# IMMUNOCHEMICAL LOCALIZATION OF CROSS-REACTIVE PROTEINS OF BLADDERWORMS WITH SPECIAL REFERENCE TO DETECTION OF SPECIFIC ANTIGEN IN CYSTIC ECHINOCOCCOSIS OF SHEEP

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## ABSTRACT

*Immunochemical localization of antigenic fractions of ovine bladder worms viz., hydatid cyst, Coenurus cerebralis and Cysticercus tenuicollis is important to identify cross reactive as well as specific proteins. SDS PAGE and western blot technique were carried out to analyze various portions of three ovine bladderworms and to identify the specific protein of cystic echinococcosis. SDS PAGE analysis of fluid antigens revealed 9,6 and 9 bands for hydatid cyst(HCFA),Coenurus cerebralis(CCFA)and Cysticercus tenuicollis(CTFA) respectively and 24, 38 and 68 kDa protein bands were common in HCFA and CCFA. The common protein band between HCFA and CTFA was identified as 28kDa.24 kDa protein which was common between CCFA and CTFA. Scolex antigen revealed 3,6 and 9 bands respectively for HPSA, CCSA and CTSA. The common protein bands between HPSA and CTSA were 29, 72 and 98 kDa. Protein bands 12, 42,98 and 112 kDa were common between CCSA and CTSA. Membrane antigen revealed 4, 4 and 6 bands respectively for HGMA, CCMA and CTMA. The common protein band between CCMA and CTMA was 16kDa only. Western blot analysis revealed that the low molecular weight protein 8 kDa from HCFA was specific for cystic echinococcosis. Cross reaction was noticed between HPSA and CTMA as well as between HGMA and CTMA..*

**Key words:** Antigens, *Coenurus cerebralis, Cysticercus tenuicollis,* Hydatid cysts, SDS PAGE, Sheep, Western blot

## INTRODUCTION

Cystic echinococcosis, Coenurosis and Cysticercosis are three important bladder worm infections of sheep caused by ingestion of ova of dog tapeworms Echinococcus granulosus, Taenia multiceps and Taenia hydatigena respectively. Among these

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Echinococcus granulosus is an important zoonotic tapeworm, hydatid cysts form in various organs of animals and man. Diagnosis of these bladderworm infections is mostly based on postmortem examination in animals as well as imaging and serodiagnosis in man and animals (Jeyathilakan *et al.,* 2011). Antigens are necessary for development of diagnostic assays and vaccines against bladderworms of sheep. Identification of antigenic profiles, sharing antigens and cross reactive proteins among these larval tapeworms is an important task for development of vaccine candidate and immunodiagnostic kits (Jeyathilakan *et al.,* 2014). In this context, the study was undertaken to localize the antigenic fractions of fluid, scolex and membrane of these three ovine bladderworms using SDS-PAGE and western blot analysis to identify the specific antigen for cystic echinococcosis.

## MATERIALS AND METHODS

### Collection of bladderworms

The hydatid, Coenurus cerebralis and Cysticercus tenuicollis cysts for this study were collected from sheep slaughtered at Corporation Slaughter House in Perambur and Department of Meat Science and Technology, Madras Veterinary College, Chennai, Tamil Nadu, India.

### Preparation fluid antigen

Fluid antigen from Hydatid cysts was prepared as per earlier report (Verastegui *et al.,* 1992).with slight modification. Briefly,hydatid cysts were thoroughly washed and fluid was aspirated. The aspirated fluid was clarified by centrifugation at 10,000 rpm for 30 min to remove the sediments,dialyzed in 1000 Da cut off membrane (Sigma, USA) against three changes of distilled water at 4$^{o}$ C and concentrated using poly ethylene glycol 6000 (SRL, India). The fluid was supplemented with 0.02 per cent sodium azide, 5 mM EDTA and 0.5 M PMSF and used as hydaid cyst fluid antigen(HCFA). Aliquots of HCFA were frozen at -20$^{o}$ C for further use. Similarly Coenurus cerebralis fluid antigen (CCFA) and Cysticercus tenuicollis fluid antigen (CTFA) was also prepared.

### Preparation of scolex antigen

Hydatid protoscolices antigen was prepared following the method of Nasrieh and Abdel-Hafez (2004) with certain modifications.Protoscolices of hydatid cysts were washed thoroughly in PBS three times to remove cyst wall debris and dead protoscolices. About 1.8 g of washed pellet was suspended in 3 ml of PBS in which 40 µl 0.2 M phenyl methyl sulphonyl Fluoride (Sigma, USA) in isopropanol were added. The mixture was homogenized using glass homogenizer (B-Braun Biotech international, Germany) for 20-30 strokes. The homogenate was sonicated at 50 cycles /s at maximum tune of 1.8 µm peak to peak during 30 s four times in an ice bath using an ultra Sonicator (B-Braun Biotech international, Germany). The sonicate was centrifuged at 15,000 rpm at 4$^{o}$C for 20 min. The supernatant was used as protoscolices antigen (HPSA) and stored at -20$^{o}$C with 0.02 per cent sodium azide as preservative. Similarly Coenurus cerebralis scolices antigen (CCSA) and Cysticercus tenuicollis scolex antigen(CTSA) was also prepared.

**Preparation of membrane antigen**

Hydatid membrane antigen was prepared by following the method of Khoo *et al.* (1997). Briefly,approximately 500 mg of cyst membrane slices were suspended in 5 ml of PBS with 5 mM EDTA and disrupted in an ultra Sonicator (B-Braun Biotech international, Germany) using 15 cycles of 15s each at maximum power. The resulting suspension was clarified by centrifugation at 10,000 rpm for 15 min. The supernatant was stored and the pellet again submitted six times to the same disruptive treatment. A pool was prepared with all the supernatants, concentrated and used as germinal membrane antigen (HGMA) and stored at -20$^{o}$ C with 0.5 M sodium azide as preservative. Similarly membranes of Coenurus cerebralis membrane antigen (CCMA) and Cysticercus tenuicollis membrane antigen (CTMA) were prepared.

**Protein estimation**

The concentration of protein of Hydatid antigens (HCFA, HPSA and HGMA), Coenurus antigens (CCFA, CCSA and CCMA) and Cysticercus antigens (CTFA, CTSA and CTMA) was determined by the method of Smith *et al.* (1985) using bicinchoninic acid protein estimation kit (Genei, Bangalore) at the absorbance of 562 nm.

**Characterization of antigens**

The fluid antigens namely HCFA, CCFA and CTMA were characterized by Sodium dodecyl sulphate poly acryl amide gel electrophoresis (SDS-PAGE) on a mini protein-3 electrophoresis apparatus (Biorad, USA) using 1 mm thickness gel using a discontinuous system in 12 per cent gel according to their molecular weights in uniform reducing conditions. Similarly scolex antigens (PSA, CCSA and CTSA) and membrane antigens (HGMA, CCMA and CTMA) were also characterized. The molecular weight of the proteins in the SDS-PAGE was determined using gel photo documentation system model DP-001 FDC with photocapture software(Laemmli,1970).

**Hyper immune sera**

Hyper immune sera were raised against hydatid antigens (HCFA, HPSA and HGMA) in adult New Zealand white rabbits (3-4 kg)separately as per standard procedure. The hyper immune sera collected were tested for antibodies titer and stored in 100 µl aliquots with Merthiolate as preservative at -20$^{o}$ C.

**Western blot analysis**

Western blot analysis was performed to find out common shared antigens and immuno dominant peptides among fluid,scolex and membrane of hydatid cyst,Coenurus cerebralis and Cysticercus tenuicollis as described by Towbin *et al.,* (1979) using Mini Trans – Blot Electrophoretic Transfer Cell (Biorad, USA). The fluid antigenic fractions were probed with 1:100 dilution of hyper immune serum of anti HCFA.Similarly scolex antigens (HPSA, CCSA and CTSA) probed with hyper immune serum of anti HPSA and membrane antigens(HGMA, CCMA and CTMA) probed with hyperimmune serum of anti HGMA.

## RESULTS AND DISCUSSION

### Preparation of bladderworm antigens

Twelve litres of hydatid fluid, 250 g of germinal membrane and 12 g of protoscolices were separated from hydatid cysts collected from slaughter house. A portion of these was used for preparation of crude hydatid cyst fluid antigen (HCFA), protoscolices antigen (HPSA) and germinal membrane antigen (HGMA). Three hundred ml of Coenurus fluid, 50 g of membrane and 5 g of scolices were collected from Coenurus cysts. A portion was used for preparation of crude Coenurus cyst fluid antigen (CCFA), scolices antigen (CCSA) and membrane antigen (CCMA). Four hundred and fifty ml of Cysticercus fluid, 40 g of membrane and 7 g of scolices were separated from Cysticercus tenuicollis cysts. A portion of these was used for preparation of crude Cysticercus fluid antigen (CTFA) scolex antigen (CTSA) and membrane antigen (CTMA). The protein concentration of the metacestode antigens was determined (Table 1).

### Characterization of fluid antigens

The three antigens (HCFA, CCFA and CTFA) were resolved by 12 per cent SDS-PAGE and the protein profile of the three antigens were as depicted in Table 2 and Fig.1. The protein profile of HCFA showed 9 band sranging from 8 to 205 kDa. The present study is almost in accordance with the findings of Ito *et al.* (1999) who reported components of 8 to 215 kDa. Shepherd and McManus (1987) also detected five major subunit antigens of 12, 16, 20, 38 and 60 kDa and 9 polypeptides were detected by Simsek and Koroglu (2004). Rajabiyoun *et al.*,(2006) found five bands Hassanain *et al.* (2016) found 4 protein bands. CCFA showed six bands ranging from 16 to 202 kDa. However Kordafshari *et al.* (2010) found 3 unclear bands. CTFA showed 9 protein bands from10 to 208 kDa. These findings in accordance with earlier report (Kordafshari *et al.* 2010).HoweverAbuseir *et al.,* (2018) and Shafiya *et al.* (2016) found 6 protein bandsonly ranging from 23 to 150 kDa with slight variation in sizes.Vaibav *et al.* (2003) found 3 bands ranging from 37 to 97 kDa only. 24, 38 and 68 kDa protein bands are common in HCFA and CCFA. The common protein band between HCFA and CTFA was identified as 28kDa.24 kDa protein was common between CCFA and CTFA.

### Scolex antigens

The scolex antigens of three bladder worm were resolved in 12 per cent SDS-PAGE (Fig. 2 and Table 1). The protein profile of HPSA showed 3 protein fractions at 29, 72 and 98 kDa which was almost coincided with identical poly peptide pattern reported by Gonzalez *et al.,* (2000). Sbihi *et al.* (1996) reported 3 protein bands of 37, 42 and 110 kDa. Kordafshari *et al.* (2010) found 4 bands only. Hassanain *et al.* (2016) found five bands ranging from 28 to 103 kDa. However Rajabiyoun *et al.*,(2006) found 12 bands ranging 15 to 120 kDa. The CCSA had 6 protein bands ranging from 12 to 112 kDa. Similarly Kordafshari *et al.,* (2010) found 6 bands only. The protein profile of CTSA had 9 bands ranging from 12to 112kDa. However Kordafshari *et al.,* (2010) found 13 bands ranging from 13 to 120 kDa.Vaibav *et al.* (2003) found 22 bands ranging from

18 to 114 kDa. The common protein bands between HPSA and CTSA were identified as 29,72 and 98 kDa. 12, 42,98 and 112 kDa protein bands were common between CCSA and CTSA. No common protein band between H PSA and CCSA was identified.

**Membrane antigens**

The three antigens viz., HGMA, CCMA and CTMA were resolved in 12 per cent SDS-PAGE(Fig.3 and Table 1). The protein fractions were ranging from 29 to 134 kDa for HGMA which is almost coincided with identical poly peptides reported by Gonzalez *et al.* (2000) who demonstrated the presence of a 29 kDa poly peptide. However Kordafshari *et al.* (2010) found 5 protein bands .CCMA showed four protein bands ranging from 16 to 116 kDa. However Kordafshari *et al.* (2010) found 6 protein bands ranging from 13 to 62 kDa. Six protein bands ranging from 16 to 210 for CTMA were detected.However Kordafshari *et al.* (2010) found 11 protein bands ranging from 12 to100 kDa.Vaibav *et al.* (2003) found 12 bands ranging from 18 to 100 kDa.The common protein band between CCMA and CTMA was 16 kDa only. No common protein band between HGMA and CCMA as well as HGMA and CTMA.

The changes observed in the protein band pattern could be attributed to many factors including different species of animals from which antigen was prepared and strain

**Table1. Protein concentration (mg/ ml) of crude antigens**

| Antigen | Fluid Antigen | Scolex Antigen | Membrane Antigen |
|---|---|---|---|
| 1. Hydatid cyst | 4.359 | 1.173 | 2.05 |
| 2. Coenurus cyst | 3.5 | 4.1 | 3.38 |
| 3. Cysticercus cyst | 3.835 | 2.4 | 2.5 |

**Table 2. Protein profile of various bladder worm antigens (kDa)**

| Antigens | Hydatid cyst | Coenurus cyst | Cysticercus cyst |
|---|---|---|---|
| Fluid antigens | HCFA 8, 16, 24, 38, 58, 68, 98, 116 & 205 | CCFA 16,24, 38, 68, 106 & 202 | CTFA 10,24,28, 32,42, 72, 116, 172 & 208 |
| Scolex antigens | HPSA 29, 72 & 98 | CCSA 12, 28,42,52,98 & 112 | CTSA 12,29,42,49,58,68,72,98 &112 |
| Membrane antigens | HGMA 29,38, 127 & 134 | CCMA 16,39,64 & 116 | CTMA 16,24,58,72,98 & 210 |

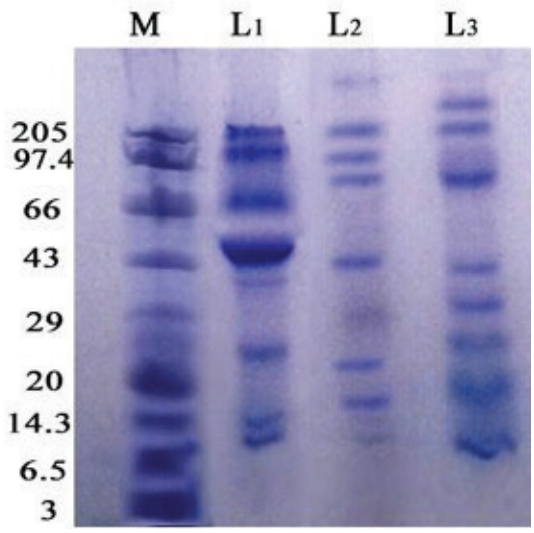


**Fig. 1. SDS PAGE analysis of fluid antigens of hydatid, *Coenurus* and *Cysticercus* cyst**

M – Molecular weight marker
L3 – *Cysticercus* fluid antigen
L2 – *Coenurus* fluid antigen
L1 – Hydatid cyst fluid antigen

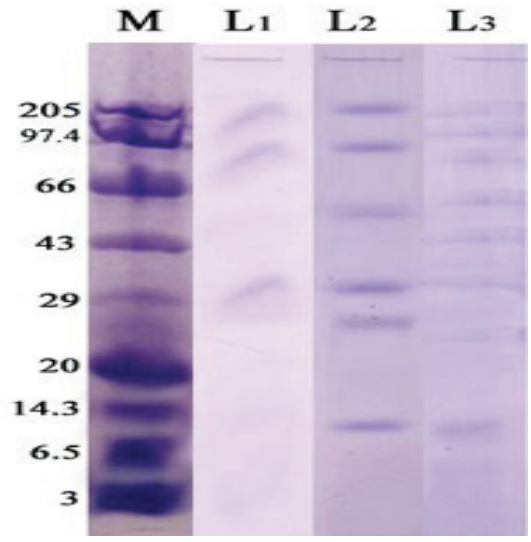


**Fig. 2. SDS PAGE analysis of scolex antigens of hydatid, Coenurus and Cysticercus cyst**

M – Molecular weight marker
L1 – Hydatid Protoscolices antigen
L2 – Coenurus scolices antigen
L3 – Cysticercus scolex antigen

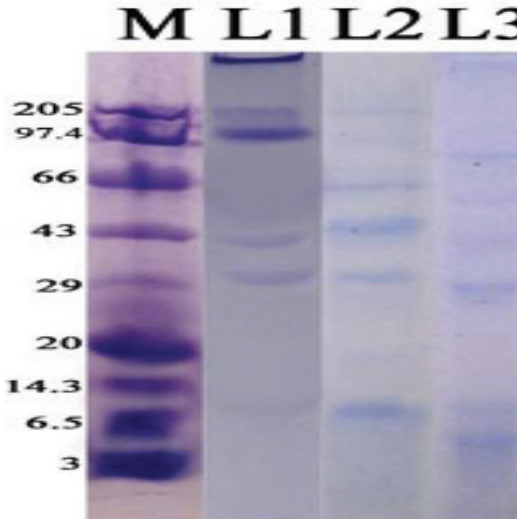


**Fig. 3. SDSPAGE analysis of membrane antigens of hydatid, Coenurus and Cysticercus cyst**

M – Molecular weight marker
L1 – Hydatid Germinal membrane antigen
L2 – Coenurus membrane antigen
L3 – Cysticercus membrane antigen

variations which have been reported in same species of animal within a country. It also depends on the different methods of antigen preparation, types of purification employed and the methods of storage (Ahmad *et al.,* 2001).This can also be attributed to the fact that very high molecular weight protein complexes in bladderworms may dissociate under reducing conditions to smaller size, two or more subunits. The presence of some smaller proteins in some samples appearing in SDS-PAGE,while with other samples these small proteins were absent or very faint. In addition, different locations of specific proteins are involved in the development of metacestodes' infestations in the animal body (Mcmanus 2014).Although HCFA is the major diagnostic antigen for cystic ecinococcosis preferred throughout the world by most scientists (Carmena *et al.,* 2006), the current study was envisaged to identify protein profile of lesser known antigens such as HPSA and HGMA in comparison with other major ovine bladderworms.

**Western blot analysis of Hydatid, *Coenurus* and *Cysticercus antigens***

Western blot analysis of fluid antigens of these three bladderworms with anti HCFA hyper immune sera revealed cross reactive protein bands in all the three antigens. The sera reacted with higher molecular weight polypeptides of HCFA (8, 68 and 116 kDa) CCFA (68, 106 and 202 kDa) and CTFA (72, 116, 172 and 208 kDa). The sera also reacted with the low molecular weight protein (8 kDa) of HCFA, however the sera did not react with low molecular weight proteins of other bladderworms (Fig.4). This indicated that the 8 kDa protein is non-cross reactive and hydatid specific protein. This finding was closely in accordance with findings of Maddison *et al.,* (1989) who found that the 8 kDa protein from hydatid fluid did not cross react with sera from any other parasitic disease. Kanwar and Kanwar (1994) also concluded that 8 kDa molecule is non cross reactive and hydatid specific.Fernandez *et al.,* (1996) found that the smallest subunit of antigen B, 8 kDa to be Echinococcus specific with potential diagnostic value. This view was also expressed by Ito *et al.,* (1999); Shambesh *et al.,* (1999); Ortona*et al.,* (2000) and Nasrieh and Abdel-Hafez (2004). Tassi *et al.,* (1981) found that higher molecular weight fractions were the best antigens for diagnosing human hydatid disease. However in the current study cross reactivity was observed at higher molecular weight proteins by Western blot analysis. Vast number of immuno diagnostic antigens for hydatidosis in man and animals has been identified. But abundant work has been generated on the diagnostic value of the 8 kDa low molecular weight component of antigen B (Carmena *et al.,* 2006).

Western blot analysis of scolex antigens (HPSA, CCSA and CTSA) with anti HPSA hyper immune serum revealed cross reaction among protein bands of HPSA (29, 72 and 98 kDa) and CTSA (58,68 and 72 kDa), where as it did not recognize the protein bands of CCSA (Fig. 5).

Western blot analysis of membrane antigens (HGMA, CCMA and CTMA) with anti HGMA hyper immune serum revealed

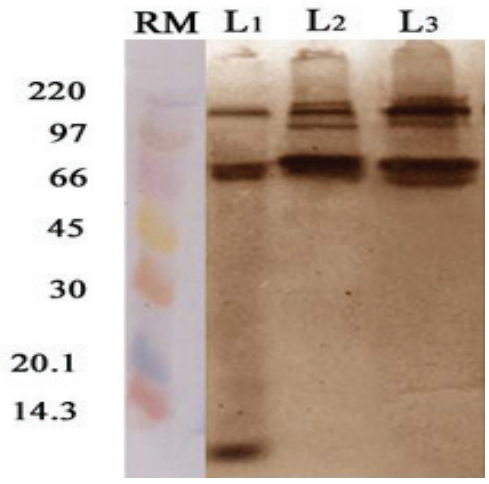


**Fig. 4. Western blot analysis of fluid antigens of hydatid , Coenurus and Cysticercus cyst probed with hyper immune serum raised against hydatid cyst fluid antigen**

RM – Rainbow molecular weight marker
L1 – Hydatid cyst fluid antigen
L2 – Coenurus fluid antigen
L3 – Cysticercus fluid antigen

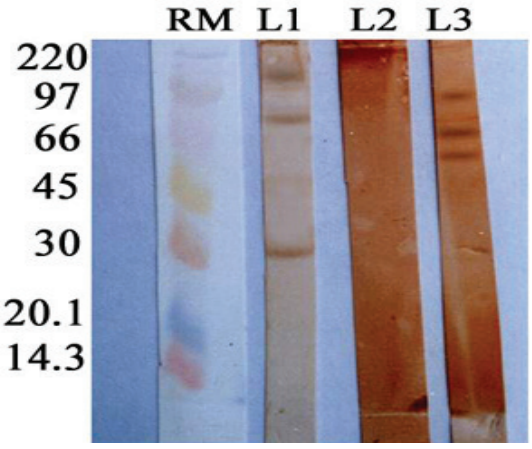


**Fig. 5. Western blot analysis of scolex antigens of hydatid, Coenurus and Cysticercus cyst probed with hyper immune sera raised against hydatid protoscolices antigen**

L3 – Cysticercus scolex antigen
RM – Rainbow molecular weight marker
L1 – Protoscolices antigen
L2 – Coenurus scolices antigen

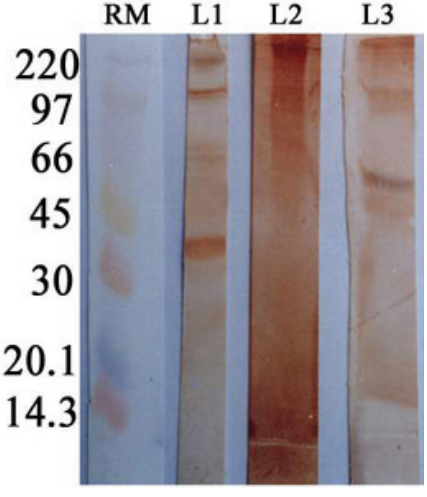


**Fig. 6. Western blot analysis of membrane antigens of hydatid, Coenurus and Cysticercus probed with hyper immune sera raisedagainst antigen**

RM – Rainbow molecular weight marker
L1 – Germinal membrane antigen
L2 – Coenurus membrane antigen
L3–Cysticercus membrane antigen

cross reaction among protein bands of HGMA (38, 127 and 134 kDa) and CTMA (58 and 98 kDa). It did not react with CCMA (Fig.6). Though various studies were carried out to find out cross reaction among fluid antigens, no work has been carried out on cross reactive studies involving Cysticercus tenuicollis and Coenurus cerebralis antigens. According to Carmena *et al.,* (2006) protoscolices and germinal membrane antigens showed poor antigenicity and therefore hydatid cyst fluid antigen always received more attention from research workers.

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