Chapter I

Identification and immunochemical characterization of diagnostically relevant antigen from parasite cyst antigens and urine
Cystic echinococcosis is a zoonotic disease of man, caused by larval stage of the dog tapeworm, *Echinococcus granulosus*. CE is common in rural population of underdeveloped countries because of their close association with domestic and wild animals. Early detection of developing hydatid cyst in patients is important to initiate appropriate chemotherapy. The infection is mostly asymptomatic and diagnosis of CE depends mainly on imaging methods and serological tests. Imaging methods are often too costly or not available in most of the endemic areas. Thus, serodiagnosis plays an important role, as it helps in the diagnosis of radiologically unclear cases and also in the determination of patient’s immune status.

The sensitivity and specificity of the serological tests depend on the stage of the disease, the localization of the parasites, the antigens and the technique used (139). The sensitivity of the test depends on differences in antigen preparation or on clinical features of the patients including the number, size, location, integrity and morphology of cysts. Moreover, qualitative and quantitative antigenic variations related for example to parasite strains or host characteristics also contributes to the difference in the sensitivity (310).

The hydatid cyst fluid is a repository of somatic and functional antigen of parasite origin. It also contains variable amounts of host proteins. Various antigenic molecules in the fluid from the parasitic cysts that develop in infected humans and the intermediate hosts of *E. granulosus* have been reported to be useful in the detection of parasite specific antibodies.
Compared with the cyst fluid antigens, rather little is studied about the usefulness of other metacestode antigens of *E. granulosus* in hydatid serology. Hence, it is suggested that there is a continued need for the identification and characterization of ‘new’ *E. granulosus* antigens since this may lead to the tests that have greater immunodiagnostic sensitivity and specificity than those currently in use and that may facilitate post-treatment immunosurveillance and studies on the immunopathology of CE (155).

*E. granulosus* protoscolex is another source of antigen, which have been evaluated recently for antibody detection in CE patients using EITB (156). But reports are scanty on the usefulness of the hydatid cyst wall as antigen for use in hydatid serology (158, 311). Rickard et al. (311) raised antisera against antigen 5 and antigen B employed the antisera to study the location of hydatid specific protein on the cyst membrane and protoscolecies of *E. granulosus* using indirect immunofluorescence techniques. The study showed that the hydatid specific protein were located on the cyst wall. In another study, *E. granulosus* alkaline phosphatase (EgAP) extracted from hydatid cyst membrane was used as antigen in ELISA and EITB. Both the tests showed a higher sensitivity and specificity using this antigen than using hydatid cyst fluid in the serodiagnosis of CE (158).

In the present study, therefore, an attempt is made to evaluate the hydatid cyst wall as well as protoscolex as source of antigen, in addition to the cyst fluid for the diagnosis of CE.

Recently, the isolation of parasite antigen excreted in the urine and their use as antigen following purification has opened a new approach in the serodiagnosis of few parasitic diseases such as schistosomiasis (163) and trypanosomiasis (164). However, till now there are no reports on isolation and purification of diagnostically relevant hydatid antigen excreted in the urine, for use in the serodiagnosis of CE.

Purification of the parasitic antigen for their use as antigen may lead to higher specificity in the serodiagnosis. The use of purified antigens for the diagnosis of CE with good sensitivity, has also been emphasized by some authors. Hydatid cyst fluid has been used either in crude form (145) or dialyzed cyst fluid antigen by
some workers (148, 159-161) while others have employed affinity chromatography to concentrate and separate individual reactive components (159, 172, 312). It is suggested that wide use of cyst fluid antigen in the serological test for diagnosis of CE is due to the presence of high levels of specific parasite antibody in the serum that is induced by cyst fluid antigens diffused out of established metacestode (267). Characterization of parasite antigen has been reported with diagnostically specific polypeptides obtained from cyst fluids (165, 166).

In the present study, an attempt is made to identify diagnostically relevant antigens from cyst wall, protoscolex and cyst fluid antigens and also from the urine of confirmed CE cases. Subsequently, the diagnostically relevant antigen from hydatid cyst wall and urine are isolated by manual elution for their use in serodiagnosis of CE. An attempt is also made to characterize immunochemically the isolated proteins from urine and hydatid cyst wall.

OBJECTIVES

- To identify and isolate diagnostically relevant antigen from hydatid cyst wall antigens.
- To identify and isolate diagnostically relevant hydatid antigen from the urine of CE confirmed patients.
- To characterize the diagnostically relevant antigen obtained from hydatid cyst wall and urine by immunochemical methods.

MATERIALS AND METHODS

Specimens

**Urine**

To identify diagnostically relevant antigen from the urine of confirmed CE cases, around 50-100 ml volume of the urine is collected from four cases of surgically confirmed and six cases of ultrasound proven CE, three cases each of amoebiasis, filariasis, neurocysticercosis and toxoplasmosis cases (disease controls) and five cases of healthy controls.
Concentration of urine

Urine samples are concentrated by ammonium sulphate precipitation (313). The urine sample (50 -100 ml) is precipitated by 100% saturation of ammonium sulphate. The precipitate is collected by centrifugation at 3000 rpm at 4°C in a cooling centrifuge for 30 minutes. The precipitate is then dialyzed against phosphate buffered saline (PBS) pH 7.2 at 4°C overnight. The clear dialysate is stored at -20°C till use.

Serum

Five milliliters of venous blood is collected from four cases of surgically confirmed and six cases of ultrasound proven CE, three cases each of amoebiasis, filariasis, neurocysticercosis and toxoplasmosis cases (disease controls) and five cases of healthy controls under aseptic precautions and is allowed to clot. The serum is separated and stored in duplicate at -20°C till use. Sodium azide (0.05 mol/l) is used as preservative.

Preparation of the parasite antigens

Collection of hydatid cyst

Hydatid cyst is removed from a case of CE at surgery by method of de-roofing of the cyst. The cyst containing daughter cysts is collected in a sterile container. The cysts are washed in several changes with sterile PBS 7.2. A part of cyst content is preserved in 10% solution of formalin for histological section to observe cyst wall morphology. The presence of laminated layer, a unique feature of hydatid cyst along with germinal layer and hooklets, demonstrated by haematoxylin (H) and eosin (E) stain helped to confirm the hydatid etiology of the cyst.

The hydatid cyst containing 30 to 40 daughter cysts are collected in a sterile container and washed in PBS 7.2 three times. The daughter cysts are found to measure from 2x3cm to 5x6cm in size and contained approximately 1ml to 5ml of hydatid fluid.
The hydatid fluid is aspirated from the cyst using a sterile syringe and collected in sterile centrifuge tube. The fluid is centrifuged at 2000rpm for 30 min at 4°C. The clear supernatant fluid is collected as hydatid fluid. The fluid is checked for sterility by inoculating on blood agar and Mac Conkey agar and incubated at 37°C for overnight. The sediment containing the protoscoleces is also collected.

**Checking the fertility of hydatid cyst**

The fertility of the cyst is detected by viability tests such as flame cell activity and vital staining (314). The histological section of hydatid cyst wall is stained by haematoxylin and eosin stain.

Flame cell activity is demonstrated by making a wet mount preparation of the cystic fluid on a slide and examining under 10x and 40x objectives. The viable protoscoleces demonstrated a faint flickering movement.

Vital staining is carried out by adding a few drops of 0.1% aqueous eosin to a drop of hydatid fluid on a slide. The viable protoscoleces do not take up the stain whereas the dead protoscoleces are readily stained with the eosin.

**Preparation of somatic hydatid antigens**

Three somatic antigen preparations are made from the same cysts and designated as hydatid cyst fluid (HCF) antigen, hydatid protoscolex (HPR) antigen, and hydatid cyst wall (HCW) antigen.

1. **Preparation of hydatid cyst fluid (HCF) antigen**

The hydatid cyst fluid antigen is prepared as per the method described by Kanwar et al. (259). The fluid is aspirated aseptically from the fertile hydatid cyst and is collected in sterile centrifuge tube. The fluid is centrifuged at 2000rpm for 30 min at 4°C, dialyzed extensively against distilled water. To this, 0.5M of phenylmethylsulfonyl fluoride (PMSF) is added at a proportion of 1/100 and stored at -20°C.
2. Preparation of hydatid protoscolex (HPR) antigen

The hydatid protoscolex antigen is prepared as per the method described by Rafiei and Craig (156). In this procedure, the cyst fluid is centrifuged at 2000rpm for 30 min and the sediment containing protoscoleces are collected in a sterile tube. The protoscoleces are washed three times with PBS 7.2. The washed protoscoleces are then subjected to three cycles of freezing and thawing and resuspended in 10 volumes of PBS 7.2 containing 0.5M PMSF (at a ratio of 1 in 100). The protoscolex suspension is then sonicated on ice in a 150w ultrasonic disintegrator, in 10-second bursts with 5 seconds intervals, until no intact protoscoleces are visible microscopically. The sonicate is left at 4°C overnight and then centrifuged at 10,000rpm for 30 min. The supernatant is collected, dispensed in small aliquots and stored as protoscolex antigen at -20°C.

3. Preparation of hydatid cyst wall (HCW) antigen

The hydatid cyst wall antigen is prepared as per the method described by Rafiei and Craig (156). After aspiration of cyst fluid, the collapsed cyst membranes are washed three times in PBS 7.2 and homogenized using mortar and pestle. The homogenized tissue is collected in a sterile container and kept at -20°C overnight. Then, it is thawed and sonicated on ice in a 150w ultrasonic disintegrator, in 60-seconds bursts with 5 second intervals, until no intact tissue bits are visible through naked eye. 0.5M of PMSF (at a ratio of 1 in 100) is added to the sonicated suspension and is left at 4°C overnight and then centrifuged at 10,000rpm for 30 min. The supernatant is collected, dispensed in small aliquots and stored as cyst wall antigen at -20°C.

4. Preparation of complete homogenate hydatid (CHH) antigen

The complete homogenate hydatid antigen is prepared by mixing equal volume of hydatid cyst fluid antigen, hydatid protoscolex antigen and hydatid cyst wall antigen.
CHAPTER I

Protein content of each antigen preparations are estimated by standard method of Lowry (315) and readings are taken at 660nm in an UV-visible spectrophotometer (Cintra 5, Australia), aliquoted to 1ml cryoprotected vials and stored at -20°C.

5. Preparation of excreted urinary hydatid antigen

The urine samples collected from confirmed cases of CE is used as the source of excreted urinary hydatid antigen. The urine samples of confirmed CE cases, disease controls (amoebiasis, filariasis, neurocysticercosis and toxoplasmosis) and healthy controls were concentrated by precipitation with ammonium sulphate method as described earlier. The dialysate obtained after ammonium sulphate precipitation of urine sample from confirmed CE patients along with control cases were subjected to protein estimation by Lowry method (315).

Approximately 20µg of urinary precipitated protein and 20µg cyst wall antigen separately is subjected to SDS-PAGE with 5% stacking gel and 12% separating gel under reducing and denaturing conditions in an electrophoretic cell at 100v for 1.30 hours. The separated urinary protein is stained in coomassie blue R-250 (0.25% staining solution) and molecular weight of the urinary protein bands is determined by comparing with standard molecular weight marker (New England, Biolabs) run along side, using the software provided with the gel documentation system (Vilver Lourmat). The diagnostically relevant hydatid antigen from urine is located by performing EITB along with cyst wall antigen as control by using sera from cases of CE and sera from other controls. The EITB confirmed diagnostically relevant hydatid antigen from urine of confirmed CE patients served as excreted urinary hydatid antigen for the study.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of somatic hydatid antigens

All the antigen preparations (cyst fluid, protoscolex, and cyst wall antigens) are subjected to SDS-PAGE separately in 12% gel for separating the antigenic proteins using a submerged gel electrophoresis apparatus (Bangalore Genei), as per
the method described by Laemmli (316). Briefly, 20µg each of cyst wall, protoscolex and cyst fluid antigen along with prestained molecular weight markers, broad range (protein of apparent Mr 175kDa-6.5kDa) (BioLabs) is subjected to electrophoresis with 5% stacking gel and 12% separating gel under reducing and denaturing conditions in an electrophoretic cell at 100v for 1.30 hours. The gel, after electrophoresis, is stained in coomassie blue R-250- 0.25% staining solution and destained to remove excess stain by appropriate destaining solution. Molecular weight of the antigen bands is determined by comparing with standard molecular weight marker (New England, BioLabs) run along side, using the software provided with the gel documentation system (Vilver Lourmat). The concentration and preparation of the reagents and buffers used in electrophoresis and staining, destaining solutions are described in appendix.

**Demonstration of immunodominant fractions of somatic hydatid antigens by EITB**

All antigenic preparations such as cyst fluid, protoscolex and cyst wall antigens are subjected to EITB separately for detecting immunodominant fractions.

For each antigen preparation, 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). Membranes containing immobilized proteins are screened with confirmed CE patient sera for detecting immunodominant antigenic fractions.

**The procedure of EITB for demonstration of immunodominant fractions**

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 is separated based on the difference in molecular weight by SDS-PAGE as per standard method described earlier.
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 volts) for 1 hour transfer time.

3. *Blocking:* The free reactive sites on the nitrocellulose membrane is blocked by PBS 7.2 containing 2% bovine serum albumin (BSA) by incubating for 3 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 7.2 containing 0.1% tween 20 (PBS-T).

5. *Sample serum dilution and incubation:* Five ml of 1:100 dilution of the patient sera are 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:* Rabbit anti-human-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 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 hydrogen peroxide (H₂O₂) is added. 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.

10. *Stop reaction:* The reaction is stopped by washing with double distilled water.
CHAPTER 1

After blocking with bovine serum albumin (2%) the membrane is cut into strips for further processing. In order to confirm the immunodominant fractions and to identify the major antigenic markers, the blotted and blocked membrane strips are subjected to immunostaining using sera from human patients with definitive diagnosis of CE and also with disease and healthy controls.

*Elution of diagnostically relevant hydatid antigen from urine of CE confirmed patient*

The diagnostically relevant hydatid antigen from the urine of CE confirmed patients is eluted as per the method described by Hager and Burgess (317) with slight modification. Briefly, a volume of 350µl (180µg) of confirmed CE patients urine sample is applied to single well by modifying the existing seven well comb by scaling the comb edge with cello tape. Concentrated urine containing diagnostically relevant hydatid antigen is resolved separately with 5% stacking gel and 12% separating gel at a constant voltage (100v) for 1.30 hours. After electrophoresis, gel is removed into a clean tray and only one lane is cut and put into staining solution containing 0.25% coomassie brilliant blue R-250 for 1 hour and destained for 30min. Meanwhile, rest of the gel is kept at 4°C for 1.30 – 2 hours.

Then the stained gel is kept along with unstained gel and the band of interest is cut out and put into a sterile homogenizer tube. The gel pieces are soaked in two changes of 1ml double distilled water for 15 min.

The liquid is decanted. 0.5ml of PSB 7.2 is added and the gel is crushed with three to four strokes in a small teflon pestle which fits snugly inside the homogenizer tube. The protein is allowed to elute for 1 hour at 25°C with occasional agitation. The mixture is centrifuged briefly to pellet the crumbled gel at 10,000rpm for 30 min at 4°C (317). The supernatant is collected in a separate vial.

A SDS-PAGE was performed with eluted diagnostically relevant hydatid proteins obtained from urine and crude cyst wall antigen along with pre stained molecular weight markers, broad range (Mw 175kDa to 6.5kDa) (New England, BioLabs), according to the method of Laemmli (316). The molecular weight of the
eluted diagnostically relevant hydatid protein is detected using the software provided with the gel documentation system (Vilber Lourmat).

**Elution of diagnostically relevant antigen from hydatid cyst wall antigen**

The diagnostically relevant hydatid protein from the hydatid cyst wall antigen is eluted as per the same method described earlier for elution of protein from the urine (317). Subsequently, molecular weight of the eluted hydatid cyst wall antigen is also detected as per the similar method described earlier for hydatid proteins of urine.

**Characterization of eluted urinary hydatid protein and cyst wall protein obtained from the urine and cyst wall**

The gel eluted antigen obtained from the urine and cyst wall is characterized by performing following tests:

1. **Detection of glycoprotein**

   The glycoprotein is detected by SDS-PAGE resolved protein and on subsequent staining by periodic acid schiff (PAS) staining method (318). The SDS-PAGE is run with diagnostically relevant hydatid protein obtained from urine and cyst wall along with crude cyst wall antigen using 12% separating gel and 5% stacking gel. The gel is subjected to glycoprotein staining method by PAS procedure. Briefly, the gel is immersed in 12.5% trichloroacetic acid (TCA) (20-25 ml) for 30 min. followed by washing. Gel is treated in 1% periodic acid in 3% aqueous acetic acid for 50 min and washed thoroughly overnight. It is transferred to fuchsin-sulphite stain in dark for 50 min and washed thoroughly with 0.5% sodium metabisulphite for 30 min. The gel is washed extensively in water and is stored in 5% acetic acid.

2. **Biochemical characterization of eluted urinary hydatid protein and cyst wall protein**

   The eluted urinary hydatid protein and cyst wall protein are characterized biochemically in terms of alteration in immunoreactivity by methods of periodate...
oxidation, by lectin binding studies, by treatment with heat, by treatment with proteolytic enzymes. All these properties are demonstrated by using the ELISA methods as described by Kanwar and Vinayak (165).

2A. Periodate oxidation

The periodate oxidation is performed to determine the presence of carbohydrate moieties in the serological reactivity.

In this study, the ELISA procedure is carried out by coating two plates, one plate coated separately with eluted urinary hydatid protein and eluted cyst wall protein, treated with periodate oxidation (treated protein) and another with eluted urinary hydatid protein and eluted cyst wall protein without subjecting to any periodate oxidation (untreated protein). The untreated protein coated plate served as control to check the alteration in immunoreactivity by significant changes in OD values.

In the ELISA plate (Nunc, Denmark), three wells in one row is coated, each well with 100μl (2μg / well) of eluted urinary hydatid proteins and three wells in another row, each well coated with 100μl (2μg / well) of eluted cyst wall proteins. The plate is then treated for periodate oxidation by incubating with 0.05M sodium metaperiodate at 4°C for 24 hours in 20mM sodium acetate buffer pH 4.5 (319).

In another ELISA plate (Nunc, Denmark), three wells in one row, each well being coated with 100μl of untreated eluted urinary hydatid proteins obtained from urine and three wells in another row, each well coated with 100μl of untreated eluted cyst wall proteins obtained from hydatid cyst wall. This plate served as controls.

Then ELISA procedure is carried out as per the method described by Kanwar and Vinayak (165) to demonstrate periodate oxidation property of the eluted protein. Briefly, the periodate treated and untreated proteins coated wells are blocked with 2% BSA in PBS 7.2 for 3 hours at 37°C and washed thrice with PBS 7.2 with 0.1% tween 20. Then the plates are incubated with 100μl (1:1000 dilution) of pooled
confirmed human sera for 1.30 hours at 37°C and washed with PBS-tween 20 for three times. After treatment with 100μl (1:2000 dilution) of antihuman HRP conjugate for an hour (Bangalore Genei Pvt Ltd, India), the colour reaction is developed with O-phenylene diamine dihydrochloride (OPD) as substrate. The reaction is stopped by addition of 50μl of 2M sulphuric acid. The absorbance is measured at 450nm in an ELISA reader (Lab systems, Finland).

2B. *Lectin binding studies*

The presence of appropriate carbohydrate moieties (sugar residues) of eluted proteins are determined by performing lectin binding studies as per the method described by Kum Kum et al. (319).

Briefly, the eluted urinary hydatid protein and cyst wall protein obtained from the urine and hydatid cyst wall are separately treated with various concentrations (50, 100, 200 or 400 μg/ml) of lectins namely, concanavalin A (con A) and Jack fruit seed agglutinin (JSA) (Jacalin) at 23°C for 1 hour. After centrifugation at 10,000rpm for 30 min, the supernatant is collected. The supernatant contained lectin-treated eluted proteins.

In this study, the ELISA procedure is carried out by coating two plates, one plate coated separately with lectin-treated eluted proteins obtained from urine and hydatid cyst wall (treated protein) and another with eluted proteins obtained from urine and hydatid cyst wall without subjecting to any lectin-treatment (untreated protein). The untreated protein coated plate served as control to check the alteration in immunoreactivity by significant changes in OD values.

The ELISA is then carried out as per the method described earlier (165) using both lectin-treated and lectin-untreated proteins, instead of using periodate treated and untreated proteins.
CHAPTER 1

2C. Treatment of eluted proteins with heat

Heat treatment is carried out to determine the sensitivity of eluted hydatid specific antigen to heat by boiling eluted proteins from the urine and hydatid cyst wall separately at 100°C for 10 min, prior to coating of antigens on wells for ELISA assay.

In this method, the ELISA procedure is carried out by coating two plates, one plate coated separately with heat-treated, eluted proteins obtained from the urine and hydatid cyst wall (treated protein) and another with eluted proteins obtained from the urine and hydatid cyst wall without subjecting to any heat treatment (untreated protein). The untreated protein coated plate served as control to check the alteration in immunoreactivity by significant changes in OD values.

The ELISA is then carried out as per the method described earlier (165) using both heat-treated and heat-untreated proteins instead of using periodate treated and untreated proteins.

2D. Treatment of eluted proteins with proteolytic enzymes

Proteolytic treatment is carried out by digestion with trypsin and proteinase K at various concentrations to know the involvement of protein moieties in the serological reactivity of eluted hydatid specific proteins.

The proteolytic treatment is done by addition of 100μl of trypsin and proteinase K at concentration of 10, 100, 500 or 1000 μg/ml in triplicate wells of ELISA plate coated with protein eluted from urine and hydatid cyst wall for 2 hours at 37°C.

In this method, the ELISA procedure is carried out by coating two plates, one plate coated separately with proteolytic agents-treated eluted proteins obtained from urine and hydatid cyst wall (treated protein) and another with eluted proteins obtained from urine and hydatid cyst wall without subjecting to any proteolytic agents treatment (untreated protein). The untreated protein coated plate served as control to check the alteration in immunoreactivity by significant changes in OD values.
The ELISA is then carried out as per the method described earlier (165) using both proteolytic agents-treated and proteolytic agents-untreated proteins, instead of using periodate treated and untreated proteins.

RESULTS

Preparation of the parasite antigens

Human hydatid cyst is used as a source of antigen for the study. The hydatid cyst fluid aspirated is checked for sterility by inoculating aerobically on blood agar and Mac Conkey agar. After overnight incubation, no bacteria were found to grow, hence it is found to be sterile.

Checking the fertility of hydatid cyst

Wet mount preparation of the hydatid cyst fluid showed no active movement of flame cells. Eosin wet mount of the fluid showed some of the protoscolex to be stained with the dye whereas other protoscolex are not stained. The results of fertility assessment were inconclusive.

However, histological section of the hydatid cyst wall showed the presence of laminated and germinal layer with few protoscolex which indicates that the hydatid cyst is fertile.

Protein content of different antigen preparations from hydatid cysts

By Lowry method, the protein content of cyst wall is estimated to be 2.75 mg/ml, protoscolex is estimated to be 2.5mg / ml, cyst fluid showed the least protein content of 2mg / ml and complete homogenate hydatid antigen with 2.35 mg/ml.
Excreted urinary hydatid antigen

The urine concentrated by precipitation with ammonium sulphate is subjected to SDS-PAGE revealed a range of protein from 70kDa to 10kDa (70kDa, 41kDa, 35kDa, 24kDa, 14kDa and 10kDa) with cases of CE. SDS-PAGE revealed Mr 70kDa, 43kDa and 37kDa in disease controls and healthy controls. The hydatid specific protein excreted in the urine is located by performing EITB using sera from confirmed CE cases and controls. The test showed 24kDa protein to be specific and present in most cases of CE. The 24kDa protein is subsequently eluted manually and used as excreted urinary hydatid antigen.

SDS-PAGE of somatic hydatid antigens

The SDS-PAGE profiles of cyst wall, protoscolex and cyst fluid antigens are shown in Figure 1. The major antigenic peptides are found to be Mr 110kDa, 69kDa, 45kDa, 36kDa and 23kDa protein fractions in cyst fluid antigen; Mr 121kDa, 92kDa, 58kDa, 43kDa, 37kDa, 30kDa and 24kDa protein fractions in protoscolex antigen and Mr 129kDa, 92kDa, 75kDa, 62kDa, 47kDa, 43kDa, 37kDa, 32kDa, 27kDa, 24kDa, 14kDa and 9kDa protein fractions in cyst wall antigen. A total of 17 bands are detected in the hydatid somatic antigens. Some of the bands are not visible with cyst fluid antigen as present in cyst wall and protoscolex antigens. The presence of Mr of 43kDa, 37kDa and 24kDa are seen in all the three components.

Demonstration of immunodominant fractions of hydatid antigens by EITB

Immunodominant peptides as identified by EITB using serum from confirmed cases of CE are shown in Figure 2. In order to identify diagnostically relevant antigens, cross reactivity is checked with serum from disease controls (cysticercosis, amoebiasis, filariasis and toxoplasmosis) and healthy controls (Figure 3).
CHAPTER I

Figure 1: SDS-PAGE profile of different components of the hydatid cyst. M- standard molecular weight marker. Lane 1- cyst wall 2- protoscoleces 3- cyst fluid.

Figure 2: EITB showing immunodominant antigen bands in various components of the hydatid cyst using serum from patient with cystic echinococcosis.

Figure 3: EITB showing cross reactive antigen bands of various components of the hydatid cyst with disease control sera. Lane 1- cyst wall 2- protoscoleces 3- cyst fluid A- serum from confirmed cystic echinococcosis B- Healthy control serum, C- Cysticercosis patient serum D- Filariasis patient serum E- Amoebiasis patient serum F- Toxoplasmosis patient serum.
Elution of urinary hydatid protein and cyst wall protein from urine and from hydatid cyst wall

SDS-PAGE is performed several times with crude extract of hydatid cyst wall and also from pooled concentrated urine of confirmed CE patients separately. The molecular weight of the eluted hydatid protein from urine of confirmed CE cases and hydatid cyst wall is found to be of Mr 24kDa (Figure 4). EITB of the eluted hydatid protein from urine and hydatid cyst wall are shown in Figure 5.

Characterization of diagnostically relevant hydatid antigen obtained from urine and hydatid cyst wall

The following characteristics were observed.

1. Detection of glycoprotein

The gel eluted antigen obtained from the urine and hydatid cyst wall is subjected to SDS-PAGE, followed by staining with PAS showed both the antigens to be glycoprotein (Figure 6).

2. Biochemical characterization of eluted antigen

2A. Periodate oxidation

The sodium meta periodate oxidation of eluted protein of both cyst wall and urine showed that immunoreactivity is altered significantly (P < 0.05). The results of periodate treatment showed the presence of carbohydrate moieties in the serological reactivity (Figure 7).

2B. Lectin binding studies

The lectin assay showed a significant decline (P < 0.05) in the immunoreactivity for hydatid cyst wall antigen and urinary protein following treatment with concanavalin A and Jacalin. It showed that eluted cyst wall and urine antigen has con A specific sugar and galactose residues (Figure 8 and 9).
CHAPTER 1

Figure 4: SDS-PAGE profile of eluted protein from the urine and hydatid cyst wall. M- standard molecular weight marker, Lane 1- crude cyst wall antigen, 2- eluted urine antigen, 3- eluted cyst wall antigen.

Figure 5: EIIB of the eluted urinary antigen and cyst wall antigen; Lane 1- crude cyst wall antigen, 2- eluted urine antigen, 3- eluted cyst wall antigen.

Figure 6: Periodic acid Schiff staining of eluted urinary antigen and cyst wall antigen; Lane 1- crude cyst wall antigen, 2- eluted urine antigen, 3- eluted cyst wall antigen.
CHAPTER 1

Figure 7: Effect on immunoreactivity of 24kDa cyst wall protein (a) and 24kDa urinary protein (b) with periodate treatment (1) and without periodate treatment (2). (Mean absorbance values of two sets tested in triplicate (P < 0.05).

Figure 8: Effect on immunoreactivity of 24kDa cyst wall protein (a) and 24kDa urinary protein (b) without Con A treatment (0) and with various concentration of Con A treatment (50µg, 100µg, 200µg and 400µg). (Mean absorbance values of two sets tested in triplicate (P < 0.05).

Figure 9: Effect on immunoreactivity of 24kDa cyst wall protein (a) and 24kDa urinary protein (b) without Jacalin treatment (0) and with various concentration of Jacalin treatment (50µg, 100µg, 200µg and 400µg). (Mean absorbance values of two sets tested in triplicate (P < 0.05).
Figure 10: Effect on immunoreactivity of 24kDa cyst wall protein (▲) and 24kDa urinary protein (●) without heat treatment (1) and with heat treatment (2). (Mean absorbance values of two sets tested in triplicate (P > 0.05).

Figure 11: Effect on immunoreactivity of 24kDa cyst wall protein (▲) and 24kDa urinary protein (●) without Trypsin treatment (0) and with various concentration of Trypsin treatment (10μg, 100μg, 500μg and 1000μg). (Mean absorbance values of two sets tested in triplicate (P < 0.05).

Figure 12: Effect on immunoreactivity of 24kDa cyst wall protein (▲) and 24kDa urinary protein (●) without Proteinase K treatment (0) and with various concentration of Proteinase K treatment (10μg, 100μg, 500μg and 1000μg). (Mean absorbance values of two sets tested in triplicate (P < 0.05).
CHAPTER I

2C. Treatment of eluted proteins with heat

Heat treatment of eluted protein of both cyst wall and urine for 10 minutes at 100°C revealed no significant reduction (P>0.05) in reactivity indicating that both are heat stable (Figure 10).

2D. Treatment of eluted proteins with proteolytic enzymes

The proteolytic treatment of eluted protein of both cyst wall and urine with enzymes, proteinase K and trypsin significantly effected the immunoreactivity (P<0.05) of both proteins. This reflects the presence of protein nature of antigen and their participation in serological reactivity (Figure 11 and 12).

DISCUSSION

CE is a chronic asymptomatic parasitic infection with viable cysts persisting throughout the life of an intermediate hosts (285). Serodiagnostic tests for specific hydatid antibodies have an important role in confirmation of clinical diagnosis and in seroepidemiological studies in CE. A variety of different antigenic preparations (whole cyst fluid and protoscolex extracts) has been used in the serological tests for detection of antibodies in serum. These antibody-based tests show varying sensitivity and specificity and thus make the diagnosis of CE still imperfect.

The sensitivity and specificity of various immunoassays has been increased by purifying crude cyst fluid either by salt fractionation (148, 159, 160), affinity chromatography (159), high performance liquid chromatography (HPLC) (146, 150, 225) or by electro elution (230). It is suggested that the purification of the crude antigens help in better diagnosis of CE (214).

In the present study, human hydatid cyst is used as a source of antigen for identification of diagnostically relevant protein and for their use in serodiagnosis. Similar to the present study, other studies have also used human hydatid cyst fluid as a source of antigen in conventional serological tests such as CFT, IHA, DD, LA and IEP with varying sensitivity and specificity (127, 141-143, 247, 248). It is suggested
that the human hydatid cyst fluids are less contaminated by serum proteins, hence they can be used in serodiagnosis (224).

Hydatid cyst fluid is a complex mixture of parasite and host-derived molecules. It contains several antigens derived from the metabolism of the parasite together with many components from the host (211). Therefore, the type and concentration of parasite-derived molecules are likely to be different in fertile and non-fertile cysts. It has been demonstrated that the highest concentration of antigenic protein was shown in batches of bovine fertile hydatid cyst fluid than that of bovine non-fertile hydatid cyst fluid. Moreover, it has also been demonstrated that the composition and antigenic performance of bovine cyst fluid are largely affected by the fertility of the cyst (140).

Thus, for an efficient diagnostic use, there is a need for the use of fertile hydatid cyst as a source of antigen in the serodiagnosis of CE. Fertile hydatid cysts from animals have been used in many studies (149-151, 153, 154, 259, 276). Kanwar et al. (259) obtained the cyst fluid from fertile hydatid cyst of sheep, goat and pigs for use as antigen in ELISA with 100% sensitivity and 76% specificity and in EITB with 100% sensitivity and specificity for serodiagnosis of CE. Craig (276) used fertile sheep hydatid cyst fluid in ELISA with good sensitivity and specificity.

Similar to this, other studies have also found that fertile hydatid cyst is ideal for use in serodiagnosis of CE (141, 142).

In the present study, fertility assessment of the surgically removed human hydatid cyst was carried out. The result of the fertility test, however, was inconclusive. This is possibly due to a month long treatment with albendazole (400mg / day) before surgery to the patient from which the cyst is taken, thus affecting viability of the cyst. The results observed in the present study is in agreement with that of Liance et al. (320) who carried out fertility assessment test with human cysts of alveolar echinococcosis and found that viability was altered by benzimidazole therapy that resulted in an abortive growth.
The histological section of hydatid cyst wall, in the present study, however was able to identify the cyst as fertile by the presence of thin germinal layer with free and attached protoscoleces and the presence of thick laminated layer, a unique feature of hydatid cyst. Garg et al. (195) also demonstrated fertility of the hydatid cyst by demonstrating germinal lining and protoscoleces.

Different antigen preparations from hydatid cysts

In the present study, different components of human hydatid cyst namely cyst fluid, protoscolex and cyst wall antigens are characterized by SDS-PAGE profile and diagnostically relevant hydatid proteins are identified by the EITB.

SDS-PAGE profile of hydatid antigen preparations

Human hydatid cyst fluid (HCF): Majority of the studies reported earlier extensively used cyst fluid as a source of antigen (145 - 154).

In the present study, human hydatid cyst fluid examined after resolution by SDS-PAGE under reducing and denaturing condition showed a range of polypeptides with Mr of 110kDa, 69kDa, 45kDa, 36kDa and 23kDa demonstrable by coomassie brilliant blue R-250 (Figure 1). Results of the present study are in agreement with studies reported by other authors (154, 267, 321). Garcia et al. (321) demonstrated seven antigenic bands located between 8kDa and 120kDa while El-Zayyat et al. (154) reported several polypeptide bands in the range of 14kDa to 200kDa using camel hydatid cyst fluid. In Leggatt et al. (267) studies, sheep cyst fluid using gradient SDS-PAGE was found to contain a major parasite bands at approximately 38kDa (larger subunit of antigen 5) and the three subunits of antigen B, at approximately 12kDa, 16kDa, 23kDa under reducing condition. A dominant and most consistently demonstrable proteins occurring as a complex was demonstrated in the 62kDa -52kDa region (267).
Results of the present study differed from the result of the SDS-PAGE profile of hydatid fluid antigens from camels (Egypt), sheep (Australia and Tunisia), moose (United states), cattle (Switzerland and Australia) and a human (United states) along with standard *Echinococcus* antigen (catalogue no52731) as reported by Maddison et al. (268). They demonstrated bands in a range of >200kDa to 14kDa under reducing conditions.

In the present study, the number of polypeptide fraction obtained with human cyst fluid is found to be less. This is possibly due to the use of human hydatid cyst fluid as source where in other studies, sheep (267, 268), camel (154, 268), cattle (268) cyst fluids were used as a source of antigen. It is demonstrated in one study that the number of antigenic polypeptides in human cyst fluid is much smaller than those of polypeptides in camel cyst fluid (322). This could be also due to the use of cyst fluid from a single cyst and not pooled cyst fluid from many cysts. Apart from this, the cyst fluid in the present study is not subjected to concentration method such as lyophilization or ammonium sulphate precipitation method followed in other studies (259, 283).

*Human hydatid protoscole antigen (HPR):* Little attention has been given to characterize the hydatid protoscole antigens and their potential utility in the serodiagnosis of CE. Rafiei and Craig (156) used protoscoleces from sheep cysts in the ELISA for antibody detection in human CE and reported a sensitivity of 90.5% and a specificity of 57%. The authors suggested that protoscoleces could also be used as source of antigen for detection of antibody in human CE, although crude extract contained cross reactive antigens. Other studies have also used protoscolex of hydatid cysts of camel (150, 217, 229) and horse (232, 233) as source of antigen in various serological assays.

In the present study, SDS-PAGE profile of crude extract of protoscolex showed a range of polypeptides with Mr of 121kDa, 92kDa, 58kDa, 43kDa, 37kDa, 30kDa and 24kDa (Figure 1). The result of the present study is in agreement with study by Rafiei and Craig (156) where they used protoscolex antigen of sheep cyst and found proteins in the range of 10kDa to 125kDa. Rafiei and Craig (156) have
also noted inter-host variation in the antigenic components of cyst fluid and protoscolex preparations of horse, camel and sheep by EITB which reflect the genotypic or strain variation within *E. granulosus*.

*Human hydatid cyst wall (HCW):* It is suggested that hydatid cyst wall could also serve as a good source of antigen (228, 323, 324). Rickard et al. (311) using ovine and equine hydatid cyst was able to demonstrate two major parasite antigen, antigen 5 and antigen B diffusely present in the parenchymal cells of laminated membrane and tegumental region of protoscolex. They suggested that due to variations in different physical characteristics of these two antigens (antigen 5 and antigen B), there is a difference in permeability of the laminated membrane to the two antigens. Varela-Diaz et al. (249) observed high prevalence of antibodies to antigen 5 in patients with CE, which possibly is due to the readily permeable nature of antigen 5.

In the present study, SDS-PAGE of crude extract of cyst wall showed a range of polypeptide fraction with Mr 129kDa, 92kDa, 75kDa, 62kDa, 47kDa, 43kDa, 37kDa, 32kDa, 27kDa, 24kDa, 14kDa and 9kDa (Figure 1). Sanchez et al. (323) used brood capsules and protoscoleces of human hydatid cyst, stained by immuogold method but failed to demonstrate antigen B in the brood capsule contents or in the tegument of the protoscoleces. They suggested that the absence of antigen B in the brood capsule contents or in the tegument of the protoscoleces might be due to the procedure involved in antigen purification, or to the specificity of the antisera used or to different antigenic expression within *E. granulosus*. The antigen B however, has been demonstrated on brood capsules and protoscoleces in other studies (228, 311), possibly due to the degradation of the components of the surrounded extra-cellular matrix of the parenchymal cells associated with the brood capsule membranes or germinal layer.

In the present study, SDS-PAGE using cyst fluid, protoscolex and cyst wall antigen showed that the dominant and most consistently demonstrable proteins appeared at approximately 43kDa, 37kDa and 24kDa in all three antigen preparations. By EITB, 24kDa of three immunodominant peptides (43kDa, 37kDa and 24kDa) is found to be hydatid specific as discussed later. A less intense,
approximately 92kDa band was found to be present in the cyst wall and protoscolex antigens but not in the cyst fluid antigen.

The absence of high molecular weight (Mr 92kDa) protein fraction from cyst fluid may be due to (i) non-secretory / non-excretory nature of the molecule, (ii) not concentrating hydatid cyst fluid by lyophilization or ammonium sulphate precipitation as carried out in other studies (259, 283) or (iii) being present in small quantity and missed during sample preparation.

In the present study, the occurrences of 14kDa and 9kDa bands were considerably weaker or barely visible with cyst wall, protoscolex and cyst fluid antigens. Some variability was observed between the occurrence and intensity of antigenic bands throughout the electrophoretic path among all three antigens, cyst wall, protoscolex and cyst fluid.

**Demonstration of immunodominant fractions of somatic hydatid antigens by EITB**

In the present study, when the crude extract of cyst fluid, protoscolex and cyst wall antigens are probed with CE confirmed patient sera by the EITB assay, reactivity is observed with bands of Mr 43kDa, 37kDa, 24kDa and 9kDa in all the three antigenic preparations (cyst fluid, protoscolex and cyst wall). Strong reactions with 43kDa, 37kDa and 24kDa are consistently observed with all the three antigens (Figure 2). Reaction in the 9kDa region is found to be variable among the three antigen preparations. In addition to all the above bands, a marked 92kDa component is present in both protoscolex and cyst wall antigens but is absent in cyst fluid antigen.

Doiz et al. (269) used purified antigen of sheep cyst fluid and detected proteins of 12kDa to 14kDa, 16kDa, 20kDa, 24kDa-26kDa, 34kDa, 39kDa and 42kDa molecular masses in 15% to 96% of patients. They reported 100% sensitivity by using the combination of two of the three proteins (20kDa, 39kDa and 42kDa). Verastegui et al. (151) used bovine cyst fluid and found that three antigenic bands of
8kDa, 16kDa and 21kDa were diagnostically important. However, cross reactions to the 8kDa, 16kDa and 21kDa band was demonstrated in 12%, 4% and 4% of sera from patients with cysticercosis.

Shepherd and Mc Manus (220) identified 5 major subunits (12kDa, 16kDa, two at 20kDa and 38kDa) from sheep cyst fluid by EITB. The 12kDa and 16kDa molecules were found to be specific. Rashed et al. (325) using EITB reported 44kDa, 34kDa, 29kDa and 8kDa as *Echinococcus* specific with 100% sensitivity and specificity. Similarly, Garcia et al. (321) found immunodominant bands located between 8kDa and 120kDa while Ramadan et al. (178) found six molecular weight antigens with molecular masses of 7kDa, 20kDa, 28kDa, 35kDa and 127kDa to be of diagnostic importance. Kanwar et al. (259) using sheep cyst fluid was able to demonstrate two hydatid specific bands of Mr 116kDa and 8kDa. High molecular proteins have also been reported to be of diagnostic value. Shambesh et al. (271) found two high molecular weight proteins with molecular masses approximately 100kDa and 130kDa that were strongly recognized by sera from CE patients.

In the present study, specificity of the polypeptides was determined by using serum from patients with other diseases such as cysticercosis, filariasis, amoebiasis, and toxoplasmosis and serum from healthy controls. The result showed that Mr 92kDa, 24kDa and 9kDa bands were highly specific proteins of diagnostic importance.

The 43kDa is found to be non-specific in the present study as it reacted with healthy controls as well as with disease controls (Figure 3). 37kDa band showed cross reaction with cysticercosis and filariasis sera. Cross reaction to 37kDa with sera from various helminthic infections was also observed in other study (220). The cross reactive nature of the 37kDa molecule has also been demonstrated by EITB or immunoprecipitation in other studies (171, 268). Leggatt et al. (267) found cross reactive nature not only with cestode, trematode and nematode parasite but also with normal control sera. The cross reaction of 37kDa is mainly attributed to the presence of antigen’s phosphorylcholine (PC) epitope (171, 220).
Demonstration of immunodominant fractions of urinary hydatid antigens by EITB

Urinary parasitic antigen as a potential source of antigen in serodiagnosis is a recent approach. The specific monoclonal antibody (MAb) has been raised against the urinary antigens collected from the urine of the cases of urinary schistosomiasis (163) and Chaga's disease (164).

Bosompem et al. (163) concentrated urine of patients in *S. haematobium* by 50% saturation with ammonium sulphate. Various fractions precipitated were purified by sephadex G-200 column chromatography, of which the fraction UP$_2$S was found to contain high concentrations of *S. haematobium* urinary antigens (ShAgs) as determined by micro-plate ELISA. The fraction UP$_2$S was used to produce specific monoclonal antibodies for detection of antigen in urine of patients infected with *S. haematobium*. In another study, *Trypanosoma cruzi* antigens in the urine of experimentally infected dogs were detected by ELISA. Subsequently, *T. cruzi* urinary antigen was purified by affinity chromatography followed by HPLC and subsequently characterized. The specific monoclonal antibodies against the purified protein was produced and used for demonstration of antigen in the urine for diagnosis of Chagas' disease (164).

In CE, earlier studies have shown that hydatid antigens appear in urine of people infected with CE (7, 181). To our knowledge, the present study constitutes the first attempt for the purification of diagnostically relevant hydatid protein from urine specimen for use in serodiagnosis of CE.

In the present study, the SDS-PAGE separated proteins, obtained from concentrated urine of confirmed CE cases and controls were subjected to EITB which revealed Mr 24kDa to be specific (Figure 4 and 5). Mr 24kDa protein was excreted in the urine of most cases of the CE. The 24kDa protein identified by coomassie brilliant blue staining was obtained by manual elution method which was done by cutting the gel containing hydatid specific band at 24kDa. The 24kDa cyst wall antigen is used as a control for 24kDa urinary hydatid antigen. Though the
method proved to be effective for isolation of 24kDa urinary hydatid antigen from the urine without further contaminants, it is nevertheless a laborious, time consuming process requiring several successive run on SDS-PAGE.

*Characterization of both the urinary hydatid protein and cyst wall protein*

The eluted proteins from urine and cyst wall are subjected to SDS-PAGE followed by PAS staining method revealed both molecules to be glycoprotein (Figure 6). The findings of the present study are in agreement with the reported study of Al-Yaman and Knobloch (166) and Chordi and Kagan (222). The glycoprotein nature of the protein Eg20 of sheep hydatid fluid, a hydrophobic 20kDa molecule was reported by using Schiff’s reagent (Al-Yaman and Knobloch (166). Chordi and Kagan (222) analyzed sheep hydatid fluid by immunoelectrophoretic method. They used Schiff’s reagent to stain the immunoelectrophoretic slides and showed that out of the 1 to 8 parasite specific bands, band no 1, 2 and 4 contained some carbohydrate components and thus showing that antigenic proteins are of glycoprotein in nature.

The characterization of purified protein from the parasite or urine of infected individual is essential since it helps not only to produce monospecific serum or recombinant antigen but also to know the origin and significance of infection. The characterization of purified protein from urine has been reported in other parasitic diseases such as trypanosomiasis (164) and schistosomiasis (163). In trypanosomiasis, urinary antigen characterization was performed by two-dimensional gel electrophoresis, lectin affinity chromatography, proteolytic digestion and EITB. The isolated urinary antigen exhibited a relative molecular size of 80kDa, with an isoelectric point of 6.2 to 6.8, binding to concanavalin A and sensitive to trypsin (164).

In canine echinococcosis, antigenic molecule present in faeces from infected dogs was characterized. The *E. granulosus* coproantigen was sensitive to periodate suggesting that carbohydrates are involved in the antigenic structure. Protease
treatment of antigenic molecules resulted in an 11% reduction in absorbance in ELISA, indicating that protein components were also present which are affected by enzyme activity. Lectin-binding ELISA assays indicated strong affinity of *E. granulosus* coproantigen to concanavalin agglutinin and *Lens culinaris* agglutinin, and moderate binding to wheat-germ agglutinin and peanut agglutinin. No binding was detectable to *Ulex europaeus* agglutinin-I, *Bandeiraea simplicifolia* or *Dolichos biflorus* agglutinin. These data indicate that *E. granulosus* coproantigen from infected dog faeces possibly contained alpha-D-mannose and/or alpha-D-glucose, beta-galactose and N-acetyl-beta-glucosamine residues (326). In CE, similar characterization of purified antigen fraction from the hydatid cyst fluid was reported by Kanwar and Vinayak (165) and Al-Yaman and Knobloch (166).

In the present study, both 24kDa urinary hydatid protein and 24kDa cyst wall protein are also characterized by periodate oxidation, lectin binding studies, heat treatment and proteolytic treatment.

On periodate oxidation of 24kDa urinary hydatid protein and 24kDa cyst wall protein, there is alteration in immunoreactivity suggesting the involvement of carbohydrate in 24kDa proteins obtained from both the urine and cyst wall (Figure 7). Similar to the present study, Al-Yaman and Knobloch (166) showed alteration in immunoreactivity of Eg20 obtained from sheep hydatid fluid while Kanwar and Vinayak (165) reported a significant alteration in the immunoreactivity of the affinity purified 8kDa molecule obtained from sheep hydatid fluid on treatment with sodium meta periodate.

Lectin binding studies showed a decline in immunoreactivity after treating the eluted fraction with respective lectins (concanavalin A and jacalin lectins) by ELISA, thus suggesting that both the 24kDa urinary hydatid protein and 24kDa cyst wall protein contained con-A specific sugar and galactose residues (Figure 8 and 9). Kanwar and Vinayak (165) carried out a lectin binding assay of affinity purified low molecular weight (Mr 8kDa) hydatid specific protein obtained from sheep hydatid fluid using lectins namely concanavalin A and *Triticum vulgaris*. They demonstrated
that Mr 8kDa is a glycoprotein polypeptide which contains alpha-D-glucose and N-acetyl-D-glucosamine as carbohydrate moieties.

In the present study, only two lectins (concalvin and jacalin) were used in the lectin binding assay of 24kDa urinary and cyst wall proteins to identify other sugar residues in the immunoreactivity of these two proteins. Other lectins such as Triticum vulgaris lectins and Dolichose biflorus lectins were not used because 24kDa proteins eluted from the urine and cyst wall was too low in quantity.

Heat treatment studies of 24kDa urinary and cyst wall protein showed that these are heat stable as there was no significant reduction in their immunoreactivity on treatment with heat (Figure 10). Kanwar and Vinayak (165) demonstrated that the exposure of affinity purified 8kDa (obtained from sheep hydatid fluid) at 100°C for 10 minutes did not alter the immunoreactivity of the protein. In another study Eg20, a hydatid specific protein obtained from sheep hydatid fluid was also not affected by boiling (166).

Treatment with proteolytic enzymes such as trypsin and proteinase K, showed loss of immunoreactivity of 24kDa proteins thereby suggesting that 24kDa molecule is protein in nature (Figure 11 and 12). Eg20 and 8kDa and 116kDa molecules reported in other studies were also found to be proteins (165, 166).

Al-Yaman and Knobloch (166) on treating Eg20 to pronase found decrease in antibody binding at a concentration of 500μg / well to human lgG. Similarly, on treatment of 8kDa and 116kDa obtained from sheep hydatid fluid with proteolytic enzymes (pepsin, trypsin and pronase), there was a significant reduction in immunological activity for both rabbit monospecific antibody and confirmed patients serum (165).

In conclusion results of the study shows that 24kDa molecules obtained from the urine and cyst wall to be a thermostable glycoprotein containing con A specific sugars and galactose residues.
Chapter 1

Summary

In the present study, human hydatid cyst is used as a source of antigen for identification of diagnostically relevant protein and for their use in serodiagnosis. The SDS-PAGE characterization of human hydatid cyst fluid revealed a range of polypeptides with Mr of 110kDa, 69kDa, 45kDa, 36kDa and 23kDa demonstrable by coomassie brilliant blue R-250. The number of polypeptide fraction in human cyst fluid is found to be less.

Reports are scanty on use of protoscolex and cyst wall as a source of antigen in the serodiagnosis. In the present study, SDS-PAGE profile of crude extract of protoscolex showed a range of polypeptides with Mr of 121kDa, 92kDa, 58kDa, 43kDa, 37kDa, 30kDa and 24kDa. The crude extract of cyst wall after resolution showed a range of polypeptide fraction with Mr 129kDa, 92kDa, 75kDa, 62kDa, 47kDa, 43kDa, 37kDa, 32kDa, 27kDa, 24kDa, 14kDa and 9kDa. The dominant and most consistently demonstrable proteins appeared at approximately 43kDa, 37kDa and 24kDa in all three antigen preparations. A less intense, approximately 92kDa band was present in the cyst wall and protoscolex antigens but not in the cyst fluid antigen. The occurrences of 14kDa and 9kDa bands were considerably weaker or barely visible with cyst wall, protoscolex and cyst fluid antigens.

In the EITB assay, when the crude extract of cyst fluid, protoscolex and cyst wall antigens are probed with CE confirmed patient sera, reactivity is observed with bands of Mr 43kDa, 37kDa, 24kDa and 9kDa in all the three components. In addition to all these bands, a marked 92kDa component is found to be present in both protoscolex and cyst wall antigens but absent in the cyst fluid antigen.

In the present study, specificity of the bands was determined by using serum from patients with other diseases such as cysticercosis, filariasis, amoebiasis, and toxoplasmosis and serum from healthy controls. Mr 92kDa, 24kDa and 9kDa bands are found to be highly specific protein of diagnostic importance. The 43kDa is found to be non-specific in the present study as it reacted with healthy controls as well as
with disease controls. 37kDa band showed cross reaction with cysticercosis and filariasis sera.

Urinary parasitic antigen as a potential source of antigen in serodiagnosis is a recent approach. To our knowledge, the present study constitutes the first attempt for the purification of diagnostically relevant hydatid protein from urine specimen for use in serodiagnosis of CE.

In the present study, the SDS-PAGE separated proteins, obtained from concentrated urine of confirmed CE cases and controls were subjected to EITB which revealed Mr 24kDa to be specific. Mr 24kDa protein was excreted in the urine of most cases of the CE.

The 24kDa molecules from urine and cyst wall on treatment with periodate oxidation showed the involvement of carbohydrate residues in the glycoprotein. Lectin binding studies with concanavalin A and jacalin lectins revealed the 24kDa glycoprotein to contain con A specific sugars and galactose residues. Heat treatment studies showed that the 24kDa glycoproteins are heat stable.