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Isolation, purification and immunobiochemical characterization of goat hydatid cyst fluid antigen

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Abstract
Hydatidosis is a neglected cyclo zoonotic disease impacting human and their livestock. In this study, Hydatid cysts from the liver and lungs of goats were collected from different slaughter houses and different butcher shops in and around Kolkata. From the Hydatid cysts, crude antigen was prepared and purification of antigen was done by gel filtration chromatography and analysis was performed by Sodium Dodecyl Sulphate Polyacrylamide gel (12.5%) electrophoresis (SDS-PAGE) under reduced conditions. The resolution of crude Goat hydatid cyst fluid antigen in SDS-PAGE revealed polypeptides with MW of 60 kDa, and 245 kDa. The purified antigen also showed the bands of polypeptides with MW of 60 kDa and 245 kDa. For purified antigens Double Immune Diffusion test was performed with antisera raised in healthy New Zealand White rabbits. The purified antigens were subjected to Western Blot technique to verify their immune reactivity with hyper immune sera which illustrated bands at 60 kDa and 245 kDa.

Keywords: Cysts, echinococcus granulosus, hydatidosis, ruminants and SDS-PAGE

1. Introduction
Echinococcosisis is one of the most significant cestode infections of man and animals caused by small tapeworms of genus Echinococcus. Echinococcosis is associated with severe morbidity and disability, and is one of the world's most geographical widespread zoonotic diseases (Craig *et al.*, 2007; Cringoil *et al.*, 2007) [1, 2]. Humans function as accidental intermediate hosts, and they are usually the dead end for the parasitic infection cycle. The life cycles of *E. granulosus can* be classified as domestic, involving the domestic dog as the main definitive host and various species of domestic ungulates as intermediate hosts, or as sylvatic, involving wild carnivores (Fofxes, wolves) and Cervidae. The sylvatic cycle is reported in limited regions of the world such as North America and Eurasia. In many endemic areas, domestic and sylvatic life cycles either coexist or overlap (Eckert and Deplazes, 2004; Thompson, 2008) [3, 4].
Hydatidosis is caused by larval stage of dog tapeworm of *Echinococcus granulosus*. It is a neglected cyclozoonotic disease impacting humans and their livestock (cattle, buffalo, sheep, goat, and pig), thereby causing significant socioeconomic and public health impacts, mostly in developing countries. The worldwide incidence of cystic echinococcosis is about 100,000-300,000 cases annually and in at least 100 countries. The highest prevalence of human hydatidosis in India has been reported from Andhra Pradesh, Maharashtra, and Tamil Nadu. The prevalence of the disease is reported to be high in food animals in India (Rao *et al.*, 2012) [5]. The infected human beings lose their normal functional capacities and have to bear more expenses for proper treatment. Hydatid disease leads to important economic loss in the form of condemnation of edible offal, by decreasing the production and vitality as a result of damage produced in the important vital organs in the body and death of the animal (Gathura and Gathuma, 1991; Jiang *et al.*, 1994) [6, 7].
In the present study, SDS-PAGE was performed to determine the polypeptide profiles of crude and purified Hydatid cyst fluid antigens of goat and analyzed by western blot technique to verify their immune reactivity with hyper immune serum raised against those antigens.

2. Materials and methods
Collection of Samples (Cyst fluid and germinal membrane)
Liver and lungs of slaughtered goats with *Echinococcus granulosus* cysts were collected from different slaughter houses and different butcher shops in and around Kolkata.

Samples were brought to the laboratory in ice-cold condition (Figures 1 and 2).

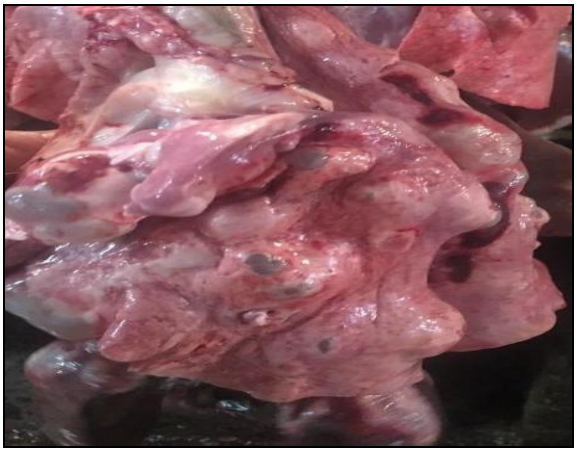


Fig 1: Hydatid cysts in lung of goat

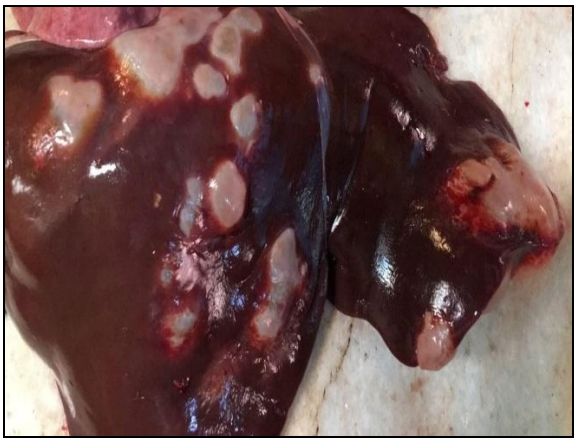


Fig 2: Hydatid cysts in liver of goat

Preparation of crude Hydatid cyst fluid antigen

The hydatid cysts were separated carefully from the host tissues, painted with 1% Iodine solution and dried. The process was repeated thrice. The hydatid fluid of each cyst was aspirated using a sterile syringe and collected in separate sterile beakers. The cyst wall was incised to examine the germinal membrane to confirm its fertility. Only the fertile hydatid cyst fluids were considered for preparation of crude antigens. Phenyl Methyl Sulphonyl Fluoride (PMSF) (0.03mM) was added in the fertile and clear hydatid cyst fluids. The globulinic antigen of hydatid fluid was obtained through precipitation with ammonium sulphate at half saturation. Then hydatid cyst fluids were centrifuged at 5,000 rpm for 30 min at 4°C (Remi c-24). The supernatants were dialyzed by dialysis membrane (cut of value 12,000) against sucrose and concentrated at 10fold. The protein concentrations of crude hydatid cyst fluid antigens were determined by Lowry *et al*. (1951) [8]. The antigens thus prepared were stored at - 20°C in aliquots for further use.

Preparation of purified hydatid cyst fluid antigens of the goat by gel filtration chromatography

The Goat Hydatid Cyst fluid (GHCF) antigen was purified by gel filtration chromatography in a column on Sephacryl S 200 (1.5 cm diameter and 60 cm in length) in a buffer containing PBS (pH7.2), PMSF (0.03mM) and 0.02% Sodium Azide at a flow rate of 20 ml per hour. The elute was collected in 35 drop fractions of 3ml each. The distribution of protein was monitored by reading the absorbance at 280 nm in a UV/VIS spectrophotometer (ELICOR-SL159). Protein fractions of GHCF were pooled together into 5 parts and named as P1 (fractions of initial part of ascending loop of first peak), P2 (fractions of rest part of ascending loop of first peak), P3 (fractions of descending loop of first peak), P4 (fractions of between first and second peak) and P5 (fractions of second peak). Then the 5-pooled fractions were concentrated by dialysis against sucrose using dialysis membrane (cut off value 12,000). The concentrated peak fractions were then preserved at -20°C in aliquots for further use. SDS-PAGE (Laemmli, 1970) [9] was performed with 12.5% gel concentration to detect the purified 61 kDa polypeptide band. The protein concentration of the part of P1 of pooled GHCF antigen was determined (Lowry *et al.*, 1951) [8].

3. Characterization of purified antigen

a) Analytical Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE): The crude and the purified hydatid cyst fluid antigen was analysed by SDS-PAGE (12.5% or 10%) according to Laemmli [9] (1970). Vertical mini slab gel electrophoresis system (Bangalore Genei) was used. The samples were mixed with sample buffer in definite proportion and heated at 100°C for 5 minutes. The quantities of proteins were 40µg per track. The electrophoresis conditions were maintained at 7 mA for half an hour followed by 9 mA for 2 hours. The gel was then stained with Coomassie blue staining solution followed by application of destaining solution after 6 hours.

b) Determination of molecular weight by SDS-PAGE

Molecular weight was determined by standard protein markers (PMW–M, Bangalore Genei). Phosphorylase (97.4kDa), BSA (66kDa), ova albumin (43kDa), carbonic anhydrase (29kDa), soyabean trypsin inhibitor (20.1 kDa) and Lysozyme (14.3kDa) were electrophoresed with other samples. The mobility patterns of all the proteins were recorded. Rm values were determined by calculating the ratio of mobility of proteins to the mobility of tracking dye.

Immunochemical Analysis

a) Raising of antisera: The antibody against GHCF antigen was raised in healthy New Zealand white rabbits with an average weight of 1.5 Kg. The antigen was mixed with equal volume of Freund's Complete Adjuvant (FCA) and was injected intramuscularly into the thigh muscle. Four booster doses of the same antigen emulsified with Freund's Incomplete Adjuvant (FIA) were given subsequently 14 days interval following the first injection, sera were collected from these rabbits 7 days after the last booster dose and were stored at -20°C in aliquots for further use with Sodium Azide (0.02%) as preservative.

b) Double immunodiffusion test (DID): The DID test was performed according to Hudson and Hay (1989) [10]. A 1% agarose was prepared with PBS (pH 7.2) and a small amount of sodium azide was added in it. The wells were filled with 20 µL of purified hydatid cyst fluid antigen, 20 µl of hyper immune serum raised against crude GHCF antigen and 20µl of normal control serum for each of Goat in central and peripheral wells respectively. The slide was then placed in a humid chamber and incubated overnight at room temperature. The slide was washed in PBS (pH 7.2), dried by blotting paper and stained with Coomassie blue staining solution.

Lastly, the stained slide was destained with destaining solution.

C) Western Blot: The antigens were characterized by western blotting technique according to Towbin *et al*. (1979) [11]. The antigens were separated by SDS-PAGE (Laemmli, 1970) [9] and then the resultant proteins were electro-blotted to nitrocellulose filter paper gel to a mini western blot apparatus (Bangalore Genei).

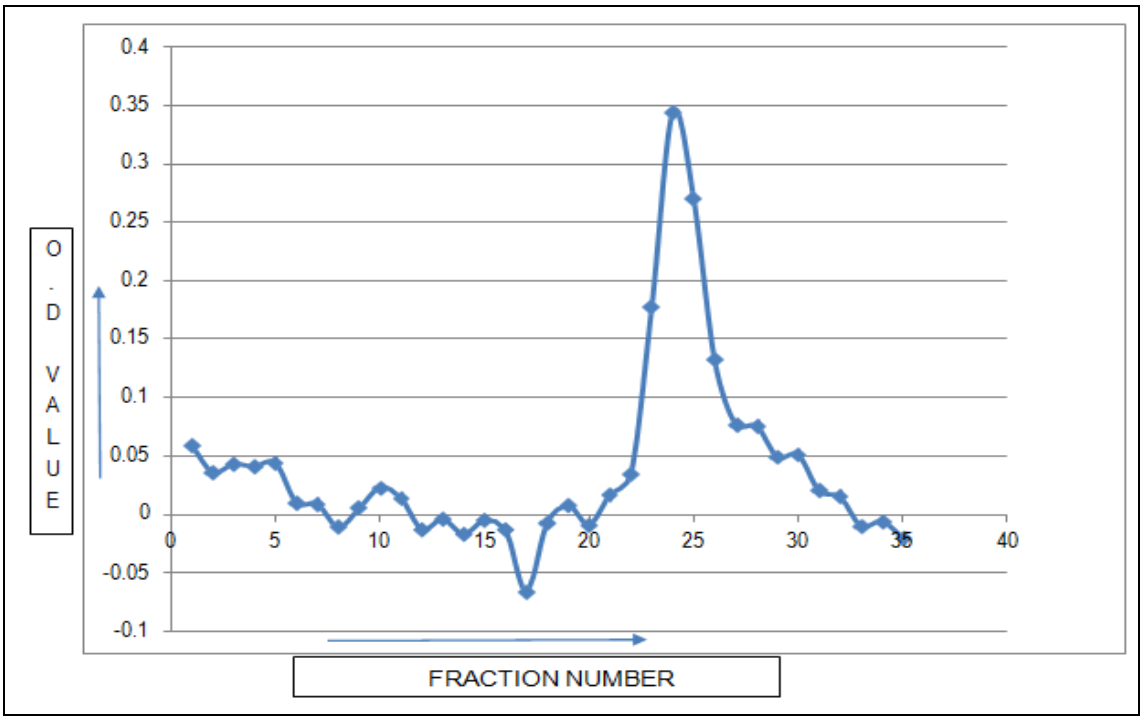
i) Transfer of the resultant proteins from the SDS-PAGE to solid support (Nitrocellulose filter paper): The gel was placed on an NCP (Nucleosome core particle) and taken in a sandwich assembly containing blotting paper pads. The sandwich assembly was submerged into the tank filled with transfer buffer, with nitrocellulose sheet facing the anode. The blot apparatus was connected with power supply and current (50 mA) applied for overnight at 4°C. After transfer, the sandwich was dismantled and the nitrocellulose sheet was processed for immunoblotting.

ii) Immunodetection: The nitrocellulose sheet was blocked by blocking solution (2% BSA in PBS) for 2 hours; NCP was then washed with PBS-T (0.05% Tween 20 in PBS) for 4 times. NCP was incubated with hyper immune serum raised against crude GHCF antigen of 1:500 dilutions in PBS against the antigen for 2 hours in a small plastic bag. After that the NCP was washed for 4 times as mentioned above.

Then the NCP was kept in another plastic bag with (1:1000 in PBS) anti-rabbit horse radish peroxidase conjugate (sigma) for two hours. After washing with PBS-T (0.05% of Tween 20 in PBS) for 4 times the NCP was rinsed with substrate solution (Tris-HCL, H2O2 and DAB). Reactive protein bands appearing after few minutes were observed. The NCP was dipped in distilled water to stop the reaction; lastly it was dried up and preserved.

4. Results

Analysis of Crude and Gel Filtration Chromatography purified hydatid cyst fluid antigens of Goat: Crude hydatid cyst fluid antigens of Goat were prepared by centrifugation and dialysis. The protein concentration of crude hydatid cyst fluid antigens, determined by Lowry *et al.*, (1951) [8] was 3.1 mg/ml. Purified hydatid cyst fluid antigens of goat were prepared by gel filtration chromatography on Sephacryl S 200 with the flow rate of 20 ml/ hr in which a major peak was obtained (Graph no.1). The fractions of major peak were pooled together. The pooled fraction was dialysed against sucrose. The protein concentration of the pooled fraction was determined by Lowry *et al.* (1951) [8]. SDS- PAGE of the dialyzed pooled fraction showed purified polypeptide bands when stained with Coomassie brilliant blue stain. The resolution of crude GHCF antigen in 12.5% SDS-PAGE revealed prominent polypeptides of MW of 60 kDa, and 245 kDa. 60 kDa, and 245 kDa polypeptides were also observed in purified GHCF antigen (Table no.1, Fig. 2).



Graph 1: Purification of crude goat hybrid cyst fluid antigen by gel filtration chromatography on sephacry S 200

Immunochemical Analysis of Purified Hydatid cyst fluid antigens of Goat: Hyper immune serum was raised in rabbit against purified GHCF antigen. Single prominent precipitin line was observed in DID test when each of purified hydatid antigens was reacted with the hyper immune serum. No precipitin lines or bands were observed between hyper immune sera and normal control serum. The purified GHCF antigens were also found to be immune reactive against hyper immune serum as studied by western blotting. The 60 and 245 kDa prominent polypeptide bands of the purified HCF antigens of goat as observed on SDS-PAGE were found to be immune reactive by western blot analysis. (Fig.4 and 5).

Table 1: Polypeptide Profiles of Crude and Purified Hydatid Cyst Fluid Antigens of Goat as Assessed By SDS- PAGE

Fig No	Separating gel (%)	Stains	Antigen Lane wise	Major bands	MW in kDa	Minor Bands	MW kDa
1	12.5%	Commassie	Lane A: CrudeGHCF Lane B: Purified GHCF	2	60 & 245	-	-
		brilliant					
		blue		2	60 & 245		

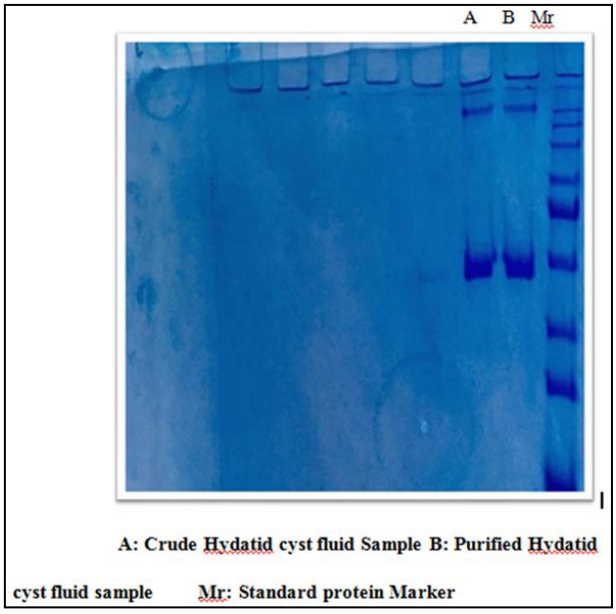


Fig 3: SDS-PAGE (12.5%) showing peptide profiles of Goat hydatid cyst fluid antigens (Coomassie blue stain).

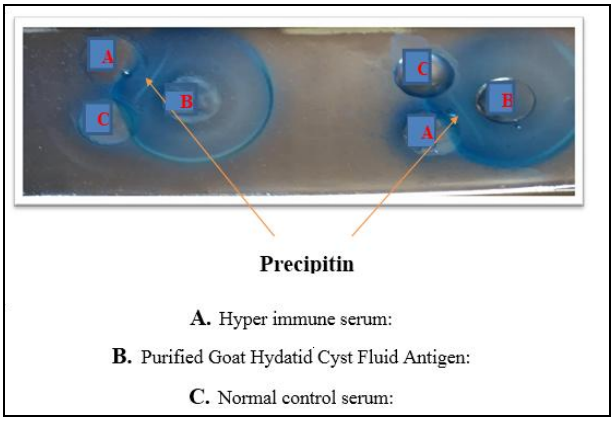


Fig 4: Double immunodiffusion test showing the precipitin line of purifieed Goat hydatid cyst fluid antigen with the hyper immune serum.

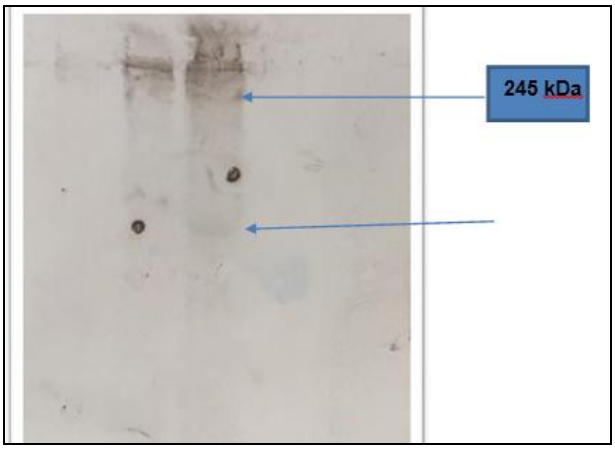


Fig 5: Western blot analysis of gel filtration chromatography purified fraction of Hydatid cyst fluid antigen of Goat.

5. Discussion

Many researchers had worked on isolation, purification and characterization of hydatid cyst fluid antigens and observed that some particular immunogenic polypeptides varied within a range of 60 to 70 kDa. In the present study, the isolation, purification and characterization of goat hydatid cyst fluid antigens was done. The results were compared with other ruminants like cattle, buffalo and sheep. Due to paucity in the information, the present study was done on goat hydatid cyst fluid antigen and was compared to other ruminants and humans also. In the present study, the resolution of crude Goat hydatid cyst fluid antigen in 12.5% SDS- PAGE revealed polypeptides with MW of 60 kDa, and 245 kDa. Latif *et al.* (2013) [12] observed 269, 166, 89, 59, and 25 kDa bands in buffalo, 46, 29, 22, and 18 kDa bands ingoat, 209, 138 and 63 kDa in sheep, 269, 89 and 59 kDa bands in cattle, Hydatid cyst fluid antigens. The earlier results were perfectly not similar to all results, but 60 kDa bands has closer to 59 kDa band of cattle, buffalo and 63 kDa band of sheep. EI-Zayyat *et al.*, (1999) [13] observed 60 kDa bands in human infected Hydatid cyst antigen fluid. The present result of the 60 kDa band of GHCFA was similar to 60 kDa of human HCFA. Burgu *et al.* (2000) [14] observed 116 kDa bands in sheep hydatid cyst fluid antigen, 68 and 8 kDa bands in infected human Hydatid cyst fluid antigen. But, the 60 kDa band observed in the present study was not similar to 116k Daband of SHCFA and 68 and 8kDa of Human HCFA. Bandyopadhyay and Singh (2000) [15] found 48 kDa and 66 kDa bands in buffalo Hydatid cyst fluid antigen. 66 kDa and 48 kDa were not similar to 60 kDa and 245 kDa bands, but 66 kDa was larger than 60 kDa bands and 48 kDa lower than 60 kDa bands.

In contrast, Ramadan *et al.* (1999) [16] detected 5, 7, 20, 28, 35, and 127 kDa bands in human hydatid cyst fluid antigen respectively. Purification of crude GHCF antigen by gel filtration chromatography showed two bands 60 and 245 kDa when resolved in 12.5% SDS-PAGE and it was found to be found to be immune reactive when analyzed by western blot.

The 60 kDa band was closely associated with 60.2 bands of sheep HCFA and nearer to 61.4 kDa band of cattle HCFA. Planchart *et al.*, (1994) [17] found 64, 58 and 30 k D a band sin sheep hydatid cyst fluid antigen but the resulting 60 kDa band of GHCF antigen was coming between 58 kDa and 64 kDa bands. The present results not similar to the findings of Shirazi *et al.* (2016) [18]. In which researchers calculated 8, 12 and 16 kDa polypeptides by Bradford assay and confirmed by SDS-PAGE. The results also not corresponding to the findings of Bandyopadhyay and singh (2000) [14] and Pal and Singh (1999) [19]. Pianteli *et al.* (1977) [20] found 4 bands greater than 400 kDa molecular weight and 5 bands 150 kDa molecular weight. The present results were not matched to the findings of Pianteli *et al.* (1977) [20]. The present study was not similar to the findings of Kanwar and Kanwar. (1994) [21] in which authors found 8 kDa polypeptide bands.

The double immune diffusion test using purified cyst fluid antigens of Goat against normal control serum and hyper immune serum raised against crude Goat Hydatid Cyst Fluid (GHCF) antigen showed single precipitin line. The band is

found between purified GHCF and hyper immune serum of rabbit. The band was very prominent. The results obtained in the present study were not a like with the finding of Raina and Singh (1997) [22]. Eight precipitin bands were detected by some workers, when rabbit hyper immune serum raised against goat Hydatid cyst fluid and reacted with GHCF antigen in DID test. Only one precipitin band was detected in the present study; the differences might be due to the fact that the authors used whole goa thydatid cyst fluid antigen but in the present study the purified HCF antigen was used. So, the antigen antibody binding being very specific, only one prominent precipitin band was obtained for crude Goat Hydatid cyst fluid antigen. Western blot of purified HCF antigens of Goat showed that, 60 kDa and 245 kDa were immune reactive.

6. Conclusion

The immunoreactive, purified polypeptides of hydatid cyst fluid antigens of goat were 60 and 245 kDA, respectively. Purified Hydatid cyst fluid antigens of goat could be used for the immunodiagnosis of echinococcosis in domestic ruminants under field condition in Hydatid prone belts.

7. References

1. Craig PS, McManus DP, Lightowlers MW, Chabalgoity JA, Garcia HH, Gavidia CM, *et al*. Prevention and control of cystic echinococcosis. Lancet. Infect. Dis 2007;7:385-394.
2. Cringoil G, Rinaldi L, Musella V, Veneziano V, Maurelli MP, *et al*. Geo-referencing livestock farm as tool for studding custic echinococcosis epidemiology in cattle and water buffaloes from south Italy. Geospatial 2007;2:105-111.
3. Eckert, Deplazes P. Biological, epidemiological, and clinical aspects of echinococcosis, zoonoses ofincreasing concern. Clin. Microbiol. Rev 2004;17(1):10715.
4. Thompson RC, Mc Manus DP. Aetiology: parasites and life cycles. In: ckert emmell MA, Meslin, Paloski ZS (eds) HOOI Manual on chinococcosis in Human and animals: A public health problem of global concern, Paris: World Organisation for Animal Health 2001, 1-1.
5. Rao SS, Mehra B, Narang R. The spectrum of hydatid disease in rural central India: An 11- year experience. Annals of Tropical Medicine and Public Health 2012.
6. Gathura PB, Gathuma JM. An evalution of the economic losses resulting from condemnation of cattle and small stock liver due to presence of hydatid cysts in Kenya. Bulletin of Animal Health and Production in Africa 19991;39:225-229
7. Jiang L, Wen H, Li X, Yan L. SDS-PAGE analysis of soluble proteins from various tissue components of Echinococcus metacestodes. Endemic Disease Bulletin 1988;13:1
8. Lowry M, Rosebrough NJ, Farr AL, Randall RJ. Protien measurement with the folin phenol reagent. J. Biol. Chem 1951;193:265.
9. Laemmli UK. Cleavage of structural protiens during the assembly of the head of bacteriophage T4. Nature 1970;227:680-685.
10. Hudson L, Hay. Practical immunology, 3rd edition Oxford: Blackwell Scientific Publication 1989.
11. Towbin H, Staehelin T, Gordan J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceeding of the National Academy of Sciences of the United States of America 1979;76:4350-4354.
12. Latif A, Tanveer A, Anjum AA, Ali AM, Rana MS, Khan MR, *et al*. Characterisation of Hydatid cyst fluid from ruminants and humans by SDS-PAGE. Journal of Animal Plant Science 2013;23(2).
13. EI Zayat EA, Ramzy RM, Abdel Baki MH, Fahmi IA, Rifat MMA, Helmy HH, *et al*. Human cystic echinococcosis: diagnostic value of different antigenic fractions of hydatid cyst fluid with different specific immunoglobulin Gsubclasses by enzyme linked immunoelectrotransfer blot. Journal of the Egyptian Society of parasitology 1999;29:817-830.
14. Burgu A, Doganay A, Gonenç B, Sarimehmetoglu HO, Kalinbacak F. Analysis of fluids of hydatid cysts from sheep by SDS-PAGE, and determination of specific antigens in protein structure by Western blotting. Turkey Journal Veterinary Animal Science 2000;24:493-500.
15. Bandopadhyay S, Singh BP. Antibody response in secondary hydatidosis by ELISA using affinity purified buffalo hydatid cyst fluid antigen. Journal of veterinary Parasitology 2000;14(1):17-20.
16. Ramadan NI, Aaty HEA, Mahmoud MSE, EI-Nori A. An enzyme- linked immunoelectro transfer blot assay for diagnosis of human cystic echinococcosis. Journal of the Egyptian Society of Parasitology 1999;29(3):849-857.
17. Planchart S, Botto Planchart S, Botto C, Alarcon de Noya B, Bonifacino R, Vivas L, *et al*. Evaluation of the double diffusion, enzyme immunoassay and immunoblottng techniques, for the diagnosis of human hydatid disease in tropical areas. Revista do Instituto de Medicine Tropical de Sao Paulo 1994;36 (3):205-210.
18. Shirazi S, Madani R, Hoghooghi Rad N, Ranjbar Bahadori S. Isolation and purification of Echinococcus granulosus antigen B from hydatid cyst fluid using three different methods 2016;71:103-108.
19. Pal S, Singh BP. Some studies on immunological characterization of sheep hydatid fluid antigens of *Echinococcus granulosus*. Indian Journal of Animal Sciences 1999;69(11):867-868.
20. Piantelli M, Pozzuoli R, Arru E, Musiani P. Echinococcus granulosus: identification of subunits of the major antigens. Journal of Immunology 1977;119(4):1382-1386.
21. Kanwar JR, Vinayak VK. Isolation & immunochemical characterization of diagnostically relevant antigens of *Echinococcus granulosus*. Indian J Med Res 1993;97:75-82.
22. Raina OK, Singh BP. Antigenic characterisation of buffalo hydatid cystic fluid. Journal of Veterinary Parasitology 1f997;11(2):185-187.