IDENTIFICATION AND CHARACTERIZATION OF PROTEIN MARKERS AND DEVELOPMENT OF IMMUNOASSAY FOR THE DETECTION OF HYDATID CYST DISEASE

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BY

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LIST OF ABBREVIATIONS

ADCC	antibody-dependent cell mediated cytotoxicity
Ag5	antigen 5
AgB	antigen B
BLAST	Basic Local Alignment Search Tool
Bp	base pair
BSA	bovine serum albumin
CIEP	counter-immune electrophoresis
Co-A	co-agglutination
CT	computerized tomography
DALYs	disability adjusted life years
DB	dot blot
DNA	deoxyribonucleic acid
E.canadansis	*Echinococcus canadensis*
E. felidis	*Echinococcus felidis*
E. granulosus	*Echinococcus granulosus*
E. multilocularis	*Echinococcus multilocularis*
E. oligarthus	*Echinococcus oligarthus*
E. shiquicus	*Echinococcus shiquicus*
E. vogeli	*Echinococcus vogeli*
E4+	carbohydrate enriched fraction of *E.granulosus*
EA21	cyclophilin
EDTA	ethylenediaminetetraacetic acid
EF1β/δ	elongation factor 1β/δ
EG95	*E. granulosus* 95 protein
EgAgB	*E. granulosus* antigen B
*Eg*ALP	*E. granulosus* alkaline phosphatase
*Eg*Teg	*E. granulosus* tegumental protein
*Eg*TPx	*E. granulosus* thioredoxin peroxidase
*Eg*Trp	*E. granulosus* tropomyosin
ELISA	enzyme-linked immunosorbent assay
ESA	excretory-secretory antigen
FAST-ELISA	falcon assay screening test-ELISA
G (1 to 10)	genotype (1 to 10)
h	hour
HCD	hydatid cyst disease
HCF	hydatid cyst fluid
HCl	hydrochloric acid
HRP	horseradish-peroxidase
HSP	heat shock protein
i.e.	id est (that is)
IEP	immunoelectrophoresis
IFAT	indirect fluorescent antibody test
Ig	immunoglobulin
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IHAT	indirect haemagglutination test
IL	interleukin

INFORMM	Institute for Research in Molecular Medicine
Interferon γ	IFN-γ
IPG	immobilized pH gradient
KCl	potassium chloride
kDa	Kilodalton
KH_2PO_4	potassium dehydrogen phosphate
K_2HPO_4	dipotassium hydrogen phosphate
L	liter
LAT	latex agglutination test
LM	light microscopy
M	molarity
mA	milliampere
MAb	Monoclonal antibodies
mg	milligram
$MgCl_2$	magnesium Chloride
min	minute
ml	milliliter
mM	millimole
MWs	molecular weights
NaN_3	sodium azide
$NaHCO_3$	sodium bicarbonate
Na_2CO_3	sodium carbonate
Na_2HPO_4	disodium hydrogen phosphate
NaCl	sodium chloride
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
NI-NTA	nickel-nitrilotriacetic acid
ng	nanogram
nm	nanometer (wave length)
NUS	National University of Singapore
OD	Optical density
OIE	Office International des Epizooties (World Organization of Animal Health)
PAIR	puncture, aspiration, injection, and reaspiration
PBS	phosphate buffered saline
PCR	polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEMED	N,N,N'.N'-tetramethylethylenediamine
V	Volt
WB	Western blot
WHO	World Health Organization
WHO-IWGE	World Health Organization-Informal Working Group on Echinococcosis
µg	microgram
µl	microliter
µm	micrometer

PENGENALPASTIAN DAN PENCIRIAN PENANDA PROTEIN DAN PEMBANGUNAN IMUNOASAI UNTUK PENGESANAN PENYAKIT SISTA HIDATID

ABSTRAK

Penyakit sista hidatid (HCD) atau hidatidosis adalah disebabkan jangkitan oleh larva *Echinococcus granulosus*. Ia boleh mengancam manusia dan banyak haiwan lain termasuklah hidupan liar serta ternakan komersil. Antigen daripada parasit yang mendorong tindakbalas humoral dan imun sel perumah berpotensi untuk bertindak sebagai penanda dalam pengesanan awal HCD. Pengesanan HCD adalah berdasarkan tanda klinikal dan kaedah pengimejan, tetapi ianya tidak spesifik. Kaedah pengimejan tidak dapat membezakan antara sista hidatid dan lesi lain, dengan itu imunodiagnosis digunakan sebagai kaedah diagnosis tambahan dalam pengesanan penyakit ini. Pada ketika ini, tiada ujian piawai dengan sensitiviti dan spesifisiti yang tinggi bagi imunodiagnosis HCD. Menerusi kajian ini, antigen daripada parasit telah disediakan daripada protoskoleks *E. granulosus* dan cecair sista hidatid (HCF) daripada sista hidatid yang diperoleh daripadai biri-biri yang dijangkiti secara semulajadi. Sampel serum diperolehi daripada 130 orang pesakit HCD, 38 individu yang dijangkiti dengan jangkitan parasit lain serta 30 individu sihat. Konjugat peroksida (HRP) IgG dan IgG4 telah digunakan sebagai antibodi sekunder. Gel elektroforesis dua dimensi dan blot Western/dot telah digunakan untuk mengenal pasti jalur protein protoskoleks dan HCF yang menunjukkan sensitiviti dan spesifisiti diagnostik yang baik.

Satu antigen protoskoleks pada jalur 60 kDa didapati mempunyai 82% (40/49) sensitiviti diagnostik dan 100% (68/68) spesifisiti diagnostik untuk

pengesanan HCD, apabila diuji dengan IgG4-HRP anti-manusia. Manakala 33% (10/30) sensitiviti dan 100% (68/68) spesifisiti diperolehi apabila diuji dengan IgG-HRP anti-manusia. Menerusi analisis jisim spektrometri menerusi MALDI TOF/TOF, jalur ini telah dikenal pasti sebagai tegumen protoskoleks paramiosin. Protein rekombinan daripada jujukan penuh paramiosin telah dihasilkan; dan sensitiviti dan spesifisiti diagnostik dalam blot IgG4 masing-masing adalah 86% (42/49) dan 98% (58/59).

Dua jalur antigenik daripada HCF telah dikenalpasti iaitu 8 kDa dan 37 kDa, dan masing-masing menunjukkan 86% (42/49) and 67% (33/49) sensitiviti diagnostik; dan 100% (67/67) and 98% (66/67) spesifisiti diagnostik apabila diuji dengan IgG4-HRP anti-manusia. Dengan IgG-HRP anti-manusia, sensitiviti dan spesifisiti bagi jalur antigen 8 kDa masing-masing adalah 88% (43/49) dan 37% (10/27). Jisim spektrometri dengan MALDI TOF/TOF telah mengenalpasti jalur 8 kDa sebagai AgB/1, manakala jalur 37 kDa pula dikenalpasti sebagai Ag5. Berdasarkan kepada jujukan AgB/1, peptida yang mempunyai 54 residu dengan berat molekul 6.415 kDa telah disintesis. Dengan menggunakan dot blot, peptida sintetik pRZ telah menunjukkan 90% (18/20) sensitiviti diagnostik dan 40% (8/20) spesifisiti diagnostik apabila diuji dengan IgG-HRP anti-manusia. Apabila diuji dengan IgG4-HRP anti-manusia, sensitiviti diagnostik ialah 88% (43/49) dan spesifisiti diagnostik ialah 100% (42/42). Kad membran nitroselulosa telah digariskan dengan pRZ yang berkepekatan pada 0.75, 1, 1.25, 1.5 dan 2 mg/ml, dan dipotong menjadi dipstik berdasarkan teknologi ‘lateral flow’. Dipstik pada dua kepekatan terakhir (1.5 dan 2 mg/ml) menunjukkan sensitiviti dan spesifisiti diagnostik yang tinggi, masing-masing adalah 98% (41/42) dan 100% (85/85).

Secara kesimpulannya, pengesanan IgG4 daripada dua protein sista hidatid iaitu paramiosin daripada protoskoleks dan AgB/1 daripada HCF didapati berkesan sebagai penanda diagnostik bagi HCD. Rekombinan paramiosin dan peptida sintetik AgB/1 pRZ menunjukkan nilai diagnostik yang baik. Kajian ini telah berjaya menghasilkan ujian pantas dipstik menggunakan pRZ, yang berasaskan pengesanan antibodi IgG4 dan teknologi 'lateral flow'. Ia menunjukkan potensi yang baik bagi pengesanan HCD secara pantas dan tepat dan sangat berguna di kawasan endemik HCD yang amnya kurang membangun.

IDENTIFICATION AND CHARACTERIZATION OF PROTEIN MARKERS AND DEVELOPMENT OF IMMUNOASSAY FOR DETECTION OF HYDATID CYST DISEASE

ABSTRACT

Hydatid cyst disease (HCD) or hydatidosis is caused by an infection with the larval stage of *Echinococcus granulosus.* It affects human and animals, including wildlife and commercial livestock. The parasite antigens induce host humoral and cellular immune responses which may serve as potential markers in early detection of HCD. Diagnosis of HCD is based on non-specific clinical signs and imaging of suspected organs such as liver and lungs. Imaging methods cannot differentiate between hydatid cysts and other lesions such as liver abscess and neoplasms, therefore, immunodiagnosis is an important additional tool to detect the infection. Currently, there is no standardized test with high sensitivity and specificity that is available for the immunodiagnosis of HCD. In this study parasite antigens were prepared from *E. granulosus* protoscolex and hydatid cyst fluid (HCF) of hydatid cysts from naturally infected sheep. Serum samples were obtained from 130 HCD patients, 38 individuals infected with other parasitic infections and 30 healthy people. Peroxidase (HRP)-conjugated anti-human IgG and IgG4 were used as secondary antibodies. Two-dimensional gel electrophoresis and western/dot blot were used to identify protein bands of protoscolex and HCF that exhibited good diagnostic sensitivity and specificity.

A protoscolex antigenic band of 60 kDa was found to be 82% (40/49) sensitive and 100% (68/68) specific for detection of HCD when probed with anti-

human IgG4-HRP. Meanwhile the diagnostic sensitivity and specificity were 33% (10/30) and 100% (68/68) respectively when probed with anti-human IgG-HRP. By mass spectrometry analysis using MALDI TOF-TOF, the band was identified as protoscolex tegument paramyosin. The full length recombinant form of the paramyosin protein was produced; and the diagnostic sensitivity and specificity in IgG4 blots were found to be 86% (42/49) and 98% (58/59) respectively.

Two antigenic bands from hydatid cyst fluid (HCF) were identified namely 8 kDa and 37 kDa; the diagnostic sensitivities were found to be 86% (42/49) and 67% (33/49) respectively; while the diagnostic specificities were determined to be 100% (67/67) and 98% (66/67) respectively when probed with anti-human IgG4-HRP. When analysed using anti-human IgG-HRP, the sensitivity and specificity of the 8kDa antigenic band was 88% (43/49) and 37% (10/27), respectively. By mass spectrometry using MALDI TOF-TOF, the 8kDa band was identified as AgB/1 and the 37 kDa band as Ag5. Based on the AgB sequence identified in this study, a peptide of 54 residues with molecular weight of 6415 Da was selected and custom-synthesized. Using dot blot, the synthetic peptide (pRZ) showed diagnostic sensitivity of 90% (18/20) and specificity of 40% (8/20) when probed with anti-human IgG-HRP; and sensitivity of 88% (43/49) and a specificity of 100% (42/42), when probed with anti-human IgG4-HRP. Nitrocellulose membrane cards were separately lined with the pRZ at 0.75, 1, 1.25, 1.5 and 2 mg/ml and cut into lateral flow dipstick strips. The dipsticks using the last two concentrations showed high diagnostic sensitivity of 95% (40/42) and 98% (41/42) respectively, and specificity of 100% (85/85).

In conclusion, IgG4 detection of two hydatid cyst protein markers i.e. paramyosin from protoscolex and AgB/1 from HCF, were found to be useful for

the diagnosis of HCD. The recombinant form of paramyosin and the synthetic peptide of AgB/1 (pRZ) showed good diagnostic value. This study has successfully produced a rapid dipstick test using pRZ based on IgG4 detection and lateral flow technology. The test shows high potential for rapid, accurate diagnosis of HCD and would be useful in low resource settings of HCD endemic areas.

CHAPTER ONE

INTRODUCTION

1.1 Overview of Hydatid Cyst Disease

Hydatid cyst disease (HCD), also called cystic echinococcosis (CE), cystic hydatid disease (CHD), or unilocular echinococcosis, is caused by the larval stage (metacestode) of the tapeworm *Echinococcus granulosus* in human, wild life and livestock. Dogs and other canids harbour the adult worms in their small intestine and serve as the definitive host. Humans as intermediate host acquire the disease by accidental ingestion of vegetables or water contaminated with ova of adult worms excreted by canids. After exposure to the gastrointestinal enzymes, the infective ovum hatches into oncosphere which reaches organs such as liver and lungs through the vascular and lymphatic systems. The larva then develops into hydatid cyst, which is gradually filled with fluid and protoscoleces. The disease is chronic, complex, and neglected (Brunetti *et al*., 2011). It results in considerable loss of disability adjusted life years (DALYs) in many under developed parts of the world and is not easy to diagnose because of the chronic course of the disease and lack of proper diagnostic facilities.

Recently, the World Health Organization (WHO) included HCD in a separate group known as “neglected zoonotic diseases” i.e. diseases which have not been given enough importance at national or international levels (WHO, 2010; Giri and Parija, 2012). HCD not only causes severe illness, but also causes economic losses due to costs related to diagnosis and surgery. This disease is scattered throughout the world with an emerging or re-emerging status in several countries. Re-emerging status with remarkable economic loss has also been

reported from developed countries (Rojo-Vazquez *et al*., 2011). Early diagnosis is based on clinical signs, which is followed by imaging of suspected organs. Clinical signs in humans are not specific and the imaging methods cannot differentiate between hydatid cysts, tumours and other lesions (Hira *et al*., 1993; Babba *et al*., 1994; Metanat *et al*., 2008). Therefore, immunodiagnosis remains an important additional tool in the primary diagnosis of the disease and for follow-up of patients after surgical or pharmacological treatment (Ortona *et al*., 2003a). Chordi and Kagan (1965) first used immunoelectrophoresis on sheep hydatid cyst fluid and identified antigenic components that were reactive with antibody in serum samples of patients with hydatid cyst. A successful immunodiagnostic test depends on the use of highly specific and sensitive antigen(s), as well as the detection of the appropriate antibody class or subclass. Detection of circulating antigens in serum was reported to be less sensitive than detection of *Echinococcus*-specific antibodies (Zhang *et al*., 2003).

1.2 History of HCD

HCD has been well known to human since ancient times. Francisco Redi in the 17th century recognised the parasitic nature of hydatid cyst and Pallas showed that the cyst was the larval stage of tapeworms. In 1801, the term‘echinococcus’ was coined by Rudolphi. Siebold showed the life cycle by feeding dogs with *Echinococcus* cysts from sheep, and in 1863 Naunyn demonstrated this by feeding dog with human *Echinococcus* cysts (Cox, 2002). In the 19th century, physicians recognized the disease and in the 20th century significant progress in the field of diagnosis and treatment, epidemiology, immunology and molecular medicine were achieved (Eckert, 2007). In Iran, *E. granulosus* was reported in stray dogs in

Tehran by Makarehchian and Janbakhsh in 1956 (Ghafarifar, 2010) and in 1971 Mobedi and Sadighian reported *Echinococcus multilocularis* for the first time in three of 30 red foxes from northwest Ardebil province in Iran (Mobedi and Sadighian, 1971).

1.3 Classification of *Echinococcus*

Echinococcus is a multicellular organism and belongs to the Kingdom Animalia, and Phylum Platyhelminthes. This parasite is in Class Cestoda (tapeworm) because of its ribbon-shaped segmented body, lack of gut and outer body covered by syncytial tegument with microtriches. It is classified under subclass Eucestoda due to it being a hermaphrodite and contains calcareous corpuscles. All tapeworms of humans are in the Order Cyclophyllidea because of the existence of two hosts within its life cycle and presence of scolex with four suckers. Non-operculated eggs, genitalia unpaired in each proglottid with marginal genital pore placed this parasite in the family of Taeniidae. However, it is substantially different from other members of this family for having no more than six segments as so compared to the species of *Taenia* which has several thousand segments (Thompson and McManus, 2001).

The full classification of the **genus** *Echinococcus* is as follows:

Kingdom *Animalia*

Phylum *Platyhelminthes*

Class *Cestoda*

Subclass *Eucestoda*

Order *Cyclophyllidea*

Family *Taeniidae*

Genus *Echinococcus* (Rudolphi, 1801)

Species: *granulosus* (Batsch, 1786)

multilocularis (Leuckart, 1863)

oligarthrus (Diesing, 1863)

vogeli (Rausch and Bernstein, 1972)

shiquicus (Xiao *et al*., 2005)

canadensis (Thompson, 2008)

E. granulosus which causes HCD and *E. multilocularis* which causes alveolar hydatid disease are the most important species due their ability to cause severe disease and economic loss. Two other species, *E. vogeli* and *E. oligarthrus*, are the causative agents of polycystic echinococcosis in central and south America and only a few cases have been reported in man (D'Alesandro, 1997; Eckert and Deplazes, 2004). *E. shiquicus* was reported only in small mammals from Tibetan plateau (Xiao *et al*., 2005), and *E. canadensis* involves wolves and large cervids in North America and Scandinavia (Thompson, 2008). *E. granulosus* is divided into 10 strains, i.e. G1- G10 and this is elaborated in section 1.10 of this thesis.

1.4 Morphology and biology of *E. granulosus*

1.4.1 Adult worm

E. granulosus is one of the smallest tapeworms and measures approximately 3.0-8.5 mm in length (Figure 1.1). It is composed of scolex, neck and strobila. Scolex or head is round in shape and consists of 4 muscular suckers and a rostellum with 28–40 hooks in 2 circular rows. Large hooks measure 30–40 μm in length and the short ones are 22–34 μm; the neck which is a regenerative region is short. Just behind the neck, the strobila or body consists of 3 to 5 proglottids.

The first or first two proglottids are immature and contain no genital organs; the last but one is mature. The mature segment contains both male and female organs. The male organ consists of 45 to 60 follicular testes. The vas efferens originate from each testis which united to build the single duct vas deferens. Vas deferens leads into the cirrus which is the male copulatory organ (Roberts and Janovy, 2009). The female organs consist of an ovary linked into a collecting tubule. Oviduct which ends in a coiled blind-ending uterus and the duct which runs into the vagina are located in the cirrus pouch and opens to the lateral genital pore. Adult worm is hermaphroditic and capable of self-semination.

The last proglottid is gravid; it is longer than broad and measures 2.0 mm × 0.5–1.0 mm. The uterus is fully filled with about 5000 eggs. The eggs are ovoid, 30–37 μm in diameter and contain a hexacanth oncosphere; and by light microscopy it cannot be differentiated from those of *Taenia* species found in dogs (Thompson and McManus, 2001; Muller and Wakelin, 2002). The eggs are presumed to be fully embryonated and infective when released from the definitive host.

A **B**

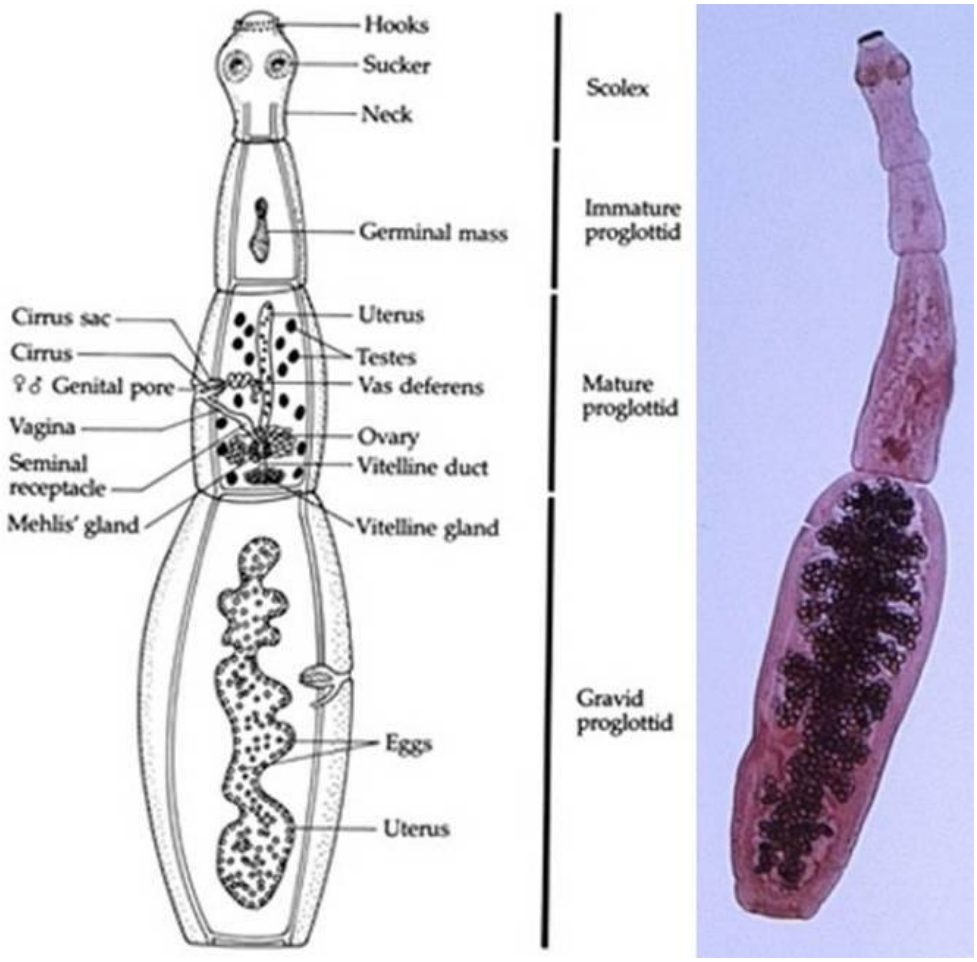

Figure 1.1 *E. granulosus* adult worm

A: Diagram of an *E. granulosus* adult worm showing the structures of the major organs (source: http://www.clem.Mscd.edu).

B: Light microscopy of *E. granulosus* adult worm stained with carmine (x100) (source: http:// www.herramientas.educa.madrid.org).

Food exchanges take place through the microtriches of the syncytial outer covering (the tegument) due to the lack of the digestive system. The adult worm has five pairs of longitudinal nerves. The motor nerve endings help to contract the suckers to assist in adherence (Flisser and Craig, 2005). Flame cells are constitutive of the osmoregulatory-excretory system of adult worm that feed ventrolateral and dorsolateral canals. These excretory canals start on each side along the strobila and a transverse canal joins them at the posterior margin of each proglottid (Roberts and Janovy, 2009).

Adult *E. granulosus* have been found in various carnivores such as stray and farm dogs, red foxes, golden jackals and wolves (Rokni, 2008). They are infected by ingestion of viscera of dead intermediate hosts containing fertile hydatid cysts (cyst with viable protoscoleces). After ingestion, protoscolex is invaginated into the posterior region until external factors which include the microtopography of the intestine, biochemical and immunological factors of the host's bile stimulate it to evaginate. At this stage, the apical region of the scolex evaginates and the organism penetrates deeply between the villi into the crypts of Lieberkuhn in the host's proximal small bowel, where it develops into adult stage in 32 to 80 days. Sexual maturity starts with unordered excretion of eggs and discharging of gravid proglottids on the surface of faeces. The definitive hosts harbour the adult worms in their intestine (Rogan and Richards, 1987; Lewall, 1998).

1.4.2 Larval stage or metacestode (hydatid cyst)

The larval stage of *E.granulosus* in intermediate hosts develops into a unilocular fluid-filled cyst which causes HCD in the human. Hydatid cysts vary in size from 1-20 cm in diameter (Eckert and Deplazes, 2004) (Figures1.2 and 1.3). The cyst consists of three layers i.e. outer pericyst or fibrous layer, ectocyst or laminated layer, and endocyst or germinal layer (Mohan, 2013) (Figure 1.4).

1.4.2.1 Pericyst (Fibrous layer)

Pericyst is an adventitial layer consisting of collagen and fibroblast tissue produced by the host tissue to surround the cyst. The cyst receives nutrients through blood vessels of pericyst by diffusion (von Sinner and Lewall**.,** 2001).

1.4.2.2 Ectocyst (Laminated layer)

Laminated layer is a polysaccharide protein complex composed of mucins bearing defined galactose rich carbohydrates, and is accompanied by calcium inositol hexakisphosphate deposits secreted by the germinal layer (Diaz *et al*., 2011b; Diaz *et al*., 2011a). This acellular, hyaline laminated layer is about 1 mm thick that forms around the germinal layer between 2 and 4 weeks post-infection in the intermediate host after ingestion of the egg and release of the oncosphere (Zhang *et al*., 2003). The process by which nutritive substances are absorbed takes place in this layer. This is not a flexible layer and stains strongly by Periodic Acid-Schiff (PAS) stain **(**Gottstein and Reichen, 2009).

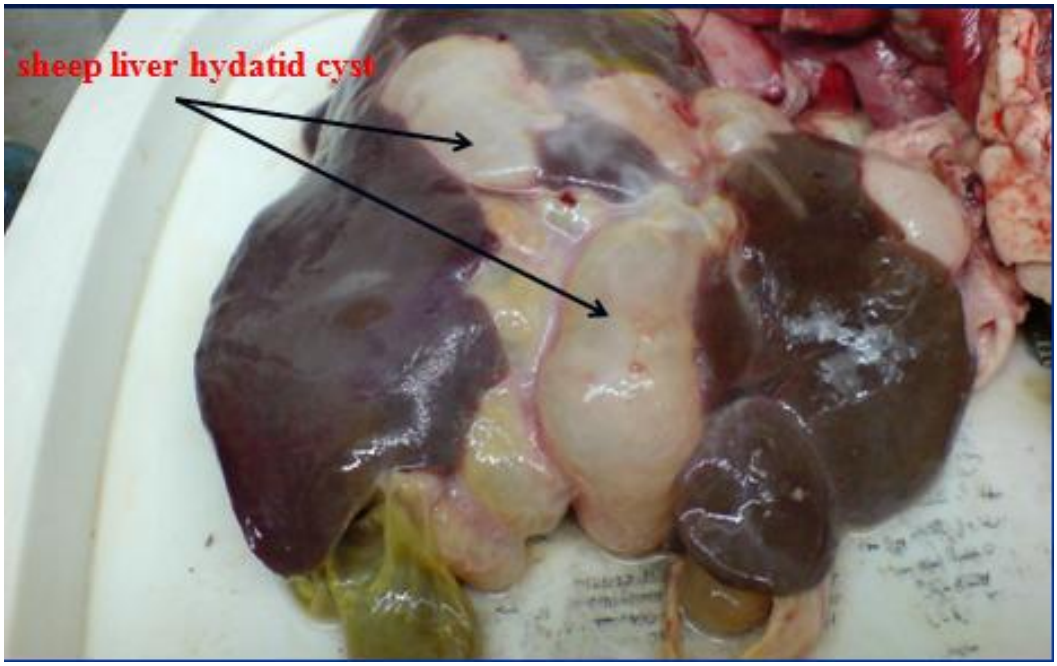

Figure 1.2 Sheep liver hydatid cyst (source: Department of Parasitology; School of Medical Sciences, Tarbiat Modares University, Tehran, Iran)

Figure 1.3 *E. granulosus* unilocular cyst gross specimen (source:http//www.Phs source.us/PH/PARA/Chapter 8.htm)

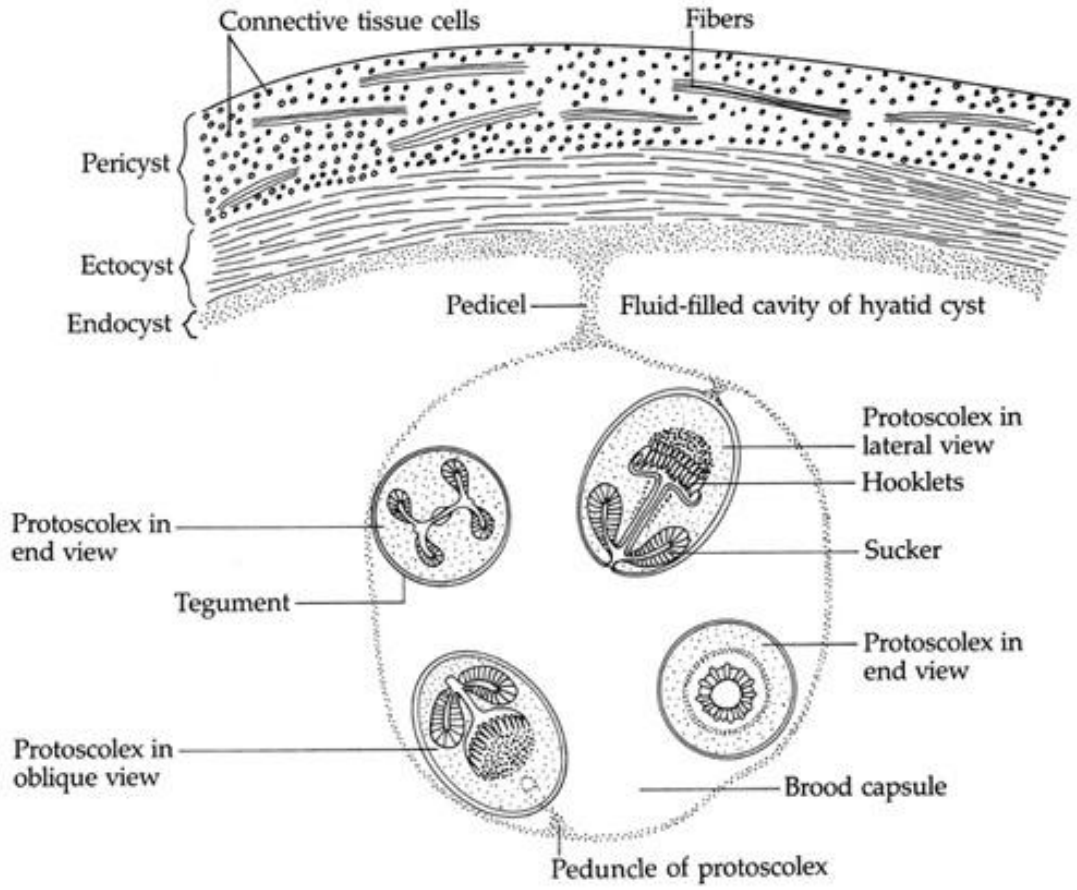

Figure 1.4 Diagram of a section through part of a unilocular hydatid cyst (source: http://www. rowdy.msudenver.edu/).

1.4.2.3 Endocyst (germinal layer)

The endocyst or cellular germinal layer with 10–25 μm thickness is the true wall of the cyst. The structure of the germinal layer shows characteristics that partially resembles the tegument of adult worm. It is composed of syncytial cytoplasm through which the microtriches enters the upper acellular cell. Germinal layer facilitates cyst proliferation and reproduction and produces brood capsules and scoleces as well as laminated layer (Galindo *et al*, 2008).

1.4.2.4 Brood capsule

Brood capsules as shown in Figure 1.4 are small vesicles (Moro and Schantz, 2009) or small secondary cysts which bud internally from the germinal layer and produce multiple protoscoleces by asexual reproduction (Siracusano *et al*., 2009). Small vesicles or daughter vesicles are attached by a stalk to the germinal layer of the mother cyst and create a scene of a bunch of grapes. These vesicles may grow through the wall of the mother cyst and produce the daughter cyst or rupture and release the protoscoleces in the hydatid fluid (Garcia, 2007).

1.4.2.5 Protoscolex

Protoscoleces emerge from the inner wall of the brood capsule which in turn emerge from the germinal layer. A protoscolex measures about 140 × 80 μm (Cheesbrough, 2004)**.** Protoscolex looks like the adult scolex with presence of rostellum containing double row of hooklets at the apical part. The hooks of the upper row with mean length of 25.9-35.0 μm contain rounded robust guards that are larger than the hooks of the lower row (22.6-27.8 μm) which have flattened guards (Antoniou and Tslentis, 1993). After ingestion by a definitive host, the

apical region of the scolex (namely suckers, rostellum and hooklets) evaginates and the organism then attaches to the gut wall and grows into an adult worm. However, if the hydatid cyst ruptures and releases protoscoleces with invaginated heads into the surrounding tissue, they may differentiate into new hydatid cysts (Figure 1.5). The whole protoscoleces and other components such as brood capsules, hooks and calcareous corpuscles suspended in a hydatid cyst fluid and form white sediment at the bottom of the cyst are called hydatid sand (John and Petri, 2006; Czermak *et al*., 2008). A human hydatid cyst usually contains 500 ml fluid floating hydatid sand with about 400,000 protoscoleces per ml.

1.4.2.6 Calcareous corpuscles

A major component of *E. granulosus* protoscoleces is calcareous corpuscles which are differentiated parenchymal cells, spherical or ovoid in shape and have a diameter ranging between 2-16 µm. They correspond to about 14% of the protoscoleces dried weight (McManus and Bryant, 1995) and contain organic base of DNA, RNA, proteins and inorganic ions (Mehlhorn, 2008). Calcium carbonate is the major component of *E. granulosus* calcareous corpuscles; in addition magnesium and phosphates are also present (Smith and Richards, 1993). The function of calcareous corpuscles is not completely clear. A hypothesis suggested that their phosphate could serve as a source of energy for metabolic requirements and carbonate content function as a buffering system. Disappearance of calcareous corpuscles in the initial establishment and development process of protoscolex to adult worm in the definitive host supports the above hypothesis (Rodrigues *et al*., 1997).

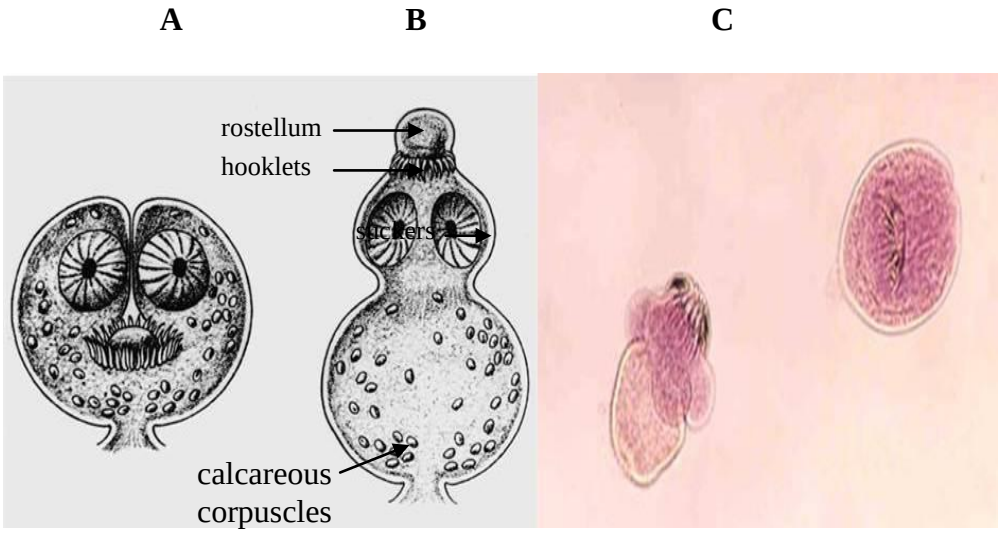

Figure 1.5 Diagram of protoscolex of *E. granulosus.* **(A)** An invaginated scolex **(B)** An evaginated scolex (source: Tropical Medicine Central Resource http://www.isradiology. org **(C)** *E. granulosus* protoscolex stained with eosin (x100) (source: http://www. medicine. cmu. ac.th).

1.4.2.7 Hydatid Cyst Fluid (HCF)

HCF is a colourless fluid with pH 6.7 and specific gravity of 1 to 1.010 and is produced by the germinal layer. It contains sodium chloride, sodium sulphate and salts of succinic acid (Parija, 2004). Biochemical analysis of HCF showed presence of glucose, calcium, creatinine, uric acids, urea, bilirubin, alkaline phosphatase, glycogen, phospholipids, cholesterol and triglycerides (Vatankhah *et al*., 2003; Radfar and Iranyar, 2004). A pressure of up to 300 mm of water keeps the endocyst in intimate contact with the pericyst (Palmer and Reeder, 2000).

1.5 Life Cycle of *E. granulosus*

The life cycle of *E. granulosus* requires two mammalian hosts (Figure 1.6). A definitive host is one in which the adult develops (in the small intestine) and the sexual phase completes; and an intermediate host in which the metacestode stage and protoscoleces usually develop (in the viscera) and the asexual phase occurs. In the natural cycle (also called the domestic cycle), the definitive hosts are dogs and other carnivores and the intermediate host are herbivores such as sheep, cattle, camel and pig. In the sylvatic cycle, the definitive and intermediate hosts are wolves and cervids respectively. The adult worm excretes eggs or gravid proglottids containing eggs along with faeces and contaminates the environment. As an intermediate host, humans acquire the disease by accidental ingestion of vegetables or water contaminated with the eggs of adult worm. Infective ovum hatches in the human duodenum; the oncosphere is released from the keratinized embryophore, probably after being activated by bile salts. The released oncosphere, possibly by secretions or using the hooklets, penetrates the small

intestinal mucosa and reach the internal organs through the vascular and lymphatic systems. The larva then develops into a hydatid cyst and is gradually fully filled with fluid and protoscoleces. Most (>90%) hydatid cysts occur in liver, lung, or both organs (Brunetti *et al*., 2011), and rarely in other organs like bones, brain, kidney (Etaiwi and Mabreh, 2008; Metanat *et al*., 2008; Mongha *et al*., 2008; Csotye *et al*., 2011; Rabbani *et al*., 2011; Vural *et al*., 2011). Hydatid cyst measures about 1 mm in diameter after 1 month and 10–55 mm after 5 months. When infected viscera of sheep or other livestock containing cysts and viable protoscoleces are ingested by dogs, protoscoleces evaginate in the stomach and are released in the small intestine. They attach to the intestinal wall through the action of the four suckers and double row of hooks, and mature into adult worms within 6-7 weeks.

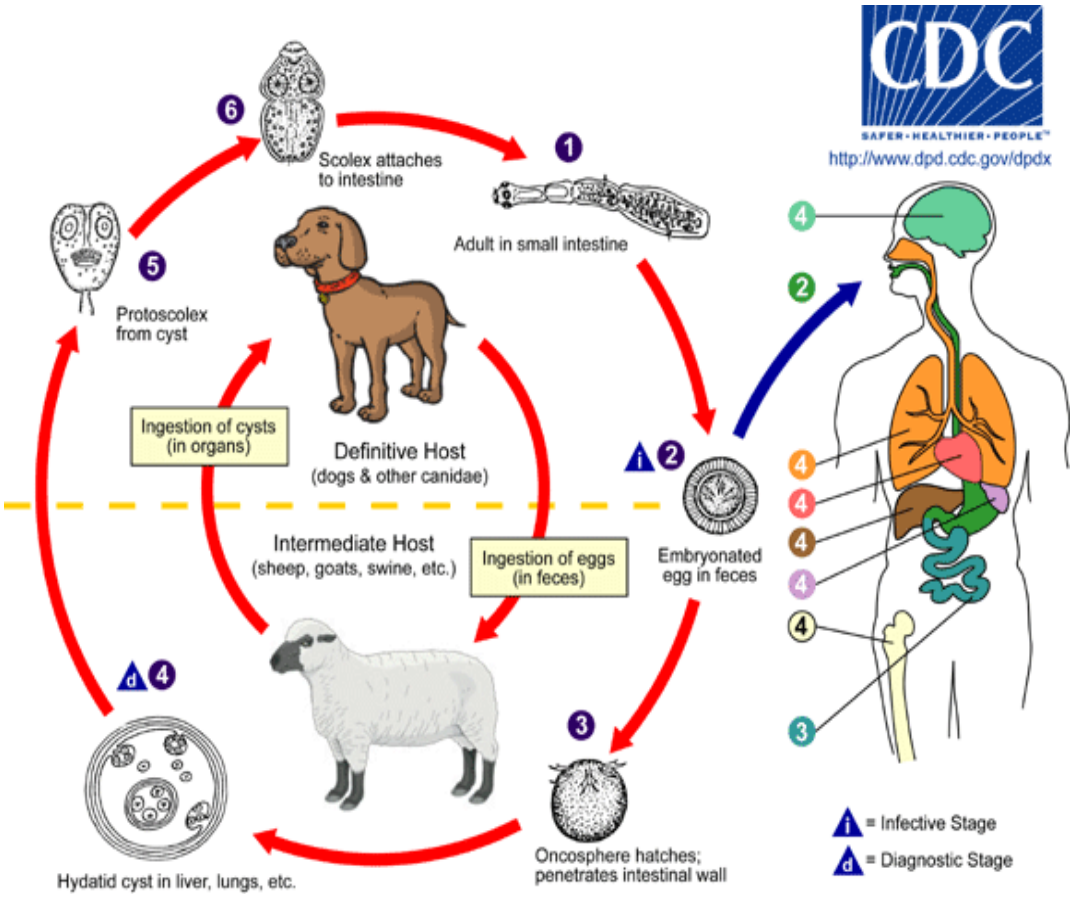

Figure 1.6 Life cycle of *E. granulosus* (source: http://www.dpd.cdc.gov/dpdx).

1.6 Brief immunology of HCD

Three significant features of *E. granulosus* infection are the fact that different mammalian species act as intermediate hosts; long-term growth of the hydatid cysts in internal organs and location of the unilocular cysts in different organs, mainly liver and lungs of humans (Zhang *et al*., 2012). Chemale *et al*. (2003), Monteiro *et al*. (2010) and Aziz *et al*. (2011) have demonstrated the similarity between the complement of host proteins found in the HCF and host plasma. This sugests that *E. granulosus* is capable of adsorbing host proteins as nutrients across its outer germinal layer or to overcome host defences either by antigen disguise or by inhibition of effectors of host immunity (Schroeder *et al*., 2009). Host age, sex, and physiological state of the intermediate host are three factors which may influence the innate susceptibility or resistance to infection with *E. granulosus*.

Human hydatid cysts are described as fertile, sterile and calcified. The protoscoleces are in the fertile cysts, but a proportion of cysts does not produce protoscoleces and are sterile (Salem *et al*., 2011). It is noticeable that hydatid cysts are generally fertile in sheep and sterile in cattle (Zhang *et al*., 2003; Daryani *et al*., 2009). Some cysts die due to hypermaturation followed by starvation and become a mummified, inert calcified cyst. Dead cysts show calcified cyst wall which can occur at any stage of the cysts life cycle (Lewall, 1998; Czermak *et al*., 2008).

1.6.1 Host immune response in pre-encystment phase

After the oncosphere locates an internal organ and develops into primary hydatid cyst, it is confronted by the host humoral and cellular immune responses to the parasite (Al-Sakee, 2011). Cell-mediated responses involve infiltration of

macrophages, eosinophils, neutrophils and fibrocytes and low-level polarized Th1 responses. Antibody responses to HCF and oncospheral antigens are low in the early infection (Zhang *et al*., 2003). A possible role for antibody-dependent cell mediated cytotoxicity (ADCC) reaction is the killing of *E. granulosus* oncospheres by neutrophils in association with antibodies (Rogan *et al*., 1992).

1.6.2 Host immune response in post-encystment phase

Parasite produces antigens that modulate the immune responses. Th1 cytokines produce IL-2, IFN- gamma and lymphotoxin, whereas Th2 cytokines express IL-4, IL-5, IL-6 and IL-10. Th1 and Th2 are cross-inhibitory and also they down-regulate each other (Tamarozzi *et al*., 2010). Antigen B family of *E. granulosus* which is implicated in Th2 induction is reported to inhibit neutrophil migration (Siracusano *et al*., 2008a; Siracusano *et al*., 2008b). Thus, Th2 responses are directly related to susceptibility to the parasitic infection, while Th1 responses are correlated with protective immunity (Ortona *et al*., 2003b; Amri *et al*., 2009). IFN- gamma inhibits Th2-cell proliferation, whereas IL-10 inhibits the synthesis of Th1 cytokines. They are at high levels in hydatid infection and may be due to the complex antigens in HCF. When a cyst dies naturally, or is killed under pharmacological treatment with albendazole or mebendazole or is removed by surgery, Th2 responses drop rapidly and Th1 responses become dominant suggesting that Th1 responses have a role in the process of cyst degeneration. When a cyst relapses and becomes active, the Th2 responses regenerate very quickly and IL-5 and IL-6 are produced in large quantities. IL-5 regulation is associated with the regulation of specific IgE and IgG4 and regulates eosinophilic response. In HCD patients, there is a correlation between production of IL-4 and

IL-10, and IgE and IgG4; therefore, both IL-4 and IFN-gamma regulate the IgE and IgG4 responses. IL-6 induces differentiation of B cells into plasma cells, therefore contributing to the development of antigen-specific humoral responses. In addition, IgG, especially IgG1 and IgG4, IgE and IgM are predominant as the cyst grows and becomes established whereas the concentrations of specific IgG1 and IgG4 decline in patients with cyst infiltration or calcification (Zhang *et al*., 2003; Zhang *et al.*, 2006; Zhang *et al*., 2012).

1.7 *E. granulosus* antigens

E. granulosus produces different antigens during its various developmental stages which modulate the host immune response and also promote parasite survival and development. These antigens are those of HCF antigens, protoscolex and adult somatic antigens and oncosphere antigens (Hewitson *et al*. 2009; Fotoohi *et al*., 2013).

1.7.1 HCF antigens

Two major HCF antigens, antigens B and 5 are lipoproteins. Both have been well characterized by immunoblotting, immunoprecipitation of radiolabeled antigen and sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Shepherd and McManus, 1987). Both antigens are located in the interstitial material between the parenchymal cells and associated with the disorganized areas in the connective area of the germinal membrane and in the parenchyma of the protoscoleces. The brood capsule wall and the brood capsule contents, the protoscoleces tegument, the parenchymal cells, the muscle cells, the calcareous corpuscles and the hooks do not contain antigen 5 or antigen B

(Sanchez *et al*., 1993). These antigens can be found and measured in the patients blood as circulating antigens (Liu *et al*, 1993). According to Oriol *et al*. (1971), by precipitation at low ionic strength (0.005 M acetate buffer, pH 5), purified HCF fraction can be obtained from crude sheep hydatid fluid.

Antigen B and 5 are not specific for *E. granulosus* as patients with infections of *E. multilocularis, E. vogeli* also have detectable levels of specific antibody to these antigens in their serum samples (Lightowlers *et al*., 1989).

1.7.1.1 Antigen B

Antigen B (AgB), the largest component of the HCF with a molecular mass of 120-160 kDa, is a polymeric thermostable lipoprotein (Oriol and Oriol., 1975) which can withstand temperatures up to 100°C for 15 min without losing antigenicity (McVie *et al*., 1997)**.** It dissociates into three main subunits at 8/12, 16 and 20 kDa in SDS-PAGE under reducing and non-reducing conditions. The previous findings suggest that 8 kDa subunit is the smallest compartment of AgB which contains at least two components (Gonzalez *et al*., 1996), namely AgB8/1 (Frosch *et al*., 1994) and Ag B8/2 (Mamuti *et al*., 2006; Mamuti *et al*., 2007). These components may constitute the building blocks of higher molecular weight subunits. Rott *et al*. (2000) evaluated the recombinant antigens *Eg*AgB8/1 and *Eg*AgB8/2 in ELISA and found that the latter was more effective than r*Eg*AgB8/1 or native AgB for diagnosis of human HCD. Virginio *et al*. (2003) evaluated these two recombinant antigens in total IgG-ELISA. They showed that r*Eg*AgB8/2 presented higher sensitivity and specificity than r*Eg*AgB8/1. On the other hand, another study reported that r*Eg*AgB8/1 performed better at detecting all subclasses, particularly IgG4 than r*Eg*AgB8/2; and N-terminal extension of the AgB8/1

subunit (p176), provided a better diagnostic performance than native AgB (Gonza´lez-Sapienza *et al*., 2000).

The 8/12 kDa subunit is the most important subunit in diagnostic studies and induces good humoral and cellular responses (Siracusano *et al*., 2008b). AgB as a protease inhibitor, inhibits neutrophil recruitment (Shepherd *et al*., 1991) and exploits the activation of T-helper cells by eliciting a non-protective Th2 cell response. Recently, findings showed that AgB induces apoptosis in polymorphonuclear cells from patients with active disease (Rigano *et al*., 2002). Until now, using phylogenetic tools there is no data on how many genes are represented in the AgB family, although it is known that AgB is encoded by a multigene family having at least five gene sites (B1–B5), each gene consisting of several minor variants that are grouped into two clusters i.e. EgAgB1/B3/B5 and EgAgB2/B4 (Frosch *et al*., 1994). These five AgB genes differ in amino acid sequences (44–81%). Recently, it was shown that the AgB family comprises at least 10 genes in five subclasses which are differentially expressed. AgB1, AgB2, AgB3 and AgB4 genes are expressed in *Echinococcus* metacestodes; while AgB5 transcripts were detected at very low levels in the larval stage, increasing its expression in the adult worm, which suggests an adult-specific function (Zhang *et al*., 2010). The parasite uses the mechanism of switching among these variants to evade the host immune response (Haag *et al*., 2004). AgB is homologous to, and shares structural similarities with helix-rich hydrophobic ligand binding proteins (HLBPs) from other cestodes and exhibits fatty acid binding properties (Chemale *et al*., 2005). There were reported sero-reactivities between the smallest subunit of AgB and antibodies in serum samples from alveolar hydatid disease and *Taenia*

solium cysticercosis patients (Lightowlers *et al*., 1989; Leggatt and McManus 1994).

1.7.1.2 Antigen 5

Antigen 5 (Ag5) with a molecular mass of 400 kDa is a polypeptide chain thermolabile lipoprotein detected by a precipitation line (arc 5) in immunoelectrophoresis assays (Carmena *et al*., 2006). It dissociates into subunits at 55 and 65 kDa in SDS-PAGE under non reducing conditions.

Under reducing conditions, it dissociates into two subunits at 38/39 and 22–24 kDa linked by disulphide bond (Di Felice *et al*., 1986). Processing of the N-terminal region of the original polypeptide chain generates the 22 kDa subunit whereas the 38 kDa component relates to the C-terminal portion (Lorenzo *et al*., 2003). The existence of a single amino acid sequence with alternative residues at some positions of the 38 kDa subunit *N*-terminal fraction and presentation of Ag5 in different isoforms proposes the hypothesis that Ag5 is similar to AgB and may be encoded by a multigene family which are variably expressed (Zhang *et al*., 2003). There is reported cross-reactivities between Ag5 and antibodies in serum of patients with alveolar hydatid disease, *T. solium* cysticercosis and other helminth infections due to the presence of phosphorylcholine bound to the 38 kDa subunit (Shepherd and McManus**,** 1987). The sites of heparan sulphate proteoglycans and calcium-binding in the 22 kDa subunit provide binding targets for the Ag5 molecule (Lorenzo *et al*., 2003) and ensure its foci in the host tissue surrounding the metacestode (Siracusano *et al*., 2011).

1.7.1.3 Other antigens of HCF

1.7.1.3.1 *E. granulosus* thioredoxin peroxidase (EgTPx)

Antioxidant protein thioredoxin peroxidase was identified by screening an *E. granulosus* cDNA library with IgG1 from patients with HCD (Margutti *el al*., 2008). It is one of the important protoscolex excretory–secretory proteins localized in tegument, subtegument and calcareous corpuscle cells of the protoscolex. EgTPx is secreted into HCF and seems to intervene in evasion mechanisms adopted by the parasite to establish infection by inducing a strong humoral response in patients with HCD (Virginio *et al*., 2012). This protein plays the role of enzymatic scavenger of hydrogen peroxide in *E. granulosus* (Li *et al.*, 2004a; Li *et al.*, 2004b).

1.7.1.3.2 *E. granulosus* tegumental antigen (EgTeg)

EgTeg is a tegumental protein located in the tegument of protoscolex, on the germinal layer of the cyst wall and in the HCF. It is an immunomodulatory molecule which induces a strong humoral response in patients with chronic HCD. EgTeg significantly inhibits polymorphonuclear cell chemotaxis, induce IL4 positive T lymphocytes and non-complement fixing antibodies (IgG4). The hypothesis of the role of EgTeg in excitation of the host immune response is due to an unclear mechanism of secretion or release of EgTeg during the degeneration of the protoscoleces into the HCF (Ortona *et al*., 2005).

1.7.1.3.3 *E. granulosus* A31 (EgA31)

EgA31 is a fibrillar protein of approximately 66 kDa with some properties of paramyosin. It is localized in the tegument of the adult and on the surface of the

protoscolex body wall; and presents in the subtegumental parenchyma, a region rich in muscle at both adult and protoscoleces stages. It is also present in the germinal layer of the cyst. There is not a large amount of EgA31 protein in the sucker regions at the time of invagination of protoscoleces in the intermediate host. The highest concentration of EgA31 is observed in the adult at the region of the suckers after the head evaginates, this suggests that EgA31 protein may be involved in the immune escape mechanisms (Fu *et al*., 1999).

1.7.1.3.4 Cyclophilin (EA21)

E. granulosus cyclophilin was identified both in protoscoleces and in HCF. Cyclophilin is an allergenic molecule that is specifically recognized by IgE from patients with HCD. It is involved in the allergic reactions manifested by the patients and does not cross-react with cyclophilins from other organisms (Ortona *et al*., 2002; Siracusano *et al*., 2008a; Siracusano *et al*., 2008b).

1.7.1.3.5 *E. granulosus* alkaline phosphatase (EgALP)

E. granulosus ALP is present in the germinal layer surface of the hydatid cyst (del Cacho *et al*., 1996) and is also a component of the HCF. Its activity in fertile HCF is significantly more than in sterile HCF (Vatankhah *et al*., 2003).

1.7.1.3.6 Heat shock proteins (HSPs)

HSPs have immunostimulatory properties and their levels increase in organisms which are exposed to conditions of stress such as heat or chemicals. The protoscoleces express several members of HSP i.e. HSP70, HSP60, HSP40 and HSP20. HSP70 has been identified as an antigen in *E. granulosus* human infection.