Preparation of Semi-purified Laminated Layer of Hydatid Cyst for Diagnosis of Infection

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Summary

A semi-purified laminated layer (LL) fraction of cyst wall of *Echinococcus granulosus* was prepared by affinity chromatography. Also its antigenic reactivity evaluated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE analysis of the LL fractions showed that the bands in the 50-55kDa region and the 25-31kDa region were greatly reduced in semi-purified fraction in comparison with unpurified sample, which presumably reflected to removal of heavy and light immunoglobulin chains. An immunoblot data also confirms considerable reduction in the presence of IgG chains within the semi-purified fraction. The current study indicated that the bands at 66, 55 and 27kDa were significant molecules for diagnosis of human cystic hydatidosis patients after purification of the crude sample.

***Keywords*:** *Echinococcus granulosus,* laminated layer, affinity purification

Introduction

The cystic echinococcosis is caused by an infection with the metacestode stage of the dog tapeworm, *Echinococcus granulosus.* The cyst wall of metacestodes consists of three layers, which the laminated layer is unique to the genus *Echinococcus.* This layer is a semi-permeable structure that allows the passage of macromolecules from the host to the parasite but prevents microorganisms and cells getting to germinal

layer. Although the mechanisms by which macromolecules penetrate into hydatid cysts are not known, Coltorti and Varela-Diaz (1975) have detected host IgG in cyst fluid from experimental animals. Gottstein and Felleisen (1995) suggested that, the survival strategy of the metacestode clearly focus on the important role played by the LL. Host serum proteins including IgG and IgM, freely penetrated the LL and reach to the germinal layer which somehow prevents or regulates their passage into the cysts (Schantz & Kagan 1980, De-Rycke *et al* 1990).

Using indirect fluorescent (IFA) technique Ali-Khan and Siboo (1981) reported that specific antibodies in alveolar hydatidosis' sera are bound to the epitopes on the LL. In terms of antigenicity, Gottstein *et al* (1983) isolated a specific fraction (Em2) from crude metacestode of the *E.multilocularis* by immunoaffinity chromatography against anti-*E.granulosus* hydatid fluid IgG coupled to CNBr-Sepharose 4B. This antigen has a molecular mass of 54 kDa and is located within the LL (Furuya *et al* 1989, Gottstein 1985, Gottstein 1992). Em2 is now confirmed as species-specific antigen and WHO recommends the Em2-ELISA as a reference immunodiagnostic test for alveolar echinococcosis. A partially purified Em 18/16 enriched fraction from *E.multilocularis* LL prepared by isoelectric focusing (Ito *et al* 1997), may have a greater specificity than the Em2 (plus)-ELISA. Since cyst fluid contains two antigens (are 5 and antigen B), both antigens have shown to have cross reactions with other helminths in particular *E.multilocularis* and *Tenia* solium (Thompson 1986, Kanwar *et al* 1992, Craig *et al* 1995).

The LL contains various host and parasite molecules it was decided to investigate the immunogenicity of this layer in *E.granulosus* with respect to its role in immunodiagnosis. The present study attempts to remove host components such as sheep serum and/or sheep IgG, by preparation of a semi-purified LL fraction using an affinity chromatography column and to evaluate its antigenic reactivity by SDS-PAGE and immunoblotting. An affinity purification of a sheep laminated layer (SLL) extract using an anti-sheep serum column was used in this study.

Materials and Methods

Parasitic material. The primary sources of hydatid cyst fluid, protoscoleces and LL were from natural infections in the liver and lungs of sheep slaughtered at a local abattoir (Manchester, UK). Cyst fluid was removed from the cysts and the cyst walls were cut and placed in phosphate buffer saline (PBS, pH 7.4). The germinal layers were carefully scraped from the LLs with forceps. The separated LL extracts were washed several times in PBS and microscopically examined to confirm the absence of traces of the germinal layers or the protoscoleces. The tissues were incubated in 1M NaCl at 4˚C for 30 min and washed with PBS. The LLs were first homogenized with an equal volume of PBS. The preparations were then disrupted by sonication in a 150W ultrasonic disintegrator, on ice for 15min (10sec on and 5sec off). Some of the sonicated materials (milky suspension) were kept in at -20˚C. The remainder of suspensions were centrifuged at 10,000 rpm at 4˚C for 10min. The suspensions and/or pellets used for SDS-PAGE and western blotting.

Protoscolex extract antigen (Px). 1ml of the protoscoleces (approximately 10^5 protoscoleces) of *E.granulosus* derived from cysts in the liver and lungs of the sheep were freeze-thawed 3 times and mixed with four volumes of PBS, pH7.4 containing aprotinin at 0.1mg/ml. The mixture was sonicated in a 150W ultrasonic disintegrator, 10sec on and 5sec off on ice until no intact protoscoleces were visible microscopically (approximately 15min). The preparation was then left on ice for one hour and centrifuged at 10,000*g* for 30min.

Semi-purified laminated layer fraction. This sample was prepared using an affinity chromatography column. In order to absorb the LL components against sheep serum molecules, this column was made according to the manufactures instruction (Pharmacia, Germany) as a follows; 600µ| of rabbit anti-sheep whole serum (Sigma, UK) containing 35mg protein was dialyzed against fresh coupling buffer. 2g of CNBr activated sepharose 6MB (Sigma, UK) was weighed out and slowly suspended into 1mM HCl (ice-cold). The swollen gel was washed on a sintered glass filter (porosity G3) with 400ml of 1mM ice-cold HCl for 15min, the supernatant being sucked off between successive additions. 1mM HCl was sucked off until cracks appeared in gel.

The washed dry gel was immediately transferred into antibody solution and incubated end over end at RT for 2h. The mixture allowed settling for few minutes. When the coupling reaction was completed then the supernatant was removed. The excess non-covalently bound ligand was washed (with high and low pH buffer) and unreacted sites were blocked with 0.2M glycine pH8.0 at RT during end over end mixing for 2h. A plug of the glass wool was placed into a 10ml syringe and gel added to its. Finally the gel was stored in 0.15M PBS pH7.2 at 4˚C. Before running column, it was checked at two stages to evaluate the coupling of protein to the beads and the absorbing of the sheep serum to an anti-sheep serum. 50ml PBS was passed through column to ensure packed down sufficiently and to equilibrate. 2ml of the supernatant LL were passed through column using a pump at a flow rate of 1ml/min. The optical density values of the solution passing through the column before and after elution was monitored by a Pharmacia LKB-optical unit UV-1 (Germany) at 280nm and printed on paper with speed of 5mm/min. The amount of the protein concentration of the sample was measured by Bradford assay (Bradford 1976).

Serum samples. Normal sheep sera (NSS) were obtained from healthy sheep.

Monoclonal antibodies to ovine IgG light chain. A mouse monoclonal antibody to ovine IgG1 isotype (specific for light chain) was commercially purchased (Serotec, USA).

Standarddization of the semi-purified laminated layer. The coupling of the protein to sepharose-6MB beads was confirmed by fluorescence microscope and the absorbing capabilities tested by passing through normal sheep serum through the column. After the LL fraction had been passed through the column the chromatographic trace showed that the very little material had bound to the column, since no peak was obtained after elution with glycine HCl. This indicated that either the amount of sheep serum components bound to the column was so small as not to be detected by the UV monitor or that preparation was inefficient. The sample was therefore passed through the column twice and also incubated with antibody coated sepharose-6MB beads in incubator twice for 2h. The final preparation was then concentrated using Amicon 10 and protein content measured by Bradford assay (Bradford 1976). In order to investigate the antigenicity of the semi-purified LL preparation at each stage

in the purification procedure, the reactivity against anti-sheep IgG, rabbit anti-sheep seram, human pooled positive and negative sera was assessed by ELISA and compared with the crude unpurified extract. In all assays the starting protein concentration was the same for each preparation prior to titration. The final preparation procedure involved incubation of the antigenic extract with antibody coated beads and produced a fraction which had lowest reactivity against anti-sheep IgG. This sample was named as a semi-purified fraction and the antigenic reactivity of that was immunologicaly evaluated.

Analysis of parasite antigens by SDS-PAGE. The SDS-PAGE technique was performed according to Laemmli (1970), under reducing conditions using 12% polyacrylamide gel. The standard molecular markers were used for each gel from range 14 up to 97KDa (Novel Experimental Technology $_{TM,}$ USA). A semi-purified LL fraction was loaded onto gels (30µg protein per minigel) and compared with the crude LL and sheep serum. Samples were boiled for 3min in sample buffer and centrifuged at 2000 rpm for 5min. The gels were run in running buffer at 20-25mA per gel until the tracking dye reached the bottom of the gels. The gels were placed in the trays containing coomassie blue and were gently rotated on an orbital shaker at RT for 2-5 h. Destain was added until clear blue bands were visible. Gels were dried using a vacuum gel drier.

Immunoblotting assessment. In order to characterize a semi-purified LL fraction prepared by an affinity chromatography, anti-sheep IgG, mouse monoclonal antibody to ovine IgG1 isotype (specific for light chain) as probes for host molecules and total IgG and IgG4 subclass antibody response of human sera to this fraction were evaluated by immunoblotting. Towbin *et al* (1979) demonstrated the electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose paper. Transfer took place at 220-250mA using a miniblotter (Hoeffer, USA) for 2h or 35mA for overnight. Antigen samples with optimized dilutions were loaded onto gels. Non specific binding sites on the NCP were blocked with PBS plus 5% skimmed milk. After washing, the NCP was probed the human pooled positive and negative sera (1/200) for 70min and incubated the appropriate alkaline phosphatase conjugates (as

used for ELISA). The mouse monoclonal antibody was used with an optimal dilution (1/300) at RT for 70min. For IgG4 subclass response, the mouse monoclonal anti-human IgG4 (RJ4) (Oxoid-Unipath, Birmingham, UK) and goat anti-mouse IgG Fc-specific were purchased (Sigma, UK). Finally blots were developed with NBT plus BCIP as substrate.

Results

In order to determine the polypeptide profile of the semi-purified LL prepared by affinity chromatography, samples were run on gels at similar protein concentration as unpurified sheep LL sample (NSLL). Results (Figure 1) showed that the semi-purified LL had difference in banding profiles in comparison with normal LL extracts. Bands in the 60-66kDa region (probably albumin) were stronger in semi-purified sample and bands in the 50-55kDa region and the 27-31kDa were more obvious in normal LL while the semi-purified LL revealed faint bands in these regions (50-55kDa and the 27-31kDa). The reduction in bands in the 50-55kDa region and the 27-31kDa region presumably reflected removal of heavy and light immunoglobulin chains.

The presence of sheep IgG in the LL was assessed by blotting with anti sheep IgG (Figure 2). This produced a different profile in the samples. Bands in the 55-60kDa region (IgG heavy chain) and the 27-29kDa region (IgG light chain) were strongly recognized in the crude LL extracts and sheep serum, while semi-purified LL antigen revealed only a weak reaction at 55kDa and a very faint reaction at 29kDa. These results indicated that at least host immunoglobulins appeared to be reduced in the semi-purified LL.

The semi-purified LL prepared was blotted against anti-human IgG and monoclonal antibody to ovine light chain IgG and compared with protoscolex (Px), normal sheep serum (NSS) and crude LL antigens. Anti-humanIgG strongly recognized IgG molecules in heavy and light chain region in both NSLL and NSS, indicating cross-reactivity between human and sheep IgG. These bands were absent in semi-purified LL (Figure 3 section a, track 3).

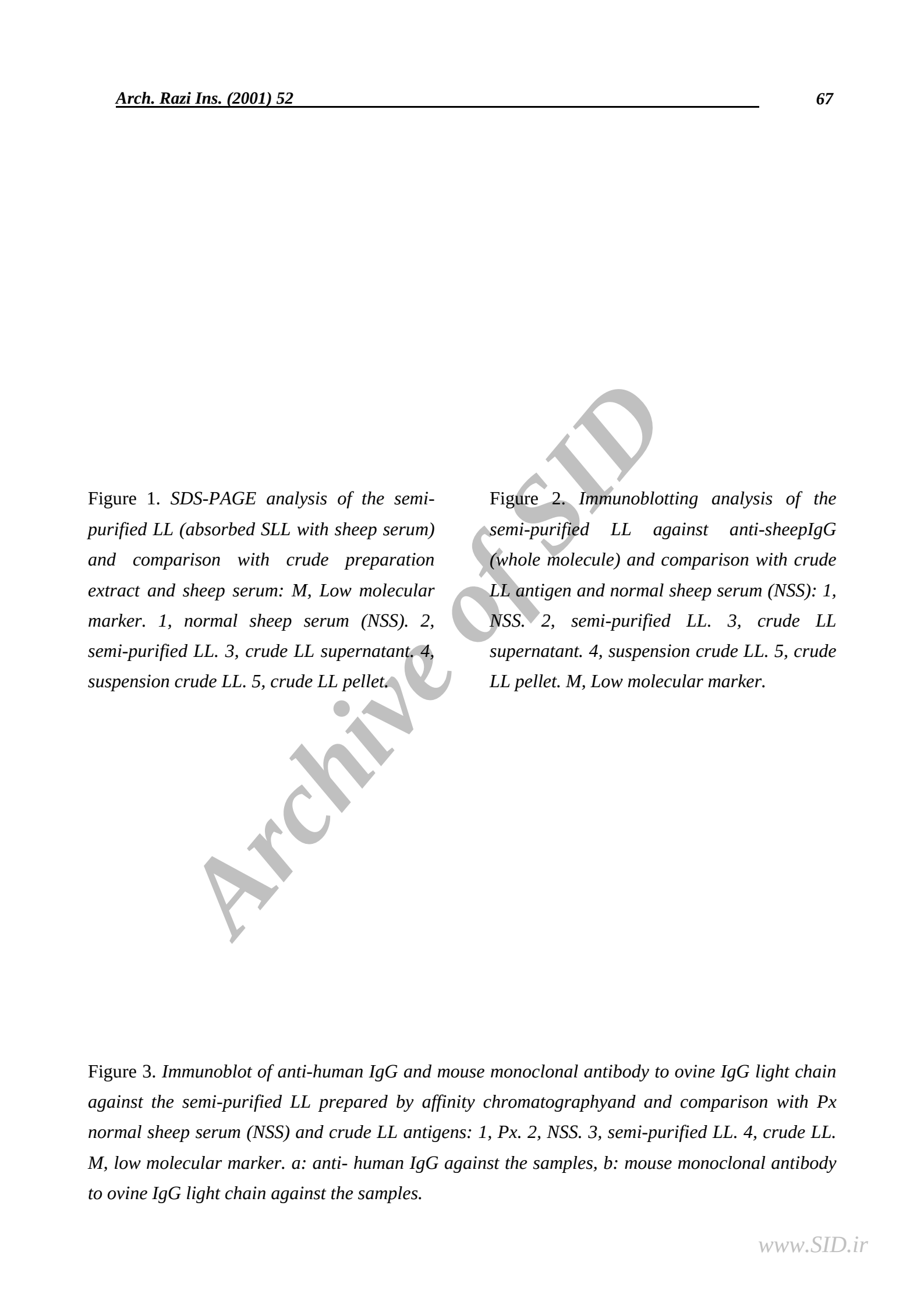

Figure 1. *SDS-PAGE analysis of the semi-purified LL (absorbed SLL with sheep serum) and comparison with crude preparation extract and sheep serum: M, Low molecular marker. 1, normal sheep serum (NSS). 2, semi-purified LL. 3, crude LL supernatant. 4, suspension crude LL. 5, crude LL pellet.*

Figure 2. *Immunoblotting analysis of the semi-purified LL against anti-sheepIgG (whole molecule) and comparison with crude LL antigen and normal sheep serum (NSS): 1, NSS. 2, semi-purified LL. 3, crude LL supernatant. 4, suspension crude LL. 5, crude LL pellet. M, Low molecular marker.*

Figure 3. *Immunoblot of anti-human IgG and mouse monoclonal antibody to ovine IgG light chain against the semi-purified LL prepared by affinity chromatographyand and comparison with Px normal sheep serum (NSS) and crude LL antigens: 1, Px. 2, NSS. 3, semi-purified LL. 4, crude LL. M, low molecular marker. a: anti- human IgG against the samples, b: mouse monoclonal antibody to ovine IgG light chain against the samples.*

This result indicates that the presence sheep IgG in the LL could add to background activity when using anti-human IgG. Results (Figure 3 section b) also showed that the monoclonal antibody to sheep IgG light chain strongly recognized bands in the 27-29kDa region in NSLL and particularly NSS, while the absorbed LL with sheep serum gave only a very faint band in this region. This again indicated a reduction in sheep IgG in semi-purified sample. It is of interest that the monoclonal antibody to sheep IgG light chain did not recognize anything in Px antigen, while anti-HuIgG gave a faint reaction in the heavy and light IgG chains region.

In order to detect specific antibody in human cystic hydatidosis serum against semi-purified LL antigen, a total IgG and IgG4 subclass antibody response in human pooled positive and negative sera to this antigen were tested and compared with Px, NSS and crude LL antigens. Results (Figure 4) showed that detection of total IgG responses gave the clearest difference between positive and negative sera to the semi-purified LL fraction. Positive serum strongly recognized three obvious bands at 66, 55 and 27kDa. Negative human serum against this antigen did not present any band at 27kDa and bands at 66 and 55kDa comparatively were very faint. The band at 66kDa was also recognized strongly in NSLL and Px but was not identified in NSS. Positive sera gave major bands in the 50up to 60kDa region against NSLL, 48up to 58kDa region against Px and 55kDa in the semi-purified sample. The pooled positive sample also recognized predominate bands in the 25 up to 31kDa (a much stronger at 29kDa) against NSLL, bands in the 27 up to 31kDa region against Px and band at 27kDa against the semi-purified LL antigen.

It is interesting to note that there was no difference between positive and negative human serum to the NSS. IgG4 subclass antibody response in pooled positive and negative sera to the semi-purified sample also gave a different profile (Figure 5). Positive sera showed a weak reaction at 55 and 27kDa, whilst negative sample did not recognized any band to the semi-purified fraction. These results indicated that the bands at 66, 55 and 27kDa were correlated to parasite antigen that total IgG and IgG4 antibody responses in positive human hydatidosis sera were able to recognize them. The strong band at 66kDa in the purified SLL may be related to presence amount of

Figure 4. *Total IgG antibody response in:a, human pooled negative serum and b, human pooled positive serum: 1, protoscolex (Px). 2, sheep serum (SS). 3, semi-purified LL. 4, crude LL. M, Low molecular marker.*

Figure 5. IgG4 antibody response in the same pattern as figure 4.

the albumin in LL. However it must be noted that IgG4 antibody response in human clinically positive serum recognized a much stronger reaction in the 25-31kDa region in the unpurified preparation, while to the semi-purified sample only identified a band at 27kDa. Bands at 29kDa and 25KDa are probably correlated to host (i.e. light SIgG chain) and band at 27kDa to be more likely to be parasite molecule.

Discussion

Recently, in a study on the general characterization of LL of *E.granulosus*, however, reported that the crude LL not only has an antigenic nature but also contains host and parasite molecule (Taherkhani & Rogan 2000). In that study an analysis of different crude LL (sheep, horse, mouse, human and gerbil) isolates by SDS-PAGE showed some similar bands between all isolates. Surprisingly, the biological composition and immunoreactivity of LL of *E.granulosus* has been poorly understood. Bortoletti and Ferretti 1978, Harris *et al* 1989, Holcman *et al* 1994 reported that this layer is of parasite origin and is secreted by the germinal layer. Kilejian and Schwabe 1971, Pezzella *et al* 1984, also reported that host material may contribute to its structure. The results of our last studies (Taherkhani & Rogan 2000) indicated that the LL contains a considerable amount of host components such as immunoglobulins, which may affect the background level of immune assays.

SDS-PAGE analysis of the fractions showed that the bands in the 50-55kDa region and the 25-31kDa region were greatly reduced in semi-purified fraction, which presumably reflected to removal of heavy and light immunoglobulin chains. Immunoblot data confirms considerable reduction in the presence of IgG chains within the semi-purified LL. In addition, blot evaluation indicated the cross-reactivity between sheep IgG with anti-human IgG that reaction of this conjugate could add to background activity in the unpurified LL and that this reduced after purification. This again confirms that host components present within the LL interfere with diagnostic assays and procedures are improved when they are removed. It is interesting to note that, there is difference between the ELISA and western blotting results. The reasons for this probably reflect to the difference between the sensitivity of two techniques and/or to the concentration effect.

However, the most useful aspect of the laminated layer in terms of serodiagnosis was evident in immunoblotting. This indicated that the bands at 66, 55, 29 and 27kDa were significant molecules for diagnosis of human cystic hydatidosis patients. Since, total IgG and IgG4 subclass antibody responses in positive sera recognized major bands in the 25 up to 29kDa region (a much stronger at 29kDa) in unpurified LL, in

the 25-31kDa (a much stronger at 27kDa) in Px and one obvious band at 27kDa in semi-purified LL. Additionally, no difference between positive and negative human serum to the NSS, no any reaction in semi-purified fraction by negative serum at 27kDa and comparatively faint reaction in unpurified LL in this region support that the 27kDa may be derived from parasite molecules. Data of SDS-PAGE and immunoblot of anti-sheep IgG, anti-human IgG and monoclonal antibody to ovine IgG light chain may are additional reasons for this. In conclusion, we found that the antigenicity of the LL of *E.granulosus* was improved after redaction of some host components within that structure and was appeared obvious bands at 66, 55 and 27kDa regions. This study has also indicated that there are potential diagnostic antigens within the LL. At no stage, however, has any attempt been made to determine the specificity of these antigens with respect to other infections. This would obviously be an important step for full characterization, in particular in relation to reactivity against sera from *E.multilocularis*, *T.solium* and *T.saginata*.

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