of 108 serum specimens, 28 were found to be T. gondii positive by capture ELISA, 57 by EITB and 21 by both techniques. Among the nine polypeptides detected by EITB, 38 recognized p14, 17 recognized p8, and 16 recognized p30 (341).

There is no report on the use of EITB for detection of hydatid antigen in serum, although other tests such as Dot-ELISA and EITB have been used more frequently for detection of circulating antigen in many parasitic diseases (341, 381, 383). The use of EITB for detection of antigen in serum for diagnosis of CE is yet to be reported. Hence, in the present study an attempt is made to evaluate the EITB for the detection of hydatid antigen in the serum for diagnosis of CE.

**OBJECTIVE**

- To detect hydatid antigen in serum by sandwich ELISA, Dot-ELISA and EITB for the diagnosis of CE.
MATERIALS AND METHODS

Study groups

Samples are collected from the patients with surgically confirmed and ultrasound proven cystic echinococcosis (CE) (n=30), from presumptive cystic echinococcosis (n = 30), from patients with other diseases (n = 30), and healthy controls as mentioned earlier (Chapter II).

Specimen

Serum

Five milliliters of venous blood is collected from patients and controls under aseptic precautions and allowed to clot. The serum is separated and stored in duplicate at -20°C till use as described earlier (Chapter I).

Detection of hydatid antigen in serum

The hydatid antigen in the serum are detected by sandwich ELISA and Dot-ELISA using both polyclonal and monoclonal hydatid antibodies; and EITB using only polyclonal antibodies as described below.

Polyclonal hydatid antibodies are raised against complete homogenate hydatid (CHH) antigen as per the method of Shariff and Parija, 1993 (129) described earlier (Chapter III). Monoclonal antibodies are prepared against F4 fractions of affinity purified hydatid cyst wall antigen as described earlier (Chapter III). The specific monoclonal antibody (MAb.v11) obtained against 24kDa as described earlier (Chapter III) is used in sandwich ELISA and Dot-ELISA in the present study for detection of hydatid antigen.

Sandwich ELISA

The sandwich ELISA using polyclonal and monoclonal hydatid antibodies separately is carried out by detection of hydatid antigen in the serum as described below.
CHAPTER IV

Sandwich ELISA using polyclonal antibodies

The sandwich ELISA is evaluated by using polyclonal hydatid antibodies for the detection of hydatid antigen in the serum of the cases of CE and controls.

The optimum concentration of coating antibody (purified polyclonal hydatid antibodies), detecting antibody dilution (i.e., rabbit anti-hydatid IgG coupled to horse radish peroxidase – an in-house preparation) and sample serum is determined by checkerboard titration curve. The sensitivity of the affinity purified polyclonal antibodies based sandwich ELISA is determined using serial dilutions (from 5000 - 10ng/ml) of artificially added cyst wall antigens in healthy serum.

Sandwich ELISA procedure consists of following steps:

1. Capturing antibody coating: Polyvinyl high binding microtiter ELISA plates (NUNC, New Zealand) are coated with 100μl (2μg well in PBS, pH 7.2) of the affinity purified polyclonal hydatid antibodies (viz., complete homogenate hydatid [CHH] antigen) in PBS 7.2 and then incubated overnight at 4°C undisturbed.

2. Washing: The un-adsorbed antibodies are removed by washing the plates with washing buffer (sterile PBS 7.2 containing 0.1% Tween-20 (PBS-T) is used as the washing buffer).

3. Blocking: The uncoated reactive sites in the wells are blocked by PBS 7.2 containing 2% BSA by incubating for three hours at 37°C.

4. Washing: Plates are washed three times with PBS-T as before.

5. Addition of Sample and incubation: 50μl of serum is added and incubated for 1.5 hours at 37°C.

6. Washing: The plates are washed three times with PBS-T as before to remove unbound materials from the sample.

7. Secondary antibody (conjugate): Rabbit anti-hydatid IgG conjugated to HRP (in-house preparation) is used at a dilution of 1:400 with PBS 7.2 containing Tween-20 (0.05%) and 100μl volume is dispensed to all the wells and incubated for 1 hour at 37°C in dark.
Rabbit anti-hydatid IgG coupled to HRP is prepared in-house as per the method described by Harinath and Reddy (384).

**Coupling of Rabbit anti-hydatid IgG to horse radish peroxidase**

A two milliliter (500μg) of rabbit anti hydatid IgG is dialyzed overnight against sodium carbonate bicarbonate buffer pH9.5. Then, a fresh 0.2M solution of sodium meta periodate (NaIO₄) is prepared. 0.1 ml of this solution is added to 0.5 ml solution of horse radish peroxidase (HRP) (2mg in 0.5ml double distilled water). The mixture immediately turns green. This mixture is kept on shaker at room temperature for 20 minutes. It is then dialyzed against 1.0mM sodium acetate buffer pH4.4 at 4°C for 5 hours with 4-5 changes. The pH is raised to 9.0-9.5 by adding 10μl of 0.2M sodium carbonate bicarbonate buffer pH 9.5 and immediately 500μg (2 ml) of rabbit anti hydatid IgG is added. This mixture is kept on shaker for 2 hours at room temperature. 100μl of freshly prepared sodium borohydride solution is added and kept at 4°C. This conjugate is dialyzed overnight against 0.1M borate buffer pH7.4 at 4°C with 2-3 changes. Finally, BSA at a final concentration of 5mg / ml is added and distributed in vials and stored at -20°C.

**Determination of optimal dilution**

Flat-bottomed microtiter plate is coated with a known concentration of 5μg / well (100μl volume) of cyst wall antigen in PBS 7.2 per well and kept overnight at 4°C. The plates are washed with PBS-tween 20 and non-specific sites are blocked with blocking solution (PBS 7.2 with 2% BSA) for 3 hours at 37°C. The plates are washed thrice with PBS-tween 20. A 100μl of optimally diluted positive and negative serum (1:1000 in PBS-tween 20) is added in each well and incubated at 37°C for 1.30 hour. After washing, 100μl of doubling dilution (100, 200, 400, 800, 1600, 2400) of rabbit anti hydatid IgG-HRP conjugate diluted in PBS-tween 20 is added and incubated at 37°C for 1 hour. Final washing is followed by addition of 100μl of OPD substrate and incubated at room temperature in the dark. The reaction is stopped by adding 50μl of 2M sulphuric acid. The OD is read using ELISA reader at 450nm (Labsystems, Finland). The conjugate in highest dilution which gave
maximum difference between antibodies coated positive and negative control wells are considered optimal dilution.

8. **Washing**- Plates are washed three times with PBS-T as mentioned before to remove unbound conjugate.

9. **Plate development**- Substrate solution is prepared freshly by adding 6 mg of OPD (S.D. Fine Chemicals, India) in 10 ml of PBS 7.2 containing 0.05% Tween-20 and 10 μl H₂O₂. 100 μl volume of the substrate solution per well is dispensed to all the wells and incubated for 15-20 minutes at 37°C in the dark for the development of optimum colour.

10. **Stop reaction**- The reaction is stopped by adding 50 μl of 2M H₂SO₄ per well. The absorbance is recorded at 450 nm using ELISA reader (Labsystems, Finland).

Various controls used are: coating antibody blank, conjugate blank, substrate blank and healthy normal serum blank.

The cut-off value for sandwich ELISA is determined by ROC curve analysis as described earlier (Chapter II).

**Sandwich ELISA using monoclonal antibodies (MAb.v11)**

The sandwich ELISA is evaluated by using monoclonal hydatid antibodies (MAb.v11) for the detection of antigens circulated in the serum of the cases of CE and controls. The optimum concentration of capturing antibody and sample serum is determined by checkerboard titration curve. The sensitivity of the monoclonal antibody (MAb.v11) based sandwich ELISA is determined using serial dilutions (from 5000 - 10 ng/ml) of artificially added cyst wall antigens in healthy serum.

Sandwich ELISA procedure consists of following steps:

1. **Capturing antibody coating**: Polyvinyl high binding microtiter ELISA plates (NUNC, New Zealand) are coated with 100 μl (2 μg/well in PBS, pH 7.2) of the affinity purified hydatid antibody that is raised against complete
homogenate hydatid (CHH) antigen in PBS 7.2 and then incubated overnight at 4°C undisturbed.

2. **Washing:** The un-adsorbed antibodies are removed by washing the plates with washing buffer (sterile PBS 7.2 containing 0.1% Tween-20 (PBS-T) is used as the washing buffer).

3. **Blocking:** The uncoated reactive sites in the wells are blocked by PBS 7.2 containing 2% BSA by incubating for 3 hours at 37°C.

4. **Washing:** Plates are washed three times with PBS-T as described before.

5. **Addition of Sample and incubation:** 50μl of serum is added and incubated for 1.5 hours at 37°C.

6. **Washing:** The plates are washed three times with PBS-T as before to remove unbound materials from the sample.

7. **Detecting antibody:** 100μl of monoclonal antibody (MAb.v11) (culture supernatant without concentration) is added in order to bind the specific antigens captured by the capturing antibodies in the plate.

8. **Washing:** The plates are washed three times with PBS-T as before to remove unbound detecting antibodies.

9. **Secondary antibody (conjugate):** Goat anti-mouse-IgG-HRP conjugated secondary antibodies (Bangalore Genei, India) at a dilution of 1:2000 with PBS 7.2 containing Tween-20 (0.1%) is used as per the manufacturer's instruction and 100μl volume is dispensed to all the wells and incubated for 1 hour at 37°C in dark.

10. **Washing:** Plates are washed three times with PBS-T as before to remove unbound conjugate.

11. **Plate development:** Substrate solution is prepared freshly by adding 6 mg of OPD (S.D. Fine Chemicals, India) in 10 ml of PBS 7.2 containing 0.1% Tween-20 and 10μl H₂O₂. 100μl volume of the substrate solution per well is dispensed to all the wells and incubated for 15-20 minutes at 37°C in the dark for the development of optimum colour.

12. **Stop reaction:** The reaction is stopped by adding 50μl of 2M H₂SO₄ per well. The absorbance is recorded at 450nm using ELISA reader (Labsystems, Finland).
Various controls used are: coating antibody blank, detecting antibody blank, conjugate blank, substrate blank and healthy normal serum blank.

The cut-off value for the sandwich ELISA is determined by ROC curve analysis as described earlier (Chapter II).

Dot-ELISA

Dot-ELISA is evaluated by using both polyclonal and monoclonal hydatid antibodies separately for detection of hydatid antigen in the serum as described below.

*Dot-ELISA using polyclonal hydatid antibodies*

The Dot-ELISA is evaluated by using polyclonal hydatid antibodies for the detection of antigens circulated in the serum of the cases of CE and controls. The optimization of Dot-ELISA conditions are performed to known the concentration of coating antibody to be dotted and the dilution of serum used. The optimum concentration of detecting antibody dilution (i.e. purified rabbit anti-hydatid IgG coupled to horse radish peroxidase –an in-house preparation) as determined earlier is used.

Dot-ELISA procedure consisted of following steps:

1. *Capturing antibody coating*: A nitrocellulose membrane (NCM) (Hybond ECL, Amersham bioscience, Germany) of size 0.5 x 0.5 cm is cut and mounted onto a plastic strip (0.5 cm x 5cm). A volume of 2μl (2μg / strip) of affinity purified polyclonal hydatid antibodies is dotted on to individual strips and air dried for 30 min.

2. *Washing*: The un-adsorbed antibodies are removed by washing the strips with washing buffer (sterile PBS 7.2 containing 0.1% Tween-20 (PBS-T) is used as the washing buffer).
3. **Blocking:** The uncoated reactive sites in the strips are blocked by PBS 7.2 containing 2% BSA by incubating for 3 hours at 37°C in rocker shaker.

4. **Washing:** Strips are washed three times with PBS-T as described before.

5. **Sample serum dilution and incubation:** One ml of 1:10 dilution of the patient sera is prepared in PBS-T and the strips are incubated for 1.5 hours at 37°C with constant shaking.

6. **Washing:** The strips are washed three times with PBS-T as before to remove unbound antibodies in sample serum.

7. **Secondary antibody (conjugate) incubation:** Rabbit anti hydatid IgG conjugated to HRP (in-house preparation) is used at a dilution of 1:400 with PBS 7.2 containing Tween-20 (0.1%) and the strips are incubated for 1 hour at 37°C in the dark with constant shaking.

8. **Washing:** Strips are washed three times with PBS-T as before to remove unbound conjugate.

9. **Colour development:** Substrate solution is prepared freshly by adding 3 mg of 3, 3’ diaminobenzidine (DAB) (Sigma, USA) in 5 ml of PBS 7.2 containing 0.1% Tween-20 and 5μl H2O2. The strips are dispensed and incubated for 15-20 minutes at 37°C in dark under constant rocking for the development of colour.

10. **Stop reaction:** The reaction is stopped by washing with double distilled water.

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

**Dot-ELISA using monoclonal hydatid antibodies (MAb.v11)**

The Dot-ELISA is evaluated by using monoclonal hydatid antibodies (MAb.v11) for the detection of antigens circulated in the serum of the cases of CE and controls. The optimization of Dot-ELISA conditions are performed to known the concentration of coating antibody to be dotted and the dilution of serum used.
Dot-ELISA procedure consists of following steps:

1. Capturing antibody coating: A nitrocellulose membrane (NCM) (Hybond ECL, Amersham bioscience, Germany) of size 0.5 x 0.5 cm is cut and mounted onto a plastic strip (0.5 cm x 5 cm). A volume of 2μl (2μg / strip) of affinity purified polyclonal antibodies is dotted on to individual strips and air dried for 30 min.

2. Washing: The un-adsorbed antibodies are removed by washing the strips with washing buffer (sterile PBS 7.2 containing 0.1% Tween-20 (PBS-T) is used as the washing buffer).

3. Blocking: The uncoated reactive sites in the strips are blocked by PBS 7.2 containing 2% BSA by incubating for 3 hours at 37°C.

4. Washing: Plates are washed three times with PBS-T as described before.

5. Sample serum dilution and incubation: One ml of 1:10 dilution of the patient sera is prepared in PBS-T and the strips are incubated for 1.5 hours at 37°C with constant shaking.

6. Washing: The strips are washed three times with PBS-T as before to remove unbound materials from the sample.

7. Detecting antibody: 100μl of monoclonal antibody (MAb.v11) (culture supernatant without concentration) is added in order to bind the specific antigens captured by the capturing antibodies in the strip and incubated for 1 hour at 37°C with constant shaking.

8. Washing: The strips are washed three times with PBS-T as before to remove unbound detecting antibodies.

9. Secondary antibody (conjugate): Goat anti-mouse-IgG-HRP conjugated secondary antibody (Bangalore Genei, India) at a dilution of 1:2000 with PBS 7.2 containing Tween-20 (0.1%) is used as per the manufacturer’s instruction and the strips are incubated for 1 hour at 37°C in the dark with constant shaking.

10. Washing: Strips are washed three times with PBS-T as before to remove unbound conjugate.
11. **Colour development:** Substrate solution is prepared freshly by adding 3 mg of 3, 3' diaminobenzidine (DAB) (Sigma, USA) in 5 ml of PBS 7.2 containing 0.1% Tween-20 and 5µl H₂O₂. The strips are dispensed and incubated for 15-20 minutes at 37°C in dark under constant rocking for the development of colour.

12. **Stop reaction:** The reaction is stopped by washing with double distilled water.

**EITB**

EITB using polyclonal hydatid antibodies is evaluated by detection of hydatid antigen in the serum as described below. The protein estimation of each serum sample is determined by Lowry method and approximately 20µg of serum is used for the separation of protein in the serum.

The procedure of the EITB for demonstrating antigenic proteins in serum consists of the following steps (See appendix for preparations of reagents and buffers for EITB):

1. **SDS-PAGE of sample serum:** Serum sample is prepared at the concentration of 20µg in sample dilution buffer. Serum antigens are separated by SDS-PAGE under reducing and denaturing conditions in a homogenous gel (12%).

2. **Electroblotting:** The separated serum antigens are blotted onto nitro cellulose membrane (NCM) by using the blotting apparatus (Pharmacia Biotech, USA). The transfer is done at constant volt (100 volts) for 1 hour transfer time.

3. **Blocking:** The free reactive sites on the NCM are blocked by PBS 7.2 containing 2% BSA by incubating for 3 hours at 37°C under constant rocking.

4. **Washing:** Membrane is washed three times with PBS-T as before.
5. **Detecting antibody dilution and incubation:** The blot is incubated with 5ml of 1:100 dilutions (in PBS-T) of polyclonal hydatid antibodies for 1.5 hours at $37^\circ C$ under constant rocking to detect hydatid antigen.

6. **Washing:** The membrane is washed three times with PBS-T as before to remove the unbound antibodies.

7. **Secondary antibody (conjugate) incubation:** Goat anti-rabbit-IgG-HRP conjugated secondary antibody (Bangalore Genei, India) at a dilution of 1:1000 with PBS 7.2 containing Tween-20 (0.05%) is used as per the manufacturer’s instruction and 10 ml volume is dispensed to the membrane in a plastic tray and incubated for 1 hour at $37^\circ C$ in dark under constant rocking.

8. **Washing:** The membrane is then washed three times with PBS-T to remove the unbound conjugate. Washing at this step is done in the dark condition.

9. **Colour development:** Substrate solution is prepared freshly by adding 6 mg of DAB (Sigma, USA) in 10 ml of PBS 7.2 containing 0.05% Tween-20 and 10$\mu$l H$_2$O$_2$. The substrate solution is added to the plastic tray carrying the membrane and incubated for 15-20 minutes (or till development of colour) at $37^\circ C$ in dark under constant rocking.

10. **Stop reaction:** The reaction is stopped by washing with double distilled water. The membrane is dried by soaking in between folds of filter paper and looked for reactive antigenic bands.

**Statistical analysis**

Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and efficiency of the diagnostic methods are calculated as per the standard method (343). Distribution of OD values between different groups are plotted using Microsoft excel worksheet. One way analysis of variance (ANOVA) is applied to compare the OD values of four groups and the test of choice for further analysis of variance (of data from unequal samples) is the Scheffe’s tests using SPSS software. Statistical difference between serological tests is compared by univariate statistical test, $\chi^2$ using Epi info 2001. In all these tests, a P-value of $< 0.05$ is considered indicative of a statistically significant difference.
CHAPTER IV

Detection of hydatid antibodies in serum

Same batch of serum from patients of CE and controls used for antigen detection by sandwich ELISA, Dot-ELISA and EITB, are also tested for serum antibodies by the ELISA, Dot-ELISA and EITB as described earlier (Chapter II).

RESULTS

Detection of hydatid antigens in the serum

Sandwich ELISA

The results of sandwich-ELISA using polyclonal and monoclonal antibodies (MAb.v11) for the detection of hydatid antigens in serum from the cases of CE and controls are summarized in Table 19.

**Sandwich ELISA using polyclonal hydatid antibodies**

The optimum concentration of coating antibody (affinity purified polyclonal antibodies) is 2μg/well and optimum serum concentration is 50μl/well. The optimal dilution of rabbit anti-hydatid IgG coupled to HRP (in-house preparation) is 1:400.

The affinity purified polyclonal antibody is capable of detecting 312ng/ml of cyst wall antigens in the serum of healthy controls (Figure 20). The ROC curve showed an area of 0.137 ± 0.066 that indicates excellent discriminating ability, established through the reaction of affinity purified polyclonal antibody with 30 healthy controls.

The sandwich ELISA using polyclonal hydatid antibodies demonstrated hydatid antigens in 23 out of 30 (76.66%) serum samples of the cases of confirmed CE (Group-I) and 22 out of 30 (73.33%) serum of suspected cases (Group-II). The test also detected hydatid antigens in 26.66% serum of parasitic disease controls (Group-III) and 16.66% serum sample of healthy controls (Group IV).
**Sandwich ELISA using monoclonal hydatid antibodies (MAb.v11)**

The optimum concentration of coating antibody (affinity purified polyclonal antibodies) is 2μg / well and the optimum serum concentration is 50μl / well. The optimum concentration of detecting antibody [monoclonal antibodies (MAb.v11)] is 100μl / well.

The monoclonal antibody (MAb.v11) is capable of detecting 500ng / ml of cyst wall antigens in the serum of healthy controls (Figure 21). The ROC curve showed an area of 0.146 ± 0.026 that indicates excellent discriminating ability, established through the reaction of affinity purified polyclonal antibody with 30 healthy controls.

![Figure 20: Titration of hydatid cyst wall antigen preparations in normal human serum by using polyclonal antibodies to know antigen detection limit in serum by ELISA (Detection limit - 312ng / ml).](image-url)
Table 19: Detection of hydatid antigen in the serum of cystic echinococcosis cases and controls by sandwich ELISA using polyclonal and monoclonal (MAb.v11) antibodies

<table>
<thead>
<tr>
<th>Subject groups</th>
<th>Detection of hydatid antigen in serum by sandwich ELISA using</th>
<th>Polyclonal antibodies</th>
<th>Monoclonal antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>*Range of OD (mean, SD)</td>
<td>No positive (%)</td>
<td>*Range of OD (mean, SD)</td>
</tr>
<tr>
<td>Confirmed cases</td>
<td>0.32-0.10 (0.23, 0.06)</td>
<td>23 (76.66%)</td>
<td>0.35-0.10 (0.21, 0.06)</td>
</tr>
<tr>
<td>(n=30)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suspected cases</td>
<td>0.24-0.10 (0.19, 0.04)</td>
<td>22 (73.33%)</td>
<td>0.38-0.12 (0.20, 0.06)</td>
</tr>
<tr>
<td>(n=30)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disease controls</td>
<td>0.21-0.10 (0.14, 0.04)</td>
<td>8 (26.66%)</td>
<td>0.17-0.10 (0.13, 0.02)</td>
</tr>
<tr>
<td>(n=30)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy controls</td>
<td>0.21-0.10 (0.13, 0.03)</td>
<td>5 (16.66%)</td>
<td>0.17-0.11 (0.14, 0.01)</td>
</tr>
<tr>
<td>(n=30)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: *Range of OD - Significant (P=0.000) by one way ANOVA between 4 groups; Scheffe's method: Significant (P=0.000), when individual comparison between confirmed cases and healthy controls were analyzed.

The sandwich ELISA using monoclonal hydatid antibodies (MAb.v11) demonstrated hydatid antigen in 20 out of 30 (66.66%) serum samples of the cases of confirmed CE (Group-I) and 18 out of 30 (60%) serum of suspected cases (Group-II). The test did not show any hydatid antigen in the serum sample of parasitic disease controls (Group-III) and healthy controls (Group IV). The results of sandwich ELISA using monoclonal antibodies (MAb.v11) for detection of circulating hydatid antigen in the serum from the cases of CE and controls is summarized in Table 19.

Figure 21: Titration of hydatid cyst wall antigen preparations in normal human serum using monoclonal antibodies to know antigen detection limit in serum by ELISA (Detection limit - 500ng/ml).
CHAPTER IV

Statistical analysis

Distribution of optical density (OD) values of sera from confirmed CE and control cases by sandwich ELISA with polyclonal and monoclonal antibodies (MAb.v11) for the detection of hydatid antigen in serum are represented in a scatter plot (Figure 22 and 23).

Figure 22: Scatter plot showing OD values at 450nm for antigen detection in serum by ELISA using polyclonal antibodies. Groups 1, 2, 3 and 4 represents confirmed cystic echinococcosis, suspected cases, disease controls and healthy controls respectively.

Figure 23: Scatter plot showing OD values at 450nm for antigen detection in serum by ELISA using monoclonal antibodies (MAb.v11). Groups 1, 2, 3 and 4 represents confirmed cystic echinococcosis, suspected cases, disease controls and healthy controls respectively.
CHAPTER IV

The OD values of the 4 groups are compared together by using one way anova for sandwich ELISA using polyclonal antibodies and sandwich ELISA using monoclonal antibodies. The test showed a highly significant difference between the sandwich ELISA using polyclonal antibodies (P=0.000) and sandwich ELISA using monoclonal antibodies (P=0.000). Of the possible individual comparisons between groups, that between confirmed cases and healthy controls, which is of interest of the study showed a highly significant difference for sandwich ELISA using polyclonal antibodies (P=0.000) and sandwich ELISA using monoclonal antibodies (P=0.000) when analyzed by Scheffe’s method.

When $\chi^2$ test applied, there is significant difference in the sensitivity between the sandwich ELISA using polyclonal antibodies and that using monoclonal antibodies (MAb.v11) (P=0.008) for the detection of hydatid antigen in serum.

The sensitivity, specificity, PPV, NPV and efficiency of the sandwich ELISA carried out with polyclonal and monoclonal antibodies for the detection of circulating hydatid antigen in serum are presented in Table 20.

Table 20: Diagnostic evaluation of sandwich ELISA for detection of hydatid antigen in serum of cases of cystic echinococcosis and controls

<table>
<thead>
<tr>
<th>DIAGNOSTIC EVALUATION CRITERIA</th>
<th>Diagnostic evaluation of sandwich ELISA for detection of hydatid antigen in serum using</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POLYCLONAL ANTIBODIES</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>76.66%</td>
</tr>
<tr>
<td>Specificity</td>
<td>83.33%</td>
</tr>
<tr>
<td>Diagnostic efficiency</td>
<td>80%</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>82.14%</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>78.12%</td>
</tr>
</tbody>
</table>

Dot- ELISA

An optimum concentration of 2µg per dot of coating antibodies could detect hydatid antigen at 1:10 serum dilutions for both Dot-ELISA using polyclonal
antibodies and Dot-ELISA using monoclonal antibodies. The results of Dot-ELISA using polyclonal and monoclonal antibodies (MAb.v11) for the detection of hydatid antigens in the serum from the cases of CE and controls are summarized in Table 21.

Table 21: Detection of hydatid antigen in serum of cystic echinococcosis cases and controls by Dot-ELISA

<table>
<thead>
<tr>
<th>Subject groups (n=120)</th>
<th>Detection of hydatid antigen in serum by Dot-ELISA using Polyclonal antibodies</th>
<th>Monoclonal antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confirmed cases (n=30)</td>
<td>18 (60%)</td>
<td>12 (40%)</td>
</tr>
<tr>
<td>Suspected cases (n=30)</td>
<td>19 (63.33%)</td>
<td>11 (36.66%)</td>
</tr>
<tr>
<td>Disease controls (n=30)</td>
<td>8 (26.66%)</td>
<td>0</td>
</tr>
<tr>
<td>Healthy controls (n=30)</td>
<td>2 (6.66%)</td>
<td>0</td>
</tr>
</tbody>
</table>

**Dot-ELISA using polyclonal hydatid antibodies**

The Dot-ELISA using polyclonal hydatid antibodies demonstrated hydatid antigen in 18 out of 30 (60%) serum samples of the cases of confirmed CE (Group-I) and 19 out of 30 (63.33%) serum of suspected cases (Group-II). The test also detected hydatid antigen in 8 out of 30 (26.66%) serum of parasitic disease controls (Group-III) and 2 out of 30 (6.66%) serum samples of healthy controls (Group IV).

**Dot-ELISA using monoclonal hydatid antibodies**

The Dot-ELISA using monoclonal hydatid antibodies demonstrated hydatid antigens in 12 out of 30 (40%) serum samples of the cases of confirmed CE (Group-I) and 11 out of 30 (36.66%) serum of suspected cases (Group-II). The test did not show any hydatid antigen in the serum sample of parasitic disease controls (Group-III) and healthy controls (Group IV).

**Statistical analysis**

When $\chi^2$ test applied, there is significant difference in the sensitivity between polyclonal and monoclonal antibodies (MAb.v11) by Dot-ELISA ($P=0.006$) for the detection of hydatid antigen in the serum.
CHAPTER IV

The sensitivity, specificity, PPV, NPV and efficiency of the Dot-ELISA carried out with polyclonal and monoclonal antibodies for the detection of circulating hydatid antigen in the serum are presented in Table 22.

Table 22: Diagnostic evaluation of Dot-ELISA for detection of hydatid antigen in serum of cases of cystic echinococcosis and controls

<table>
<thead>
<tr>
<th>DIAGNOSTIC EVALUATION CRITERIA</th>
<th>Diagnostic evaluation of Dot-ELISA for detection of hydatid antigen in serum using</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Polyclonal antibodies</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>60%</td>
</tr>
<tr>
<td>Specificity</td>
<td>93.33%</td>
</tr>
<tr>
<td>Diagnostic efficiency</td>
<td>76.66%</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>90%</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>70%</td>
</tr>
</tbody>
</table>

Table 23: Comparative evaluation of Sandwich ELISA and Dot-ELISA for detection of hydatid antigen in serum using polyclonal antibodies

<table>
<thead>
<tr>
<th>DIAGNOSTIC EVALUATION CRITERIA</th>
<th>Diagnostic evaluation of polyclonal antibodies for detection of hydatid antigen in serum using</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sandwich ELISA</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>76.66%</td>
</tr>
<tr>
<td>Specificity</td>
<td>83.33%</td>
</tr>
<tr>
<td>Diagnostic efficiency</td>
<td>80%</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>82.14%</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>78.12%</td>
</tr>
</tbody>
</table>

Table 24: Comparative evaluation of Sandwich ELISA and Dot-ELISA for detection of hydatid antigen in serum using monoclonal antibodies

<table>
<thead>
<tr>
<th>DIAGNOSTIC EVALUATION CRITERIA</th>
<th>Diagnostic evaluation of monoclonal antibodies (MAb v11) for detection of hydatid antigen in serum using</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sandwich ELISA</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>66.66%</td>
</tr>
<tr>
<td>Specificity</td>
<td>100%</td>
</tr>
<tr>
<td>Diagnostic efficiency</td>
<td>83.33%</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>100%</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>75%</td>
</tr>
</tbody>
</table>
CHAPTER IV

Table 25: Serum hydatid antibodies and serum hydatid antigen in cystic echinococcosis confirmed cases by various immunoassays

<table>
<thead>
<tr>
<th>Confirmed CE cases (n=30)</th>
<th>Serum antibody detection by</th>
<th>Serum antigen detection by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ELISA</td>
<td>Sandwich ELISA using polyclonal antibodies</td>
</tr>
<tr>
<td></td>
<td>Dot-ELISA</td>
<td>Sandwich ELISA using monoclonal antibodies</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dot-ELISA using polyclonal antibodies</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dot ELISA using monoclonal antibodies</td>
</tr>
<tr>
<td>Positive</td>
<td>29</td>
<td>23</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>7</td>
</tr>
</tbody>
</table>

EITB

The results of EITB using polyclonal hydatid antibodies for the detection of hydatid antigen in the serum from CE patients and controls are provided in the Table 26.

A total of five antigenic peptides of Mr 92kDa, 40kDa, 35kDa, 30kDa and 24kDa are demonstrated by the EITB in serum samples. Of these, three antigenic peptides (viz., 92kDa, 30kDa and 24kDa) are found to be of diagnostic value (Figure 24).

The polyclonal hydatid antibodies reacting with one or more of the three peptides in the serum is considered positive by the test. The EITB for hydatid antigen is positive in the serum from 25 of 30 (83.33%) confirmed CE cases (Group I) and in 22 of 30 (73.33%) suspected cases (Group II). The test showed a false positive reaction in 2 sera of 30 (6.66%) parasitic disease controls. The test is negative with sera from healthy controls (Table 26).
Figure 24: Detection of antigen in serum from cases of cystic echinococcosis and controls by EITB using polyclonal hydatid antibody. P: Standard positive serum, N: known negative serum, 1 to 30 depicts the number of cases/controls.
Table 26: Detection of hydatid antigen in serum of cystic echinococcosis cases and controls by EITB

<table>
<thead>
<tr>
<th>Subject groups (n=120)</th>
<th>EITB for hydatid antigen in serum detected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>92kDa</td>
</tr>
<tr>
<td>Confirmed cases (n=30)</td>
<td>5 (16.66%)</td>
</tr>
<tr>
<td>Suspected cases (n=30)</td>
<td>0</td>
</tr>
<tr>
<td>Disease controls (n=30)</td>
<td>0</td>
</tr>
<tr>
<td>Healthy controls (n=30)</td>
<td>0</td>
</tr>
</tbody>
</table>

The 92kDa, 30kDa and 24kDa antigenic peptides in the serum samples from confirmed CE cases (Group I) using polyclonal hydatid antibodies are recognized with a frequency of 16.66%, 63.33%, and 70% respectively. None of the suspected cases (Group II), disease controls (Group-III) and healthy controls (Group IV) are reactive to the 92kDa antigenic peptide. The 30kDa antigenic peptide is found reactive to polyclonal hydatid antibodies in the serum samples from suspected cases (Group-II) and disease controls (Group-III) with a frequency of 36.66% and 6.66% respectively. None of the healthy controls are reactive (Group IV) to the 30kDa antigenic peptide (Table 26). The 24kDa antigenic peptide is found reactive in the serum samples from suspected cases (Group-II) with a frequency of 56.66%. None of the disease controls (Group-III) and healthy controls (Group IV) are reactive to the 24kDa antigenic peptide. In addition polyclonal hydatid antibodies reacted with the 40kDa (90%), and 35kDa (60%) antigenic peptides in the serum of cases of confirmed CE. Moreover the polyclonal hydatid antibodies recognized the 40kDa antigenic peptide in 17 control sera (11 disease control sera and 6 healthy control sera) and 35kDa antigen in 15 control sera (9 disease control sera and 6 healthy control sera) nonspecifically.

**Statistical analysis**

The sensitivity, specificity, PPV, NPV and efficiency of the EITB carried out with polyclonal antibodies for the detection of circulating hydatid antigen in the serum are presented in Table 27.
Comparative evaluation of sandwich ELISA, Dot-ELISA and EITB for hydatid antigen detection in serum

The results of the comparative evaluation of sandwich ELISA and Dot-ELISA using polyclonal and monoclonal antibodies and EITB is summarized in the Table 28.

Table 27: Diagnostic evaluation of EITB for detection of hydatid antigen in serum of cases of cystic echinococcosis and controls

<table>
<thead>
<tr>
<th>DIAGNOSTIC EVALUATION CRITERIA</th>
<th>Diagnostic evaluation of EITB for detection of hydatid antigen in serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>83.33%</td>
</tr>
<tr>
<td>Specificity</td>
<td>100%</td>
</tr>
<tr>
<td>Diagnostic efficiency</td>
<td>91.66%</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>100%</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>85.71%</td>
</tr>
</tbody>
</table>

Table 28: Comparative diagnostic evaluations of ELISA, Dot-ELISA and EITB for the detection of hydatid antigen in the serum

<table>
<thead>
<tr>
<th>Diagnostic evaluation criterion</th>
<th>Sandwich ELISA</th>
<th>Dot-ELISA</th>
<th>EITB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Polyclonal antibodies</td>
<td>Monoclonal antibodies</td>
<td>Polyclonal antibodies</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>76.66%</td>
<td>66.66%</td>
<td>60%</td>
</tr>
<tr>
<td>Specificity</td>
<td>83.33%</td>
<td>100%</td>
<td>93.33%</td>
</tr>
<tr>
<td>Diagnostic efficiency</td>
<td>80%</td>
<td>83.33%</td>
<td>76.66%</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>82.14%</td>
<td>100%</td>
<td>90%</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>78.12%</td>
<td>75%</td>
<td>70%</td>
</tr>
</tbody>
</table>

Detection of hydatid antibodies in the serum

The result of sandwich ELISA, Dot-ELISA and EITB for the serum antibodies is mentioned in the Chapter II.
DISCUSSION

Most antibody detection assays in CE are neither sensitive nor very specific and there is no correlation between the levels of anti-hydatid antibodies and the size or location of hydatid cyst (159). Moreover, anti-hydatid antibodies persist for years after surgical removal of the cyst (159). The non-specificity of the antibody detection assays is thought to be due to sharing with other helminths (385).

The antibody detection assays in CE fails to differentiate the current infection from past due to the residential nature of antibodies in circulation (137). Hydatid antigen is present in serum possibly due to the biological permeability of minute volume of fluid antigen through the hydatid cyst wall, or secreted / excreted metabolic products of the hydatid cyst or due to deformed hydatid cyst induced by chemotherapy (109). The antigen is present during active infection, hence detection of serum hydatid antigen always indicates recent infection (137). Some studies have also shown the usefulness of the serum antigen detection for monitoring the post-treatment evaluation of the CE cases (273, 275, 287).

Many studies have evaluated various antigen-based tests for detection of hydatid antigen in the serum for diagnosis of CE. These include the CIEP (137), Co-A (129), LAT (170), ELISA (168, 259, 273, 287) and Dot-ELISA (176).

In the present study, ELISA, Dot-ELISA and EITB using polyclonal and monoclonal antibodies have been evaluated for the detection of hydatid antigen in sera for diagnosis of CE.

Sandwich ELISA

Sandwich ELISA is a highly sensitive test which enables qualitative or quantitative determination of specific antibody or antigen through chromogenic reaction (168). The assay is fast and accurate and is used to determine the antigen concentration in unknown samples. Sandwich 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.

In the present study, the sandwich ELISA using polyclonal antibodies showed a sensitivity of 76.66% and specificity of 83.33% (Table 20). The test demonstrated circulating antigens in 23 of 30 (76.66%) confirmed CE cases. At a cut-off point of 0.203, the sensitivity of the assay is found to be moderate (76.66%), which was low in comparison to higher sensitivity shown by the ELISA using monospecific antibodies against specific hydatid antigens (8kDa antigen and 116kDa antigen) reported by Kanwar and Vinayak (259). In their study, ELISA showed a sensitivity of 85% and 90 % respectively for detection of 8kDa antigen and 116kDa antigen in the serum for the diagnosis of CE.

The possible reason for increased sensitivity obtained in the study by Kanwar and Vinayak, (259), in comparison to the present study may be due to lack of adopting proper pretreatment of the serum samples as followed in other studies (259, 362). The use of monospecific anti-8kDa or anti-116kDa antibodies was 10% more efficient in detecting hydatid specific antigen in acid treated sera than in untreated sera. This may be due to the availability of relatively more free antigen after dissociation of immune complexes by glycine-HCl. It has been reported earlier (386) that the treatment of serum with polyethylene-glycol (PEG) preferentially precipitates larger immune complexes. Acid-treated sera are likely to contain a relatively greater quantity of antigen after dissociation of circulating immune complexes (259). This may explain the higher detection efficiency with sera treated with glycine-HCl than with untreated sera (259).

In other studies, sandwich ELISA using polyclonal antibodies for detection of hydatid antigen in the serum showed a low sensitivity compared to moderate sensitivity of 76.66% observed in the present study. Gottstein (168) employing double antibody sandwich ELISA using polyclonal antibodies demonstrated circulating antigen in concentrations of 355-580ng / ml in four of 10 patients (40%) with CE before operation. Candolfi et al. (287) demonstrated circulating antigens in
21% of sera form patients with CE. The author suggested that low sensitivity obtained by sandwich ELISA using polyclonal antibodies for the detection of hydatid antigen in the serum is due to the level of passage of parasite proteins from fissuration of cyst wall. Craig (276), in his study observed that the sensitivity of ELISA was 90% for Turkana patients but as low as 50% for UK patients.

The hydatid antigens in the serum have also been demonstrated by other simple assays such as CIEP (137), Co-A (129) and LAT (170) using polyclonal antibodies. In CIEP, a moderate sensitivity of 55.55% in surgically proved and high sensitivity of 100% in ultrasound proved CE cases was reported for the diagnosis of CE (137). 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 (129). The LAT another serum hydatid antigen-based test, showed a sensitivity of 72% and a specificity of 98% for the diagnosis of CE (170).

In the present study, the antigen detection limit of sandwich ELISA using polyclonal antibodies was 312ng / ml. This was lower than the antigen detection limit of 270ng / ml reported by Gottstein (168). The possible reason for difference in the detection limit of antigen might be due to qualitative and quantitative difference in the concentration of binding coating antibody and use of the serum sample dilution (276).

The sandwich ELISA showed a false positive reaction in 5 of 30 healthy controls (16.66%) (Table 19). In spite of using affinity purified polyclonal antibodies, the specificity of the sandwich ELISA is moderate (83.33%) (Table 20) in the present study and this may be due to interference with host components contaminating the antigen (168).

Ferragut et al. (387) analyzed 148 sera provided by retrospective post-surgical follow-up of 19 patients by ELISA. The results showed that 14 patients developed new cysts one to ten years after surgery while no new disease was observed in the other five. Some of the former patients showed circulating antigens,
as early as two months after surgery while no circulating antigens were observed in the other five patients at any time. In addition, a collection of 38 sera obtained before surgery were similarly tested and five of them showed only circulating antigens, while 18 showed only antibodies and 12 sera showed antibodies and circulating antigens. Based on this, the authors suggested that circulating antigen may be an early marker of CE, thus being relevant for post-surgical follow-up.

It is suggested that the use of monoclonal antibodies, being of single specificity, provides unique opportunities for improving circulating antigen detection in the serum when compared with polyclonal antibodies (239).

In the present study, the sandwich ELISA using monoclonal antibodies (MAb.v11) derived from native 24kDa antigen is used for the detection of hydatid antigen in CE. At a cut-off point of 0.172, circulating antigen levels are measured by sandwich ELISA using monoclonal antibodies (MAb.v11) with a sensitivity of 66.66% and specificity of 100%, with no evidence of cross-reactivity with other parasites (Table 20).

The sandwich ELISA using monoclonal antibodies (MAb.v11), in the present study, demonstrated relatively a high sensitivity (66.66%) than the sandwich ELISA using monoclonal antibodies for demonstration of hydatid antigen in the serum, as reported by Liu et al. (239). In their study, 200 sera from 42 CE patients were tested for circulating antigen by ELISA using four separate monoclonal antibodies against antigen 5 and antigen B. Of the 200 sera from 42 CE patients examined, 11 (5.5%) were shown to have circulating antigen detectable by MAb 24.14, 16 (8%) by MAb 61A12, 15 (7.5%) by MAb 31.15 and 15 (7.5%) by MAb 39B3. The combined detection rate using these four monoclonal antibody was 19% (38/200) (239).

The potential use of sandwich ELISA using monoclonal antibodies (MAb.v11) is limited by its low sensitivity (500ng / ml) when compared with sandwich ELISA using polyclonal antibodies (312ng / ml), as observed in the present study and in other study (239). A significant difference (P= 0.008) is
observed in the sensitivity between purified polyclonal antibodies (76.66%) and monoclonal antibodies (MAb.v11) (66.66%) for the detection of hydatid antigen in the serum with sandwich-ELISA.

It is suggested that the polyclonal antibodies tend to provide better sensitivity than monoclonal antibodies because polyclonal antibodies can react with multiple epitopes of the antigen. Another reason of low sensitivity with monoclonal antibodies is possibly due to the interfering effect of normal human serum components on the binding of monoclonal antibody with hydatid antigen (168, 272).

Craig and Nelson (272) were able to reduce the background binding in circulating antigen assay through acid treatment of patient sera, which resulted in an increase in non-specific binding of monoclonal antibodies. They suggested that this enhanced non-specific binding of monoclonal antibodies is possibly due to exposure of previously unavailable non-specific binding sites of serum by acid treatment.

Comparative analysis of the antigen detection data in relation to serum antibody data in the present study (Table 25) showed that the presence of circulating antigen detected by sandwich ELISA using polyclonal antibodies is negative in 7 of 30 confirmed CE cases. Similarly, the circulating antigen detected by sandwich ELISA using monoclonal antibodies is negative in 10 of 30 confirmed CE cases. Those sera that are negative for the circulating antigen by sandwich ELISA using polyclonal and monoclonal antibodies are all positive for the serum antibodies using ELISA with either of cyst wall antigen, protoscolex antigen and cyst fluid antigen. The failure to detect the circulating antigen in serum by sandwich ELISA either using polyclonal or monoclonal antibodies, as observed in the present study, is possibly due to antigens being present in the serum as the immune complexes and being not available as free antigen for detection (168). This is also supported by a study of D'Amelio et al. (142), in which they could able to detect immune complexes in patients with echinococcosis, but they were unable to identify free hydatid antigen in the serum.
CHAPTER IV

The result of present study suggests that sandwich ELISA can be used for detection of hydatid antigen in serum for diagnosis of CE in a well equipped laboratory.

The ELISA and its modifications are the most commonly used immunoassays so far available to detect circulating antigens in the serum of patients with CE (168). However, ELISA can be performed only in well equipped laboratories. Though the test is sensitive, it is expensive, requires technical expertise, perishable reagents and is difficult to adopt in small poorly equipped laboratories. In poorly equipped laboratories in the developing countries, there is a need for equally sensitive and specific, but simpler enzyme immunoassays such as Dot-ELISA that can be used in less well equipped laboratories.

Dot-ELISA

Dot-ELISA is a simple, rapid microassay which needs minute volume of reagents to perform, has a potential for the detection of hydatid antigen in the serum for diagnosis of CE.

In the present study, the Dot-ELISA is evaluated using affinity purified polyclonal antibodies and monoclonal antibodies (MAb.v11) for the detection of hydatid antigen in the serum. Dot-ELISA using polyclonal antibodies detected circulating antigens in 18 of 30 confirmed CE cases. The sensitivity of the assay is found to be 60 % (Table 22). Result of the study is in agreement with the study by Romia et al. (176). In their study, hydatid cyst fluid antigen and anti-echinococcal hyperimmune rabbit sera were used for the detection of antibodies and antigens respectively. They detected circulating echinococcal antigens using Dot-ELISA in 18 patients with hydatid disease and 32 healthy blood donors as controls. Their results showed that the Dot-ELISA was sensitive (55.6%) and rapid for detecting hydatid antigens. Low sensitivity was attributed to the small amounts of circulating antigens and / or immune complexes formation (176).
In bancroftian filariasis, Dot-ELISA was compared with sandwich ELISA for the detection of antigen in the sera. The result showed that in Dot-ELISA, 67 of 70 serum samples from microfilaremic patients were positive at a dilution of 1:50. End titers ranged from 1:80 to 1:1280. In sandwich ELISA, 64 of the 70 serum samples were positive at a dilution of 1:10. End titers ranged from 1:10 to 1:320. The specificity of both assays was over 91%, but their sensitivity was markedly different. Dot-ELISA could detect as little as 0.055ng / ml microfilarial antigens added to normal human serum, whereas the lower limit of detection by sandwich-ELISA was 10ng / ml parasite antigens (382).

In the present study, the sensitivity of the Dot-ELISA using polyclonal antibodies (60%) for the detection of hydatid antigen in the serum is relatively lower when compared to sandwich ELISA using polyclonal antibodies (76.66%). Two of 30 (6.66%) healthy controls showed false positive reactions (Table 21). Thus, the specificity of the test is 93.33% which is comparatively higher than the specificity obtained by sandwich-ELISA using polyclonal antibodies (83.33%) (Table 23).

In the present study, the Dot-ELISA using mouse monoclonal antibody (MAb.v11) identified 12 of 30 confirmed CE cases. The test showed a sensitivity of 40%, which is comparatively lower than that of 66.66% sensitivity of the sandwich ELISA using monoclonal antibody (MAb.v11) (Table 24). None of the healthy controls are positive for circulating antigen in the serum by the test. Thus the specificity of the assay is 100% which is similar to the 100% specificity of sandwich-ELISA using monoclonal antibodies (Table 24).

The difference in sensitivity between the Dot-ELISA and sandwich ELISA using either polyclonal antibodies or monoclonal antibodies is possibly due to the qualitative and quantitative difference in the performance of the assay.

The hydatid antigen detection data in relation to antibody in the serum is analyzed. It revealed that 12 of 30 confirmed CE cases are negative for the circulating antigen by Dot-ELISA using polyclonal antibodies. Similar analysis for circulating antigen detected by Dot-ELISA using monoclonal antibodies is negative in 18 of 30
confirmed CE cases (Table 25). Those sera which are negative for the circulating antigen by polyclonal and monoclonal antibodies based Dot-ELISA are all positive for antibody detection by either of cyst wall, protoscolex and cyst fluid antigens using Dot-ELISA.

In conclusion, Dot-ELISA using polyclonal antibodies for the detection of hydatid antigen in the serum showed a moderate sensitivity (60%) and relatively higher specificity (93.33%) while Dot-ELISA using monoclonal antibodies (MAb.v11) for the detection of hydatid antigen in the serum showed a sensitivity of 40% and specificity of 100% (Table 22). Though the assay is highly specific, the sensitivity is very low when compared with that of sandwich ELISA. As the test is easy to perform and there is no need of skilled person, it can be used in poorly equipped laboratories, and is suitable to screen large number of samples for seroepidemiological studies in the CE.

EITB

EITB is a sensitive method for visualizing specific proteins in complex antigenic mixtures. This technique involves separation of protein on SDS-PAGE, followed by immobilization on nitrocellulose sheet and subsequently identified by immunological reaction with antibody and enzyme labeled second antibody probe (385).

In the present study, EITB is used to analyze circulating hydatid antigen in the serum and to best of our knowledge, the first attempt made to detect the presence of hydatid antigen in the serum for diagnosis of CE.

SDS–PAGE separation of serum revealed 10 fractions in the range of 120kDa to 24kDa (120kDa, 105kDa, 100kDa, 92kDa, 83kDa, 77kDa, 40kDa, 35kDa, 30kDa and 24kDa) after staining with coomassie brilliant blue R-250.

The results of EITB revealed five antigenic peptides of Mr 92kDa, 40kDa, 35kDa, 30kDa and 24kDa as immunodominant peptides (Figure 24). Of these, three
antigenic peptides (viz., 92kDa, 30kDa and 24kDa) are found to be non-reactive with individuals having other parasitic diseases including filariasis ($n = 11$), neurocysticercosis ($n = 11$), toxoplasmosis ($n=3$), amoebiasis ($n=5$) and healthy controls ($n = 30$) indicating the potential of this antigenic peptides in serum as more specific for the diagnosis of active cases of CE.

In EITB, the Mr 24kDa is recognized as the most common antigens in the serum by majority of surgically confirmed and ultrasound proven CE patients. The Mr 24kDa antigen is detected in 21 of 30 (70%) serum samples of surgically confirmed and ultrasound proven CE patients by EITB (Table 26). EITB for the detection of hydatid antibodies in serum also showed 24kDa as more specific polypeptides with 86.66% sensitivity (Table 14).

The predominant presence of 24kDa protein secreted in the serum exhibited strong antigenicity as it is recognized by wide variety serum of confirmed CE cases. This is not surprising since this antigen has been recognized as the immunodominant epitope from cyst wall, protoscolex and cyst fluid antigens using serum of surgically confirmed and ultrasound proven CE patients, as observed in the present study. The presence of more specific and highly sensitive 24kDa in the serum shows that hydatid specific serum antigen (24kDa) is secreted from the parasite and reinforces the hypothesis that smaller macromolecules can be transported through hydatid cyst wall (284).

Next to Mr 24kDa, the EITB showed higher sensitivity for 30kDa polypeptide. The 30kDa polypeptide is found to be reactive in 19 of 30 (63.33%) CE confirmed patients (Table 26). The 30kDa antigenic molecule in the serum of CE confirmed patients is not recognized as immunodominant epitope from cyst wall, protoscolex and cyst fluid antigens by hydatid antibodies in the serum of confirmed CE cases. Thus, the presence of 30kDa antigenic polypeptide in the serum may be due to few sequestrated hydatid cyst or secreted / excreted metabolic products of the hydatid cyst or a protein produced by deformed parasite induced by chemotherapy that are not immunogenic in the host.
EITB also revealed a high molecular weight antigenic polypeptide of Mr 92kDa in 5 of 30 (16.66%) CE confirmed patients sera (Table 26). The sensitivity of Mr92kDa was lower and the presence of 92kDa only in 5 of 30 surgically confirmed and ultrasound proven CE patients might be possibly due to the strain variations of *E. granulosus* which needs further study for validation. Such strain variations in *E. granulosus* strains of different animal hosts has also been observed by Rafiei and Craig (156). In their study, they found differences in the reorganization of protein molecules in protoscoleces extracts of hydatid cysts obtained from different hosts such as sheep, horse and camel by the EITB (156).

The specific fractions of 92kDa and 24kDa are not identified in the serum of healthy or disease controls. Though the 30kDa molecule is not identified by healthy controls, but identified by 2 of the filarial sera. In the present study, two antigenic polypeptides of Mr40kDa and 35kDa are found to be non-specific as it exhibited cross reactions with sera of filariasis, neurocysticercosis and toxoplasmosis.

In the present study, very low molecular weight antigenic polypeptides are not found in the serum of confirmed CE patients. Reports of EITB for demonstration of the serum antigen in parasitic diseases are scanty (341). The test has only been used in toxoplasmosis for the detection of serum antigen. In toxoplasmosis, studies have shown the presence of only low molecular weight protein in the sera from the patients. Hafid et al. (341) have demonstrated low molecular weight proteins of 14kDa and 8kDa in the serum by EITB in a larger number of sera of pregnant women (79) and immunocompromised patients (29) suffering from toxoplasmosis. In another study, Hassl et al. (388) demonstrated low molecular weight polypeptides of Mr 27kDa, 32kDa and 57kDa in the serum of toxoplasmosis patients with AIDS by EITB. Moir et al. (389) have also reported low molecular weight proteins of Mr 6kDa, 22kDa, 25kDa, 28kDa, 29kDa, 36kDa and 37kDa in their study, in the serum of patients with acute and previous *T. gondii* infection by EITB.

In the present study, only polypeptides of high molecular weight to medium (92kDa, 30kDa and 24kDa) are recognized to be of diagnostic importance by EITB.
CHAPTER IV

Very low molecular weight antibodies such as 6kDa and other molecular weight polypeptides (22kDa, 25kDa, 28kDa, 29kDa, 36kDa and 37kDa) (389) and 14kDa and 8kDa (341), which were demonstrated by EITB in other parasitic disease such as toxoplasmosis is not found in the present study. The reason for the absence of such very low molecular weight antigenic polypeptides in the serum of CE cases might be due to alteration in structural conformation of the protein of hydatid antigen during circulation.

In the present study, the EITB showed an overall high sensitivity (83.33%) than sandwich ELISA and Dot-ELISA for the detection of hydatid antigen in the serum (Table 28). The EITB technique is a sophisticated procedure which needs highly skilled personnel, sophisticated instruments and expensive reagents to carry out the assay, therefore, is highly suitable for adaptation in a well equipped reference laboratory for confirming the diagnosis of CE.

Comparative evaluation of sandwich ELISA, Dot-ELISA and EITB for hydatid antigen detection in serum

Circulating hydatid antigen in the serum is detected in cases of CE and controls with sandwich ELISA and Dot-ELISA using polyclonal and monoclonal antibodies (MAb.v11); and EITB using polyclonal antibodies (Table 28). The results of the present study showed that sandwich ELISA with a diagnostic efficacy of 80% using polyclonal antibodies and 83.33% using monoclonal antibodies (MAb.v11) for detection of hydatid antigen in the serum. Dot-ELISA using polyclonal antibodies showed a diagnostic efficacy of 76.66% and that using for monoclonal antibodies showed a diagnostic efficacy of 70%. The EITB showed a diagnostic efficacy of 91.66%. The EITB was found to be the most specific test with 100% specificity for the detection of specific polypeptides in the serum. When the serum samples from 30 cases of CE examined by sandwich-ELISA, Dot-ELISA and EITB using polyclonal antibodies are combined, the positive rates is 83.33% whereas a overall false positive rate of 6.66% of antigen is detected in the serum of 30 healthy controls.
SUMMARY

The present study demonstrated the presence of circulating hydatid antigen in the sera of patients with CE by sandwich ELISA, Dot-ELISA using polyclonal and monoclonal antibodies and EITB using polyclonal antibodies.

The sandwich ELISA using polyclonal antibodies showed a sensitivity of 76.66%, specificity of 83.33%, diagnostic efficiency of 80%, positive predictive value of 82.14% and negative predictive value of 78.12%. The sandwich ELISA using monoclonal antibodies (MAb.v11) showed a sensitivity of 66.66%, specificity of 100%, diagnostic efficiency of 83.33%, positive predictive value of 100% and negative predictive value of 75% for the detection of hydatid antigen in CE.

The sera from 7 of 30 confirmed CE cases were negative for antigen by sandwich ELISA using polyclonal antibodies, while 10 sera of 30 confirmed CE cases were negative for antigen by sandwich ELISA using monoclonal antibodies. However all 30 sera from confirmed CE cases were all positive for the serum antibodies by ELISA using either cyst wall antigen, protoscolex antigen or cyst fluid antigen.

The Dot-ELISA using polyclonal antibodies showed a sensitivity of 60%, specificity of 93.33%, diagnostic efficiency of 76.66%, positive predictive value of 90% and negative predictive value of 70% for the detection of hydatid antigen in the serum. The sensitivity and specificity of Dot-ELISA using mouse monoclonal antibody (MAb.v11) is found to be 40% and 100% respectively. The test showed a diagnostic efficiency of 70%, positive predictive value of 100% and negative predictive value of 62.5%. The Dot-ELISA using polyclonal antibodies failed to detect circulating antigen in sera from 12 of 30 confirmed CE cases while the Dot-ELISA using monoclonal antibodies failed to detect antigen in sera from 18 of 30 confirmed CE cases. Those sera which were negative for the serum antigen by the polyclonal and monoclonal antibodies based Dot-ELISA were all positive for
antibody detection by Dot-ELISA using either cyst wall, protoscolex or cyst fluid antigens.

To best of our knowledge, this is the first attempt where EITB is used to detect circulating hydatid antigen in the serum for diagnosis of CE. The EITB revealed Mr 92kDa, 40kDa, 35kDa, 30kDa and 24kDa proteins as five immunodominant peptides. Of these, three antigenic peptides of Mr 92kDa, 30kDa and 24kDa are found to be non-reactive with individuals having other parasitic diseases and healthy controls, hence are considered as highly specific for the diagnosis of CE.

The Mr 24kDa antigen is detected in 21of 30 (70%) serum samples of surgically confirmed and ultrasound proven CE patients by EITB. Next to 24kDa, the EITB showed higher sensitivity for 30kDa polypeptide (63.33%). The high molecular weight antigenic polypeptide of Mr 92kDa was present in 5 of 30 (16.66%) CE confirmed patients sera.

The specific fractions of 92kDa and 24kDa are not identified in the serum of healthy or disease controls. Though the 30kDa molecule is not identified by healthy controls, 2 of the filarial sera are reactive.

The EITB for the presence of either one or combination of two or three of the diagnostically relevant peptides (92kDa, 24kDa and 30kDa) showed a sensitivity of 83.33%, specificity of 100%, diagnostic efficiency of 91.66%, positive predictive value of 100% and negative predictive value of 85.71%.

In the present study, two antigenic polypeptides of Mr40kDa and 35kDa are found to be non-specific as it exhibited cross reactions with sera of filariasis, neurocysticercosis and toxoplasmosis.