Chapter II

Detection of hydatid antibodies in the serum
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5. CHAPTER II

DETECTION OF HYDATID ANTIBODIES IN THE SERUM

Cystic echinococcosis is mostly asymptomatic, in its early stages and many cases go unreported in endemic countries (109). CE can be diagnosed only late in the symptomatic stages when significant pathology has already occurred (135). Hence, lack of pronounced clinical symptoms and weak immune response to larval antigens has generated interest to evaluate reliable serodiagnostic tests in CE.

The first attempt to devise a serological test for detection of circulating anti-
Echinococcus antibodies in serum was complement fixation test (244). After that, a number of tests have been developed for the detection of antibodies in the serum that includes agar-gel-precipitation test (244), bentonite flocculation test (205, 327), immunoelectrodiffusion (250), indirect immunofluorescent test (IIFT) (244) and radioimmunoassay (250).

The need for an immunodiagnostic test which is simple, rapid and yet sensitive in laboratory and field conditions has resulted in the development of various tests which includes: indirect haemagglutination test (IHAT) (143, 205, 245 – 252, 327), rapid IHA (115, 127), latex agglutination test (LAT) (245, 253, 327) and counter-current immuno electrophoresis (CIEP) (117, 244, 245, 246).

Most of these serodiagnostic tests show varying sensitivity and specificity for the demonstration of antibodies in the diagnosis of CE. Low sensitivity or false negative reaction is the major problem associated with the serological tests detecting the circulating antibodies. The possible factors contributing to the high incidence of false negative reactions are due to the presence of immune complexes, the location, the size and the fertility of hydatid cysts in the host as well as low antibody responses in the human hosts. The low specificity of the test is being mostly related
to cross reactions and false positive reactions either due to malignancies, cirrhosis of 
 liver or the presence of anti-P1 antibodies (220).

The need for improving specificity of the assay has involved research on cyst 
 fluid fractions (159). Attempts to identify the cyst fluid components that are 
 important in eliciting an antibody response have led to the identification of two 
 major parasite antigens: antigen 5 and antigen B. The demonstration of serum 
 antibodies precipitating Capron's antigen 5, by immunoelectrophoresis was earlier 
 considered to be specific for the diagnosis of CE. Despite its usefulness for various 
 serological tests, antigen 5 shows limitation in species specificity and diagnostic 
 sensitivity (227, 228). Antibodies to antigen 5 have been reported to occur in 
 patients with alveolar echinococcosis and neurocysticercosis. Antigen B, another 
 component of cyst fluid has been reported to be species specific by Shepherd and 
 McManus (220), but yet to be validated by other studies.

ELISA

During past two decades the serodiagnosis of CE has largely been improved 
 by the application of enzyme linked immunoassay (EIA) techniques such as standard 
 and rapid ELISA for IgG antibodies (254, 255), IgM-ELISA (256) and IgE- ELISA 
 (257) for detection of antibodies in the serum.

Sorice et al. (328) evaluated ELISA, and compared with IHA and CIEP 
 tests with 31 sera of patients with hydatid cysts, 17 sera of patients with other 
 infectious and parasitic diseases and 7 sera of blood donors for diagnosis of CE. The 
 result showed that ELISA was positive in 30 out of the 31 sera of the patients with 
 active CE (96.7%), while the IHA and CIEP were positive in 29 cases (93.5%). The 
 control sera from patients with other diseases proved to be all negative, except one 
 from a patient with active schistosomiasis.

ELISA and radioallergosorbent test (RAST) were evaluated for detection of 
 specific IgE antibodies in the serum of CE cases (329). Sera from 43 patients with
CE, eight with schistosomiasis, two with taeniasis, two with cancer and 16 healthy blood donors were tested by both the tests. The results showed that the ELISA was as sensitive as RAST (both the tests showed a sensitivity of 77%), but the RAST showed a higher rate of false positive reactions with sera from patients with other helminthic diseases (80% vs. 20%). Results of the study also revealed that specific IgE evaluation represents a useful addition to the conventional serodiagnostic tests (329).

In another study (251) ELISA using "Antigen 880" (which is believed to be similar to Antigen B) showed a sensitivity of 88% and specificity of 100% with 50 sera from Kenyan patients with surgically confirmed CE and 130 sera from individuals without CE. It was suggested that "Antigen 880" may be more useful than "Arc 5" antigen in the diagnosis of human CE in Kenya due to the high sensitivity obtained with the antigen.

ELISA, therefore, has been used more frequently than other serological tests because of its high sensitivity in CE (152, 153, 255, 330). However, ELISA is a technically cumbersome procedure, time consuming, needs technically skilled personnel and can be carried out only in well equipped laboratories. Although, promising results have been reported for ELISA in the detection of serum antibodies, still some difficulties exist with serological diagnosis of CE (212).

Dot-ELISA

Dot-ELISA is a highly versatile solid phase immunoassay, being increasingly used for detection of antibody in serum for the diagnosis of leishmaniasis (331), toxoplasmosis (332), malaria (332), trypanosomiasis (333), cysticercosis (334) and CE (173). The principle of Dot-ELISA is same as that of ELISA except that matrix used here is nitrocellulose membrane (NCM) glued to plastic strip instead of microtiter plate to carry out the test (258).
Rapid Dot-ELISA as a field test has been evaluated by many workers for the diagnosis of CE (147, 174 – 176, 265). The Dot-ELISA was evaluated for the detection of antibodies to purified antigen B by using 50 microliters of whole blood in a field assessment in the Turkana region of north-west Kenya (174). The ELISA showed a sensitivity of 94% and specificity of 90.3% for CE. They suggested that the Dot-ELISA is a rapid field test, inexpensive and simple to perform and recommended it to be a useful back-up to ultrasound scanning.

Dot-ELISA was compared with ELISA in a study reported by Sorice et al. (335). The two techniques were shown to be closely related (p greater than 0.001) and were highly sensitive (92.0% of positive results in 75 sera from patients with hepatic or pulmonary hydatidosis) and specific (93.5% of negative results in 31 sera from patients affected by other parasitic diseases and 100% of negative results in 30 normal controls). The authors observed that Dot-ELISA was more economical and more rapid (takes only 4 hours) than the conventional plate ELISA. They suggested that the Dot-ELISA could be a useful tool in field epidemiological surveys since it is sensitive, specific and simple to perform as it does not require any expensive apparatus or high technical expertise (335).

EITB

The EITB assay for the diagnosis of CE have been reported to be more specific than ELISA and Dot-ELISA. The latter two tests although are sensitive but are not highly specific and often show cross reaction with sera from patients with other cestode infections (166, 175, 212, 220, 259, 268, 270).

The EITB using cyst fluid from hydatid cysts of human (177, 178) and from animals such as camel (152-154), sheep (148-150), cattle (145-147) and swine (151) revealed various polypeptides of different molecular weight, of diagnostic importance.
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The EITB using cyst fluid revealed 8kDa, 29kDa and 34kDa as diagnostically relevant bands and exhibited 91% sensitivity and overall specificity of 97%. The test provided 99% discrimination between seropositive pre-operative CE cases and cross-reactive non-cestode parasitic infections or malignancies (336). EITB with cyst fluid revealed Mr 44kDa, 34kDa, 29kDa and 8kDa proteins to be Echinococcus specific and provided 100% sensitivity and specificity in CE. Cross-reactions were found with 27kDa, 21kDa, 16kDa and 13kDa proteins in sera of tumor patients and with 200kDa, 175kDa, 62kDa, 52kDa and 40kDa proteins in schistosomiasis (325). Kaddah et al. (152) in their study demonstrated EITB as a good confirmatory test showing 100% sensitivity, 100% specificity and 100% diagnostic efficacy.

Sbihi et al. (150) evaluated EITB for detection of antibodies in serum of CE patients with crude sheep hydatid cyst antigen preparations (total sheep hydatid fluid and homogenates of protoscoleces), purified fractions enriched in antigens 5 and antigen B, and glycoproteins from hydatid fluid. Polypeptide bands of 12kDa to 14kDa, 20kDa, and 34kDa, obtained by purified fractions, yielded a sensitivity of 95% and specificity of 100% when assayed with sera from noninfected humans and from patients suffering from other parasitic diseases. Glycoproteins obtained by subjecting hydatid fluid to chromatography through concanavalin A column revealed 42kDa band to be sensitive (95%) as well as highly specific (100%) for CE. The authors suggested that the purification procedures can strongly affect the diagnostic value of antigens with identical electrophoretic behaviour in SDS-PAGE.

Hydatid cyst fluid is primarily used as antigen in ELISA, Dot-ELISA and EITB for detection of serum antibodies in CE (146, 149, 153, 175, 176, 229, 287, 337). Most of these tests use crude, semi crude or purified fractions such as antigen 5 and antigen B of hydatid cyst fluid for diagnosis of CE (145, 148, 152, 159, 160, 172, 174, 225, 230, 312, 330). Reports, however, are very few on the use of both hydatid protoscolex (HPR) (156) and hydatid cyst wall (HCW) (158) as source of antigens in the diagnosis of CE.
In the present study as mentioned earlier (Chapter I), the SDS-PAGE analysis of crude cyst wall antigen showed twelve polypeptides of Mr 129kDa, 92kDa, 75kDa, 62kDa, 47kDa, 43kDa, 37kDa, 32kDa, 27kDa, 24kDa, 14kDa and 9kDa. The EITB revealed 92kDa, 24kDa and 9kDa as diagnostically relevant peptides. Among these three polypeptides, 24kDa was found to be markedly antigenic as it was recognized by sera of most of the cases of CE. The purified 24kDa cyst wall molecule is found to be glycoprotein, containing con A specific sugar and galactose residues, sensitive to periodate, heat stable and sensitive to proteolytic treatment. Therefore, in the present study, 24kDa cyst wall protein is used for the first time as a source of antigen in the Dot-ELISA for the diagnosis of CE.

In addition to this, attempt is made to evaluate the hydatid protoscolex, hydatid cyst wall and also hydatid cyst fluid antigens in the ELISA and Dot-ELISA but only the hydatid cyst wall antigen in the EITB for the diagnosis of CE.

So far somatic parasitic antigen obtained from different stages of the parasite was used in the serodiagnosis of many parasitic infections such as filariasis (338), cysticercosis (339, 340) and toxoplasmosis (341). Currently, one of the recent approaches in serodiagnosis of parasitic diseases is the use of urine as source of parasitic antigen (163, 164). Such an attempt has been made to identify species specific parasite antigen of diagnostic potential from urine and use them as antigen in serodiagnosis of schistosomiasis (163) and trypanosomiasis (164).

In CE, although the presence of hydatid antigen in urine has been demonstrated by CIEP (181) and Co-A (7), no reports are so far available in the literature on the use of urine as source of hydatid antigen for diagnostic use. The urinary hydatid antigen excreted is neither characterized nor studied for its possible use in serology of CE.

In the present study as mentioned earlier (Chapter I), for the first time we have analyzed the urine of confirmed CE cases by SDS-PAGE that showed six polypeptides of Mr 70kDa, 41kDa, 35kDa, 24kDa, 14kDa and 10kDa. The EITB revealed three polypeptides of Mr 24kDa, 14kDa and 10kDa to be of diagnostic
value. Of these three polypeptides, 24kDa is found to be present in the urine of majority of CE confirmed cases. This 24kDa urinary hydatid protein characterized immunochemically is found to be a glycoprotein, containing con A specific sugar and galactose residues, periodate-sensitive, heat stable and sensitive to proteolytic treatment. In the present study, therefore, an attempt is made to evaluate 24kDa urinary hydatid protein as source of antigen for use in the diagnosis of CE.

OBJECTIVES

- To evaluate protoscolex and cyst wall of hydatid cyst for their use as antigens in the serological diagnosis of CE.
- To detect hydatid antibodies in serum by ELISA, Dot-ELISA using cyst fluid, protoscolex and cyst wall antigens and by EITB using only cyst wall antigen for diagnosis of CE.
- To evaluate the Dot-ELISA using 24kDa urinary hydatid protein and 24kDa cyst wall proteins eluted from urine and hydatid cyst wall respectively, as antigens for serodiagnosis of CE.

MATERIALS AND METHODS

Study groups

The present study is conducted in the Department of Microbiology of Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER) Hospital, Pondicherry. Samples are collected from the patients with cystic echinococcosis (CE), patients with other diseases, healthy students and blood donors. The informed consent is obtained from all human adult participants.

The Groups included:

Group 1- Surgically confirmed and ultrasound proven cystic echinococcosis (n=30): The group included 10 cases of surgically confirmed and 20 cases of ultrasound-proved cases of CE.
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The cysts removed during surgery are confirmed to be of hydatid cyst etiology by histopathological evidence of germinal layer in the wall of the cyst and demonstration of sclerises and hooklets in the aspirated cyst fluid.

Ultrasound-proved CE cases included un-operated cases of CE but proved by ultrasonography. The cysts, which showed daughter cysts or prominent septation, and pathognomonic hydatid sands in cysts by ultrasound, are diagnosed as ultrasound-proven CE.

**Group 2-** Presumptive cystic echinococcosis (n = 30): This group included clinically diagnosed (presumptive) cases of CE. These patients presented with the clinical signs and symptoms of CE but are not operated; hence they are not proved surgically.

**Group 3-** Controls with other parasitic diseases (n = 30): This group included patients with other parasitic diseases other than CE like amoebiasis, filariasis, neurocysticercosis and toxoplasmosis.

**Group 4-** 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.

**Specimen**

**Serum**

Five milliliters of venous blood is collected with aseptic precautions from CE patients and controls as mentioned earlier (Chapter I).

**Hydatid antigens**

1. **Hydatid cyst fluid (HCF) antigen**

   The hydatid cyst fluid antigen is prepared from aspirated fluid of fertile hydatid cyst after centrifugation and dialysis as per the method described by Kanwar
et al. (259) (Chapter I). The protein content of cyst fluid antigen estimated by Lowry method is found to be 2mg/ml.

2. **Hydatid protoscolex (HPR) antigen**
   
The hydatid protoscolex (HPR) antigen is prepared by centrifuging the cyst fluid, collecting the sediment containing protoscoleces, washing and sonicating as per the method of Rafiei and Craig (156) described earlier (Chapter I). The protein content of protoscolex antigen estimated by Lowry method is found to be 2.5mg/ml.

3. **Hydatid cyst wall (HCW) antigen**
   
The hydatid cyst wall (HCW) antigen is prepared by homogenizing the collapsed cyst membranes and subsequently sonicating as per the method of Rafiei and Craig (156) described earlier (Chapter I). The protein content of cyst wall antigen is found to be 2.75mg/ml by Lowry method.

4. **24kDa urinary hydatid protein**
   
The diagnostically relevant hydatid protein from the urine of confirmed cases is identified by EITB using sera from cases of CE, disease controls and healthy controls as described earlier in the Chapter I. The molecular weight of the specific protein of the urine is found to be 24kDa as determined by SDS-PAGE. The 24kDa urinary hydatid protein is purified by manual elution and used as a source of antigen in serodiagnosis. The eluted urinary hydatid protein is estimated to be 20µg/ml.

5. **24kDa cyst wall protein**
   
The EITB of hydatid cyst wall antigen revealed three diagnostically relevant polypeptides of Mr92kDa, 24kDa and 9kDa using sera from cases of CE, disease controls and healthy controls as described earlier in the Chapter I. Of these three peptides, 24kDa hydatid cyst wall protein is markedly antigenic as it is recognized by most of the confirmed CE cases. Hence, the 24kDa hydatid cyst wall protein is taken for their use in serodiagnosis. The protein content of eluted cyst wall protein is found to be 18µg/ml.
Serological tests

ELISA

The ELISA is performed by using the crude extract of cyst fluid antigen, protoscolex antigen and cyst wall antigen separately for the detection of IgG antibodies in the same batch of serum samples from cases of CE and controls.

The optimum concentration of antigen and serum dilution is standardized by checkerboard titration. Serial dilutions of sera from healthy persons and confirmed CE cases are tested to produce a dilution curve. The optimum dilution is that which fell on the linear phase of the plot and gave best discrimination between positive and negative sera. The optimum antigen concentration was 5μg / well. All the test serum samples are analyzed at the optimum dilution of 1:1000 in sterile PBS 7.2 containing 0.1% Tween-20 (PBS-T).

ELISA using the cyst fluid antigen for detection of serum antibodies consisted of following steps:

1. *Antigen coating*: Polyvinyl high binding microtiter ELISA plates (NUNC, New Zealand) are coated with a volume of 100μl / well (5μg / well) of the cyst fluid antigen in PBS 7.2 and then incubated overnight at 4°C undisturbed.

2. *Washing*: The un-adsorbed antigen is 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. *Sample serum dilution and incubation*: 1:1000 dilution of the patient sera is prepared in PBS-T. 100μl of each diluted 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 antibodies in sample serum.
7. **Secondary antibody (conjugate) incubation:** 1:2000 dilution of rabbit anti-human-IgG-HRP conjugated secondary antibody (Bangalore Genei, India) is used as per the manufacturer's instruction with PBS 7.2 containing Tween-20 (0.05%) and 100μl volume is dispensed to all the wells and incubated for 0.5 hour at 37°C in dark.

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

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

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

Various controls used are: antigen blank, primary antibody blank, conjugate blank, substrate blank, healthy normal serum blank.

**The cut-off value for ELISA is determined by receiver operating characteristic (ROC) curve analysis**

The ROC curve is produced as described by El-morshedy et al. (342). The ROC curves are generated by varying the cut-off point for a positive test result and plotting the true-positive rate (TPR) (sensitivity) against the false positive rate (FPR) (1-specificity) for each cutoff. Curves that are closer to ideal point (TPR=1, FPR=0) that is closer to the upper left corner of the ROC curve are superior to curves that are lower and towards the right.

The same ELISA procedure is repeated by using separately protoscolex antigen and cyst wall antigen and tested for IgG antibodies in the same batch of serum samples from cases of CE and control groups as mentioned earlier.
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Dot-ELISA

The Dot-ELISA is performed by using five different antigens (crude extract of cyst fluid antigen, protoscolex antigen, cyst wall antigen, 24kDa urinary hydatid protein and 24kDa cyst wall protein) for the detection of IgG antibodies in the same batch of serum samples from cases of CE and controls. The optimization of Dot-ELISA conditions are performed to known the concentration of antigen to be dotted and the dilution of serum used.

Dot-ELISA using the cyst fluid antigen for detection of antibodies consisted of following steps:

1. **Antigen 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 /strip (5μg / strip) of cyst fluid antigen is dotted on to individual strips and air dried for 30 min.

2. **Washing:** The un-adsorbed antigen is 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 three hours at 37°C with constant shaking.

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

5. **Sample serum dilution and incubation:** 1:1000 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 the sample serum.

7. **Secondary antibody (conjugate) incubation:** 1:2000 dilution of rabbit anti-human-IgG-HRP conjugated secondary antibody (Bangalore Genie, India) is used as per the manufacturer's instruction with PBS 7.2 containing Tween-20 (0.1%) and incubated for 0.5 hours at 37°C in the dark in a rocker shaker.
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 is added just before adding to the plastic tray. 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 development of brown coloured spot in 2 – 5 minutes. When the reaction is complete, the strips are washed with distilled water and allowed to dry. Once dried, the strips are stored in the dark.

The same Dot-ELISA procedure is repeated by using separately crude antigens of protoscolex, cyst wall, 24kDa urinary hydatid protein and 24kDa cyst wall protein except that 4μl of 24kDa urinary hydatid protein and 24kDa cyst wall protein is dotted instead of 2μl of crude antigens of cyst fluid, protoscolex and cyst wall dotted on NCM. The Dot-ELISA using all these antigens are tested for IgG antibodies in the same batch of serum samples from cases of CE and controls as mentioned earlier.

**EITB**

Only hydatid cyst wall antigen is used in the EITB for detecting hydatid antibodies (IgG) in the same batch of serum samples from cases of CE and controls.

Briefly, the separated antigenic protein fractions in SDS-PAGE are blotted electrophoretically onto nitrocellulose membrane (NCM) (Hybond ECL, Amersham bioscience, Germany) as per the method devised by Towbin (266). The blotting is done using the blotting apparatus (Pharmacia Biotech, USA).
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The procedure of EITB for demonstrating reactive antigenic proteins consists of the following steps (See appendix for preparations of reagents and buffers for EITB):

1. **SDS-PAGE**: The antigenic mixture in the crude preparation of cyst wall is separated based on the difference in molecular weight by SDS-PAGE as per standard method described earlier (Chapter I).

2. **Electroblotting**: The separated antigens are blotted onto nitro cellulose membrane (NCM) (0.22 μm) by using the blotting apparatus (Pharmacia Biotech, USA). The transfer is done at constant volt (100 v) for 1 hour transfer time.

3. **Blocking**: The free reactive sites on the NCM (cut to strips) are blocked by PBS 7.2 containing 2% BSA by incubating for three hours at 37°C under constant rocking. This step enables the blocking of non-specific sites.

4. **Washing**: Membrane strips are washed three times with PBS-T as before.

5. **Sample serum dilution and incubation**: Five ml of 1:100 dilutions of the patient sera is prepared in PBS-T and the membrane strips are incubated for 1.5 hours at 37°C under constant rocking.

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

7. **Secondary antibody (conjugate) incubation**: 1:1000 dilution of rabbit anti-human-IgG-HRP conjugated secondary antibody (Bangalore Genie, India) is used as per the manufacturer's instruction with PBS 7.2 containing Tween-20 (0.05%) and the strips are placed in a plastic tray. This is incubated for 0.5 hour at 37°C in dark under constant rocking.

8. **Washing**: The membrane strips are washed three times with PBS-T as before to remove the unbound conjugate.

9. **Colour development**: Substrate solution is prepared freshly by adding 6 mg of 3, 3’ diaminobenzidine (DAB) (Sigma, USA) in 10 ml of PBS 7.2 containing 0.05% Tween-20 and 10μl H₂O₂ is added just before adding to the plastic tray. Five ml volume of the substrate solution per strip is dispensed and incubated for 15-20 minutes at 37°C in dark under constant rocking for the development of colour.

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

After blocking with BSA (2%), the membranes containing immobilized proteins are cut into strips and are screened with individual serum from confirmed CE cases and controls. After treating separately with individual serum, strips are subjected to immunostaining to identify the immunodominant fractions.

**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.

**RESULTS**

**ELISA**

The results of ELISA carried out with crude extract of cyst fluid antigen, protoscolex antigen and cyst wall antigen for detection of hydatid IgG antibodies in serum from the cases of CE and controls is summarized in Table 8.

An optimum concentration of $5\mu g$ / well of cyst fluid, protoscolex and cyst wall antigen could detect anti-hydatid antibodies at 1:1000 dilution of serum from cases of CE.
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ELISA using cyst fluid antigen demonstrated diagnostic level of antibodies in 24 out of 30 (80%) sera of confirmed cases of CE (Group-I) and 16 out of 30 (53.33%) sera of suspected cases (Group-II). The test also detected antibodies in 43.33% sera of parasitic disease controls (Group-III) and 6.66% sera of healthy controls (Group IV).

ELISA using protoscolex antigen demonstrated diagnostic level of antibodies in 21 out of 30 (70%) sera of confirmed cases of CE (Group-I) and 18 out of 30 (60%) sera of suspected cases (Group-II). The test also detected antibodies in 33.33% sera of parasitic disease controls (Group-III) and 10% sera of healthy controls (Group IV).

Table 8: Detection of total IgG antibodies in serum of cystic echinococcosis and controls by ELISA using cyst fluid, protoscolex and cyst wall antigens

<table>
<thead>
<tr>
<th>SUBJECT GROUPS (n=120)</th>
<th>Antibody detection in serum by ELISA using</th>
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<tbody>
<tr>
<td></td>
<td>CYST FLUID ANTIGEN</td>
</tr>
<tr>
<td></td>
<td>No positive (%)</td>
</tr>
<tr>
<td>Confirmed cases</td>
<td>24 (80%)</td>
</tr>
<tr>
<td>(n=30)</td>
<td></td>
</tr>
<tr>
<td>Suspected cases</td>
<td>16 (53.33%)</td>
</tr>
<tr>
<td>(n=30)</td>
<td></td>
</tr>
<tr>
<td>Disease controls</td>
<td>13 (43.33%)</td>
</tr>
<tr>
<td>(n=30)</td>
<td></td>
</tr>
<tr>
<td>Healthy controls</td>
<td>2 (6.66%)</td>
</tr>
<tr>
<td>(n=30)</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.
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ELISA using cyst wall antigen demonstrated diagnostic level of antibodies in 26 out of 30 (86.66%) sera of the cases with confirmed CE (Group-I) and 20 out of 30 (66.66%) sera of suspected cases (Group-II). The test also detected antibodies in 30% sera of parasitic disease controls (Group-III) and 10% sera of healthy controls (Group IV).

The cut-off value determination by receiver operating characteristic (ROC) curve analysis

The ROC curve showed an area of $0.235 \pm 0.212$ using cyst fluid antigen, $0.259 \pm 0.106$ using protoscolex antigen and $0.349 \pm 0.206$ using cyst wall antigen with ELISA that displayed the trade off in sensitivity and specificity as the cut off point is varied.

Statistical analysis

Distribution of optical density (OD) values of sera from confirmed CE cases and controls by ELISA with cyst wall antigen, protoscolex antigen and cyst fluid antigen for detection of IgG antibodies are represented in a scatter plot (Figure 13, 14 and 15).

![Figure 13](image)

Figure 13: Scatter plot showing OD values at 450nm for antibody detection in serum by ELISA using cyst fluid antigen. Groups 1, 2, 3 and 4 represents confirmed cystic echinococcosis, suspected cases, disease controls and healthy controls respectively.
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Figure 14: Scatter plot showing OD values at 450nm for antibody detection in serum by ELISA using protoscolex antigen. Groups 1, 2, 3 and 4 represents confirmed cystic echinococcosis, suspected cases, disease controls and healthy controls respectively.

Figure 15: Scatter plot showing OD values at 450nm for antibody detection in serum by ELISA using cyst wall antigen. Groups 1, 2, 3 and 4 represents confirmed cystic echinococcosis, suspected cases, disease controls and healthy controls respectively.

The OD values of the 4 groups are compared jointly by using one way anova for ELISA with cyst wall antigen, protoscolex antigen and cyst fluid antigen respectively. The test ($P=0.000$) showed a highly significant difference between the 4 groups using ELISA with either of cyst wall antigen, protoscolex antigen and cyst fluid antigen. 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 when analyzed by Scheffe’s method ($P=0.000$) for ELISA with cyst wall antigen, protoscolex antigen and cyst fluid antigen respectively.

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The sensitivity, specificity, PPV, NPV and efficiency of the ELISA carried out with crude extract of cyst wall antigen, protoscolex antigen and cyst fluid antigen for detection of hydatid IgG antibodies in serum are presented in Table 9.

Table 9: Diagnostic evaluation of ELISA for diagnosis of cystic echinococcosis

<table>
<thead>
<tr>
<th>Diagnostic Evaluation Criteria</th>
<th>Diagnostic evaluation of ELISA for detection of hydatid antibodies in serum using</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CYST FLUID ANTIGEN</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>80%</td>
</tr>
<tr>
<td>Specificity</td>
<td>93.33%</td>
</tr>
<tr>
<td>Diagnostic efficiency</td>
<td>86.66%</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>92.30%</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>82.35%</td>
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</tbody>
</table>

Dot-ELISA

The results of Dot-ELISA using crude extract of cyst wall antigen, protoscolex antigen and cyst fluid antigen and with 24kDa urinary hydatid protein and 24kDa cyst wall protein for detection of hydatid antibodies in serum from the cases of CE and controls is summarized in Table 10 and 11.

An optimum concentration of 5μg separately of cyst fluid, protoscolex and cyst wall antigen per dot could detect specific anti-hydatid IgG antibodies at 1:1000 serum dilutions of sera from CE cases.
Table 10: Detection of total Ig G antibodies in serum of CE cases and controls by Dot-ELISA using cyst fluid, protoscolex and cyst wall antigens for diagnosis of cystic echinococciosis

<table>
<thead>
<tr>
<th>SUBJECT GROUPS (n=120)</th>
<th>Antibody detection in serum by Dot-ELISA using</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CYST FLUID ANTIGEN</td>
</tr>
<tr>
<td>Confirmed cases (n=30)</td>
<td>28 (93.33%)</td>
</tr>
<tr>
<td>Suspected cases (n=30)</td>
<td>26 (86.66%)</td>
</tr>
<tr>
<td>Disease controls (n=30)</td>
<td>13 (43.33%)</td>
</tr>
<tr>
<td>Healthy controls (n=30)</td>
<td>5 (16.66%)</td>
</tr>
</tbody>
</table>

Dot-ELISA using cyst fluid antigen demonstrated diagnostic level of antibodies in 28 out of 30 (93.33%) sera of confirmed cases of CE (Group-I) and 26 out of 30 (86.66%) sera of suspected cases (Group-II). The test also detected antibodies in 43.33% sera of parasitic disease controls (Group-III) and 16.66% sera of healthy controls (Group IV).

Dot-ELISA using protoscolex antigen demonstrated diagnostic level of antibodies in 26 out of 30 (86.66%) sera of confirmed cases of CE (Group-I) and 24 out of 30 (80%) sera of suspected cases (Group-II). The test also detected antibodies in 40% sera of parasitic disease controls (Group-III) and 20% sera of healthy controls (Group IV).

Dot-ELISA using cyst wall antigen demonstrated diagnostic level of antibodies in 29 out of 30 (96.66%) sera of the cases with confirmed CE (Group-I) and 26 out of 30 (86.66%) sera of suspected cases (Group-II). The test also detected antibodies in 43.33% sera of parasitic disease controls (Group-III) and 16.66% sera of healthy controls (Group IV).
Table 11: Detection of total IgG antibodies in serum of cystic echinococcosis cases and controls by Dot-ELISA using eluted protein from hydatid cyst wall and urine

<table>
<thead>
<tr>
<th>SUBJECT GROUPS (n=120)</th>
<th>Dot-ELISA using eluted specific protein from</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CYST WALL ANTIGEN</td>
</tr>
<tr>
<td>Confirmed cases (n=30)</td>
<td>23 (76.66%)</td>
</tr>
<tr>
<td>Suspected cases (n=30)</td>
<td>9 (30%)</td>
</tr>
<tr>
<td>Disease controls (n=30)</td>
<td>3 (10%)</td>
</tr>
<tr>
<td>Healthy controls (n=30)</td>
<td>1 (3.33%)</td>
</tr>
</tbody>
</table>

Dot-ELISA using 24kDa cyst wall protein demonstrated diagnostic level of antibodies in 23 out of 30 (76.66%) sera of the cases with confirmed CE (Group-I) and 9 out of 30 (30%) sera of suspected cases (Group-II). The test also detected antibodies in 10% sera of parasitic disease controls (Group-III) and 3.33 % sera of healthy controls (Group IV).

Dot-ELISA using 24kDa urinary hydatid protein demonstrated diagnostic level of antibodies in 24 out of 30 (80%) sera of the cases with confirmed CE (Group-I) and 12 out of 30 (40%) sera of suspected cases (Group-II). The test also detected antibodies in16.66% sera of parasitic disease controls (Group-III) and 3.33 % sera of healthy controls (Group IV).

**Statistical analysis**

The sensitivity, specificity, PPV, NPV and efficiency of the Dot-ELISA carried out with crude extract of cyst wall antigen, protoscolex antigen and cyst fluid antigen and with 24kDa urinary hydatid protein, 24kDa cyst wall protein obtained from urine and hydatid cyst wall separately for detection of hydatid IgG antibodies in serum are presented in Table 12 and 13.
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Table 12: Diagnostic evaluation of Dot-ELISA for diagnosis of cystic echinococcosis

<table>
<thead>
<tr>
<th>DIAGNOSTIC EVALUATION CRITERIA</th>
<th>Diagnostic evaluation of Dot-ELISA for detection of hydatid antibodies in serum using</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CYST FLUID ANTIGEN</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>93.33%</td>
</tr>
<tr>
<td>Specificity</td>
<td>83.33%</td>
</tr>
<tr>
<td>Diagnostic efficiency</td>
<td>88.33%</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>84.84%</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>92.59%</td>
</tr>
</tbody>
</table>

Table 13: Diagnostic evaluation of Dot-ELISA using eluted specific protein from cyst wall and urine for diagnosis of cystic echinococcosis

<table>
<thead>
<tr>
<th>DIAGNOSTIC EVALUATION CRITERIA</th>
<th>Diagnostic evaluation of Dot-ELISA using eluted specific protein from</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CYST WALL ANTIGEN</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>76.66%</td>
</tr>
<tr>
<td>Specificity</td>
<td>96.66%</td>
</tr>
<tr>
<td>Diagnostic efficiency</td>
<td>86.66%</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>95.83%</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>80.55%</td>
</tr>
</tbody>
</table>

EITB

The results of EITB using cyst wall antigen for detection of IgG antibody in serum from CE patients and controls are provided in the Table 14.

A total of five antigenic peptides of Mr 92kDa, 43kDa, 37kDa, 24kDa and 9kDa are demonstrated by the EITB using serum samples. Of these, three antigenic peptides (viz., 92kDa, 24kDa and 9kDa) are found to be of diagnostic value. The sera reacting with one or more of these peptides is considered positive by the test.
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The EITB for hydatid antibodies is positive in 27 of 30 (90%) confirmed CE cases (Group I) and 20 of 30 (66.66%) suspected cases (Group II). The test showed a false positive reaction in 8 of 30 (26.66%) disease controls and 1 of 30 (3.33%) healthy controls.

Table 14: Detection of antibodies in serum of cystic echinococcosis cases and controls by EITB

<table>
<thead>
<tr>
<th>SUBJECT GROUPS (n=120)</th>
<th>EITB using hydatid cyst wall antigen detected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>92kDa</td>
</tr>
<tr>
<td>CONFIRMED CASES (n=30)</td>
<td>9 (30%)</td>
</tr>
<tr>
<td>SUSPECTED CASES (n=30)</td>
<td>1 (3.33%)</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 24kDa and 9kDa peptides are found to be two predominant peptides. The 24kDa peptide is found to be reactive with 26 out of 30 (86.66%) sera of confirmed CE cases (Group I) and 11 out of 30 (36.66%) sera of suspected cases (Group II). The test showed a false positive reaction in 2 out of 30 (6.66%) sera of disease controls (Group III). The test is negative for healthy controls (Group IV). The 9kDa peptide is found to be reactive with 22 out of 30 (73.33%) sera of confirmed CE cases (Group I) and 16 of 30 (53.33%) sera of suspected cases (Group II). The test showed a false positive reaction in 6 of 30 (20%) sera of disease controls (Group III) and one healthy control (Group IV) is reactive.

Another peptide of 92kDa is highly specific but it is less predominant as it found to react with 9 out of 30 (30%) sera of confirmed CE cases (Group I) and 1 out of 30 (3.33%) sera of suspected cases (Group II). This peptide (92kDa) did not react with any of the control sera. EITB using crude cyst wall antigen picture shown in the blot (Figure 16). The 43kDa and 37kDa peptides are the commonly recognized cross-reactive peptides detected by negative control sera (Figure 16).
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Confirmed cases of cystic echinococcosis

Suspected cases of cystic echinococcosis

Disease controls

Healthy controls

Figure 16: Detection of antibody in serum from cases of cystic echinococcosis and controls by EITB using hydatid cyst wall antigen. P= Standard positive serum, N= known negative serum. 1 to 30 depicts the number of cases/controls.
Statistical analysis

The sensitivity, specificity, PPV, NPV and efficiency of the EITB carried out with crude extract of cyst wall antigen for detection of hydatid IgG antibodies in serum are presented in Table 15.

Table 15: Diagnostic evaluation of EITB using hydatid cyst wall antigen for diagnosis of cystic echinococcosis

<table>
<thead>
<tr>
<th>DIAGNOSTIC EVALUATION CRITERIA</th>
<th>Diagnostic evaluation of EITB using hydatid cyst wall antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>90%</td>
</tr>
<tr>
<td>Specificity</td>
<td>96.66%</td>
</tr>
<tr>
<td>Diagnostic efficacy</td>
<td>93.33%</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>96.42%</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>90.62%</td>
</tr>
</tbody>
</table>

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

All cases are subjected to detection of hydatid antibodies in the serum by ELISA and Dot-ELISA using hydatid cyst wall, hydatid protoscolex and hydatid cyst fluid antigens, Dot-ELISA using 24kDa urinary hydatid protein and 24kDa cyst wall protein and EITB using hydatid cyst wall antigen.

The results showed that sensitivity of the Dot-ELISA using protoscolex and cyst fluid antigens is significantly higher (P= 0.03; P=0.02) than the ELISA using protoscolex and cyst fluid antigens. There is no significant difference (P=0.05) observed in the sensitivity of Dot-ELISA using cyst wall, ELISA using cyst wall antigen, Dot-ELISA using 24kDa urinary hydatid protein and 24kDa cyst wall protein for the serodiagnosis of CE. A significant difference in the sensitivity is
noted for EITB using diagnostically relevant polypeptides such as 92kDa, 24kDa and 9kDa for their use in the serodiagnosis (Table 16).

Table 16: Comparative diagnostic evaluations of ELISA, Dot-ELISA and EITB for detection of antibodies in serum for the diagnosis of cystic echinococcosis

<table>
<thead>
<tr>
<th>Diagnostic evaluation criterion</th>
<th>ELISA</th>
<th>Dot-ELISA</th>
<th>Dot-ELISA WITH SPECIFIC PROTEIN</th>
<th>EITB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCW</td>
<td>HPR</td>
<td>HCF</td>
<td>HCW</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>86.66%</td>
<td>70%</td>
<td>80%</td>
<td>96.66%</td>
</tr>
<tr>
<td>Specificity</td>
<td>90%</td>
<td>90%</td>
<td>93.33%</td>
<td>83.33%</td>
</tr>
<tr>
<td>Diagnostic efficacy</td>
<td>88.33%</td>
<td>80%</td>
<td>86.66%</td>
<td>90%</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>89.65%</td>
<td>87.5%</td>
<td>92.30%</td>
<td>85.29%</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>87.09%</td>
<td>75%</td>
<td>82.35%</td>
<td>96.15%</td>
</tr>
</tbody>
</table>

Correlation of sensitivity of serological test with number of hydatid cysts in various organs

The sensitivity of serologic tests is compared between 21 surgically confirmed and ultrasound proven CE patients with only one cyst and 9 surgically confirmed and ultrasound proven CE patients with multiple cysts. Statistically significant differences (P<0.05) between single and multiple cysts are observed by all the serological assays (ELISA using protoscolex and cyst fluid antigen, Dot-ELISA using cyst wall and cyst fluid antigen, Dot-ELISA using 24kDa urinary protein, 24kDa cyst wall protein separately and EITB). However the ELISA using hydatid cyst wall antigen (P=0.521) and Dot ELISA using protoscolex antigen (P=0.521) did not show any significant difference between single and multiple cysts.
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DISCUSSION

CE is a chronic zoonosis, usually characterized by slow growing fluid-filled cysts in the liver, lungs or other organs (135). Clinical diagnosis is invariably made when the disease is relatively advanced and already causing symptoms related to hydatid cyst induced pressure effects.

CE is a serious disease, which poses important diagnostic and follow up problems. Early diagnosis and treatment of CE thereby reduces morbidity and mortality due to disease to a great extent. Serological tests form the basis of diagnosis in CE, which not only helps to confirm clinical findings but also helps to identify radiologically unclear cases and to determine patients immune status. The tests also provide confirmation of CE in community screening studies (109). Immunodiagnosis of CE plays a major role in endemic areas because of their low cost and ease of performance.

Demonstration of circulating hydatid antibodies which occur frequently in established CE was the earliest approach for diagnosis of CE. The production of specific antibodies in CE may also depend on the number, size, location and condition of hydatid cyst and only approximately 60% to 80% of confirmed CE patients are seropositive for antibodies (139). However, the diagnostic utility of most serologic tests has frequently been questioned because of their low sensitivity and specificity, particularly in specific cyst localizations such as in the lung (330, 344).

ELISA

EIA techniques such as standard and rapid ELISA for IgG ELISA (254, 255), IgM-ELISA (256) and IgE ELISA (257), are being increasingly used because of their relative high sensitivity and specificity. The present study is designed to assess the usefulness of cyst fluid, protoscolex and cyst wall antigens for detection of hydatid antibodies in serum for the diagnosis of CE.
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Hydatid cyst fluid (HCF): The findings of crude cyst fluid antigens based ELISA showed a sensitivity of 80% and a specificity of 93.33% for the detection of serum IgG antibodies (Table 9). The sensitivity obtained with ELISA using cyst fluid antigens are in agreement with Gottstein et al. (322) (85% in hepatic cyst and 50% in pulmonary CE using camel cyst fluid antigen) and Daeiki et al. (155) (sensitivity of 60% - 80%).

Results of the present study however are not in agreement with results of those reported by other workers. The ELISA using sheep hydatid cyst fluid showed a very low sensitivity of 57%. The predictive values of ELISA tests were 82% and 90% for positive tests and 86% and 82% for negative tests, respectively with the two cut-off points (274). Iacona et al. (212) evaluated ELISA (tube method) using purified hydatid fluid fraction for demonstration of specific antibodies in human CE. The ELISA showed a sensitivity of 64%. They recommended that ELISA should be considered not as alternative but as a useful addition to the range of immunodiagnostic tests available for serodiagnosis of CE.

False positive reactions are frequently noted problem in serological tests in patients infested by tapeworm and other helminths (both larval and adult worms). Guisantes et al. (345) found false positive results when testing with a specific ELISA for CE in patients with T. saginata, Enterobius vermicularis and Fasciola hepatica. False positives results were also observed with sera from the patients with alveolar echinococcosis, schistosomiasis and trichinosis (330, 346), sera from onchocerciasis, schistosomiasis, and ascariasis (346) and sera from cysticercosis (268, 347).

In the present study, the ELISA using cyst fluid antigens showed 25% false positive reactions. Two of 30 healthy controls, 3 of 11 filariasis, 7 of 11 neurocysticercosis, 1 of 3 toxoplasmosis and 2 of 5 amoebiasis are positive by the ELISA. Similar observations have also been documented in a study conducted by Ramzy et al. (153). In their study, with ELISA using camel hydatid cyst fluid observed a false positive reaction of 34.9% with sera from other parasitic infections. However, Iacona et al. (212) in their study found that ELISA showed a low false
positive reaction of 4.8% with non-hydatid cases including normal controls and cross reaction was observed only with fascioliasis. Similarly Sorice et al. (328) reported a 4% false positive reaction with the ELISA using the crude hydatid fluid antigen due to cross reactivity with schistosomiasis.

Several factors were attributed to cross reactivity and false positive reactions. Similarity of one of the component of hydatid cyst fluid to P1 blood group antigen (259), existence of lipoprotein antigen (Ag B) common to many helminths (212) and complex of related antigens in different parasite that stimulates the production of overlapping antibodies in their hosts which in turn is reflected in the frequency of cross reactions to the antigens of parasites in serological tests (348). The false positives obtained in the present study with ELISA using cyst fluid antigens are probably due to the use of crude cyst fluid antigen in the study. Hence, it has been suggested that use of purified fractions might reduce false positive or non-specific reactions (212, 349).

*Hydatid protoscolex (HPR):* In the present study, the ELISA using protoscolex antigens showed a sensitivity of 70% and specificity of 90% for diagnosis of CE (Table 9). In the present study, sensitivity of ELISA using protoscolex antigens is found to be moderate. The test showed a non-specific reaction of 22% with sera from various endemic parasitic infections which included 4 from 11 cases of filariasis, 4 from 11 cases of neurocysticercosis, 1 from 3 cases of toxoplasmosis and 1 from 5 cases of amoebiasis and 3 from 30 healthy controls. Raifei and Craig (156) obtained a reasonable sensitivity of 90.5% but a very low level of specificity (57%) in ELISA using extract of *E. granulosus* protoscoleces from sheep cysts. Barring this, study reports are lacking on the use of protoscolex as antigen in the diagnosis of CE.

*Hydatid cyst wall (HCW):* Reports are lacking, except for one, on the use of cyst wall as antigen for demonstration of serum antibodies. Mahmoud and Gamra (158) employed ELISA with *E. granulosus* alkaline phosphatase (EgAP) extracted from hydatid cyst membranes of sheep liver cysts origin and obtained 100% of sensitivity.
and specificity for diagnosis of CE. The present study, therefore, is designed to evaluate the ELISA using cyst wall antigens for the presence of serum hydatid antibodies.

The findings of the study showed that ELISA using cyst wall antigens could demonstrate hydatid antibodies in serum with a sensitivity of 86.66% and specificity of 90% (Table 9). The test showed a false positive reaction of 20% which includes 3 of 11 filarial sera, 6 of 11 neurocysticercosis sera and 3 of 30 healthy control sera.

In conclusion, the ELISA, in the present study, using either cyst fluid, protoscolex or cyst wall antigen has proved to be a sensitive procedure for the serodiagnosis of CE as shown in the earlier studies.

**Dot-ELISA**

Dot-ELISA is a highly versatile solid-phase immunoassay for antibody detection in the diagnosis of leishmaniasis (331), toxoplasmosis (332), malaria (332), trypanosomiasis (333), cysticercosis (334) and CE (173). The assay uses minute amounts of reagents dotted onto solid surfaces such as nitrocellulose or cellulose acetate membranes which avidly bind proteins (332). After incubation with antigen-specific antibody and enzyme-conjugated anti-antibody, the addition of a precipitable, chromogenic substrate causes the formation of a colored dot on the solid phase which is visually read.

In the present study, the Dot-ELISA using cyst wall, protoscolex and cyst fluid antigens separately, showed a sensitivity of 93.33%, 86.66% and 96.66% respectively, for detection of specific hydatid antibodies in serum (Table 12).

The sensitivity of Dot-ELISA obtained using cyst fluid (93.33%), protoscolex (86.66%) and cyst wall (96.66%) antigens separately in the present study is in agreement with the results of Pappas et al. (331). In their study, Dot-ELISA using sheep hydatid cyst fluid antigens showed a sensitivity of 96% and
specificity of 98%. Cross-reactions were observed with sera from patients with cysticercosis, filariasis, toxocariasis, trichinosis, visceral larval migrans, and liver cirrhosis. Only one false-positive reaction was observed by the Dot-ELISA when 52 sera from healthy subjects were assayed. They observed that the Dot ELISA is a rapid and economical enzyme immunoassay which is very antigen-conservative, requires only nanogram quantities of parasite antigen and serum conservative and needs only 50 microliter of diluted patient serum (175).

In another study, the bovine hydatid antigen was used to develop a simple and fast in vitro diagnostic assay for CE (147). The procedure of Dot-ELISA consisted of incubation of the serum sample with a textile colloidal dye (pink) and a nitrocellulose stick to which the hydatid antigen was bound. The presence of parasite-specific antibodies results in dyeing of the stick reactive area and appearance of a coloured spot. Dot-ELISA showed positive results in all the patient sera and in none of the control sera. The test showed a good predictive value, allowing a speedy diagnosis of CE (147). Dot-ELISA was also found to be 88.9% sensitive, 96.9% specific for rapid diagnosis of CE by demonstration of hydatid antibodies in the sera (176).

In the present study, the specificity of Dot-ELISA is 83.33% using cyst wall and cyst fluid antigen whereas 80% using protoscolex antigen (Table 12). The moderate specificity obtained in the present study is due to cross reaction with sera from neurocysticercosis, filariasis and amoebiasis. Rogan et al. (174) reported lower specificity (52%) and higher sensitivity (97%) with the use of crude sheep hydatid cyst fluid. They have also observed cross reactions with sera from patients with cysticercosis, filariasis, toxocariasis and trichinosis. It is suggested that the cross reaction was due to the use of a crude and complex hydatid fluid preparation containing shared helminth antigens (173).

In the present study, the use of cyst wall and protoscolex antigens along with cyst fluid antigens in ELISA and Dot-ELISA for the diagnosis of CE showed that there is no significant difference in the sensitivity of ELISA(P=0.739) or Dot-
CHAPTER II

ELISA (P = 0.785) using either of cyst wall, protoscolex and cyst fluid antigens. No significant difference in the sensitivity of the ELISA or Dot-ELISA by using cyst wall, protoscolex and cyst fluid antigens, suggests that protoscolex and cyst wall could also be used as antigen apart from cyst fluid which is routinely used in the serodiagnosis of CE.

It is suggested that the use of more purified antigen preparation which lacks common antigens can greatly improve assay reliability by reducing false-positive reactions (173, 174, 347). Other workers have reported that the ELISA using crude cyst fluid antigens are as sensitive as the ELISA using purified antigens, although the former lacks specificity (350, 351). Ramzy et al. (153) observed that the ELISA using crude camel hydatid cyst fluid antigen is more practical for use in endemic areas.

**Dot-ELISA with 24kDa urinary protein and 24kDa cyst wall protein**

The 24kDa urinary protein identified from the urine of confirmed CE cases by EITB and subsequently eluted manually, as mentioned earlier (Chapter I) is employed in Dot-ELISA for the detection of hydatid antibodies in serum. Similarly, 24kDa cyst wall protein, also obtained from the hydatid cyst wall is used in Dot-ELISA for demonstration of serum antibodies.

The Dot-ELISA using 24kDa urinary protein showed a sensitivity of 80% and specificity of 96.66% which is nearly similar to that of the ELISA using 24kDa cyst wall protein with 76.66% sensitivity and 96.66% specificity for the detection of total IgG antibodies in the sera of confirmed CE patients and controls (Table 13). Serum antibodies against 24kDa urinary protein are detected in 24 of 30 sera of confirmed CE patients. The reason for moderate sensitivity obtained with Dot-ELISA using 24kDa urinary protein and 24kDa cyst wall protein in the present study possibly is due to the manually eluted antigen extracted from the urine and cyst wall which could have lost some of the functional epitopes. The specificity of the Dot-ELISA using either 24kDa urinary protein (96.66%) or 24kDa cyst wall protein
(96.66%) is more than that of the Dot-ELISA using crude antigens of cyst fluid (83.33%), protoscolex (80%) and cyst wall (83.33%) as observed in the present study (Table 13).

Results of the Dot-ELISA using 24kDa urinary protein and 24kDa cyst wall protein compares well that of a study of Romia et al. (176) who reported a sensitivity of 88.9% and specificity of 96.9% by Dot-ELISA but using antigen B instead. Similarly, the Dot-ELISA using antigen B, showed a sensitivity of 95% and specificity of 100% (150).

Results of the present study showed that there is no significant difference in the sensitivity of Dot ELISA (P=0.408) using either 24kDa urinary protein or 24kDa cyst wall protein. The present results suggest that 24kDa urinary protein as well as 24kDa cyst wall protein could be used as antigen in the Dot-ELISA for the diagnosis of CE.

Some of the technical advantage of Dot-ELISA observed in the present study includes (i) reagent conservation (ii) many samples assayed in a short period (iii) no need for specific metric reading, as the result can be read visually and is field applicable (iv) performance of test at room temperature.

In the present study, 24kDa urinary protein and 24kDa cyst wall protein is used in Dot-ELISA only but not in ELISA and EITB. This is due to the fact that the volume of eluted proteins either from urine or cyst wall is too low for use in both ELISA and EITB. Liance et al. (352) made an effort to identify and purify *Echinococcus* species specific antigens for use in ELISA and EITB, but found it difficult to obtain the antigen in sufficient amounts for extensive use. Similarly, Oriol et al. (283) also observed difficulties with an adequate supply of antigens from naturally infected animals and thus found difficulty in the use of the minute volume of antigen in the serological techniques for the diagnosis of CE.
EITB

The EITB is a test which combines the high sensitivity and specificity of the immuno enzymatic tests with the high resolution of specific proteins in SDS-PAGE. This makes EITB to be more useful in the diagnosis of many parasitic diseases including cysticercosis (353), fascioliasis (354), schistosomiasis (355) and CE (171, 220, 259, 268).

In the present study, EITB is performed using sera from different groups to identify diagnostically relevant bands. Proteins of diagnostic relevance (Mr 92kDa, 24kDa and 9kDa) are present in the protoscolex and cyst wall whereas cyst fluid contained only 24kDa and 9kDa but not 92kDa. The cyst wall which showed all three of the diagnostically relevant bands (Mr 92kDa, 24kDa and 9kDa) are used as the antigen in the EITB for demonstration of serum antibodies in the CE.

24kDa polypeptide: In the present study, Mr 24kDa is observed to be protein of diagnostically relevance with 26 out of 30 (86.66%) sera reactive by EITB (Table 14). Similar low molecular weight proteins in other antigen preparations have also been reported in other studies. These are 24kDa protein of antigen B (171), 23kDa protein (220, 267) and 20kDa (166) of hydatid cyst fluid.

The EITB using E. granulosus specific antigens revealed 48 different antigenic bands in a study reported by Ayadi et al. (356). Of these, a 35kDa antigen was recognized by only 68% of the sera whereas 8kDa antigens corresponding to the specific E. granulosus antigen was recognized by 80% of the sera coming from patients with CE and not by any 86 control sera. Bands of 21kDa, 30kDa and 92kDa were also specific and were recognized by at least 50% of tested sera. Sera from patients with CE were also found to recognize at least one of the 8kDa, 21kDa, 30kDa, 35kDa or 92kDa specific antigens.

EITB with a purified antigen from sheep hydatid fluid recognized proteins of 12kDa to 14kDa, 16kDa, 20kDa, 24kDa to 26kDa, 34kDa, 39kDa and 42kDa as
diagnostic value in another study (269). The combination involving two of the three proteins (20kDa, 39kDa and 42kDa) made the EITB possible to diagnose 100% of the cases. The antibodies specific to proteins 39kDa and 42kDa disappeared in less than one year in the patients cured after surgery, while in patients with persistent or recurrent parasitism the bands present before surgery persisted or other new ones appeared. They found EITB with these antigen bands to be highly useful in the diagnosis and post-surgical monitoring of CE patients (269).

Two antigens, Antigen 5 and Antigen B are considered to be specific for genus *Echinococcus*. In the present study, the protein of Mr 24kDa revealed by EITB using hydatid cyst wall antigen may probably represent a larger subunit of antigen B complex (consists of 8kDa or 12kDa, 16kDa and 24kDa) as observed during the partial characterization of protein mentioned earlier (Chapter I). But, no experiment is carried out during the present study to substantiate this observation. Hence, further investigations are needed to support the observation.

9kDa polypeptide: The sensitivity of the EITB assay for the Mr 9kDa band obtained in the present study is 73.33% (Table 14). Similar diagnostically relevant low molecular weight proteins have also been reported in other studies by EITB. These include 8kDa (171, 268) and 12kDa (220, 268, 270). 8kDa and 12kDa bands reported in other studies in contrast to 9kDa bands reported in the present study, possibly is due to use of low-molecular mass standards, which are inaccurate below 14kDa when used and the possibility of 8kDa band appearing as 12kDa. An accurate description of a band of this size can be made only with the use of ultra-low molecular mass weight markers. Similar observations have been made by Verastegui et al. (151), who suggested that the 12kDa identified by Shepherd and McManus (220) would appear to be the 8kDa, since they used prestained high molecular mass markers to measure the molecular mass (151).

The sensitivity of the EITB for the 9kDa band (73.33%) in the present study is relatively lower when compared with results of Lightowlers et al. (171), Kanwar et al. (259) and Maddison et al. (268). EITB using *Echinococcus* antigen with an
apparent molecular weight of 8kDa showed a sensitivity of 91% for surgically confirmed CE and specificity of 100% for echinococcosis. Marked cross-reactivity was observed with serum specimens from patients with *E. multilocularis* and *E. vogeli* infections (268).

The hydatid cyst fluid of sheep, goat, pig and human were analyzed by EITB with sera from 20 surgically confirmed cases of CE which revealed twelve polypeptides bands ranging between 8kDa to 116kDa (259). The polypeptides of 16kDa, 24kDa, 38kDa, 45kDa and 58kDa were not only recognized by all CE sera but also by many sera from patients with other infections. However, polypeptides of 8kDa and 116kDa were very specific because these were recognized by only CE sera but not by any sera from patients with cysticercosis, other parasitic infections or viral hepatitis or from healthy controls (259).

The polypeptide bands, 8kDa to 12kDa, the smallest subunit of antigen B (which is not altered by reduction with 2-mercaptoethanol), was suggested to be 100% specific for antibodies against the genus *Echinococcus* (357). Lowest molecular weight subunit of antigen B (8kDa-12kDa), of diagnostic value has been also reported in other studies (174, 220).

In contrast to these studies as mentioned above, Siracusano et al. (270) reported a lower sensitivity (33.7%) of EITB for the 12kDa band. This low sensitivity, possible may be due to the use of blotted antigen preparation or due to the use of peroxidase conjugate.

The specificity of the EITB for the 8kDa band remains controversial. Poretti et al. (336) reported that the specificity of immunodiagnosis can be improved by using the EITB to detect antibody to the 8kDa subunit of the antigen B. Ito et al. (214) found that 92% of CE sera as well as 79% of alveolar echinococcosis sera reacted with 8kDa subunit of antigen B. The antigen, however, did not cross react with sera from patients with other parasitic diseases. Hence, the authors suggested that the antigen B is not specific for *E. granulosus* species but is genus specific for
Echinococcus. The observation by Ortona et al. (310) was also in accordance with Ito et al. (214).

The reason for earlier reported specificity of the EITB for the 8kDa or 12kDa molecule (171, 267) might be due to the use of insufficient number of sera or the use of pooled sera. Moreover, cross reactive antibody might be elicited by taeniid antigens which possess a shared epitope with the 12kDa subunit of E. granulosus, thus diminishing the diagnostic specificity of the molecule.

92kDa polypeptide: In the present study, the EITB assay showed a relatively high specificity (100%) and lower sensitivity (30%) for Mr 92kDa band (Table 14). Similar to the present study, Ayadi et al. (356) reported bands of 21kDa, 30kDa, and 92kDa which appeared to be specific and were recognized by at least 50% of tested sera using E. granulosus specific antigens by EITB. Lingelbach and Hinz (358) also identified low level of antibody binding to specific Echinococcus antigen of molecular weight 20kDa and 93kDa protoscolex antigens by EITB.

In conclusion, in the present study, EITB using hydatid cyst wall antigen have shown maximum specificity for Mr 24kDa and 92kDa bands followed by Mr9kDa for detection of antibodies in the diagnosis of CE. In other studies, EITB have shown maximal specificity for 8kDa or 10kDa and 16kDa polypeptides apart from 24kDa in the diagnosis of CE (210, 259, 271, 268, 359). However, Verastegui et al. (151) found that the EITB showed maximal specificity for 16kDa and 21kDa followed by 8kDa for the diagnosis of CE.

Overall sensitivity and specificity of the EITB for either one of the three immunodominant bands or combination of either one (92kDa, 24kDa and 9kDa) is 90% and 96.66% respectively, as observed in the present study (Table 15). Results of the present study are in agreement with the observation of other reported studies (230, 270). The EITB using hydatid fluid showed sensitivity to range from 50% to 91% (336), using hydatid fluid fraction showed a sensitivity of 90% (270) and using native antigen B showed a sensitivity of 80% (230). It is suggested that true
specificity of the EITB for sera from individuals living in a zone where cysticercosis is endemic would be less than 100%. In seroepidemiological studies in areas endemic for both CE and cysticercosis, EITB is found to be more suitable than other serological test because of its higher sensitivity and fewer cross reactions (151).

Despite the high sensitivity and specificity of the EITB with some single band results such as 24kDa, as observed in the present and other studies, the analysis of different bands (9kDa, 24kDa and 92kDa) combined together, the sensitivity of EITB is enhanced, but without a notable loss of specificity (357). Poretti et al. (336) found that by using the two bands 29kDa and 34kDa, as diagnostic markers in addition to the 8kDa band obtained by the EITB, an increase in diagnostic sensitivity was obtained for preoperative CE sera from 71% to 91% without losing specificity concerning non-cestode cross reactivities.

In the present study, the EITB for Mr 37kDa and 43kDa bands are found to be non specific, as these two bands reacted with sera from filariasis, neurocysticercosis and healthy controls.

37kDa polypeptide: 37kDa band cross reacted with 4 of 11 filarial sera and 5 of 11 neurocysticercosis sera. Similar to the present study, other studies have all demonstrated cross reactive nature of 38kDa molecule by EITB or immunoprecipitation (171, 220, 268). Leggatt et al. (267) found that 38kDa was cross reactive not only with human antibody to other cestode, trematode and nematode parasites but also with human antibody from normal control sera. It is suggested that wide spread reactivity of 38kDa may be partly attributed to antigens phosphorylcholine (PC) epitope (171, 220). In another study by Zhang et al. (280), 40.47% of the healthy population contained antibodies to reduced 36kDa protein. This is not surprising because the antigen 5 molecule contains the phosphorylcholine epitope, a widely distributed hapten and also cross reacts with blood group p1 antigen.
In contrast to our observation and observations of other studies as mentioned above, many other studies have shown that EITB for 39kDa is very specific. Siracusano et al. (270) found that 39kDa subunit was not cross reactive with antibody from healthy controls or non hydatid patients. In another study, EITB using polypeptide bands of 12kDa, 14kDa, 20kDa and 34kDa, obtained from purified sheep hydatid fluid, yielded a sensitivity of 95% and specificity of 100% (150). The EITB for 32kDa unreduced protein that corresponds to the 38kDa protein reported by Verastegui et al. (151) showed a sensitivity of 92% and a specificity of 100%. In a study reported from Italy, EITB for 39kDa band which was derived from antigen 5 was 90% sensitive and 100% specific. Cross reactions did not occur (270).

43kDa polypeptide: EITB showed another non-specific band of Mr 43kDa in the present study. But this antigen is found to be specific in other studies (150, 218). Piantelli et al. (218) demonstrated that 47kDa and 20kDa, two subunits present in the antigen 5 is species specific for *E. granulosus*. The EITB for 42kDa, the polypeptide obtained from sheep hydatid cyst fluid purified through concanavalin A column, was 95% sensitive and 100% specific (150).

The diagnosis of CE in humans is normally achieved through detection of specific antibodies using an assay of high sensitivity (e.g. ELISA) for initial screening followed by a test of relatively high specificity for confirmation (159). Results of this study supports results of other studies further that EITB could be useful for the diagnosis of CE with high specificity. EITB assay, nevertheless, has the drawbacks of needing sophisticated equipment and highly trained personnel.

Though antibody detection assays such as ELISA, Dot-ELISA and EITB, as demonstrated in the present study are found to be useful for serodiagnosis of the CE, nevertheless, low sensitivity and inability to discriminate between recent and old infections are the major disadvantages of these and other antibody based immunoassays in the diagnosis of CE. Some of the reason for the occurrence of such false negative reactions due to (i) secretion/ excretion by the parasite of high levels of circulating antigen which generate circulating immune complexes after reacting
with antibodies, interfering in this way with antibody detection (272, 273), (ii) the parasite with dormant or calcified cyst that may cease to produce antibodies (274), (iii) antibody mopping by circulating antigen (272), and iv) some of the cysts of lung is associated with low serum antibodies and hydatid cysts of the eye and brain produce no or very low serum antibodies (268, 270).

**Comparative evaluation of ELISA, Dot-ELISA and EITB for detection of hydatid antibodies in serum**

The results showed that higher sensitivity is obtained with Dot-ELISA using protoscolex and cyst fluid antigens whereas ELISA or Dot-ELISA using cyst wall antigen was equally sensitive for antibody detection. This suggests that Dot-ELISA or ELISA can be adopted for primary serological test for diagnosis of CE to maximize case detection. The more specific, EITB may be used to confirm seropositives that helps in clinical follow-up of the cases (Table 16).

**Correlation of sensitivity of serological test with number of hydatid cysts in various organs**

Sensitivity of serological test varies depending on the presence of single and multiple cysts. Very few studies have reported the influence of cyst number on test sensitivity (330, 344, 360). Similar to other studies, in the present study, all serological tests except ELISA using cyst wall antigen and Dot-ELISA using protoscolex antigen showed higher sensitivity with sera from patients with multiple hydatid cysts than with sera from patients with single hydatid cyst.

**SUMMARY**

In the present study, ELISA and Dot-ELISA using cyst fluid, protoscolex and cyst wall antigens, Dot-ELISA using 24kDa urinary and cyst wall protein; and EITB using cyst wall antigen were used for the detection of hydatid antibodies in serum.

The ELISA using crude cyst fluid antigens showed a sensitivity of 80%, specificity of 93.33%, diagnostic efficiency of 86.66%, positive predictive value of
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92.30%, and negative predictive value of 82.35% for the detection of serum IgG antibodies. The ELISA using protoscolex antigens showed a sensitivity of 70%, specificity of 90%, diagnostic efficiency of 80%, positive predictive value of 87.5%, and negative predictive value of 75%. The ELISA using cyst wall antigens could demonstrate hydatid antibodies in serum with a sensitivity of 86.66%, specificity of 90%, diagnostic efficiency of 88.33%, positive predictive value of 89.65%, and negative predictive value of 87.09%.

In the present study, Dot-ELISA using cyst fluid, protoscolex and cyst wall antigens separately, showed a sensitivity of 93.33%, 86.66% and 96.66% respectively, for detection of specific hydatid antibodies in serum. The specificity of Dot-ELISA using cyst wall and cyst fluid antigen was 83.33% whereas that of Dot-ELISA using protoscolex antigen was 80%. The diagnostic efficiency of Dot-ELISA using cyst fluid, protoscolex and cyst wall antigens was 88.33%, 83.33% and 90% respectively and positive predictive value of cyst fluid, protoscolex and cyst wall antigens was 84.84%, 81.25% and 85.29% respectively. The Dot-ELISA using cyst fluid, protoscolex and cyst wall antigens showed a negative predictive value of 92.59%, 85.71% and 96.15% respectively.

The sensitivity of ELISA and Dot-ELISA using cyst fluid, protoscolex and cyst wall antigens showed no significant difference, suggesting that protoscolex and cyst wall can also be used as good source of antigen in serodiagnosis of CE.

Dot-ELISA using 24kDa urinary protein showed a sensitivity of 80% and specificity of 96.66%, nearly similar to that of Dot-ELISA showing the sensitivity (76.66%) and specificity (96.66%) by using 24kDa cyst wall proteins. The results suggest that 24kDa urinary protein can also serve as alternate source of antigen. The Dot-ELISA using 24kDa urinary protein showed a diagnostic efficiency of 88.33%, positive predictive value of 96%, and negative predictive value of 82.85%. The Dot-ELISA using 24kDa cyst wall protein showed a diagnostic efficiency of 86.66%, positive predictive value of 95.83%, and negative predictive value of 80.55%.
In the present study, EITB using the cyst wall antigen showed three diagnostically relevant bands of Mr 92kDa, 24kDa and 9kDa. Of the three bands, Mr 24kDa is observed to be present in 26 out of 30 (86.66%) sera by EITB. The sensitivity of the EITB assay for the Mr 9kDa band is 73.33% and for Mr 92kDa band is 30%. EITB showed maximum specificity for Mr 24kDa and 92kDa bands followed by Mr9kDa for detection of antibodies in the diagnosis of CE. Overall sensitivity and specificity of the EITB for either one of the three immunodominant antigens or combination of either one (92kDa, 24kDa and 9kDa) is 90% and 96.66% respectively. The EITB using cyst wall showed two nonspecific bands of Mr 37kDa and 43kDa that reacted with sera from filariasis, neurocysticercosis and healthy controls.

Both the ELISA or Dot-ELISA using cyst fluid, protoscolex and cyst wall antigens are found to be equally sensitive for detection of antibodies in the serodiagnosis of CE. The data suggests that that both the tests can be used as screening tests for initial serodiagnosis of CE to maximize case detection. The more specific, EITB may be used to confirm seropositives that helps in clinical follow-up of the cases.

In the present study, all serological tests except ELISA using cyst wall antigen and Dot-ELISA using protoscolex antigen showed higher sensitivity with sera from patients with multiple hydatid cysts than with sera from patients with single hydatid cyst.