DEVELOPMENT AND EVALUATION OF AN ANTIGEN DETECTION TEST FOR HYDATID CYST DISEASE

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DEVELOPMENT AND EVALUATION OF AN ANTIGEN DETECTION TEST FOR HYDATID CYST DISEASE

by

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

±	more or less
~	around (molecular mass)
µg	microgram
µL	microliter
µm	micrometer
ºC	degree Celsius
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
Ag5	antigen 5
AgB	antigen B
APS	ammonium persulphate
AUC	Area under ROC curve
BSA	bovine serum albumin
CIEP	counter-immune electrophoresis
cm	centimeter
Co-A	co-agglutination
CT-Scan	computerized tomography scan
DALYs	disability adjusted life years
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
E. felidis	*Echinococcus felidis*
e.g.	*exempli gratia* (for example)
E. granulosus	*Echinococcus granulosus*
E. multilocularis	*Echinococcus multilocularis*
E. oligarthus	*Echinococcus oligarthus*
E. shiquicus	*Echinococcus shiquicus*
E. vogeli	*Echinococcus vogeli*
E.canadansis	*Echinococcus canadensis*
ECL	enhanced chemiluminescent
EDTA	ethylene diamine tetra acetic acid
*EG*95	*E. granulosus* 95 protein
EF-1β/δ	elongation factor 1β/δ

*Eg*AgB	*E. granulosus* antigen B
*Eg*Teg	*E. granulosus* tegumental protein
*Eg*Trp	*E. granulosus* tropomyosin
ELISA	enzyme-linked immunosorbent assay
ESA	excretory/secretory antigen
g	gravity
G	genotype
HCD	hydatid cyst disease
HCF	hydatid cyst fluid
HCl	hydrochloric acid
HRP	horseradish-peroxidase
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
IPTG	Isopropyl β-D-1-thiogalactopyranoside
K_2HPO_4	dipotassium hydrogen phosphate
KCl	potassium chloride
kDa	Kilodalton
KH_2PO_4	potassium dehydrogen phosphate
L	liter
LAT	latex agglutination test
LFD	Lateral flow dipstick
LM	light microscopy
M	molarity
mA	milliampere
MAb	Monoclonal antibodies
mg	milligram

$MgCl_2$	magnesium Chloride
mL	millilitre
mm	millimeter
mM	millimole
mV	millivolt
MWCO	molecular weights cut-off
Na_2CO_3	sodium carbonate
Na_2HPO_4	disodium hydrogen phosphate
NaCL	sodium chloride
nAgB	native Antigen B
$NaHCO_3$	sodium bicarbonate
NaN_3	sodium azide
NaOH	sodium hydroxide
NCM	nitrocellulose membrane
ng	nanogram
$(NH_4)_2SO_4$	ammonium sulphate
NI-NTA	*nickel-nitrilotriacetic acid*
nm	nanometers (wave length)
NTDs	neglected tropical diseases
OD	Optical density
OI	Optical intensity
OIE	Office International des Epizooties (World Organization of Animal Health)
PAIR	puncture, aspiration, injection, and reaspiration
PBS	phosphate buffered saline
PCR	polymerase chain reaction
psi	pound-force per square inch
rAgB	recombinant antigen B
ROC	Receiver operating characteristic
RT	room temperature
SDS	Sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TBS-T	Tris buffered saline/0.05% Tween

TEMED	N,N,N’,N’-tetramethylethylenediamine
U	Unit
US	Ultrasonography
USM	Univesiti Sains Malaysia
V	Volt
v/v	volume/volume
v/w	volume/weight
WB	Western blot
WHO	World Health Organization
WHO-IWGE	World Health Organization-Informal Working Group on Echinococcosis

PEMBANGUNAN DAN PENILAIAN UJIAN PENGESANAN ANTIGEN UNTUK PENYAKIT SISTA HIDATID

ABSTRAK

Penyakit sista hidatid (HCD) adalah penyakit parasit zoonotik yang boleh menjangkiti manusia dan haiwan. Ujian pengimejan dan pengesanan antibodi telah digunakan untuk mendiagnosis HCD, yang terdahulu tidak dapat membezakan antara sista hidatid dan patologi lain; manakala yang lain mungkin tidak dapat membezakan antara jangkitan lampau dan kini, dan mungkin juga tidak sesuai untuk menilai keberkesanan rawatan. Dalam hal ini, ujian pengesanan antigen dapat membantu menangani isu-isu di atas. Sebelum ini telah dilaporkan bahawa antigen sista hidatid dapat dikesan dalam pesakit HCD menggunakan pelbagai assai. Walau bagaimanapun keputusan yang dilaporkan tidak memuaskan, dengan kepekaan diagnostik yang berubah-ubah (25%-83%). Oleh itu, kajian ini bertujuan untuk membangunkan ujian pengesanan antigen untuk penyakit HCD. Satu panel sampel yang terdiri daripada serum pesakit HCD (n = 35), individu yang sihat (n = 38) dan pesakit yang dijangkiti parasit lain (n = 13) telah digunakan dalam kajian ini. Tiga jenis antigen *E. granulosus* disediakan iaitu cecair sista hidatid (HCF), antigen B natif (nAgB), dan antigen B rekombinan (rAgB). Antibodi poliklonal terhadap setiap antigen dihasilkan dalam arnab, diikuti dengan penulenan antibodi IgG. Seterusnya, sebahagian daripada IgG tersebut dilabelkan dengan enzim 'horseradish peroxidase' (HRP). Dengan menggunakan tiga jenis IgG dan tiga jenis antibodi terkonjugat dengan HRP, pelbagai gabungan sandwic ELISA telah diuji. Gabungan ELISA yang terbaik telah dioptimumkan lagi dan dinilai menggunakan panel sampel serum. Seterusnya, sebahagian daripada IgG yang ditulenkan telah konjugatkan dengan

nanopartikel emas untuk pembangunan dipstik aliran sisi (LFD). Dipstik aliran sisi dititis dengan tiga IgG yang berlainan, dan assai LFD menggunakan pelbagai kombinasi antibodi tangkapan dan pengesan diuji. Gabungan asai LFD terbaik kemudiannya dioptimumkan dan dinilai secara kualitatif (secara visual) dan secara kuantitatif menggunakan alat pembaca aliran sisi. Hasil keputusan menunjukkan bahawa gabungan ELISA yang menggunakan IgG anti-HCF sebagai antibodi tangkapan, dan anti-nAgB IgG-HRP sebagai antibodi pengesan mempamerkan isyarat yang terbaik di antara semua kombinasi lain. Walau bagaimanapun, ujian selanjutnya menunjukkan bahawa nilai diagnostiknya rendah. Sementara itu, ujian gabungan LFD dipstik yang dititiskan dengan anti-nAgB IgG dan nanopartikel emas yang terkonjugat dengan anti-HCF IgG sebagai pengesan adalah yang terbaik. Selanjutnya, ujian LFA telah menunjukkan 77.14% sensitiviti diagnostik dan 82.35% spesifisiti diagnostik. AUC daripada ujian LFD ialah 0.849, ini menunjukkan ketepatan diagnostik yang baik. Kesimpulannya, pengesanan antigen ujian LFD menunjukkan sensitiviti dan spesifisiti diagnostik yang memuaskan. Ujian LFD ini berpotensi digunakan seiring dengan ujian pengesanan antibodi untuk diagnosis pesakit HCD dan susulan selepas rawatan.

DEVELOPMENT AND EVALUATION OF AN ANTIGEN DETECTION TEST FOR HYDATID CYST DISEASE

ABSTRACT

Hydatid cyst disease (HCD) is a zoonotic parasitic disease which can infect both humans and animals. Imaging and antibody detection tests have been used to diagnose HCD, the former cannot differentiate between hydatid cyst and other pathologies; while the latter may not be able to differentiate between past and present infections, and may also not be suitable for evaluating treatment efficacy. In this regard, antigen detection tests may be able to assist in addressing the above issues. Previously it has been reported that hydatid cyst antigens can be detected in HCD patients using various assays. However the reported results were not satisfactory, with variable diagnostic sensitivities (25% - 83%). Therefore, this study was aimed at developing an antigen detection test for HCD. A panel of samples comprising serum from HCD patients (n=35), healthy individuals (n=38) and patients with other parasitic diseases (n=13) were used. Three types of *E. granulosus* antigens were prepared i.e. hydatid cyst fluid (HCF), native antigen B (nAgB) and recombinant antigen B (rAgB). Polyclonal antibodies against each of the antigens were produced in rabbits, followed by purification of their IgG fractions. Next, a portion of the purified IgGs were labelled with horseradish peroxidase (HRP). Using the three types of IgGs and three types of HRP-conjugated antibodies, various ELISA sandwich combinations were tested. The best ELISA combination was further optimized and evaluated using the panel of serum samples. Another portion of the purified IgGs were conjugated to colloidal gold nanoparticles for development of lateral flow dipstick (LFD). The dipsticks were dotted with three different IgGs, and

LFD assay using various combinations of capture and detector antibodies were tested. The best LFD assay combination was then further optimized and evaluated, both qualitatively (visually) and quantitatively using a lateral flow reader. Results showed that ELISA combination that used anti-HCF-IgG as the capture antibody, and anti-nAgB-IgG-HRP as the detector antibody exhibited the best signal among all other combinations. However, further testing revealed low diagnostic value of the assay. Meanwhile LFD assay using dipstick dotted with anti-nAgB-IgG and gold conjugated anti-HCF-IgG was the best LFD assay combination. On further testing, the LFD assay demonstrated 77.14% diagnostic sensitivity and 82.35% diagnostic specificity. The AUC of the LFD assay was determined to be 0.849, which indicated good diagnostic accuracy. In conclusion, the antigen detection LFD assay showed satisfactory diagnostic sensitivity and specificity. It is envisaged that the LFD assay is useful to be used in tandem with an antibody detection test for HCD patient diagnosis and post-treatment follow-up.

CHAPTER 1 : INTRODUCTION

1.1 Overview of hydatid cyst disease (HCD)

HCD which is also known as cystic echinococcosis, unilocular echinococcosis or cystic hydatid disease is a zoonotic disease and it is caused by the metacestode (larval stage) of the tapeworm *Echinococcus granulosus* which can infect human, livestock and wildlife. Adult cestodes live in the small intestines of dogs or other canids as their definitive hosts and shed their ova through their faeces. Sheep, goat, cow and other herbivores act as their intermediate hosts and get infected by ingestion of water or plants which are contaminated with ova of the adult worm. Humans are accidental intermediate hosts and become infected by ingestion of *E. granulosus* ova. After peroral infection of humans, the ova that comes in contact with gastrointestinal enzymes will hatch into oncospheres and they migrate to different organs mostly liver and lungs through the vascular or lymphatic systems. Then, the oncospheres will develop into metacestodes and form hydatid cysts. The hydatid cyst gradually increases in size and is filled with hydatid fluid and protoscoleces. HCD is a chronic and complex disease (Moro and Schantz, 2009). Morbidity and mortality of HCD are due to failure or dysfunction of affected organs, rupture of the hydatid cyst, sepsis and anaphylactic shock (Thompson, 2017).

HCD is distributed worldwide with an emerging or re-emerging status with reported incidences from South America, East Asia, Middle East, North Africa, and Mediterranean countries in mostly undeveloped areas (Romig, 2003). However, its re-emerging status with noticeable economic loss has also been reported in developed countries (Rojo-Vazquez *et al.*, 2011). HCD causes considerable loss of disability adjusted life years (DALYs) in many under developed areas and has a big

impact on the economy because of complications in treatment and resultant disabilities (Budke *et al.*, 2006). Recently, HCD was included in a diverse group known as "neglected tropical diseases (NTDs)" by the World Health Organization (WHO). The NTDs is a group of communicable diseases that prevails in tropical and subtropical regions with prevalence among the most impoverished areas and have not been given enough importance at the national or international levels (WHO, 2010).

The diagnosis of HCD is based on the clinical signs followed by imaging and serodiagnosis. Clinical symptoms such as abdominal pain and vomiting is non-specific and imaging of suspected organs cannot differentiate between hydatid cyst, abscess or tumour (Zhang *et al.*, 2003a). Thus, imaging along with serological test are used to confirm HCD. However, serodiagnosis are probably more useful in early diagnosis and post-treatment follow-up of the disease (Craig *et al.*, 2007a).

Previously, many serological tests based on the detection of specific antibodies or antigens have been reported for diagnosis of HCD (Sarkari and Rezaei, 2015). There are various serological tests available for detection of antibodies against HCD in the format of enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence antibody test (IFAT), immunoelectrophoresis (IEP), and immunoblotting (IB) (Zhang *et al.*, 2012b). However these tests have several limitations e.g. cross-reaction with other infections, variable sensitivity, inability to differentiate between past and present infections as well as to evaluate treatment efficacy (Mariconti *et al.*, 2014). In this regard, an antigen detection test may be able to assist in addressing the issues stated above (Devi and Parija, 2003b; Sadjjadi *et al.*, 2009). Currently, commercial tests for diagnosis of HCD are only available in the

form of antibody detection and there is no antigen detection kit available in the market.

1.2 History of HCD

Hydatid cysts were well known in ancient times. This kind of cyst was mentioned in the Babylonian Talmud when seen in animals which were slaughtered in religious ceremonies. Hippocrates and Galen also reported the same condition in animals that were butchered for food. Hydatid cysts in humans were described in old European medical texts as enlarged glands or bags of mucosa connected to blood vessels with rapid growth in size. Francisco Redi in year 1600 proposed the parasitic nature of hydatid cyst (Ridley, 2012) and the German clinician, Pierre Simon Pallas in 1766, for the first time showed that *E. granulosus* was linked to the cyst. The first valid name, "*Hydatigena garnulosa*" was given by Batch in 1786 based on recognized cysts in sheep from Germany. A few years later in 1801, the genus *Echinococcus* was named by Rudolphi. However, the link between the cyst and the metacestode was not yet recognized. Siebold and Küchenmeister in 1853 demonstrated that hydatid cysts from sheep are the origin of adult tapeworms in dogs and shortly thereafter in 1863 Bernhard Naunyn showed adult tapeworms developed in dogs which were fed with human hydatid cysts (Cox, 2002; Tappe *et al.*, 2010).

Although this disease was discovered in the olden times, only in the present century was significant progress made in biology, immunology, diagnosis, treatment and epidemiology of HCD (Eckert and Thompson, 2017). In Iran, *E. granulosus* was identified in stray dogs in Tehran for the first time by Makarehchian and Janbakhsh in 1956 and two decades later Mobedi and Sadeghian reported *Echinococcus multilocularis* in red foxes in the northeast part of Iran (Ghafarifar, 2010). To the

best of our knowledge, the history of HCD in Malaysia has not been documented and Malaysia is a non-endemic area for HCD with just a few cases of human HCD reported. These cases were imported from neighbouring or other countries which are endemic for HCD. The first and latest documented case of HCD in Malaysia were reported by Kutty *et al.* (1970) and Suria Hayati *et al.* (2015) respectively.

1.3 Classification of the genus *Echinococcus*

Echinococcus is a small endoparasitic flatworm belonging to the Kingdom Animalia. This parasite is classified under Phylum Platyhelminthes (flatworms) in the Class Cestoda (tapeworm) because of its flat ribbon-like body, with anterior scolex and posterior tape made of segments. The adult worm do not have alimentary canal and body cavity and a syncytial tegument cover the outer body. The parasite is a true tapeworm in the subclass Eucestoda because its elongated body consist of a linear set of proglottids (male and female reproductive organs) and hermaphroditic characteristics. All the parasitic tapeworms which can infect humans are classified in the Order Cyclophyllidea because they have indirect life cycles involving larval form in intermediate host and are transmitted to the definitive host by carnivorism where they develop into adult worms with four suckers on the scolex. *Echinococcus* is placed in the *Taeniidae* family due to its operculated eggs with two covering, unpaired genitalia in each proglottid with irregularly alternating lateral genital pores. However, *Echinococcus* has only up to six proglottids compared with other members of this family such as *Taenia* which may have more than a thousand proglottids (Thompson and McManus, 2001; Thompson, 2017). Table 1.1 shows the full scientific classification of the genus *Echinococcus*.

Table 1.1 The scientific classification of the genus *Echinococcus* (Thompson and McManus, 2001).

Domain:	*Eucaryota*
Kingdom:	*Animalia*
Phylum:	*Platyhelminthes*
Class:	*Cestoda*
Subclass:	*Eucestoda*
Order:	*Cyclophyllidea*
Family:	*Taeniidae*
Genus:	*Echinococcus*

1.4 Species of the genus *Echinococcus*

In the past, there was a debate on whether echinococcosis (hydatidosis) might be caused by one or more species. Meanwhile more species were described and identified based on the differences in adult worm morphology such as rostellar hook shape, number of proglottids and position of the genital pore (Romig *et al.*, 2015). However, some of these newly identified species were not considered valid and separate species. Thus far, nine species were accepted as valid species of *Echinococcus*. However, just four of them are common and have high importance (Nakao *et al.*, 2013). Table 1.2 shows the valid species in the genus *Echinococcus.* The four most common species of the genus *Echinococcus* with valid taxonomy and well recognized are namely: *Echinococcus granulosus* (Batsch, 1786), *Echinococcus multilocularis* (Leuckart, 1863), *Echinococcus oligarthrus* (Diesing, 1863) and *Echinococcus vogeli* (Rausch and Bernstein, 1972). These four species have distinct morphology in both their larval and adult stages (Thompson and McManus, 2001). *E. granulosus* and *E. multilocularis* are the most important species as they cause human HCD and human alveolar hydatid disease respectively. Infection with these two species is severe and leads to economic loss. Figure 1.1 displays the morphology differences of adult *Echinococcus* species.

Table 1.2 A list of valid species in the genus *Echinococcus* (Nakao *et al.*, 2013).

Species	Synonym and first identified	Distribution	Definitive hosts **	Intermediate hosts **	Hydatid features	Human infection
Echinococcus granulosus	*Hydatigena granulosa* (Batsch, 1786)	Worldwide	Dog	Sheep, goat and cattle	Unilocular	Common
Echinococcus multilocularis	*Taenia multilocularis* (Leukart, 1863)	Holarctic	Red fox and arctic fox	Arvicoline rodents	Alveolar	Common
Echinococcus oligarthrus	*Taenia oligarthra* (Diesing, 1863)	Neotropical	Wild felids	Agoutis	Unilocular	Uncommon
Echinococcus vogeli	–	Neotropical	Bush dog	Paca	Polycystic	Uncommon
Echinococcus ortleppi	–	Worldwide*	Dog	Cattle	Unilocular	Uncommon
*Echinococcus Canadensis****	*Echinococcus granulosus Canadensis* (Webster and Cameron, 1961)	Worldwide* /Northern arctic and boreal	Dog/Wolf	Pig, camel, cattle, goat and sheep/ Moose, reindeer and wapiti	Unilocular	Uncommon
Echinococcus equinus	*Echinococcus granulosus equinus* (Williams and Sweatman, 1963)	Worldwide*	Dog	Horse	Unilocular	Unknown
Echinococcus shiquicus	–	Tibetan plateau	Tibetan fox	Pika	Unilocular	Unknown
Echinococcus felidis	–	Africa	Lion	Unknown	Unknown	Unknown

* Sporadic distribution

** Only the most significant hosts are listed

*** Three different genotypes with different distribution and hosts, refer to the source.

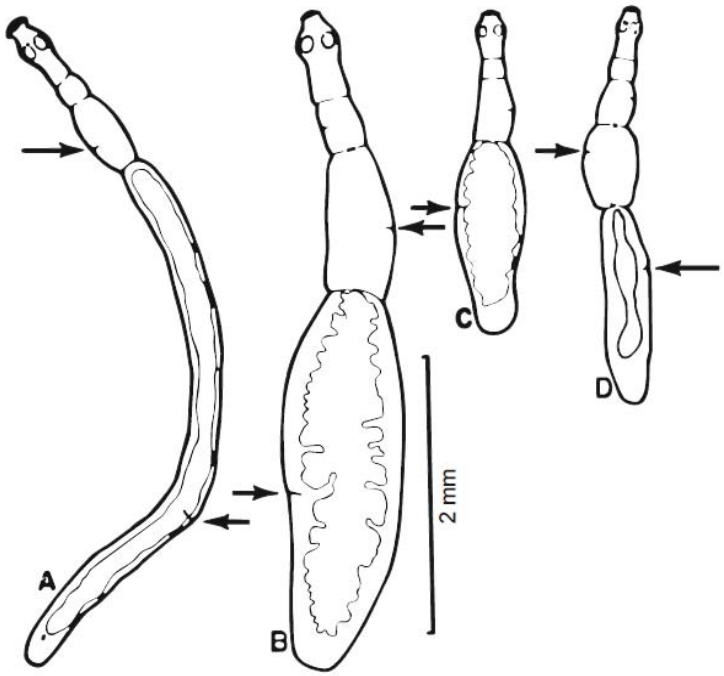

Figure 1.1 Comparative general morphology of the most important adult *Echinococcus* species (Thompson and McManus, 2001).

A: *Echinococcus vogeli*

B: *Echinococcus granulosus*

C: *Echinococcus oligarthrus*

D: *Echinococcus multilocularis*

← and →: Genital pore

1.5 Morphology and biology of *E. granulosus*

1.5.1 Adult stage

Adult *E. granulosus* measures approximately 2 to 7 millimeters (mm) in length and is one of the smallest endoparasite worms among all tapeworms. The worm is composed of the scolex (head), narrow neck and body. The head or spherical scolex is equipped with specialized attaching tools consisting of four muscular suckers and two circular rows of hooks (22-40 μm), one row with big hooks and another row with small hooks which all placed on the rostellum. The narrow neck is a regenerative part and separates the scolex from the rest of the body. The body or storbila is segmented which consists of three to four proglottids (reproductive units) and in rare cases up to six proglottids (Thompson and McManus, 2001).

The first segment is not mature and no genital organ will develop inside. The penultimate segment is sexually mature and both male and female organs will develop. In the male organ, usually around 50 follicular testes are scattered in the mature proglottid. Each testis links to a single vas deferens and all vas deferens become united to form one main vas deferens which connects to the male copulatory organ called cirrus. The female organ is composed of the ovary, ootype, coiled uterus and vitellaria in the cirrus sac which opens to the lateral genital pore. *E. granulosus* is hermaphroditic and is capable of both self or cross fertilization (Roberts *et al.*, 2013; Mandal, 2015) .

The last proglottid is gravid and measures approximately 2.0 mm x 1.0 mm with a uterus filled with approximately 5000 eggs. The eggs that are released from the proglottid are oval shaped measuring 30-37 μm in diameter and each egg contains a single hexacanth oncosphere (embryo). Under the light microscope, the

eggs of *E. granulosus* are morphologically indistinguishable from the eggs of *Taenia* species. The eggs are covered with a thick and resistant outer layer which is capable of surviving in the environment for long periods (6-12 months) and are infective to humans and animals (Thompson, 2017). The figure 1.2 displays the body structure and organs of an adult *E. granulosus.*

This tapeworm has no digestive tract and it absorbs food through the outer body layer (tegument) when in contact with nutrients in the intestine of their definitive host. The microtriches on the tegument increases the surface area for food absorption. The nervous system of an adult worm consists of five pairs of longitudinal nerves. The motor nerves is responsible for the contraction of the suckers on the scolex for the purpose of attachment to the intestine wall of the host (Flisser and Craig, 2010). The excretion system consists of Flame cells and canals. The Flame cells are found in all endoparasitic tapeworms and they maintain the organ's osmotic balance by excreting wastes through the ventrolateral and dorsolateral canals. There are canals on each side of the worm's body along the storbila which conduct wastes to the external environment (Roberts *et al.*, 2009).

The key definitive host of adult *E. granulosus* are domestic dogs, but it can also be found in other canids such as wolves, golden jackals and red foxes (Rokni, 2008; Craig *et al.*, 2015). They acquire infection by ingesting the infected organs of intermediate host (herbivores or omnivores) with fertile hydatid cysts containing viable protoscoleces. After ingestion, the apical region of scolex invaginate within the basal region of protoscolex tegument to protect the suckers, hooks and rostellum until it is stimulated to evaginate by external factors i.e. proteolytic enzymes (pepsin and pancreatin), host's bile salt and the microtopography of the intestine. Following evagination, the protoscolex is very active and it penetrates deeply into the mucosal

surface between the villi and the crypts of Lieberkuhn. The protoscolex then develops into the adult worm in 34-58 days. Sexual maturity starts with egg production in the proglottid and release of gravid proglottid into the small intestine which is excreted with the faeces. The infected definitive host carries adult *E. granulosus* in their intestines (Lewall, 1998; Thompson, 2017). Figure 1.3 shows the development stages of *E. granulosus* from protoscolex to an adult worm.

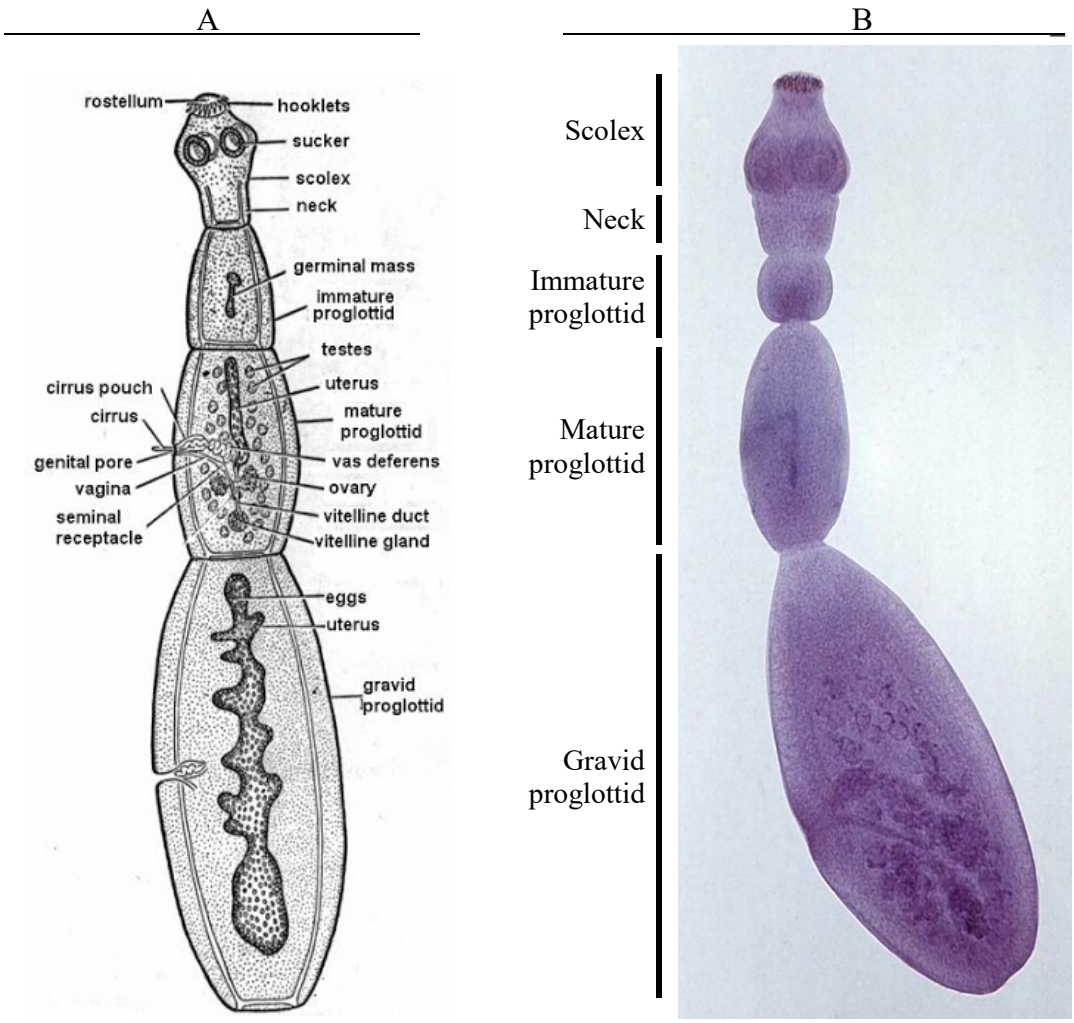

Figure 1.2 Adult worm of *E. granulosus.*

A: Schematic diagram of body structure and organs (nscu.edu, 2017).

B: Light microscopic (100X) image of an adult worm (www.k-state.edu, 2017).

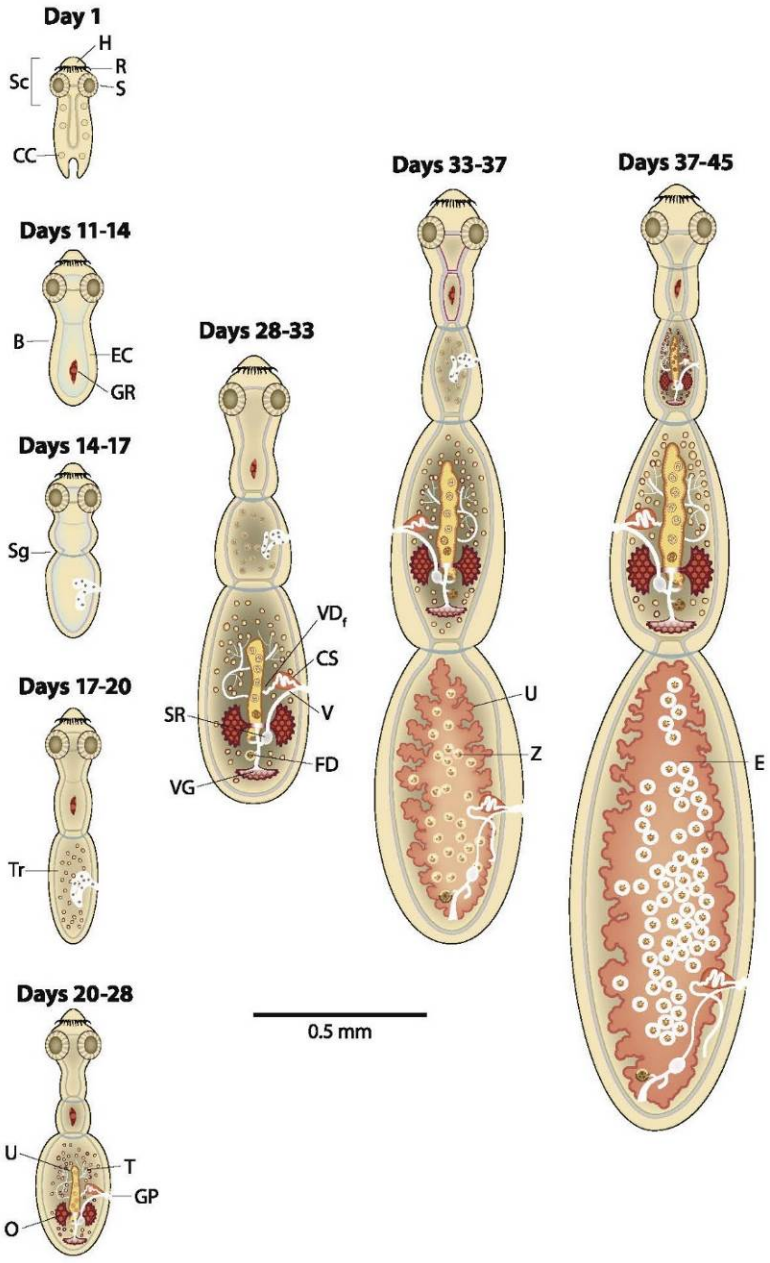

Figure 1.3 Stages of development of *E. granulosus* from protoscolex to adult mature worm (Thompson, 2017).

B: band, CC: calcareous corpuscles, CS: cirrus sac, E: embryonated eggs, EC: excretory canal, FD: female reproductive ducts, GP: genital pore, GR: genital rudiment, H: hooks, O: ovary, R: rostellum, S: suckers, Sc: scolex, Sg: segment, SR: seminal receptacle, Tr: rudimentary testes, T: testes, U: uterus V: vagina, VD: vas deferens, VG: vitelline gland, Z: zygotes.

1.5.2 Larva stage (metacestode)

The metacestode of *E. granulosus* develops into unilocular cyst (hydatid cyst) in their intermediate hosts. The cyst is spherical in shape, filled with clear fluid (hydatid fluid) and contains complex structures of the *E. granulosus.* The size of the cyst may vary from 1 to 15 centimeter (cm) in diameter, however larger cyst may also be observed (Eckert and Deplazes, 2004; Moro and Schantz, 2009). Figures 1.4 and 1.5 show the pictures of hydatid cysts in the liver and an isolated unilocular hydatid cyst respectively. The hydatid cyst comprises three layers: 1.Adventitial layer, 2.Laminar layer and 3.Germinal layer.

1.5.2(a) Adventitial layer (pericyst or pseudocyst)

The adventitial layer or host fibrous layer is produced by host tissue and surrounds the hydatid cyst. This layer comprises collagen and fibroblast cells. The hydatid cyst receives nutrients through blood vessels in this layer by diffusion (Vanek, 1980).

1.5.2(b) Laminar layer (ectocyst)

The laminar layer or laminated layer is formed as a result of the host's cellular inflammatory reaction which surrounds the viable hydatid cyst (Thompson, 2017). This layer is acellular, tough and elastic and varies in thickness. The laminar layer is formed around the germinal layer between 2 to 4 weeks after peroral infection of the intermediate host with *E. granulosus* eggs and oncosphere liberation (Zhang *et al.*, 2003a).

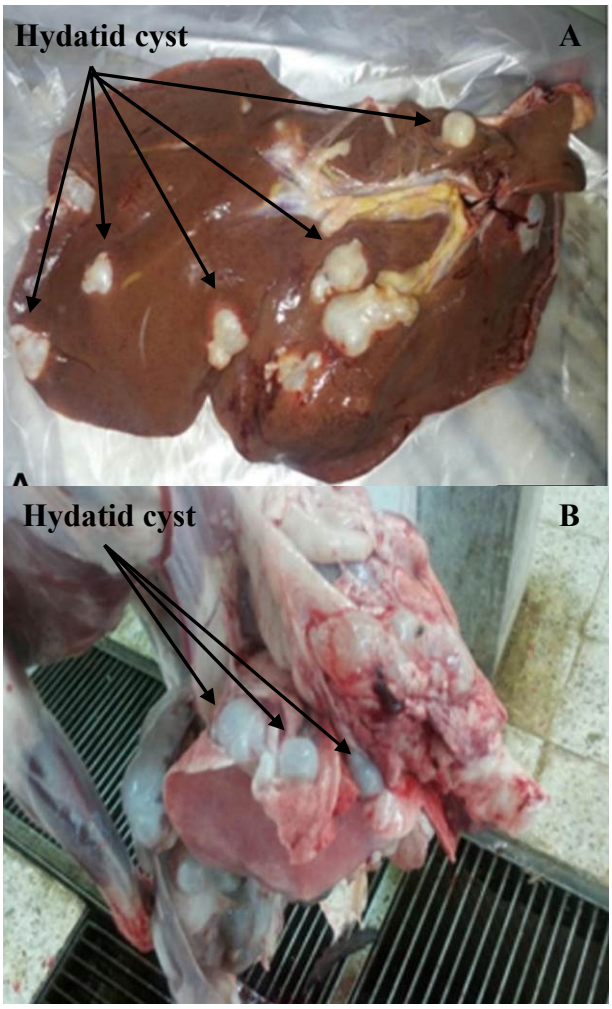

Figure 1.4 Hydatid cysts in liver (A) and lung (B) of sheep infected with *E. granulosus* (Almalki *et al*., 2017).

Figure 1.5 Spherical and unilocular hydatid cyst of *E. granulosus*. (southhampton.ac.uk, 2017).

1.5.2(c) Germinal layer (endocyst)

This layer is a single layer of cells lining the inner area of the cyst. The structure of the germinal layer or germinal epithelium is similar to the outer body layer (tegument) of the adult worm. This layer is the true wall of the cyst with a thickness of 10-20 µm. This layer comprises several cell types i.e. muscle cells, glycogen storing cells, tegumental cells and undifferentiated cells. The undifferentiated cells are the proliferative layer which produces the laminated layer, brood capsules and scoleces. The brood capsule is formed as a small bud and proliferates toward the cyst cavity (Galindo *et al.*, 2008; Thompson, 2017).

1.5.2(d) Brood capsules

The brood capsules are small vesicles or small secondary cysts which grow large and asexual buds form within their lumen from the germinal layer to produce many protoscoleces (Moro and Schantz, 2009; Siracusano *et al.*, 2009). Protoscoleces develop asynchronously and at the final stage of development, the hooks are positioned on the invaginated rostellum (Thompson, 2017). The small vesicles are stalked to the germinal layer of the larger mother cyst and grow big to form secondary cysts (daughter cysts) or may release the protoscoleces into the hydatid fluid by rupturing (Garcia *et al.*, 2011). Figures 1.6 shows a cross-section of a hydatid cyst and figure 1.7 shows *E. granulosus* protoscolex.

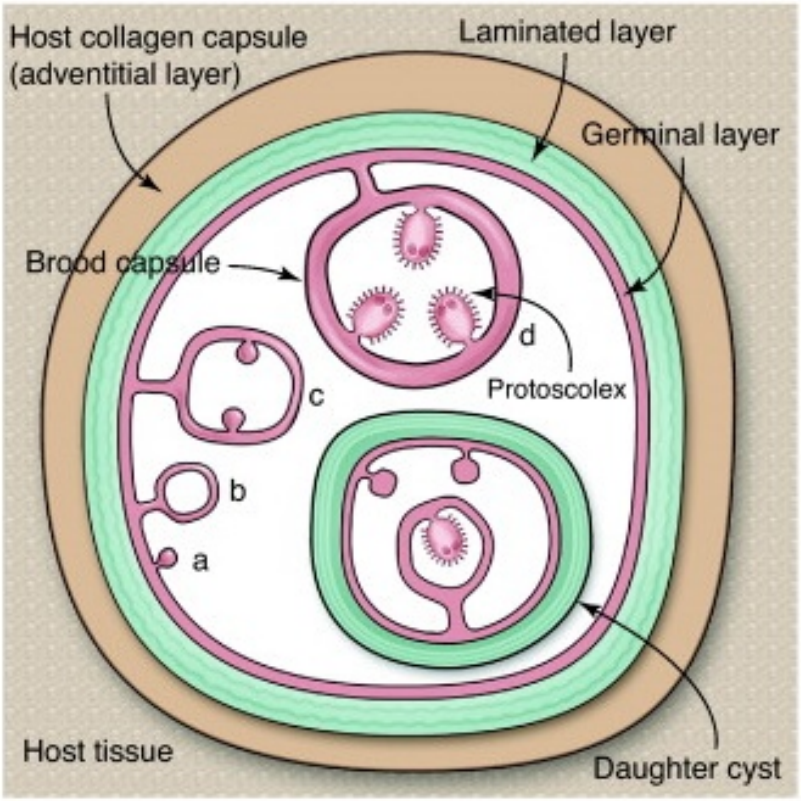

Figure 1.6 Diagrammatic representation of a cross section of unilocular *E. granulosus* hydatid cyst (Diaz *et al.*, 2011).

a: Small vesicle is formed from inner layer of the germinal layer

b: Small vesicle vacuolated and performed a cavity inside.

c: Protoscoleces are initially formed in small vesicle

d: Fully developed protoscoleces (with hooks) are formed within vesicle

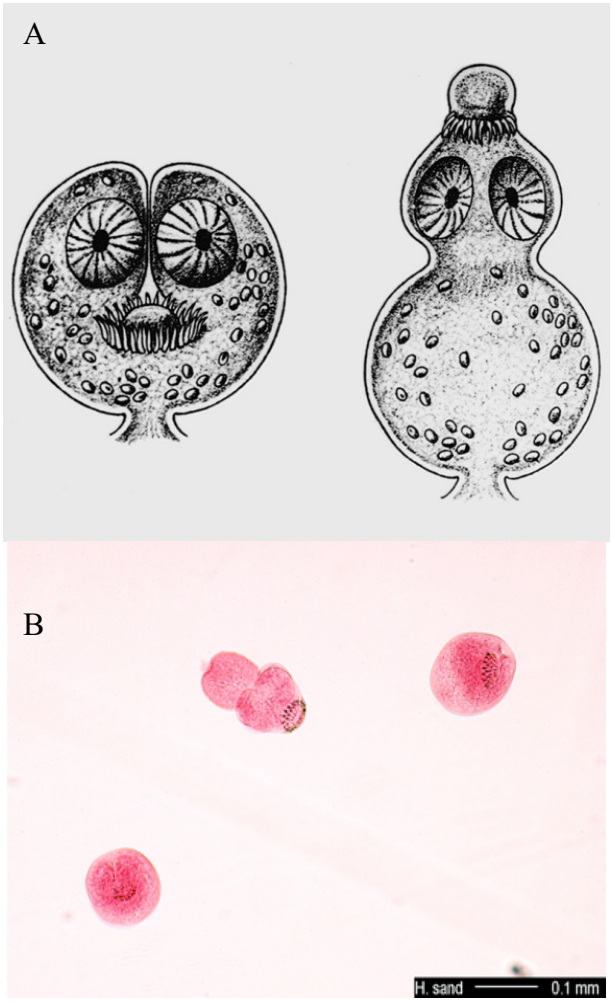

Figure 1.7 *E. granulosus* protoscolex , (A) Diagram of invaginated protoscolex (left) and evaginated protoscolex (right) (www.isradiology.org, 2017); (B) light microscopic (x200) picture of hydatid sands (atlas.or.kr, 2017).

1.5.2(e) Protoscolex

Protoscolex become apparent from the inner wall of brood capsule. The exact time of the development of protoscolex within the hydatid cyst in human is not clear, but it is estimated to be more than 10 months after infection. Usually protoscoleces are observed in cysts with a size of 5 to 20 mm in diameter. Also, some proportion of cysts may remain sterile as they do not produce protoscoleces (Eckert and Deplazes, 2004). A fully developed protoscolex measures about 3 mm in length and it is similar to an adult scolex. It has many hooks in two parallel rows on the rostellum (Muller and Wakelin, 2002). After digestion of hydatid cyst containing protoscoleces by their definitive hosts, the invaginated protoscolex evaginates in the gastro-intestine tract of the definitive host and attaches to the intestine wall using hooks and suckers on the rostellum and scolex. Finally, it develops into an adult worm. If there is hydatid cyst spillage or ruptures in the intermediate host, the released protoscoleces may develop new cysts in the surrounding tissues. The protoscoleces with other components in the hydatid cyst including hooks, calcareous corpuscles and brood capsules form a white sediment called hydatid sand at the bottom of the hydatid cyst (Eckert and Deplazes, 2004; Czermak *et al.*, 2008).

1.5.2(f) Calcareous corpuscle

Calcareous corpuscle (CC) is the main component (14% of dried weight) of *E. granulosus* protoscoleces. The CCs are oval in shape and they measure between 2 µm to 15 µm in diameter (McManus and Bryant, 1995). The exact function of CCs is not completely clear. But it is thought that CC is a ready source of calcium carbonate and it is responsible for lipid metabolism, osmotic balance and tissue repair. (Li *et al.*, 2004).

1.5.2(g) Hydatid cyst fluid

Hydatid fluid is a clear or clear yellow fluid with pH of 6.7 and specific gravity of about 1.0. This fluid is secreted by germinal layer of hydatid cyst and it provides nutritional requirements for larval growth and development. This fluid contains inorganic material, biochemical metabolites and proteins (Juyi *et al.*, 2013). The pressure of hydatid fluid in the cyst is about 0.5 psi of water which keeps the endocyst in close contact with the pericyst (Palmer and Reeder, 2001).

1.6 Life cycle of *E. granulosus*

E. granulosus requires two hosts which belong to the bovid and canid families, in order to complete life cycle. A carnivorous animal as a definitive host which harbours the adult worm in the small intestine and produce ova/eggs (sexual phase), and a herbivorous animal as an intermediate host in which the metacestode develops into cyst containing protoscoleces in the viscera (asexual phase). There are two type of life cycle for *E. granulosus* called the natural cycle and sylvatic cycle. In the natural cycle or domestic cycle, domestic dogs act as the definitive host and domestic herbivores or omnivores such as sheep, goat, cow, camel and pig are the intermediate host. The sylvatic cycle or wild cycle involves wild carnivores such as wolf as the definitive host and wild ungulates like moose and reindeer as the intermediate host. The adult *E. granulosus* releases the gravid proglottids filled with eggs into the intestine of the definitive host and the definitive host sheds the eggs with their faeces to the environment. The intermediate hosts become infected by ingestion of water or plants which are contaminated with the eggs. Humans get infected by accidental ingestion of *E. granulosus* eggs and are dead-end host (Eckert *et al.*, 2001; Eckert and Deplazes, 2004).

After oral uptake of *E. granulosus* egg by a human, the fully embryonated and infective egg hatches in the stomach and small intestine. The oncosphere is liberated from the oncospheral membrane presumably after being activated by bile salts. The free oncosphere migrates toward the intestinal wall and penetrates deeply into the tips of the intestinal villi using both hooks and secretion before reaching the vascular and lymphatic system. The oncosphere migrates to the internal organs through the vascular system and localizes. Once the oncosphere localizes in an organ, it develops into the metacestode and forms a cyst. The cyst is gradually filled with hydatid fluid and protoscoleces (Thompson, 2017). In an infected human, hydatid cyst may be observed in one or more organs mostly in the liver and lungs, and in rare cases the brain, kidneys and bone (Brunetti *et al.*, 2011). The size of hydatid cyst measures about 1 mm in diameter at 1 month post-infection and after 5 months it grows to 10-55 mm. The life cycle of *E. granulosus* will be completed when the definitive host ingest infected organs and viscera of the intermediate host containing hydatid cyst with viable protoscoleces. The protoscoleces evaginate in the stomach of the definitive hosts and attach to the wall of the small intestine using the suckers and hooks on the scolex where they develop into mature adult worms within seven weeks (Muller and Wakelin, 2002). The figure 1.8 illustrates the life cycle of *E. granulosus.*

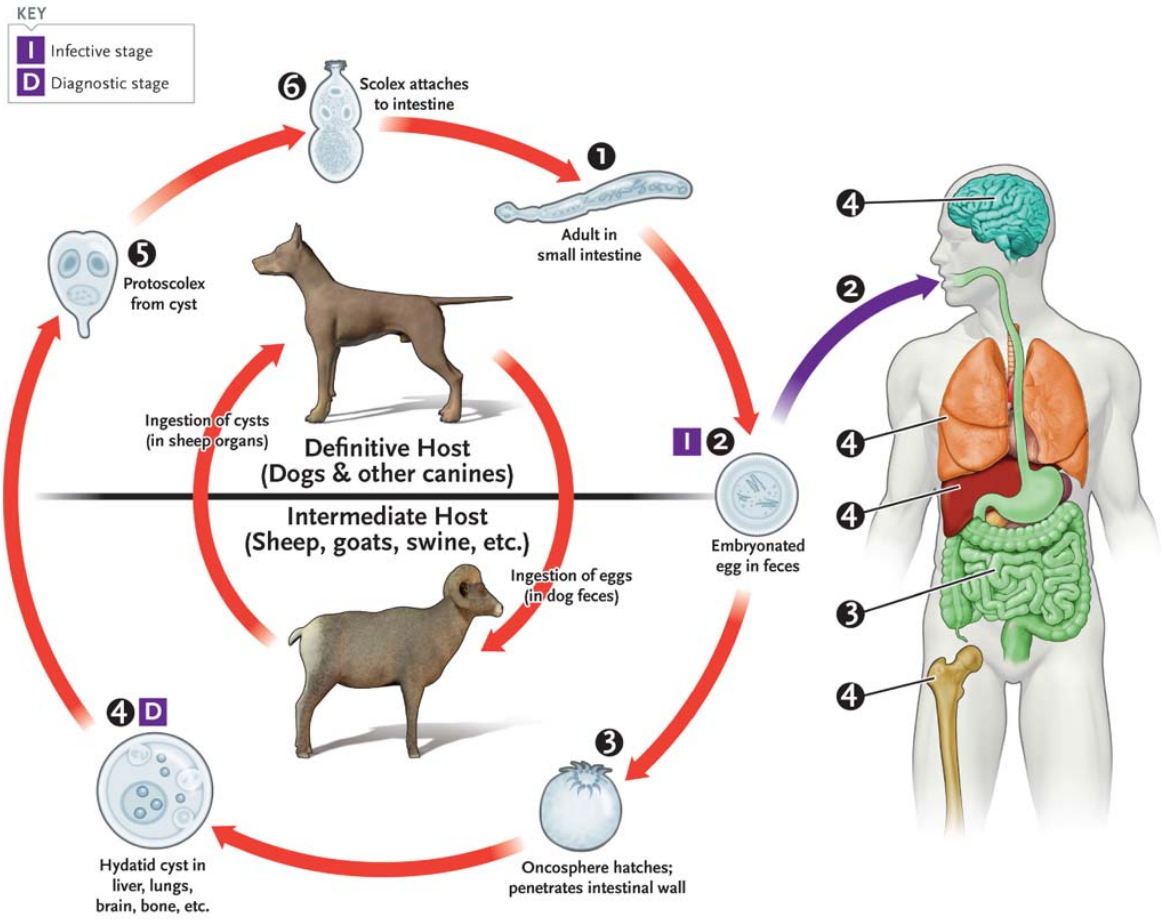

Figure 1.8 Life cycle of *E. granulosus* (Levinson, 2016).

1.7 Epidemiology of *E. granulosus*

The distribution of *E. granulosus* globally is generally related to animal husbandry (domestic life cycle). There are only a few places in North America and Eurasia where the sylvatic life cycle (wild life cycle) is established (Thompson, 2008). The specific roles of various host species in the cycle of the disease, might be notably different between endemic regions. Domestic and sylvatic life cycles could co-exist or overlap in many endemic areas (Eckert and Deplazes, 2004). Using molecular biology techniques, McManus and Thompson (2003) described ten distinct strains (genotypes) of *E. granulosus* namely G1 to G10. They used mitochondrial DNA sequences to identify genotypes and it appears that each genotype has adopted a particular life cycle pattern which involves specific hosts. These ten genotypes include two sheep strains (G1 and G2), two cattle strains (G3 and G5), one horse strain (G4), one camelid strain (G6), one pig strain (G7), one cervid strain (G8) ,one Poland swine strain (G9) and one Eurasia reindeer strain (G10). The G1 strain is the common sheep-dog strain which is the cause of most human infections. This strain is geographically distributed specifically in North Africa. The G2 strain is the Tasmanian sheep strain which is distributed in Tasmania and Argentina. The emergence of this strain in some endemic European countries (Italy, Spain, Portugal, France and Bulgaria) have been reported recently. The G3 and G5 strains (cattle strains) are both distributed in Asia, but G5 (like the G4 horse strain) is also endemic in Europe. The G8 strain is maintained in the sylvatic cycle in North America and Eurasia where wolves or dogs act as the definitive host and reindeer and moose act as the intermediate host. Human infection with this strain is rare. In the new classification, the old classified genotypes G1 to G5 were reclassified as follows: G1 to G3 into *E. granulosus sensu strict*; G4 into *E. equines*

and G5 into *E. ortleppi* (McManus and Thompson, 2003; Siracusano *et al.*, 2012b; Romig *et al.*, 2015).

With respect to the human infections, the G1 strain (sheep strain) and G5 strain (bovid strain) are the most important strains (Stojkovic *et al.*, 2014). In Iran the presence of the G1, G3 and G6 genotypes have been reported by Sharafi *et al.* (2014).

1.7.1 Risk of hydatid cyst infection in human

Humans become infected by peroral ingestion of *E. granulosus* eggs which infected dogs or other canids (definitive host) passed with their faeces into the environment. The identification of risk factors for infection in humans is complicated because of the long latent period between the initial infection, and appearance of clinical symptoms and diagnosis of the disease. In addition, humans and dogs are capable of widespread movements. The infection can be transmitted to humans by direct and indirect ways. The direct infection or hand-to-mouth route happens particularly where there is intimate contact of humans with dogs. The eggs attach to the hairs around the anus of infected dogs and can also be found attached to the paws and muzzle which may transfer to humans who touch the dog. Indirect transfer happens via handling or consuming water or food such as vegetables, salad and raw meat contaminated with *E. granulosus* eggs. The indirect transmission can also happen through intermediaries such as flies or other arthropods (Moro and Schantz, 2009). The prenatal transfer of the disease has still not been reported (Conn, 1994).

Humans of all ages are susceptible to HCD. The disease has been reported in children younger than 1 year old as well as adults older than 75 years old and there is no differences in disease prevalence between males and females. About 60% of the