Pathological and Biomolecular Study on Hydatid Disease in Camels, Cattle and Sheep

By

Ahmed Mohammed Ahmed Osman
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Supervisor:

Ahmed Abdelrahim Gameel; BVSc, MSc, DVM, PhD
Professor of Veterinary Pathology

Co- Supervisor:

Imadeldin E. Aradaib; BVSc, MSc, MPVM, PhD
Professor of Molecular Medicine

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To My Family

For Their Support, Patience and Understanding

I Dedicate This Work
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ABSTRACT

A pathological and biomolecular study on hydatid disease in animals was conducted. 3415 animals (31 camels, 898 cattle and 2486 sheep) were examined for presence of hydatid cysts. The highest prevalence rate (58.06%) was recorded in camels followed by cattle (1.67%) and sheep (0.4%). The lung was the main organ involved in camels and cattle, while in sheep the liver was the target organ. Camel and cattle cysts were larger in size (2-6 cm diameter) than those of sheep (< 2cm diameter). The amount of cyst fluid varied from 3.5- 25 ml in camel, 0.5- 25 ml in cattle and 0.3- 2.7 ml in sheep. The highest fertility rate was found in cattle cysts (66.67%) followed by camels (50%). All liver cysts from sheep and camels were sterile and mostly calcified. Cysts fluid collected from sheep had high levels of total proteins, cholesterol, sodium and potassium. In camels and cattle, total protein and cholesterol were highest in fluid with thick consistency or in caseated cyst contents. Calcium concentration was high in both camel and sheep cysts, while phosphorus was high in cattle.

Sections from infected lungs and liver showed distinct hydatid cyst structures surrounded by cellular reaction (macrophages, lymphocytes and plasma cells) and fibrous tissue capsule. Caseated cysts showed intense cellular reaction with neutrophil infiltration. Surrounding lung and hepatic tissue showed pressure atrophy and necrosis of variable degrees. Scolices were only seen in lung sections of camel and cattle.

Periodic acid Schiff (PAS) and Masson's trichrome stains clearly demonstrated the cyst membranes, the fibrous tissue reaction and the protoscolices.

The nested polymerase chain reaction (PCR) assays were performed for genotyping cysts extracted DNA (79 cysts) using NADH dehydrogenase1 as a target gene. All 79 samples resulted in amplification of 276 bases pair PCR products specific for the camel strain (G6) of *Echinococcus granulosus*.

It was concluded that the camel strain (G6) of *E. granulosus* is the most prevalent in Sudan and that the camels are most susceptible to infection. Nested PCR assay is recommended as a reliable diagnostic test for molecular epidemiological surveys of various animals strains of *E. granulosus*. 
ملخص الظروحة

تم إجراء دراسة مرضية ودراسة حيوية جزئية على مرض الأكياس العداري في الحيوانات. تم فحص 3415 حيوان (31 رأس من الأيل، 898 رأس من الأبقار و 2486 رأس من الضأن). أظهرت النتائج ان اعلى معدل إصابة بالمرض كان في الأيل (58.06%) ثليها الأبقار (1.67%) ثم الضأن (0.4%). كانت الرنة هي أكثر الأعضا عرضاً للإصابة في كل من الأيل والأبقار بينما كان الكبد هو العضو الأكثر إصابة في الضأن. الأكياس العدارية المجمعة من الأيل والأبقار كانت هي الأكبر حجماً (2-6 سم) بينما كانت أقل من 2 سم في الضأن. تراوت كميات السائل العداري ما بين 3.5 – 25 ملليتر، 0.5 – 2.7 ملليتر في كل من الأيل، الأبقار والضأن على التوالي. نسبة الأكياس العدارية المعدية كانت عالية في الأبقار (66.67 %) مقارناً مع الضأن (50 %). كل الأكياس العدارية التي وجدت في الكبد في كل من الإيل والضأن كانت غير معدية مع وجود بداية للتهلاك.

وحدثت نسبة البروتينات الكلية، الكولسترول، الصوديوم والبوتاسيوم عالية في السائل العداري للضأن.

كما لوحظ ارتفاع نسبة البروتينات الكلية والكولسترول في الأكياس العدارية التي يكون السائل فيها فلثياً أو التي بها نوع من التغيرات في كل من الإيل والأبقار. كانت نسبة الكالسيوم عالية في السائل العداري للإيل والضأن بينما لوحظ ارتفاع نسبة السفور في السائل العداري للأبقار.

المقاطع النسيجية أظهرت طبقات الكيس العداري واضحة مع إحساسها بارتفاع خلوبي يتكون من الخلايا الممتدة الكبيرة، الخلايا الليمفاوية والخلايا البلازماية بالإضافة لوجود المحفظة الليفية. كان الارتفاع الخلوبي كثيفاً في الأكياس العدارية المنتجه حيث حيوي على ارتفاع للعدالت. كان ضمن الضغط و بعض التغيرات التخريبية واضحة في النسيج المجاور للكيس العداري، كما شوهدت بعض المخاط السفلي (روس) في نسيج الرئة في الإيل والأبقار.

أظهرت طبقات الكيس (Masson's trichrome) PAS والماسون ثلاثية الألوان العداري و المحفظة الليفية تفاصيل واضحة.

تم استخدام التفاعل التلقيمي المتسلسل الداخلي لتحديد نوع عراتات الدودة الكلبية على 79 كيس عداري، حيث كان جين المترددة (G6PD) من نوع الهالوين هو الجين المستهدف في التكبير. كل العينات كانت موجبة لعتره الجمال (G6).

خلصت الدراسة إلى أن عترة الجمال (G6) من الدودة الكلبية E. granulosus هي العته السائدة في السودان وان الإيل هي الأكثر عرضة للإصابة بالمرض. كما توصي الدراسة بإجراء فحص التفاعل التلقيمي المتسلسل الداخلي لنشر عراتات الدودة الكلبية في مختلف الحيوانات.
INTRODUCTION

Hydatidosis, a problem of worldwide importance, is caused by adult or larval (metacestode) stages of cestodes belonging to the genus *Echinococcus* of the family *Taeniidae*. At present, taxonomically it has four valid species, namely: *E. granulosus*, *E. multilocularis*, *E. oligarthrus* and *E. vogeli* (FAO/UNEP/WHO, 1981; Soulsby, 1982; Thompson and McManus, 2001). These four species are morphologically distinct in both adult and larval stages (OIE, 2004).

Adult parasites are usually attached to the mucosa of the anterior part of the small intestine of definitive hosts. The larval stages are found in the inner organs (mainly lungs and liver) of a wide variety of domestic and wild intermediate hosts (Thompson and Lymbery, 1988; Thompson, 1995). Among these four species *E. granulosus* and *E. multilocularis* are the most important from a public health and economic point of view (McManus, 1996). *E. oligarthrus* and *E. vogeli* are restricted to the South American and Arctic regions in sylvatic areas, respectively.

*E. granulosus* is of wide distribution. The parasite is adapted to definitive hosts of the family *canidae* and a wide range of intermediate hosts (humans, domestic and wild herbivores/omnivores) (Thompson and McManus, 2001). It is widely spread in sheep-rearing areas and where definitive hosts exist (Torgerson and Health, 2003). The adult parasite hardly causes disease in the definitive host (Thompson, 1995). The clinical and economic, as well as zoonotic, significances of these parasites are almost completely confined to infection of intermediate hosts.

*Echinococcus granulosus* exhibits substantial genetic diversity that has important implications for the design and development of vaccines, diagnostic reagents and drugs effective against this parasite. DNA approaches have been used for accurate identification of these genetic variants and then have been applied in molecular epidemiological surveys of cystic echinococcosis in different geographical settings and host assemblages (McManus and Thompson, 2003).
Based on genetic variations, ten distinct genotypes of *E. granulosus* (G1-G10) have been described (Thompson and McManus, 2002; Lavikainen et al., 2003).

Several molecular techniques have been applied in diagnosis and genotyping of *E. granulosus* (Bowles et al., 1992; Bowles and McManus, 1993 a & b; Gasser and Chilton, 1995). Utility of the PCR would be more accurate in diagnosis of echinococcosis even in atypical cases (Georges et al., 2004). Identification of *E. granulosus* strains in Sudan is still limited in two reports (Dinkel et al., 2004; Omer et al., 2004). Both indicated the presence of camel strain or genotype 6 (G6) and cattle strain or genotype 5 (G5) of *E. granulosus*; the former was found to be the most prevalent strain.

The objectives of this study are:

- To carry out a pathological investigation on the hydatid disease in camels, cattle and sheep including: infectivity rates; cysts sizes; fluid amounts; status of cysts; chemical analysis of cystic fluids; histopathological and histochemical examinations on cyst wall and surrounding tissue.
- Genotyping of *Echinococcus granulosus* of hydatid cysts collected from different intermediate hosts using the nested polymerase chain reaction (nPCR).
CHAPTER ONE
Literature Review

1.1 Morphology of the Adult Parasite

The adult *E. granulosus* is only a few millimeters long, varying between 2-7 mm in length with 3-4 segments (rarely up to six segments) (Eckert and Deplazes, 2004; OIE, 2004). Anteriorly, the adult parasites possess a scolex, which has four muscular suckers and two rows of sickle shaped hooks, one large and one small, on the rostellum. The mean length of the large hook ranges from 22-49 µm, and that of the small hook ranges from 17-39 µm (Thompson and Lymbery, 1988; OIE, 2004).

The adult worm is hermaphrodite. The body or strobila has a number of reproductive units (proglottids) (Chatterjee, 1980; Smyth, 1994; Hendrix, 1998), the mature penultimate proglottid and the terminal proglottid. The latter is gravid and is usually more than half the length of the worm (Soulsby, 1982). This gravid proglottid/uterus has 12-15 short lateral diverticuli (Bhatia and Pathak, 1990) and, is usually filled with 100-1500 thick-shelled eggs (Thompson, 1995). The ratio of the anterior part of the strobilar to the gravid segment ranges between 1:0.86-1:1.30 (FAO/UNEP/WHO, 1981; Thompson, 1995).

The gravid proglottids and or eggs are shed in the faeces (McManus et al., 2003). The eggs are brown in color and morphologically indistinguishable from those of other tapeworms of the genus *Taenia* (Smyth, 1994; Eckert et al., 2001b). The size of the eggs ranges from 30-40 µm and they have a thick radially striated shell. The egg has a single hexacanth embryo, the oncosphere, which has three pairs of hooks (Chatterjee, 1980; Kassai, 1999; Thompson and McManus, 2001).
1.2 Life Cycle

_E. granulosus_ is an obligatory heterogeneous parasite with a complex life cycle. It requires two mammalian hosts to complete its life cycle. This involves the definitive hosts (for example, domestic dogs and wild canids) and the intermediate hosts (for example domestic and wild ungulates, humans) (OIE, 2004). The definitive host is infected by ingestion of offal containing fertile hydatid cysts (i.e. cysts with viable protoscolices). The protoscolices evaginate and attach to the intestinal mucosa and develop into adult stages (McManus et al., 2003). The pre-patent period of _E. granulosus_ in the definitive host ranges from 34- 58 days (Thompson, 1995). The adult worm passes out gravid proglottids containing eggs, or free eggs are passed out with the faeces. These gravid proglottids, or eggs, are dispersed and contaminate the environment, feed, grass or water, etc, which are sources of infection to many intermediate hosts, including humans (Soulsby, 1982; Gemmell and Lawson, 1986; Thompson, 1995). Humans are normally accidental intermediate hosts because they are rarely involved in the transmission cycle. They can be considered as ecological aberrant hosts (Torgerson and Heath, 2003).

The infective eggs in grass feed or in water are ingested by the intermediate hosts and hatch into oncospheres (larvae) inside the stomach and intestines (Horton, 2003). The liberated larvae penetrate the small intestine and reach their final localization passing through vascular and lymphatic systems to the liver and lungs (Soulsby, 1982), but they rarely spread to other unusual sites including kidney, spleen (Al- Jawhar and Yaseen, 2007).

Once the oncosphere has reached its final location (liver and lungs), it develops into the metacestode stage (primary cyst). The time for development of the cysts varies between 6- 12 months (Thompson, 1995). The developed cyst is unilocular, thick-walled, spherical fluid-filled cavity surrounded by two parasite-derived layers, an inner nucleated germinal layer and an outer acellular laminated
layer surrounded by a host produced fibrous capsule (Zhang et al., 2004) and exo-adventitial fibrous membrane (Peng et al., 2004).

The cyst size ranges from 2 to 30 cm in diameter, but in areas where there is unrestricted growth, the cyst may be very large and contains several liters of fluid (Urquhart et al., 1988).

1.3 The cyst wall

1.3.1 The acellular laminated layer

The acellular laminar layer is a carbohydrate-protein complex with galactose, galactosamine and glucosamine as the principal component of the polysaccharide portion (Kilejian and Schwabe, 1971). This layer is not present in very young cysts until it is about 14-18 days old when it first appears as a thin, clear layer on its outer margin (Taherkhani, 2001).

1.3.2 The germinal layer

The germinal layer consists of distal cytoplasmic syncytium and a perinuclear layer containing tegument, muscle, glycogen, undifferentiated cells (Lascano et al., 1975; Thompson, 1995). The inner germinal membrane produces brood capsules, and on the inner wall of the brood capsules, an asexual budding process of protoscolices enhances the infectivity and compensates for low sexual reproduction, producing thousands of protoscolices within a single cyst. Each single protoscolex is capable of developing into a sexually mature adult worm. Once the definitive hosts consume the organs with fertile and viable cysts they become infected and the life cycle is completed (Thompson and McManus, 2001).
**1.4 The hydatid cyst fluid (HCF)**

Hydatid cyst fluid (HCF) is a complex mixture of parasite-derived and host-derived molecules. It contains several antigens derived from the metabolism of the parasite together with many components from the host (Rickard and Lightowlers, 1986). Therefore, the type and concentration of parasite-derived molecules are likely to be very different in fertile and non-fertile cysts. Just like serum, the hydatid fluid is containing proteins, cholesterol, glucose, urea, uric acid, creatinine, triglyceride, minerals (Sodium, Potassium, Calcium, and Chloride) and trace elements (Copper, Zinc, Magnesium, Phosphorous, and Selenium) (Shaafie et al., 1999; Radfar and Iranyar, 2004).

Biochemical substances within hydatid cysts play a definitive role in the metabolism, physiology, and immunology of cystic echinococcosis (Thompson and Lymbery, 1995; Shaafie et al., 1999).

The quantitative differences in the metabolism of *E. granulosus* and variation in the biochemical composition of hydatid fluids reflect strain variation in different intermediate hosts (Shaafie et al., 1999; Thompson and Lymbery, 1995; McManus and Macpherson, 1984). Moreover, the development of the same strain or species of *E. granulosus* in different species of intermediate hosts, the organs carrying the cyst and the cyst fertility status may also cause shifts in the metabolism essential for parasite survival in different environments (Thompson, 1991; Anwar, 1994; Thompson and Lymbery, 1995).

The levels of triglycerides and proteins were found to be significantly lower in the cyst fluids of goats, camels, cattle, and humans compared with sheep, indicating that the levels of these parameters are not influenced by the hosts (Shaafie et al., 1999).

Higher cholesterol levels observed in hydatid cysts fluid collected from camels, cattle, and sheep (Shaafie et al., 1999), though comparatively low levels of cholesterol in sheep and human hydatid fluids were reported Sheriff and Ghwarsha (1985). Sheriff et al. (1989) suggested that cholesterol increases as hydatid cysts degenerate.
The camel origin hydatid cyst has large amounts of glucose, creatinine compared with those from other species, while the human hydatid cyst has a significantly more uric acid than the cyst from other intermediate hosts (Radfar and Iranyar, 2004).

The study by Kadir (2006) revealed that, the hydatid cyst fluid of camel has low concentration of total protein and uric acid and high level of urea nitrogen when compared to sheep, cattle and goats. The level of cholesterol was lower than that of cattle and goats but higher than that of sheep. The triglyceride concentration was lower than in sheep and goats and similar to cattle.

In cattle, Anwar (1994) found that the proteins, lipids and glucose were found to be significantly different in each case of fertile and infertile (sterile) hydatid cystic fluid. A significantly higher amount of protein contents were found in lungs as compared to liver cyst fluids, while lipids were found significantly lower in liver infertile (sterile) hydatid cystic fluid. Similarly, the contents of glucose were found significantly higher in livers than the lungs cystic fluid.

Among the electrolytes, camel origin hydatid cysts were found to have significantly large amounts of calcium than those of other intermediate hosts (Radfar and Iranyar, 2004), but Kadir (2006) showed that, the calcium concentration in camels was lower than in sheep but higher than that of cattle and goats. Potassium levels in both cattle and camel cysts were higher than that of sheep and goats (Radfar and Iranyar, 2004).

In cattle, sodium contents were found significantly higher in sterile hydatid cystic fluid of livers, while significantly lower concentrations of potassium were observed in sterile hydatid cystic fluid of lungs (Anwar, 1994).

On the trace elements, the concentration of copper and manganese in camel cyst fluid was lower than those of sheep and cattle but higher than that of goats, while zinc concentration was lower than that of sheep but higher than those of cattle and goats (Kadir, 2006) In cattle, phosphorous, copper and iron were found in significantly higher concentrations in the fertile cystic fluid of livers and lungs as compared with the sterile ones. Magnesium contents were found nearly
constant in both fertile and sterile hydatid cystic fluid of livers and lungs (Anwar, 1994).

1.5 Protoscoleces formation

Development of protoscoleces only takes place in the fertile cysts. As reported by Galindo et al. (2002), protoscoleces formation can be achieved in seven steps. Cellular buds formed by a clustering of cells emerge from the germinal layer of hydatid cysts. The buds elongate and the cells at their bases seem to diminish in number. Very early on, a furrow appears in the elongated buds, delimiting anterior (scolex) and caudal (body) regions. Hooks are the first fully-differentiated structures formed at the apical region of the nascent scolex. In a more advanced stage, the scolex shows circular projections and depressions that develop into suckers. A cone can later be seen at the center of the hooks, the body is expanded and a structured neck is evident between the scolex and the body. During protoscoleces development this parasitic form remains attached to the germinative layer through a stalk. When fully differentiated, the stalk is cut off and the infective protoscoleces is now free in the hydatid fluid.

1.6 Histochemical studies on the hydatid disease

In order to increase knowledge in the hydatid cyst formation and its constituents and to seek a more confirmatory diagnosis, histochemical studies on the cyst wall were conducted using special stains.

Since the laminated layer of the hydatid cyst is an acellular, polysaccharide protein complex, it stains strongly by periodic acid, Schiff's reagent (PAS) and that provides a useful diagnostic marker in histological studies (Kilejian et al., 1962).

Mahmoud and El-Garhy (2002) carried out a histochemical study on hydatid cyst wall of *E. granulosus* from goat and sheep. They reported that the cyst wall
contains a carbohydrate-protein substrate complex, collagen and possibly calcium. Calcium is also reported in protoscolices of hydatid sand.

Sections from a liver of Najdi sheep with severe hydatidosis, stained with hematoxlin and eosin (H&E), PAS and Masson's trichrome stain, revealed that the cyst wall was composed of three layers of variable thickness (germinal, laminated and fibrous layers). Fibrosis in portal areas and foci of precancerous changes were also observed (Rashed et al., 2004)

Staining of human origin hydatid cyst was done by Handa et al., (2005) using H&E and Masson’s trichrome stains. The study revealed numerous hooklets, laminated membrane and scolices. No inflammatory cells were seen. The laminated membrane appeared as fragments of acellular material with delicate parallel striations staining deep blue. Many scolices which were round to oval structures about 100µm in diameter with one or two rows of characteristic radially arranged hooklets were seen. The hooklets were about 20-40um in size, semi-translucent, refractile, triangular or sickle-shaped with an inner semi-translucent core of the same shape. The hooklets were better highlighted by Masson’s trichrome stain. Some scolices showed flame cells and were without hooklets.

1.7 Pathogenicity and Clinical Signs

There are no pathogenic effects in definitive hosts even if the animals are heavily infected with *E. granulosus* (Eckert and Deplazes, 2004). Therefore, infected final hosts (mostly dogs), show no clinical signs except itching on the back (sledge-like position) but if a large number of parasites are present, they may have diarrhoea.

The pathogenicity of the hydatid cyst in the intermediate hosts depends on the severity of the infection and the organs involved. The clinical signs are not obvious (Eckert and Deplazes, 2004), and the disease is rarely diagnosed before slaughter of the animals. Sometimes animals show clinical symptoms, such as bronchopneumonia, hepatic disorders leading to ascitis; jaundice; heart failure;
slow growth; weakness and lameness, but symptoms depend on the location of the cysts (OIE, 2005).

Most humans infected with hydatidosis are asymptomatic. In general, more than 90% of the cysts are found in the liver, lungs, or both, but are rarely (2-3%) located in the brain, bone, heart, kidney and other tissues (McManus et al., 2003). These cysts are not destroyed by the body’s defense mechanism, and may develop into large hydatid cysts. Clinical signs can take months to years to develop and become more apparent as the cysts grow. As the cysts increase they can cause pain in the upper abdominal region, occlusion of ducts, pressure atrophy or dysfunction of the affected organs. The most serious development is if the cyst ruptures. The cysts may rupture into the thoracic or peritoneal cavity, causing anaphylaxis or secondary cystic echinococcosis, or into the biliary tree, leading to cholangitis and cholestasis (Eckert and Deplazes, 2004).

1.8 Immunity in the intermediate hosts

The immunology of hydatid disease has been divided conceptually into pre-encystment and post-encystment phases (Rickard and Williams, 1982; Zhang et al., 2004), which are differentiated by the formation of the laminated layer around the hydatid cyst. This occurs between 2 and 4 weeks post infection in the animal intermediate or human host following ingestion of the egg and release of the oncosphere (Rickard and Williams, 1982).

1.8.1 Innate Resistance and Early Immunity

1.8.1.1 Primary infection

Very little is known about the factors affecting innate susceptibility to infection with *E. granulosus* following ingestion of the infective egg stage and establishment of the primary cyst. Host age, sex, and physiological state may
influence the innate susceptibility or resistance to infection (Rickard and Williams, 1982).

After infection, the earliest detectable immunoglobin G (IgG) response to hydatid cyst fluid (HCF) antigens occurs after 2 to 11 weeks in mice and sheep, respectively (Torres Rodriguez and Wisnivesky, 1978; Yong et al., 1984), and after 4 weeks in vervet monkeys (Rogan et al., 1993). Early infections may be associated with a significant cellular inflammatory response (Rickard and Williams, 1982; Lloyd, 1987) that may cause pathologic changes (Allen, and Maizels, 1996; Finkelman et al., 1991). Leukocytosis, mainly of eosinophils, lymphocytes, and macrophages will be clear (Petrova, 1968). Experiments in vitro have shown also that neutrophils, in association with antibody, can kill oncospheres of *E. granulosus*, suggesting a possible role for antibody-dependent cell-mediated cytotoxicity reactions (Yong et al., 1984). At the early stages of disease, there is a marked activation of cell-mediated immunity to the parasite (Fotiadis et al., 1999).

1.8.1.2 Secondary infection.

In experimentally induced secondary infections in mice, intraperitoneally injected protoscolices were surrounded by a considerable cellular infiltration within three days, initially involving activated macrophages and subsequently including neutrophils, eosinophils, and lymphocytes (Richards et al., 1983; Riley et al., 1986; Riley et al., 1985). Interleukin-10 (IL-10), IL-4, and IL-5 secreted in vitro by splenocytes can be detected as early as one week post infection (Dematteis et al., 1999). High levels of tumor necrosis factor alpha (TNFα), gamma interferon (IFNγ), IL-6, and specific IgG1 were detectable in serum, and IgG3 was measurable in the peritoneal cavity using protoscolex somatic antigens (Haralabidis et al., 1995; Dematteis et al., 1999). These data suggest that polarized T- helper cells (Th2) reactions are evoked at the very beginning of the immune response to secondary infection. *E. granulosus* protoscolices contain immunogenic T-independent antigens (Baz et al., 1999). Primary antibody
responses to protoscolex somatic antigens and the production of IgM and IgG3 in early infection appear to be stimulated mainly by a T-independent mechanism (Baz et al., 1999).

1.8.2 Established Cysts

Compared to events occurring during early infection, the immune response to established cysts has received much more attention. In humans there is frequent occurrence of elevated antibody levels, particularly of the IgG, IgM, and IgE isotypes (Craig, 1986; Daeki et al., 2000; Dessaint et al., 1975). In seropositive individuals, there are trends to be a predominance of IgG1 and IgG4 antibody recognition of protoscolices antigen 5 (Ag5) and protoscolices antigen B (AgB), respectively (Aceti et al., 1993; Ioppolo et al., 1996; Shambesh et al., 1997).

Also involved in the establishment phase is cellular infiltration, which includes eosinophils, neutrophils, macrophages, and fibrocytes (Archer et al., 1977; Slais and Vanek, 1980; Richards et al., 1983; Riley et al., 1985; Rigano et al., 1996). However, this generally does not result in a severe inflammatory response and aged cysts tend to become surrounded by a fibrous layer that separates the laminated layer from host tissue. Eosinophilia and the production of high levels of IgE are the common consequences of infection by helminths (Bell, 1996).

1.9 Diagnosis

The diagnosis of hydatidosis in humans is based on clinical, radiological, microscopic, computed axial tomography (CT scanning), immunological (Bhatia and Pathak, 1990; McManus et al., 2003) and magnetic resonance imaging (MRI) methods (Khuroo, 2002). Clinically it is difficult to diagnose without the aid of laboratory investigation. If the CT scan shows a cyst regardless of confirmation by serology, a diagnosis should be made (King, 2000). The diagnosis of cystic echinococcosis in livestock is mainly based on necropsy findings or postmortem
examinations at the slaughterhouses (Eckert et al., 2001 a). Diagnosis by clinical examination is rarely made. Ultrasound examination for cystic structures may be used for the diagnosis in smaller animals, such as sheep and goats, but it has been also used in the horse (Eckert et al., 2001 a). Diagnosis of hydatidosis by post-mortem examination of organs or tissues, especially livers and lungs, is based on the presence of fluid-filled cysts. The size of cysts may vary from about a ping-pong ball to a cyst comprising several liters of fluid. More than 90% of the cysts occur in the liver and lungs but may occasionally occur in other organs such as kidneys, spleen, omentum, heart etc. (McManus et al., 2003). Cysts may contain many protoscolices (fertile cyst) or only fluid (sterile cyst). These cysts are differentiated from other diseases like tuberculosis, tumors, pulmonary sequestration, which are seen as a parenchymatous opacity (Pawlowski et al., 2001) and can also be diagnosed by immunological tests and molecular methods.

Immunological tests, although useful in humans, are less sensitive and specific in livestock and at present cannot replace necropsy (Craig et al., 1996; OIE, 2004).

In clinical practice in humans it is noted that enzyme-linked immunosorbent assays (ELISA) using crude hydatid cyst fluid have a high sensitivity (over 90%) but specificity is often unsatisfactory (Eckert and Deplazes, 2004). Currently there is no suitably sensitive and specific serological test available for hydatidosis for any livestock species (Eckert et al., 2001 a), but contrary, a study by (Kittelberger et al. 2002) evaluated three ELISA tests employing a purified Antigen B sub unit, a recombinant EG95 oncosphere protein (OncELISA) and a crude protoscolex antigen (ProtELISA) in naturally or experimentally infected sheep with E. granulosus. The highest diagnostic sensitivity was 63% with the protoscolex antigen, but specificities were high at around 96%. Although these sensitivities are relatively low in terms of individual cases, the assay could be beneficial if used on a flock basis. Recently, (Simsek and Koroglu, 2004) investigated antigenic characteristics of hydatid cyst fluid in sheep by the SDS-PAGE method, to evaluate sensitivity and specificity of ELISA and Enzyme-
Linked Immuno-electro Transfer Blot (EITB) assay for the diagnosis of sheep hydatidosis, and to determine sero-prevalence of hydatidosis in the sheep population. The result showed that 116kDa band was specific in sheep tested by EITB by using the antigen prepared from sheep hydatid cyst fluid. Sensitivity and specificity of EITB assay were determined as 88% and 84% whereas corresponding rates for ELISA were 60% and 94% respectively. Also sero-prevalence of hydatidosis in sheep was found as 62% by ELISA and 66.4% by EITB. Recent studies suggest that EITB has potential to serve as an inexpensive diagnostic, surveillance and research tool in sheep. The cysts of different strains can be distinguished by molecular methods by PCR and different primers.

The disease in definitive hosts is mainly diagnosed by the presence of adult tapeworms attached to the dog’s small intestine. But, in post-mortem examination of dogs, the small tapeworms lie deeply between the villi of the intestinal mucosa, and are often overlooked. In living dogs, examinations of faecal samples are done by coproscopy, but it hardly to differentiate *E. granulosus* onchospheres from other *Taenia* eggs, except when proglottids resembling rice-corn are excreted. Nowadays, *Echinococcus* species can be diagnosed by several indirect methods such as: serum antibodies, copro-antigens and parasite DNA in fecal samples (Craig *et al.*, 2003; OIE, 2004). ELISAs for specific copro-antigens have been developed, that have sufficient specificity and sensitivity for detecting *Echinococcus* species in faecal material from infected dogs and definitive hosts. Also, ELISA based on detection of antibodies has been developed for diagnosing *Echinococcus* species in the serum of infected final hosts. ELISA based on the detection of specific antibodies has not reached a practical stage as it does not differentiate between current and previous infections (OIE, 2004; Craig *et al.*, 2003).

Moe recently, Benito and Carmen (2005) reported that double-antibody sandwich ELISA can be used for the detection of *E. granulosus*. Also DNA analytical techniques have been developed such as PCR-restriction fragment length polymorphism (PCR-RFLP), random amplified polymorphic DNA-PCR (RAPD-PCR), and southern blotting (SB) offering the most direct approach to the
characterization of distinct species and strains of *Echinococcus* (McManus, 1996).

The copro PCR test is aimed at detecting *Echinococcus* DNA present in *Taenia* eggs in fecal samples. It gives more accurate estimation of the potential risk for human infection than ELISA-based methods. An *E. granulosus*-specific PCR was described that utilized the mitochondrial CO1 gene with reported sensitivity equivalent to the detection of 200 eggs and no cross-reactions were observed with DNA from *E. multilocularis*, *T. hydatigena*, *Taenia ovis* or *Dipylidium caninum* cestodes (Cabrera et al., 2002). Also Abbasi et al., (2003) reported that this test enabled species-specific identification of *E. granulosus* isolated ova, but with a sensitivity of only several hundred eggs. The sensitivity limit was at least 100 eggs per gram of faeces.

### 1.10 Molecular approach of cystic echinococciosis

*Echinococcus granulosus* exhibits substantial genetic diversity that has important implications for the design and development of vaccines, diagnostic reagents and drugs effective against this parasite. DNA approaches have been used for accurate identification of these genetic variants and have been applied in molecular epidemiological surveys of cystic echinococciosis in different geographical settings and host assemblages (McManus and Thompson, 2003). The recent publication of the complete sequences of the mitochondrial (mt) genomes of the horse and sheep strains of *E. granulosus*, and the availability of mt DNA sequences for a number of other *E. granulosus* genotypes, has provided additional genetic information that can be used for more in depth strain characterization and taxonomic studies of these parasites. (McManus and (Thompson, 2003).
1.10.1 Phenotypic variation

During the past 40 years, observations in the laboratory and the field have revealed considerable phenotypic variability among isolates of *Echinococcus*. This variation has largely been observed in *E. granulosus*, and between isolates of the parasite from different species of intermediate host. Of most significance was the initial research by Smyth and Davies (1974) that demonstrated fundamental differences in the developmental biology of *E. granulosus* of horse and sheep origin. These seminal studies provided a platform of understanding for both earlier and subsequent observations on differences in the development and infectivity and the pathogenicity between isolates of the parasite from various host species in different parts of the world.

1.10.2 Concept of a strain

Given the epidemiological significance of such intra-specific variation in *E. granulosus* and the international efforts to establish control programs in different endemic regions, an informal nomenclature was needed to reflect the phenotypic variability evident between host-derived isolates of *E. granulosus*. Thus, the concept of a ‘strain’ was developed and defined as variant which differ statistically from other groups of the same species in gene frequencies, and in one or more characters of actual or potential significance to the pathology, epidemiology and control of echinococcosis (Thompson and Lymbery, 1988). This variability may be delineated on the basis of differences in nucleic acid sequences, and reflected in phenotypic characters that affect the life cycle pattern, host specificity, development rate, pathogenicity, antigenicity and sensitivity to chemotherapeutic agents, transmission dynamics, epidemiology and control of cystic hydatid disease/ echinococcosis (Thompson and McManus, 2001). From a practical point of view, the recognition of strain variation is a major prerequisite for control efforts aimed at limiting transmission in endemic regions. Cystic echinococcosis caused by *E. granulosus* continues to rank as one of the
most important parasitic zoonoses worldwide, especially where there is a close association between humans and livestock (Thompson, 1995). Under such circumstances, human behaviour reflected in inadequate husbandry practices is largely responsible for sustaining cycles of transmission. Education and surveillance underpin control efforts to break these cycles, and it is therefore essential to recognize which strains are present and which hosts support the perpetuation of the life cycle. The concept of a strain also grew from increasing evidence of genetic differences between \textit{E. granulosus} from different species of intermediate host (Thompson, 1995).

Increasingly, genetic variation has been found to correlate with phenotypic variability and, from this, the concept of ten genotypes of host adapted strains of \textit{E. granulosus} was developed (Table 1), (Thompson and McManus, 2002; Lavikainen et al., 2003).
Table 1: *Echinococcus* species, strains, isolates and genotypes.

<table>
<thead>
<tr>
<th>Species strain/isolate (genotype)</th>
<th>Known intermediate hosts</th>
<th>Known definitive hosts</th>
<th>Proposed taxonomic designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep strain (G1)</td>
<td>Sheep, cattle, pigs, camels, goats, macropods</td>
<td>Dog, fox, dingo, jackal and hyena</td>
<td><em>Echinococcus granulosus</em></td>
</tr>
<tr>
<td>Tasmanian sheep strain (G2)</td>
<td>Sheep, cattle</td>
<td>Dog, fox</td>
<td><em>E. granulosus</em></td>
</tr>
<tr>
<td>Buffalo strain (G3)</td>
<td>Buffalo, cattle</td>
<td>Dog, fox</td>
<td><em>E. granulosus</em></td>
</tr>
<tr>
<td>Horse strain (G4)</td>
<td>Horses and other equines</td>
<td>Dog</td>
<td><em>Echinococcus equinus</em></td>
</tr>
<tr>
<td>Cattle strain (G5)</td>
<td>Cattle</td>
<td>Dog</td>
<td><em>Echinococcus ortleppi</em></td>
</tr>
<tr>
<td>Camel strain (G6)</td>
<td>Camels, goats, sheep and Cattle.</td>
<td>Dog</td>
<td><em>E. granulosus</em></td>
</tr>
<tr>
<td>Pig strain (G7)</td>
<td>Pigs</td>
<td>Dog</td>
<td><em>E. granulosus</em></td>
</tr>
<tr>
<td>Cervid strain (G8)</td>
<td>North American cervids</td>
<td>Wolf, dog</td>
<td><em>E. granulosus</em></td>
</tr>
<tr>
<td></td>
<td>Zebra, wildbeests, Warthog, bushpig, buffalo, various antelope, giraffe, hippopotamus.</td>
<td>Lion</td>
<td>E. granulosus</td>
</tr>
<tr>
<td>---------------------</td>
<td>-------------------------------------------------------------------------------------</td>
<td>--------------------</td>
<td>---------------</td>
</tr>
<tr>
<td><strong>Lion strain (G9)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>G10</strong></td>
<td>Finnish cervids moose and reindeer</td>
<td>Wolf, dog</td>
<td>E. granulosus</td>
</tr>
<tr>
<td><strong>(Fennoscandien cervid strain)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.10.3 Genetic basis for phenotypic variation

The application of molecular tools for characterizing isolates of *Echinococcus* has had a major impact on understanding the population genetics, epidemiology and taxonomy of the parasite. DNA analysis has also provided some insights into the genetic basis for the phenotypic variation that is characteristic for *E. granulosus*. In early studies, techniques used for *Echinococcus* strain and species characterization employed morphological (e.g. rostellar hook) traits (Lymbery, 1998), and biochemical and isoenzyme markers (McManus and Bryant, 1995). As they became available, more sensitive molecular approaches were used, and these have been successful in discriminating species and strains, and have confirmed the complexity of genetic variation within *E. granulosus* (Thompson and McManus, 2001; McManus and Bryant, 1995; McManus, 2002).

1.10.4 Molecular typing methods

While epidemiological data on the regional occurrence and relative public health importance of different genotypes/ species are urgently required, no method for screening of large numbers of samples or for application as coprodiagnostic tools to final hosts are yet available. Methods used previously for strain typing include sequencing of partial mitochondrial cytochrome c oxidase subunit 1 (cox1) and of NADH dehydrogenase 1 (nad1) genes (Bowles *et al.*, 1992; Bowles and McManus, 1993b). Internal transcribed spacer-1 and Internal transcribed spacer- 2 (ITS-1 and ITS-2) regions of ribosomal DNA (rDNA) were used to characterize isolates of *E. granulosus* from different hosts and geographical areas by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Bowles and McManus, 1993a; Gasser and Chilton, 1995). Other studies have analyzed heterogeneity between *Echinococcus* strains and species using the random amplified polymorphic DNA-PCR (RAPD-PCR) (Scott and McManus, 1994; Siles-Lucas *et al.*, 1994).
1.10.5 The Polymerase Chain Reaction (PCR)

1.10.5.1 The polymerase chain reaction

There have been number of key developments in molecular biological techniques. However, the one that has had the most impact in recent years is the polymerase chain reaction (PCR). One of the reasons of the adoption of the PCR is the elegant simplicity of the reaction and relative ease of the practical manipulation steps. Frequently this is one of the first techniques used when DNA is analyzed; it has opened up the analysis of cellular and molecular processes to those outside the field of molecular biology. The PCR is used to amplify precise fragment of DNA from a complex mixture of starting material usually termed the template DNA and in many cases requires a little DNA purification. It does require some knowledge of the DNA sequence information, which flanks the fragment of DNA to be amplified (target DNA). From this information two oligonucleotides primers may be chemically synthesized, each complementary to a stretch of DNA to the 3`-side of the DNA, one oligonucleotide for each of the two DNA strands. It may be thought of as a technique analogous to the DNA replication that takes place in cells, since the outcome is the same, the generation of new complementary DNA stretches based upon existing one. It is also a technique that has replaced, in many cases, traditional DNA cloning methods, since it fulfils the same function, the production of large amounts of DNA from limited starting material. This is achieved, however, in a fraction of time needed to clone DNA fragment. Although not without its drawback, the PCR is a remarkable development that is changing the approach of many scientists to the analysis of nucleic acid and continues to have profound impact on core bioscience and biotechnology (Wilson and walker, 2000).
1.10.5.2 Stages of the polymerase chain reaction

The PCR consists of three defined sets of times and temperature, termed steps: (1) denaturation (2) annealing and (3) extension. Each of these steps is repeated 30 – 40 times or cycles. In the first cycle, the double stranded template DNA is first denatured by heating the reaction to above 90°C. Within the complex DNA the region to be specifically amplified (target) is made accessible. The temperature is then cooled to between 40°C to 60°C. The precise temperature is critical and each PCR system has to be defined and optimized. Reaction that is not optimized may give rise to other DNA product in addition to the specific target or may not produce any amplified products. The second annealing step allows the hybridization of the two oligonucleotide primers, present in excess, to bind to their complementary sites, which flank the target DNA. The annealed oligonucleotides act as primers for DNA synthesis, since they provide a free 3'-hydroxyl group for the DNA polymerase. The third step, DNA synthesis or extension, is carried out by a thermostable DNA polymerase, most common Taq DNA polymerase. (Wilson and Walker, 2000).

DNA synthesis proceeds from both of the primers until the new strands have been extended along and beyond the target DNA to be amplified. It is important to note that, since the new strand extend beyond the target DNA they will contain region near their 3' end that is complementary to the other primer, thus if another round of DNA synthesis is allowed to take place, not only will the original strands be used as templates but also the new strands. Most interestingly, the products obtained from the new strands will have a precise length, delimited exactly by the two regions complementary to the primers. As the system is taken through successive cycle of denaturation, annealing and extension, all the new strands will act as templates and so there will be an exponential increase in the amount of DNA produced. The net effect is to selectively amplify the target DNA flanked by the primers.

One problem with the early PCR reactions was that the temperature needed to denature the DNA also denatured the DNA polymerase. However, the
availability of a thermostable DNA polymerase enzyme isolated from the thermophilic bacteria *Themus aquaticus* found in hot springs provided the mean to automate the reaction. Taq DNA polymerase has a temperature optimum of 72°C and survives prolonged exposure to a temperature as high as 96°C and so still active after each of the denaturation steps. The wide spread utility of the technique is also due to the ability to automate the reaction and as such many cyclers have been produced in which it is possible to programme—in the temperature and the times for a particular PCR reaction. (Wilson and walker, 2000).

### 1.10.6 NADH dehydrogenase

Respiratory-chain NADH dehydrogenase (also known as complex I or NADH-ubiquinone oxidoreductase) is an oligomeric enzymatic complex located in the inner mitochondrial membrane and it has 42 subunits (Weiss et al., 1991).

Nucleotide sequences of a 471 bp region of the mitochondrial NADH dehydrogenase 1 gene were obtained and had shown inter and intra-specific variations within *Echinococcus* (Bowel and McManus, 1993b). This region of the rapidly evolving mitochondrial genome is useful as a marker of species and strain identity and as a preliminary indication of evolutionary divergence within the genus.

### 1.10.7 Molecular epidemiology

A description follows of the utility of DNA-based approaches in helping to clarify the complex issue of strain variation in *E. granulosus* and their value for molecular epidemiological studies of cystic echinococcosis. The various genotypes of *E. granulosus* that have been identified together with their host and geographical ranges are presented in table 2 (McManus and Thompson, 2003; Lavikainen *et al.*, 2003; Dinkel *et al.*, 2004; Omer *et al.*, 2004).
Table 2: Genotypes/strains of *E. granulosus* categorised by DNA analysis with their host and geographical range.

<table>
<thead>
<tr>
<th>Genotype (strain)</th>
<th>Host Origin</th>
<th>Geographic origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1 (common sheep strain)</td>
<td>Sheep</td>
<td>UK, Spain, China, Australian, mainland, Tasmania, Kenya, Uruguay, Turkey, Jordan, Lebanon, Italy, Argentina, Brazil, Iran, Nepal.</td>
</tr>
<tr>
<td></td>
<td>Cattle</td>
<td>UK, Spain, Kenya, Tasmania, Jordan, China.</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>Australian mainland, Tasmania, Jordan, Lebanon, Holland, Kenya, China, Argentina, Spain</td>
</tr>
<tr>
<td></td>
<td>Goat</td>
<td>Kenya, China, Nepal.</td>
</tr>
<tr>
<td></td>
<td>Buffalo</td>
<td>India, Nepal.</td>
</tr>
<tr>
<td></td>
<td>Camel</td>
<td>China.</td>
</tr>
<tr>
<td>Strain Type</td>
<td>Species</td>
<td>Locations</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>------------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>G2 (Tasmanian sheep strain)</td>
<td>Sheep, Human</td>
<td>Tasmania, Argentina.</td>
</tr>
<tr>
<td>G3 (buffalo strain?)</td>
<td>Buffalo</td>
<td>India.</td>
</tr>
<tr>
<td>G4 (horse strain)</td>
<td>Horse, Donkey</td>
<td>UK, Ireland, Switzerland.</td>
</tr>
<tr>
<td>G5 (cattle strain)</td>
<td>Sheep, Goat, Cattle, Buffalo, Human</td>
<td>Nepal, Switzerland, Holland, Brazil, Sudan, India, Nepal, Holland.</td>
</tr>
<tr>
<td>G6 (camel strain)</td>
<td>Camel, Cattle, Human</td>
<td>Kenya, Somalia, Kenya, Sudan, China, Iran, Mauritania, China, Iran, Mauritania, Sudan, Argentina, Nepal, Iran,</td>
</tr>
<tr>
<td></td>
<td>Animal</td>
<td>Country</td>
</tr>
<tr>
<td>----------------</td>
<td>--------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>Sheep</td>
<td></td>
<td>Mauritania, Sudan.</td>
</tr>
<tr>
<td>Iran, Sudan</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>G7 (pig strain)</strong></td>
<td>Pig</td>
<td>Poland, Slovakia, Ukraine, Argentina, Spain.</td>
</tr>
<tr>
<td></td>
<td>Wild boar</td>
<td>Ukraine.</td>
</tr>
<tr>
<td></td>
<td>Beaver</td>
<td>Poland.</td>
</tr>
<tr>
<td></td>
<td>Cattle</td>
<td>Slovakia.</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>Poland, Slovakia.</td>
</tr>
<tr>
<td><strong>G8 (cervid strain)</strong></td>
<td>Moose</td>
<td>USA.</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>USA.</td>
</tr>
<tr>
<td><strong>G9 (cervid strain)</strong></td>
<td>Human</td>
<td>Poland.</td>
</tr>
<tr>
<td><strong>G10 (Fennoscandien cervid strain)</strong></td>
<td>Moose</td>
<td>Finland.</td>
</tr>
<tr>
<td></td>
<td>Reindeer</td>
<td>Finland.</td>
</tr>
</tbody>
</table>
1.11 Studies on hydatidosis in Sudan

In the Sudan hydatidosis is caused by the larval state of the cestode *E. granulosus*. The adult parasite is found in the dog, while the larval stages observed in camels, cattle, sheep, goats and man.

Most of the reports on the hydatid disease in Sudan were as epidemiological studies on the prevalence rates, but also some histopathological studies were done (Ahmed, 1985; Saad *et al.*, 1985; Adam, 1997).

The first report of the disease in Sudan was in 1908 in a camel slaughtered in Khartoum state (Annual report of the Sudan Vet. Services, 1908).

Studies on animal echinococcosis and hydatidosis in Sudan were carried out by Abdel Malek, (1959) who reported one case of bovine liver hydatidosis in Kosti, four cases of *E. granulosus* in the small intestine of dogs in Khartoum and Kosti and three cases of lungs, liver and spleen hydatid cysts in camel in Khartoum and Omdurman.

Eisa *et al.*, (1962) further reported prevalence rates of 25%, 19.4% and 33.33% in cattle, sheep and goats, respectively in Equatoria and Upper Nile state.

Prevalence rates of 6.2% and 9.3% in cattle and goats, respectively in Equatoria state El Khawad *et al.*, (1976).

The study by El Khwad *et al.*, (1978) on hydatidosis in cattle, sheep and goats in western Sudan resulted in 24.8%, 12.2% and 10%, respectively, as prevalence rates of the disease. A high prevalence rate of 67.74% reported in camels in El Obeid (Saad *et al.*, 1989). In southern Darfur state, among 763 cattle and 205 camels the prevalence rates was 6.42% and 79.51%, respectively, (Adam, 1997).

In central Sudan a survey on hydatidosis in camels, cattle, sheep and goats conducted by El Khwad *et al.*, (1979) revealed prevalence rates of 35.3%, 8.16%, 4.28% and 3.71%, respectively.

In Butana area Tola, (1987) found prevalence rates of 56.4%, 2.1% and 2% in camels, cattle and sheep, respectively, while Saad and Magzoub, (1986) found
among 141 camels slaughtered in Tambool 64 (45.41%) were infected with hydatid cysts.

Saad and Magzoub, (1989 a & b) carried out a survey on hydatidosis in area representing all regions of the Sudan except the southern Sudan. The overall prevalence rates were 48.69%, 3.84%, 12.9% and 4.4% in camels, cattle, sheep and goats. Also the results showed that, all cysts from sheep and goats were calcified and semi-calcified except two lung cysts which were fertile (0.81%). 42.4% and 29% of camel and cattle hydatid cysts were fertile, respectively; also the liver was the preferable site in cattle. In sheep the majority of cases were in liver. The cyst sizes were small in both lungs and liver.

In northern Sudan, 37% prevalence rate was reported in camels (El Hussein and Ali, 1990). El Sawi, (1994) conducted a study on 1362 and 164 sheep and goats, respectively, slaughtered at Omdurman abattoir and the prevalence rates were 8.9% in sheep and 4.21% in goats.

In the study by Adam, (1997) on 763 cattle and 205 camels, 28.57% of cattle cysts were small (< 2 cm) and the rest were medium (2 – 6 cm) and the fluid volume ranged from 2- 41 ml. The liver was the main predilection site (58.73%). In camels, 26.62% of the cysts were small, 72.4% were medium and 1.04% were large (7-10 cm), the fluid volume ranged from 0.3- 124 ml.

Histopathological changes were studied by Adam, (1997) on four hydatid cases (two camels and two cattle) compromising lungs and liver tissues. Cellular infiltration of lymphocytes and plasma cells, alveolar edema, atelectasis mild congestion and compression of bronchioles adjacent the cyst wall were seen in lung sections, while in the liver atrophy of hepatic tissue around the cyst, hyperplasia of the bile ducts and dilatation in the sinusoids were observed.

Saad, (1985) also reported a tissue cellular reaction around the cyst wall and he suggested that immunological response of the host to the infection to be delayed hypersensitivity type of reaction.

Concerning human hydatidosis, the first report described six cases including two Egyptians (Christopherson, 1909). Eisa et al., (1962) reported the
occurrence of hydatidosis as an endemic disease among people of the Taposa tribe in Kapoeta district and Equatoria state in the southern Sudan.

Cahill et al., (1965) conducted serological study on hydatidosis in people of southern Sudan and found that 13.1% were positive.

1.11.1 Molecular epidemiology of hydatidosis in Sudan

Hydatidosis is a major public health problem in the Sudan, being endemic in many areas of the country and numerous livestock and human cases are reported (Eisa et al., 1962). Despite the importance of the disease, strain identification and characterization studies were, until recently, limited to two reports. The camel strain G6 and Cattle strain G5 (*E. Ortleppi*) were identified in 46 isolates from camel, cattle and sheep origin (Dinkel et al., 2004). Camel strain of *E. granulosus* has been reported in isolates from camel and cattle origin hydatid cysts as well human origin hydatid cyst (Omer et al., 2004).

1.12 Therapy and Treatment

1.12.1 Treatment against Tapeworms in Dogs

A wide variety of anthelmentics (arecolin, niclosamide, benzimidazole compounds and praziquantel) are available for the treatment of echinococcosis in dogs (Bhatia and Pathak, 1990). Praziquantel, the drug of choice, is effective against both juvenile and adult *Echinococcus* parasites. The dosage rate of praziquantel is 5 mg/kg (Eckert and Deplazes, 2004; OIE, 2005).

1.12.2 Treatment of Cystic Stages

Surgical removal of the hydatid cyst is the treatment of choice for symptomatic cysts in humans (Safioeas et al., 1999). Several of the benzimidazole compounds have been shown to have efficacy against hydatid cysts. Long term treatment with albendazole has a particularly marked effect on
the cysts, and is used as pretreatment before surgery (Morris et al, 1990) (Horton, 2003). The albendazole sulphone oxide was shown to be an active anthelminthic (Horton, 2003). The current recommendation by the World Health Organization is percutaneous puncture under sonographic guidance, aspiration of cystic fluid, injection of a protoscolicidal agent (alcohol) and reaspiration of cyst content. This procedure needs to be further evaluated in large scale studies (Riengchan et al., 2004).

More recently, herbal extracts mainly garlic (*Allium sativum*) extracts were used as a protoscolicidal agent (Sadjjadi et al., 2004).

### 1.13 Prevention and Vaccination

There is no drug treatment in herbivores or omnivores. The control procedure used to eliminate echinococcosis from Iceland, the Falkland Islands, Tasmania and New Zealand is not appropriate to continental environments. A vaccine to protect grazing animals against infection is an additional control method that focuses on grazing animals instead of the dog (Health et al., 2003). Vaccination with a recombinant oncospheral *E. granulosus* antigen (EG95) induces high degrees of protection, reducing the cyst numbers in vaccinated sheep by approximately 90-100% (Health et al., 2003). A high degree of immunity (about 80%), persists for six months (in absence of re-infection), and pregnant ewes vaccinated before lambing transfer high levels of antibody to their lambs (Health and Lightowlers, 1997). Considerable research has been undertaken with EG95 antigens in China to protect dogs from echinococcosis, and until now significant inhibition of egg production has been achieved (Zhang et al., 2007).

Currently, EG95 has been used in combination with live attenuated strains of *Salmonella enterica serovar Typhimurium* and the parapox virus as a recombinant vaccine (Health et al., 2007; Petavey et al., 2007).
CHAPTER TWO

MATERIALS AND METHODS

2.1 The pathological investigation

The pathological work was divided into four parts. The first one was general parasitological examinations on the cyst. The second part was to estimate the concentration of the chemical components of the hydatid fluid mainly, total proteins, cholesterol and minerals (sodium, potassium, calcium and phosphorus). The other two parts were the histopathological and histochemical studies on the cyst wall and surrounded host tissue.

2.1.1 Samples

3415 animals were targeted to perform this study. 31 camels, 898 cattle and 2486 sheep. These animals were slaughtered at Tambool, Ghanawa and Al-Kadro abattoirs, respectively.

2.1.2 The parasitological investigation

Four parasitological tests were included. These were: measuring the cysts sizes, amounts of aspirated fluid, status of the cyst fluid and cyst.

2.1.2.1 The cyst size

The cyst size was determined by measuring the diameter of the cyst in centimeters using a measuring tape. Then according to the size the cysts were categorized as follows:
- From 1 cm to 5 cm = small cyst.
- From 6 cm to 10 cm = medium cyst.
- From 11 cm to 15 cm = large cyst.
2.1.2.2 The fluid amount

The cyst fluid was aspirated using sterile syringes then placed into measuring tubes to estimate the fluid amount in milliliters.

According to the fluid amount, five groups (G_A - G_E) were designated:

- Group A (G_A): From 0 ml to 5 ml.
- Group B (G_B): From 6 ml to 10 ml.
- Group C (G_C): From 11 ml to 15 ml.
- Group D (G_D): From 16 ml to 20 ml.
- Group E (G_E): From 21 ml to 25 ml.

2.1.2.3 Status of the cysts fluid

The status of the cyst fluid was evaluated visually by observing the consistency of the fluid. Accordingly, the fluid was described as watery, thick or caseated.

2.1.2.4 Cysts status

To determine the cyst status, cysts were divided into fertile cysts, sterile cysts or calcified cysts.

Fertility of collected cysts was examined as follows:

One drop of each hydatid fluid was placed on glass slide and covered with cover slip. The slide was examined under the light microscope using the objective lens 100x.
2.1.3 Estimation of fluid chemical components

2.1.3.1 Estimation of total proteins

The concentrations of the total proteins were estimated by colorimetric method using total proteins liquicolor kits (Human, Germany).

**Principles:**

Cupric ions react with protein in alkaline solution to form a purple colour. The absorbance of this complex is proportional to the protein concentration in the sample.

**Contents:**

**Reagents:**

- Sodium hydroxide ..................200mmol/l
- Potassium sodium tartrate ..........32mmol/l
- Copper sulfate .....................12mmol/l
- Potassium iodide .................30mmol/l

**Standard:**

- Protein.................................8 g/dl
- Sodium azide .........................0.095%

**Assay:**

1000µl of the reagent was pipetted into cuvettes. 20µl of the standard was added to one cuvette tube and 20µl of each hydatid fluid was added to the rest cuvettes. All cuvettes was mixed and incubated at 25°C for 10 minutes.
One cuvette containing only the reagent was used as blank for zero adjustment of the colorimeter with wavelength of Hg 546nm and optical path of 1 cm. The absorbance of the samples and standard was measured against the reagent blank to determine the optical density.

**Calculation of the protein concentration with standard:**

Protein concentration of the Sample = \( \frac{\text{optical density of the sample}}{\text{optical density of standard}} \) g/dl.

**2.1.3.2 Estimation of cholesterol**

The cholesterol was determined using an enzymatic colorimetric test (CHOD-PAP) (Plasmatec Ltd, UK).

**Principle:**

Cholesterol and its esters are released from lipoproteins by detergents. Cholesterol esterase hydrolysis the esters and \( \text{H}_2\text{O}_2 \) is formed in the subsequent enzymatic oxidation of cholesterol- oxidase according to the following reaction:

\[
\text{Cholesterol ester} + \text{H}_2\text{O} \rightarrow \text{Cholesterol Esterase} \rightarrow \text{Cholesterol} + \text{fatty acids.}
\]

\[
\text{Cholesterol} + \text{O}_2 \rightarrow \text{Cholesterol Oxidase} \rightarrow \text{Cholest-3-0n} + \text{H}_2\text{O}_2
\]

\[
2\ \text{H}_2\text{O}_2 + \text{Phenol} + 4\text{-aminoantipyrine} \rightarrow \text{Peroxidase} \rightarrow \text{quinoneimine dye} + 4\ \text{H}_2\text{O}
\]

**Reagent concentration:**

**Buffer:**

- Pipes buffer, pH 6.9.........................90mmol/l
- Phenol........................................26mmol/l
**Enzymatic reagent:**

- Cholesterol oxidase ...................... 200 U/l
- Cholesterol esterase ..................... 300 U/l
- Peroxidase ................................ 1250 U/l
- 4-aminoantipyrine ....................... 0.4mmol/l

**Standard:**

- Cholesterol .......................... 5.16mmol/l

**Assay:**

1000µl of the working reagent was pipetted into cuvettes. 10µl of the standard was added to one cuvette tube and 10µl of each hydatid fluid was added to the rest cuvettes. All cuvettes were mixed and incubated at 25°C for 10 minutes.

One cuvette containing only the reagent was used as blank for zero adjustment of the colorimeter with wavelength of Hg 546nm and optical path of 1 cm. The absorbance of the samples and standard was measured against the reagent blank to determine the optical density.

**Calculation:**

Cholesterol concentration = 5.16 (optical density of the sample/ optical density of standard) mmol/l.
2.1.3.3 Determination of minerals

2.1.3.3.1 Determination of sodium and potassium (Na, K)

0.1ml of the hydatid fluid was added to 9.9 ml distilled water as well as 0.1 of the standard solution in concentrations of 140mmol/l and 5mmol/l for sodium and potassium, respectively. The optical density of the hydatid fluid and the standard solution was read against blank using flame photometer.

**Calculation:**

\[ \text{Na concentration} = 140 \left( \frac{\text{reading of sample}}{\text{reading of standard}} \right) \text{ mmol/l} \]

\[ \text{K concentration} = 5 \left( \frac{\text{optical density of sample}}{\text{standard concentration}} \right) \text{ mmol/l}. \]

2.1.3.3.2 Determination of inorganic phosphorus

**Reagents:**

10% TCA:

10g of Trichloroacetic acid (TCA) dissolved in 100 ml distilled water.

**Ammonium Molybdate solution:**

15g of Ammonium Molybdate dissolved in 400 ml distilled water. 100 ml of 10N sulphuric acid added. The volume made up to 800 ml with distilled water.

**Metol solution:**

1g of P-methyl amino-phenol added to 100 ml of 3% solution of sodium bisulphite.
**Standard:**

0.2197g of potassium dihydrogen phosphate dissolved in one liter of distilled water. Few drops of chloroform were added.

**The test:**

1 ml of the hydatid fluid was added to 9 ml of 10% TCA and 0.5 ml of working standard was added to 4.5 ml of 10% TCA. The reagent blank was 5 ml of 10% TCA. 1 ml of ammonium molybdate solution was added to all tubes. After mixing 1 ml of metol solution was added either. All tubes as mixed and allowed to stand for 30 min. at the room temperature then all tubes were read in the colorimeter at wavelength 680.

**Calculation:**

Phosphorus concentration = 5(optical density of sample/optical density of standard) mg/100ml.

**2.1.3.3.3 Determination of calcium**

A stock solution was prepared by dissolving 0.25g of calcium carbonate in 0.1 N hydrochloric acid (HCl) and made up to 100 ml with the acid. The working standard was prepared by diluting 4 ml of the stock solution with 100 ml distilled water. 0.5 ml working standard added to 1 ml of 0.5% chloronillic acid in a tube, used as standard. 0.5 ml of the hydatid fluid was placed into a centrifuge tubes then 1 ml of 0.5% chloronillic acid was added to each.

All tubes were allowed to stand for 15 min. and centrifuged at 3000 rpm for 5 minutes. The supernatant was decanted, and tubes were drained on a filter paper. The precipitate was washed with 0.5 ml distilled water and centrifuged again, the supernatant decanted and the tubes drained on a filter paper. The precipitate was dissolved in 4 ml of 4% Ferric nitrate then the tubes were allowed
to stand for 5 minutes then read using the colorimeter at 500 wave length after
adjusted at zero with Ferric chloride as a blank solution.

Calculation:

Calcium concentration = 10 (optical density of sample/optical density of
standard) mg/100ml.

2.1.4 Histopathological study

Immediately after aspiration of the cyst fluid, the cysts and the surrounding
tissues were fixed in 10% neutral formal saline to be used in the histopathological
and the histochemical studies.

After fixation, small samples of the hydatid cyst wall and the surrounding
tissue were trimmed and processed for routine histopathology. Samples were
dehydrated in ascending grades of alcohol, cleared in xylene and embedded in
paraffin wax. After blocking sections, 5µm thick were cut using a rotary
microtome. Tissue sections were floated in a water bath at 48°C and collected on
glass slides. Sections were further deparaffinized in xylene and rehydrated in
descending grades of alcohol then washed in distilled water (Drury and
Wallington, 1967)

2.1.4.1 Staining

Sections were stained using hematoxylin & eosin (H&E) as a routine
histopathological stain (Drury and Wallington, 1967).

2.1.5 Histochemical study

In this part, already prepared blocks in 2.1.4 were used. Sections were cut in
5µm thickness then deparaffinized in xylene and rehydrated in descending
grades of alcohol then washed in distilled water and stained with special
histochemical stains. These were: Masson’s trichrome stain, Periodic Acid Schiff stain (PAS), Burstone’s method for alkaline phosphatase and Barka’s method for acid phosphatase.

2.1.5.1 Periodic Acid Schiff (PAS) stain Pearse (1959)

Schiff’s reagent

One gram (1g) of basic fuchsin was added to 200 ml boiled distilled water. When dissolved the solution was cooled and filtered. SO₂ gas was slowly bubbled through the solution. The solution was shaked until become clear transparent red in color then it was let to stand in a dark cupboard overnight.

Procedure

Sections were deparaffinized and rehydrated then oxidized for 5 minutes in 1% aqueous periodic acid. Sections were washed in running water for 5 minutes and rinsed in distilled water then placed in Schiff’s reagent for 10 minutes and again washed in running water for 30 minutes. Finally sections were dehydrated in alcohol and cleared in xyelene.

2.1.5.2 Masson’s trichrome stain Masson (1929)

Solutions:

Cytoplasmic stain

1% Ponceau de xylidine (ponceau 2 R) in 1% acetic acid.
1% Acid fuchs in 1% acetic acid.
Differentiator and mordant

1% Phosphomolybdic acid in distilled water.

Fiber stain

2% Light green in 2% acetic acid.

Acid alcohol

0.5% hydrochloric acid in 70% alcohol.

Procedure

Sections were deparaffinized and rehydrated then stained with hematoxylin and washed well in water. Section were differentiated acid alcohol and washed again in tap water then rinsed in distilled water, then sections were stained in the red cytoplasmic stain for 5 minutes then washed and rinsed in tap water and distilled water, respectively. After that sections were differentiated in 1% phosphomolybdic acid and rinsed in distilled water. Sections were counterstained in light green for 2 minutes and washed well in 1% acetic acid for 1 minute. Finally sections were dehydrated in alcohol and cleared in xyylene.
2.2 The biomolecular study

2.2.1 Sampling

Total of 79 hydatid cysts were collected from slaughtered sheep, cattle and camels at Al Kadaro, Ghanawa and Tambool abattoirs, respectively. The cysts were recovered mainly from lungs and livers (Figures 1-3).

2.2.2 Samples

The cyst fluid of all samples containing protoscoleces was aspirated using sterile syringes and then transferred to 1.5 ml eppendorf tube. The tubes then centrifuged on microcentrifuge at 12000 rpm for 3 min. the supernatant was discarded, the deposit was preserved by adding 300µl of 70% ethanol alcohol, then all tubes were kept in -20°C to be used for the DNA extraction.

2.2.3 DNA extraction

The genomic DNA of all samples was isolated using two DNA extraction methods. The first was the phenol DNA extraction method and the other by using commercial DNA extraction kits (QIAGEN Inc. Chatsworth, CA).

2.2.3.1 Phenol DNA extraction

Solutions:

Tris lysing buffer 0.1mmol.
Phenol, Chloroform and Isoamyl alcohol solution at concentrations 25 ml, 24 ml and 1 ml, respectively.
Free nucleic acid water or double distilled water (dd H₂O).
Procedure:

500µl of each sample was taken and dropped into new eppendorf tubes. The tubes were then centrifuged in the microcentrifuge at 12000 rpm for 3 min to evacuate the supernatant mainly containing the 70% alcohol. The deposit containing protoscoleces was washed with nucleic acid free water by centrifugation at 12000 rpm for 2 min. 300µl of Tris lysing buffer 0.1mmol was added to the deposit of each tube and vortexed for 15 sec for proper mixing then incubated in the water path at 37°C for 10 min. The tubes were then centrifuged at 12000 rpm for 2 min, and the supernatant containing DNA of each tube was pipetted off and transferred to new eppendorf tubes. The DNA was purified by adding 500µl of Phenol, Chloroform and Isoamyl alcohol solution to each tube. The DNA was then precipitated by centrifugation at 12000 rpm for 10 min after adding 1000µl of cold absolute ethyl alcohol (at -20°C). The alcohol was pipetted off carefully from each tube without disturbing the DNA pallet at the bottom of the tube. All tubes were kept overnight to dry at room temperature before resuspending the DNA pallet in 100µl free nucleic acid water or double distilled water (dd H₂O). All tubes containing the extracted DNA were then kept at -20°C.

2.2.3.2 DNA extraction with Qiagen commercial kits

After elimination of 70% ethyl alcohol, each sample was washed with nucleic acid free water and the DNA isolated using QIAamp blood kit (QIAGEN Inc. Chatsworth, CA). The manufacturer's instructions were carefully followed, briefly, 200µl of sample, 20µl of proteinase K stock solution and 200 µl of lysing buffer were pipetted into 1.5 ml eppendorf tube and the mixture was incubated at 56°C for 10 min. 200µl of absolute ethanol was added to the sample and the mixture was mixed by vortexing and spinning. The mixture was transferred to the QIAspin column and placed in a clean 2 ml collection tube and centrifuged at 12000 rpm for 1 min. The QIAspin column was washed twice using 500µl of washing buffers W1 and W2, respectively, by spinning for 1 min with washing
buffer W1 and for 3 min with washing buffer W2. The QIA spin column was then placed in a clean 1.5 ml eppendorf tube and the DNA was eluted with 200µl double distilled water preheated at 70°C.

2.2.4 Primers selection

For the first amplification step, a pair of outer primers (EGL1 and EGR2) was selected from the published sequences of NADH dehydrogenase 1 gene of *E. granulossus* and used in the PCR assay (Bowles and McManus, 1993; Osman et al., 2007). This pair of outer primers was designed for the synthesis of the primary *E. granulossus*-specific PCR product. Primer EGL1 included bases 32- 53 of the positive sense strand (5' TGA AGT TAG TAA TTA AGT TTA A '3). EGR2 included bases 447- 466 of the complementary strand (5 'AAT CAA ATG GAG TAC GAT TA '3). Using primers EGL1 and EGR2, the primary PCR amplification will produce a 435-bp PCR product from *E. granulossus*-complex DNA.

For specific identification of the camel genotype (G6), a pair of nested primers (EGL3 and EGR4) was designed from a variable region within the same sequence of NADH dehydrogenase 1 gene cited above (Osman et al., 2007). EGL3 included bases 162- 181 of the positive sense strand (5' TTA TAG TAT GCT TTC TGT GT '3). EGR4 included bases 420- 437 of the complementary strand (5' AAC ACA CAC ACC ACC AAG AAT '3). The nested primers resulted in amplification of a 276-bp PCR product from the camel genotype.

**Table 3: Selected Primers**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>No. of bases</th>
<th>PCR products(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGL1</td>
<td>5'-TGAAGTTAGTAATTAAGTTA-3’</td>
<td>22</td>
<td>435</td>
</tr>
<tr>
<td>EGR2</td>
<td>5'-AATCAATGGAGTGATTTA-3’</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>EGL3</td>
<td>5'-TTATAGTAGTCTTCTGTGT-3’</td>
<td>20</td>
<td>276</td>
</tr>
<tr>
<td>EGR4</td>
<td>5'-AACACACACACCCAAGAAT-3’</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>
2.2.5 Master Mix preparation

A stock buffered solution containing 250 µl 10x PCR buffer, 100µl of MgCl₂, 12.5µl each of adenosine triphosphate (dATP), thymidine triphosphate (dTTP), guanidine triphosphate (dGTP) and cytidine triphosphate (dCTP) was prepared in 1.5 ml eppendorf tube. Double distilled water was added to bring the volume of the stock buffer solution to 1.5 ml.

2.2.6 Polymerase Chain Reaction (PCR) assays

2.2.6.1 Polymerase Chain Reaction (PCR) assay specific for *Echinococcus granulosus* complex

2.2.6.1.1 Preparation of the PCR tubes

The primers EGL1 and EGR2 were used at a concentration of 20 pg µl⁻¹. Two microliters of the primers, 5µl of the target DNA (from the extracted samples) and 42 µl of the already prepared master mix were added onto 0.5 ml PCR tubes and mixed by vortexing. One microliter of *Taq* DNA polymerase (Perkin Elmer) at concentration of 5 U µl⁻¹ was added to each tube. All PCR amplification reactions were carried out in a final reaction volume of 50µl.

2.2.6.1.2 Thermal cycling profiles

Thermal cycling profiles were as follows: 2 min initial incubation or denaturation at 94°C followed by 35 cycles of 94°C for 1 min, 55°C for 30 sec and 72°C for 45 sec and a final extension at 72°C for 10 min. Thermal profiles were performed on thermal cycler (Techne, Germany) (Figure 4).
2.2.6.1.3 Gel electrophoresis

Following the amplification, 12µl from each PCR containing amplified products were loaded onto 1.0% SeaKem agarose (FMC Bioproduct, Rockland ME) after mixing with the loading dye and electrophoresed on 1% Tris-Borate-EDTA (TBE) running buffer containing ethidium bromide at 90 volts for 30 to 40 minutes (Figure 5).

2.2.6.1.4 Gel documentation

The gels and the PCR products were visualized and photographed under the ultraviolet (UV) light.

2.2.6.2 Specificity test

The specificity of the specific *Echinococcus granulosus* primers EGL1 and EGR2 was examined using a DNA target isolated from protoscoleces of the hydatid cyst and DNA from the *Cystisercus taeniocolis* (the larval stage of *Taenia hydatigena*) and *Coenurus celberalis* (the larval stage of *Taenia multiceps*) as related cestodes. The PCR assay was carried out as follows:

The primers EGL1 and EGR2 were used at a concentration of 20 pg µl⁻¹. Two microliters of the primers, 5µl of the target DNA and 42µl of the already prepared master mix were added onto 0.5 ml PCR tubes and mixed by vortexing. One microliter of *Taq* DNA polymerase (Perkin Elmer) at concentration of 5 U µl⁻¹ was added to each tube. All PCR amplification reactions were carried out in a final reaction volume of 50µl.

2.2.6.2.1 Thermal cycling profiles

Thermal cycling profiles were achieved as mentioned in 2.2.6.1.2
2.2.6.2.2 Gel electrophoresis

Gel electrophoresis was done as in 2.2.6.1.3

2.2.6.2.3 Gel documentation

Gel documentation was carried out as in 2.2.6.1.4

2.2.6.3 Sensitivity test

Serial dilutions of 10 fold dilution was done to test the sensitivity of the PCR assay using primers EGL1 and EGR2, that was as follows:

90µl of free nucleic acid free water was pipetted into four eppindorf tubes. 10µl of the target DNA was transferred to the first eppindorf tube, the DNA was mixed with the free nucleic acid free water by sucking up and down using the micro pipette then 10µl was pipetted off and transferred to the second tube and mixed with the nucleic acid free water. These procedures were done with the rest of the tubes. The PCR assay was done using primers EGL1 and EGR2 as mentioned in 2.2.6.1.2

2.2.6.3.1 Gel electrophoresis

Gel electrophoresis was done as in 2.2.6.1.3

2.2.6.3.2 Gel documentation

Gel documentation was carried out as in 2.2.6.1.4

2.2.6.4 Nested Polymerase Chain Reaction (nPCR)

2.2.6.4.1 Preparation of the nested PCR tubes

For the nested PCR amplification, 2.0 µl of the primary PCR product produced by EGL1 and EGR2 were transferred to 0.5 ml PCR tube containing (2 µl of nested primers and; 45 µl of stock PCR buffer and 1µl Taq DNA polymerase at a
concentration of 5.0 U/µl. The nested pair of primers (EGL3 and EGR4) was expected to amplify a 276 bp PCR amplicons, internal to the annealing sites of primers EGL1 and EGR2.

2.2.6.4.2 Thermal cycling profiles

All PCR amplifications were carried out in a final volume of 50 µl. The thermal cycling profiles were as follows: a 2 min incubation at 95° C, followed by 30 cycles of 94° C for 1 min, 57° C for 30 sec and 72° C for 45 sec, and a final incubation at 72° C for 10 min. Thermal profiles were performed on thermal cycler (Techne, Germany)

2.2.6.4.3 Agrose gel electrophoresis

Following amplification, 12 µl from each nested PCR containing amplicon were loaded onto gels of 1. % SeaKem agarose (FMC Bioproduct, Rockland ME) and electrophoresed. The gels were stained with ethidium bromide and the bovineine-specific PCR amplicons were easily identified following visualization under UV light.

2.2.6.4.4 Gel documentation

Gel documentation was carried out as in 2.2.6.1.4
Figure 1: Camel lungs infected with multiple hydatid cysts.

Figure 2: Camel liver infected with two hydatid cysts
Figure 3: Camel spleenic hydatid cyst.

Figure 4: PCR thermal cycler Techne (TC 312).
Figure 5: Electrophoresis apparatus.
CHAPTER THREE

Results

3.1 The pathological findings

3.1.1 The infectivity rate (Tables 4-6)

Out of 31 camels, 898 cattle and 2486 sheep examined, 18 (58.06%), 15 (1.67%) and 10 (0.4%) animal were infected with hydatid cysts, respectively.

In camels, 13 (72.22%) cysts were in lungs and 5 (27.78%) were in the liver.

In cattle, 15 (100%) hydatid cysts were in the lungs, and no cyst was found in the liver, while in sheep, 10 (100%) hydatid cysts were in the liver, while no cyst was observed in lungs.

3.1.2 The cyst size (Tables 4-6)

All collected cysts were small or medium in size, no large cysts were found among the collected samples.

In camels, 13 (72.22%) cysts were small and 5 (27.78%) cysts were medium in size.

For cattle origin hydatid cysts, 11 (73.33%) cysts were small and 4 (26.67%) cysts were medium in size.

Sheep origin hydatid cysts, 9 (90%) cysts were small in size and 1 (10%) cyst was less than 1 cm (0.8 cm).

3.1.3 The fluid amount (Tables 4-6)

Based on the fluid measuring criteria, 5 (27.78%) camel cysts, 5 (33.33%) cattle cysts and 10 (100%) sheep cysts were found to fall within group A (G_A) (0-5 ml).

10 (55.56%) camel cysts and 2 (13.33%) cattle cysts were in group B (G_B) (6-10 ml).
One (5.55%) camel hydatid cyst and 5 (33.33%) cattle hydatid cysts were in group C (G_C) (11- 15 ml). Just one (6.67%) cattle hydatid cyst was in group D (G_D) (16- 20 ml). Two (11.11%) camel hydatid cysts and 2 (13.33%) cattle hydatid cysts were in group E (G_E) (21- 25 ml).

3.1.4 Status of the hydatid cysts fluid (Tables 4- 6)

11 (61.11%) camel origin hydatid cysts, 10 (66.67%) cattle origin hydatid cysts and 5 (50%) sheep origin hydatid cysts had watery fluid consistency. 3 (16.67%) camel cysts, one (6.67%) cattle cyst and 2 (20%) sheep cysts had thick consistency. Two (11.11%) camel cysts and 3 (20%) cattle cysts were caseated (Figure 6).

Watery fluids were clear, whereas thick and caseated fluids were turbid or had yellowish color.

3.1.5 Cysts status (Tables 4- 6)

9 (50%) camel cysts and 10 (66.67%) cattle cysts were fertile, while no sheep origin hydatid cyst was found fertile.

5 (27.78%) cysts, 2 (13.33%) cysts and 6 (60%) cysts were sterile in camels, cattle and sheep, respectively. Camel sterile hydatid cysts, three cysts were in liver while two cysts were in lungs.

2 (11.11%) cysts, one (6.67%) cyst and 3 (30%) cysts were under calcification in camels, cattle and sheep, respectively.
Figure 6: Caseated hydatid cyst.
3.1.6 Results of fluid chemical components

3.1.6.1 Total proteins (Tables 7-9)

In camel hydatid cysts fluid, the concentrations of the total proteins ranged from 0.21 g/dl to 8.0 g/dl (average = 1.13 g/l). The low concentrations were found mostly in the sterile cysts, while the higher concentrations were in cysts with thick fluid consistency.

In cattle hydatid cysts, the total protein concentrations varied from 0.42 g/dl to 8.0 g/dl (average = 1.74 g/dl). The higher concentrations were in cysts with thick or caseated fluid consistency.

In sheep, the total proteins concentrations of collected hydatid cysts fluids ranged from 0.84 g/dl to 5.09 g/dl (average = 2.23 g/dl). The higher concentrations were also in cysts with thick fluid consistency.

3.1.6.2 Cholesterol (Tables 7-9)

In camel origin hydatid cysts, the cholesterol concentrations were from 0.41 mmol/l to 2.68 mmol/l (average = 0.75 mmol/l). The higher concentrations were observed in cysts which are thick in fluid consistency.

For hydatid cysts of cattle origin, cholesterol concentrations were from 0.62 mmol/l to 2.89 mmol/l (average = 0.95 mmol/l). The higher concentrations were also seen in cysts with thick or caseated fluid consistency.

In sheep origin hydatid cysts, the amounts of cholesterol were from 0.70 mmol/l to 2.54 mmol/l (average = 1.28 mmol/l). The higher concentrations were mostly seen in cysts with thick fluid consistency.

3.1.6.3 Minerals (Tables 7-9)

3.1.6.3.1 Sodium and potassium

In camel origin hydatid cysts, the concentrations of sodium and potassium were from 30.8 mmol/l to 109.2 mmol/l (average = 53.11 mmol/l) and from 0.4 mmol/l to 2.3 mmol/l (average = 1.49mmol/l), respectively.
In cattle origin hydatid cysts, the concentrations of sodium and potassium were from 18.5 mmol/l to 77.0 mmol/l (average = 38.61 mmol/l) and from 0.8 mmol/l to 2.2 mmol/l (average = 1.47 mmol/l), respectively.

For sheep hydatid cysts, the concentrations of sodium and potassium were from 68.5 mmol/l to 77.6 mmol/l (average = 72.94 mmol/l) and from 3.83 mmol/l to 8.53 mmol/l (average = 5.95 mmol/l), respectively.

### 3.1.6.3.2 Inorganic phosphorus

In camel origin hydatid cysts, phosphorus concentrations were from 0.013 mg/100ml to 4.8 mg/100ml (average = 1.31 mg/100ml).

For the hydatid cyst of cattle origin, phosphorus concentrations were from 0.5 mg/100ml to 5.6 mg/100ml (average = 2.05 mg/100ml).

In sheep origin hydatid cysts, the amounts of phosphorus were found from 0.04 mg/100ml to 1.9 mg/100ml (average = 1.02 mg/100ml).

### 3.1.6.3.3 Calcium

In camel origin hydatid cysts, calcium concentrations were from 10.0 mg/100ml to 190 mg/100ml (average = 64.07 mg/100ml).

For the hydatid cyst of cattle origin, calcium concentrations were from 15 mg/100ml to 100 mg/100ml (average = 39.67 mg/100ml).

In sheep origin hydatid cysts, the amounts of calcium were from 41.0 mg/100ml to 111.1 mg/100ml (average = 66.96 mg/100ml).
Table 4: Particular of hydatid cysts from camels slaughtered at Tambool abattoir.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Abattoir</th>
<th>Organ</th>
<th>Cyst Size. Cm</th>
<th>Fluid Amount. ml</th>
<th>Fluid Status</th>
<th>Fluid Color</th>
<th>Cyst Status</th>
<th>Total Slaughtered Animal</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHP1</td>
<td>Tambool</td>
<td>Lungs</td>
<td>8</td>
<td>22</td>
<td>Watery</td>
<td>Clear</td>
<td>Fertile</td>
<td>31</td>
</tr>
<tr>
<td>CHP2</td>
<td>Tambool</td>
<td>Lungs</td>
<td>5</td>
<td>8</td>
<td>Watery</td>
<td>Clear</td>
<td>Fertile</td>
<td>31</td>
</tr>
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<td>CHP4</td>
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<tr>
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<td>31</td>
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<td>-</td>
<td>calcified</td>
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<td>Clear</td>
<td>Sterile</td>
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<td>Tambool</td>
<td>Lungs</td>
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<td>25</td>
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<td>Clear</td>
<td>Fertile</td>
<td>31</td>
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<td>Lungs</td>
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<td>15</td>
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<td>Clear</td>
<td>Fertile</td>
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<td>-</td>
<td>31</td>
</tr>
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<td>Range</td>
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<td>0-25</td>
<td>-</td>
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</tr>
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</table>

CHP = Camel hydatid cyst used for the pathological study.
Table 5: Particular of hydatid cysts collected from cattle slaughtered at Ghanawa abattoir.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Abattoir</th>
<th>Organ</th>
<th>Cyst Size. Cm</th>
<th>Fluid Amount. Ml</th>
<th>Fluid Status</th>
<th>Fluid color</th>
<th>Cyst Status</th>
<th>Total Slaughtered Animal</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHP1</td>
<td>Ghanawa</td>
<td>Lungs</td>
<td>7</td>
<td>0.5</td>
<td>Caseated</td>
<td>Yellowish</td>
<td>Fertile</td>
<td>313</td>
</tr>
<tr>
<td>BHP2</td>
<td>Ghanawa</td>
<td>Lungs</td>
<td>7</td>
<td>13</td>
<td>Watery</td>
<td>Clear</td>
<td>Fertile</td>
<td>313</td>
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<td>Ghanawa</td>
<td>Lungs</td>
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<td>11</td>
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<td>Clear</td>
<td>Sterile</td>
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<td>12</td>
<td>Thick</td>
<td>Yellowish</td>
<td>Fertile</td>
<td>313</td>
</tr>
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<td>Ghanawa</td>
<td>Lungs</td>
<td>6</td>
<td>17.5</td>
<td>Watery</td>
<td>Clear</td>
<td>Fertile</td>
<td>251</td>
</tr>
<tr>
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<td>Ghanawa</td>
<td>Lungs</td>
<td>5</td>
<td>24</td>
<td>Watery</td>
<td>Clear</td>
<td>Fertile</td>
<td>251</td>
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<td>Clear</td>
<td>Fertile</td>
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<td>Clear</td>
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<td>Yellowish</td>
<td>Fertile</td>
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<td>Ghanawa</td>
<td>Lungs</td>
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<td>9</td>
<td>Watery</td>
<td>Clear</td>
<td>Fertile</td>
<td>251</td>
</tr>
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<td>Lungs</td>
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<td>12.5</td>
<td>Watery</td>
<td>Clear</td>
<td>Fertile</td>
<td>251</td>
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<td>2</td>
<td>-</td>
<td>Caseated</td>
<td>-</td>
<td>-</td>
<td>251</td>
</tr>
<tr>
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<td>-</td>
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<td>-</td>
<td>Caseated</td>
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<td>-</td>
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<td>4</td>
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<td>Clear</td>
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**BHP** = Cattle hydatid cyst used for the pathological study.
Table 6: Particular of hydatid cysts obtained from sheep slaughtered at Al-Kadaro abattoir.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Abattoir</th>
<th>Organ</th>
<th>Cyst Size. cm</th>
<th>Fluid Amount. ml</th>
<th>Fluid Status</th>
<th>Fluid Color</th>
<th>Cyst Status</th>
<th>Total Slaughtered Animal</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHP1</td>
<td>Al Kadaro</td>
<td>Liver</td>
<td>1.3</td>
<td>0.8</td>
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<td>Turbid</td>
<td>Sterile</td>
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<td>Al Kadaro</td>
<td>Liver</td>
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<td>0.3</td>
<td>Thick</td>
<td>Yellowish</td>
<td>Sterile</td>
<td>623</td>
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<td>SHP3</td>
<td>Al Kadaro</td>
<td>Liver</td>
<td>3</td>
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<td>Yellowish</td>
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<td>Sterile</td>
<td>846</td>
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<td>SHP5</td>
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<td>Liver</td>
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<td>0.9</td>
<td>Watery</td>
<td>Turbid</td>
<td>Sterile</td>
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</tr>
<tr>
<td>SHP6</td>
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</table>

SHP = sheep hydatid cyst used for the pathological study.
Table 7: Chemical analysis of hydatid fluid of camel origin.

<table>
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<th>Samples</th>
<th>Cyst Status</th>
<th>Total Protein, g/dl</th>
<th>Cholesterol mmol/l</th>
<th>Sodium mmol/l</th>
<th>Potassium mmol/l</th>
<th>Calcium mg/100ml</th>
<th>Phosphorus mg/100ml</th>
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<td>CHP1</td>
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CHP = Camel hydatid cyst used for the pathological study.
**Table 8: Chemical analysis of hydatid fluid of cattle origin.**

<table>
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<tr>
<th>Samples</th>
<th>Cyst Condition</th>
<th>Total Protein. g/dl</th>
<th>Cholesterol mmol/l</th>
<th>Sodium mmol/l</th>
<th>Potassium mmol/l</th>
<th>Calcium mg/100ml</th>
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**Average**

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**Standard DEVIATION**

|   |   | 2.740141 | 0.706568211 | 14.11063 | 0.445856343 | 24.242462 | 2.14378948 |

**BHP =** Cattle hydatid cyst used for the pathological study.
**Table 9: Chemical analysis of hydatid fluid of sheep origin.**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Cyst Condition</th>
<th>Total Protein. g/dl</th>
<th>Cholesterol mmol/l</th>
<th>Sodium mmol/l</th>
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SHP = sheep hydatid cyst used for the pathological study.
3.1.7 Histopathological findings

In the histopathological study, all lung sections showed fibrous tissue reaction (capsules), cellular reaction, necrosis and collapsed lung of tissue neighbouring the cyst wall, while liver sections showed fibrous tissue reaction, cellular infiltration atrophy and necrosis of hepatocytes neighbouring the cyst wall.

Parasitic membranes (laminated membranes and germinal layers) were obvious in most examined sections, some were continuous and intact containing brood capsules and protoscolices and the others were disrupted. Some sections showed only remnants of these membranes in both lung and liver sections (Figures 7-10).

Laminated membranes varied in thickness and number of laminations (Figure 11).

Parasitic sections (scolices) were seen in lung tissue, in most sections in different numbers, whereas such feature was not observed in liver sections.

Cellular infiltrations were in different manner; intense, slight (scanty) or focal, but occasionally some sections showed a tendency of forming granulomas with giant cells formation. Types of infiltrated inflammatory cells were mainly mononuclear cells, macrophages, lymphocytes, plasma cells and neutrophils (Figures 12-14). The later cell was seen in caseated cysts.

In camel lung sections, fibrous tissue reaction (capsule) was moderate and the cellular reaction was minimal except in caseated cysts where intense cellular infiltrations were found and extended to the inner wall of the cyst. In such cases neutrophils were the predominant cell type and parts of them were degenerating or dead forming pus in the cyst cavity. Laminated membranes were mostly moderate in thickness, but were very wide and multi-laminated in caseated cysts (Figures 15-19). In some sections, big zones of collapsed lung tissue accompanied with emphysema and compressed bronchioles were seen and that was quite obvious in larger cysts. Most of the sections showed parasitic sections (scolices) spread in lung tissue in different numbers and one section had a big cyst neighboured by daughter cyst (Figure 20). Proliferation of the bronchial
epithelium was marked in some sections, while some slides showed calcification and fibrosis in lung tissue and alveolar congestion (Figures 21-22).

In camel liver sections, fibrous tissue reaction (capsule) was moderate as well as the cellular reaction, but one section showed heavy cellular reaction. Laminated membranes were disrupted in most sections and vacuolated in some. All sections showed atrophy, degenerative changes and necrosis in hepatocytes nearby the cyst wall. Sinusoidal dilatation, proliferation of capillaries and fibroblasts, fibrosis in portal areas, rings of hemorrhages and fatty change were occasionally seen in liver sections. Calcifications were marked in some cases (Figures 23-30).

In lung sections of cattle with hydatid cysts, fibrous tissue reaction (capsule) was well developed and supplied with many small blood vessels. Vacuolated round structures containing cells were observed in the most inner end of the fibrous tissue reaction. Cellular infiltrations were rather intensive or diffuse, but some sections showed assemblage of focal mononuclear cells infiltration as granulomas. Cellular infiltrations were seen in most sections in the most inner side of fibrous tissue capsules before the laminated membranes. Neutrophils were seen invading the laminated membrane particularly in caseated cysts. Organized cellular structures resembling giant cells were seen in most sections at the inner part of fibrous tissue capsules just before the laminated membranes. Cyst walls were observed to be irregular in most sections. As in camel lung cysts, laminated membranes were found to be broad and multi-laminated in the caseated cysts. Zones of collapse, emphysema, dilated bronchi and necrosis of lung tissue neighbouring the cyst wall were also seen. Parasitic sections (scolices) were also seen, but less than what appeared in camels. Fibrosis in lung tissue was marked in addition to some calcifications. The rest of the lung tissue, some sections showed chronic pneumonia, suppurative bronchitis, peribronchial lymphoid hyperplasia, proliferation of the peribronchial glands and fetalization of alveolar cells (Figures 31-43).

In sheep origin hydatid cysts, all liver sections showed very wide zone of fibrous tissue reaction capsules. Cellular infiltrations were diffuse mostly, but a
number of sections showed scanty cellular infiltration and others showed assemblage of mononuclear cells in a manner of granulomas. Macrophages were found to be the predominant type of cells in addition to presence of Langhans giant cells. The parasitic membranes were not seen, but some debris was obvious in cyst cavity. Perivascular cellular infiltration of inflammatory cells composed mainly of mononuclear cells and neutrophils were noticed. Calcifications were marked in most sections. Neighbouring the cyst wall, many degenerated, necrotic hepatocytes with nuclear pyknosis, some mitotic figures and loss of lobular structure were seen. Sinusoidal dilatations were occasionally marked as well as strangulated hepatic tissue surrounded by the thickened and fibrosed interlobular tissue. Parts of sections showed fibroplasia surrounding necrotic liver parenchyma, epithelial hyperplasia in bile ducts, bile ductules hyperplasia, vacuolar degeneration (regular & irregular), accumulation of leukocytes in the blood vessels, fibrosis in portal areas, with fibers arranged in different directions (Figures 44- 50).
Figure 7: Lung section showing fibrous tissue capsule infiltrated with inflammatory cells, parasitic membranes (laminated membrane & germinal layer), scolices and collapse in lung tissue neighboring the cyst wall (H&E 50X).

Figure 8: Lung section showing fibrous tissue capsule, zone of infiltrated inflammatory cells, collapsed lung tissue and compressed bronchiole (H&E 100X).
Figure 9: Section showing the laminated membrane, germinal layer and protoscolices (H&E 100X).

Figure 10: Liver section showing interrupted laminated membrane, moderate fibrous tissue reaction capsule infiltrated with scanty inflammatory cells (H&E 50X).
Figure 11: Section showing broad laminated membrane and heavy cellular reaction in caseated hydatid cyst (H&E 50X).

Figure 12: Severe cellular reaction in caseated hydrated cyst (H&E 100X).
Figure 13: Lung section showing collection of inflammatory cells forming granuloma and collapsed lung tissue (H&E 50X).

Figure 14: Infiltrated inflammatory cells of different types mononuclear cells, macrophages, plasma cells and lymphocytes (H&E 400X).
Figure 15: Camel lung section of camel origin hydatid cyst showing the laminated membrane, the fibrous tissue capsule infiltrated with inflammatory cells, compressed bronchiole and collapsed alveoli (H&E 50X).

Figure 16: Section showing the laminated membrane, germinal layer and brood capsule containing protoscolices (H&E 100X).
Figure 17: Section showing severe cellular reaction in caseated hydatid cyst (H&E 100X).

Figure 18: Section of caseated hydatid cyst showing severe infiltration of neutrophils which extending to the laminated membrane. Degenerated and dead neutrophils also appear inside the cyst cavity (pus) (H&E 50X).
**Figure 19:** Section showing interrupted broad laminated membrane in caseated hydatid cyst (H&E 50X).

**Figure 20:** Lung section of camel origin showing presence of parasitic sections (scolices) in the lung tissue (H&E 100X).
Figure 21: Camel lung section showing proliferation of bronchial epithelium and congestion in the alveolar walls (H&E 100X).

Figure 22: Section from caseated hydatid cyst showing calcification and heavy cellular reaction (H&E 50X).
Figure 23: Camel liver section showing interrupted laminated membrane, moderated fibrous tissue capsule with slight cellular reaction and some hepatocytes vacuolations (H&E 50X).

Figure 24: Liver section showing degenerated and necrotic hepatocytes with loss of hepatic structure (H&E 50X).
Figure 25: Liver section showing proliferation of bile ductules and hemorrhage (H&E 100X).

Figure 26: Liver section showing proliferation of capillaries and hemorrhage (H&E 100X).
Figure 27: Liver section showing sinusoidal dilatation (H&E 50X).

Figure 28: Liver section showing areas of hemorrhage (H&E 100X).
Figure 29: Liver section showing disrupted laminated membrane, calcification in the cyst wall and proliferation of capillaries (H&E 50X).

Figure 30: Liver section showing centrolobular fatty change (H&E 100X).
Figure 31: Lung section of cattle origin hydatid cyst showing the laminated membrane and broad fibrous tissue capsule (H&E 50X).

Figure 32: Lung section of cattle origin hydatid cyst showing very broad fibrous tissue capsule (H&E 50X).
Figure 33: Lung section of cattle origin hydatid cyst showing focal cellular infiltration as a granuloma (H&E 100X).

Figure 34: Lung section of cattle origin hydatid cyst showing the laminated membrane, broad fibrous tissue capsule infiltrated with inflammatory cells (H&E 100X).
Figure 35: Lung section of cattle origin hydatid cyst showing organized cellular structures in the inner side of the fibrous tissue capsule and cellular infiltration (H&E 100X).

Figure 36: Lung section of cattle origin hydatid cyst showing irregular cyst wall (H&E 50X).
Figure 37: Lung section of cattle origin hydatid cyst showing very broad laminated membrane (H&E 50X).

Figure 38: Lung section of cattle origin hydatid cyst showing calcification in the cyst wall (H&E 50X).
Figure 39: Lung section of cattle origin showing parasitic section (scolex) in the lung tissue and areas of emphysema (H&E 100X).

Figure 40: Lung section showing interlobular fibrosis (H&E 50X).
Figure 41: Lung section showing suppurative bronchitis and heavy cellular reaction (H&E 100X).

Figure 42: Lung section showing proliferation of peribronchial lymphoid tissue and peribronchial glands (H&E 50X).
Figure 43: Lung section showing fetalization in the alveolar epithelium (H&E 100X).

Figure 44: Liver section of sheep origin hydatid cyst showing very broad fibrous tissue capsule (H&E 50X).
Figure 45: Liver section showing heavy cellular reaction (H&E 100X).

Figure 46: Liver section of sheep origin hydatid cyst showing formation of giant cells (H&E 400X).
Figure 47: Liver section of sheep origin hydatid cyst showing calcification in the cyst wall (H&E 50X).

Figure 48: Liver section showing nuclear changes (pyknosis and mitotic figures) (H&E 400X).
**Figure 49:** Liver section showing fibrosis in the portal area with proliferation of small capillaries (H&E 100X).

**Figure 50:** Liver section showing accumulation of leukocytes inside blood vessel (H&E 100X).
3.1.8 Histochemical study

3.1.8.1 Periodic Acid Schiff (PAS) stain

Generally, laminated membranes, germinal layers, brood capsules and the protoscolices took very deep positive PAS magenta color (Figures 51- 53).

In camel origin hydatid cysts, laminated membranes and germinal layers appeared intact, interrupted or in a remnant forms. Laminated membranes were very distinct and broad in the caseated cysts. Parasitic sections (scolices) were also PAS positive in the lung tissues (Figure 54).

In cattle origin hydatid cysts, the laminated membranes and the germinal layers were seen intact, disrupted or as remnants. Parasitic sections (scolices) were many in the lung tissue in some cases (Figures 55- 57).

In sheep hydatid cysts, the parasitic membranes were not obvious, and centro-lobular glycogen infiltration was seen in some cases (Figures 58- 59).

3.1.8.2 Masson’s trichrome stain

Generally, the fibrous tissue capsules, laminated membranes and germinal layers were distinct. The fibrous tissue stained green in color, laminated membranes appeared as a cellular layers with slightly gray to green color, whereas germinal layers appeared as nucleated and dark purplish in color (Figures 60- 61).

In camels, lung cysts showed thin and moderate fibrous tissue reaction. Laminated membranes were sometimes continuous, in some sections disrupted, and were very broad in the caseated cysts (Figures 62- 63).

Neighbouring the cyst wall, there was a lot of damage, necrotic cells and collapsed alveoli. Parasitic sections (scolices) were also seen in lung tissue taking slight green color in most sections but in different shapes. Peribronchial and perivascular fibrosis were also seen in some sections (Figures 64- 65).

In the liver cysts, the fibrous tissue reaction was also moderate and the laminated membranes were interrupted in most sections and broad and irregular in some. Atrophy and necrosis of hepatocytes were also seen neighboring the cyst wall. Extensive fibrosis was observed in the portal areas,
fibrosis around the central veins and strangulation in the liver lobules in some sections. Part of sections showed centric fatty change (Figures 66-69).

In the cattle origin hydatid cysts, the fibrous tissue capsules appeared very broad and wide and in most sections. Their inner sides nearby the laminated membranes consisted of large vacuolated cells and giant like cells reaction. Laminated membranes were either continuous or interrupted and in some sections were seen as remnants. Broad laminated membranes were observed in casiated cysts. Filamentous rods, reddish in color were seen in laminated membrane of one of the caseated cysts. Filamentous rods, reddish in color were seen in laminated membrane of one of the caseated cysts. Filamentous rods, reddish in color were seen in laminated membrane of one of the caseated cysts. Alveolar collapse and necrosis were also seen in lung tissue near the cyst wall. Parasitic sections (scolices) took a slight green color in the lung tissue in different forms. Parts of the sections showed slight fibrous tissue formation (strands) in irregular arrangements. Interlobular fibrosis was quite obvious in most sections (Figures 70-74).

In sheep origin hydatid cysts, the fibrous tissue reaction was distinct and very broad in most sections and also found in surrounding liver tissue (strangulation). The parasitic membranes were not distinct in all sections. Atrophied necrotic hepatic tissues were seen neighbouring the cyst wall. Regular and irregular Vacuolations could be seen in some sections (Figures 75-76).
Figure 51: Lung section showing PAS positive laminated membrane, the germinal layer and brood capsules (taking deep magenta color) (PAS 50X).

Figure 52: Section showing PAS positive laminated membrane, the germinal layer and brood capsules (PAS 100X).
Figure 53: Section showing broad and coiled laminated membrane of camel origin caseated hydatid cyst (PAS 50X).

Figure 54: Lung section of camel origin showing PAS positive parasitic sections (scolices) in the lung tissue (PAS 50X).
Figure 55: Lung section of cattle origin hydatid cyst showing PAS positive laminated membrane taking deep magenta color (PAS 100X).

Figure 56: Lung section of cattle origin hydatid cyst showing very broad laminated membrane (PAS 50X).
Figure 57: Lung section of cattle origin showing PAS positive parasitic sections (scolices) in the lung tissue (PAS 100X).

Figure 58: Liver section of sheep origin hydatid cyst, parasitic membranes (the laminated membrane and the germinal layer) are not appearing (PAS 50X).
Figure 59: Liver section of sheep origin showing centro-lobular glycogen accumulation (PAS 100X).

Figure 60: Lung section showing the fibrous tissue capsule taking green color, the laminated membrane taking slight green color, the germinal layer taking purple color and the brood capsules are taking slight green color (Masson's trichrome 50X).
Figure 61: Lung section showing the laminated membrane appears slightly greenish, while the germinal layer appears nucleated and purplish in color (Masson's trichrome 100X).

Figure 62: Lung section of camel origin hydatid cyst showing moderate fibrous tissue capsule taking green color (Masson's trichrome 50X).
Figure 63: Lung section of caseated camel origin hydatid cyst showing broad and interrupted laminated membrane (Masson's trichrome 50X).

Figure 64: Lung section of camel origin hydatid cyst showing parasitic sections (scolices) in the lung tissue (Masson's trichrome 100X).
Figure 65: Lung section of camel origin showing peribronchial and perivascular fibrosis (Masson's trichrome 50X).

Figure 66: Liver section of camel origin hydatid cyst showing the fibrous tissue reaction capsule, the laminated membrane. Fibrosis extending to the liver tissue (Masson's trichrome 50X).
Figure 67: Liver section of camel origin hydatid cyst showing fibrosis in the portal area (Masson's trichrome 100X).

Figure 68: Liver section of camel origin showing severe fibrosis strangulating necrotic liver tissue (Masson's trichrome 50X).
Figure 69: Liver section of camel origin showing centric vacuolations (fatty change) (Masson's trichrome 100X).

Figure 70: Lung section of cattle origin hydatid cyst showing the fibrous tissue capsule containing organized cellular structures in the most inner side (Masson's trichrome 100X).
Figure 71: Lung section of caseated cattle origin hydatid cyst showing broad laminated membrane (Masson's trichrome 50X).

Figure 72: Lung section of cattle origin hydatid cyst showing very broad fibrous tissue capsule (Masson's trichrome 50X).
Figure 73: Lung section of cattle origin hydatid cyst showing interlobular fibrosis (Masson's trichrome 50X).

Figure 74: Lung section of caseated cattle origin hydatid cyst showing filamentous rods reddish in color spreading on very broad laminated membrane (Masson's trichrome 100X).
Figure 75: Liver section of sheep origin hydatid cyst showing very broad zone of fibrous tissue capsule (Masson's trichrome 50X).

Figure 76: Liver section of sheep origin hydatid cyst showing extensive fibrosis which appears strangulating necrotic liver tissue (Masson's trichrome 50X).
3.2 The biomolecular findings

3.2.1 Polymerase Chain Reaction (PCR) assay specific for *Echinococcus granulosus-* complex

Using the outer primers EGL1 and EGR2 the polymerase chain reaction (PCR) was made possible to amplify the *Echinococcus granulosus-* complex extracted DNA and afforded the expected PCR products of 435-bp (Figure 77).

3.2.2 Specificity test

When using the outer pair of primers (EGL1 and EGR2), the PCR- based assay was able to amplify *Echinococcus granulosus-* complex extracted DNA and produced specific 435-bp PCR products, while no PCR products were detected when the mentioned primers were applied to DNA extracted from the metacestodes of other related *Taenia* spp. including *Taenia hydatigena* and *Taenia multiceps* (Figure 78).

3.2.4 Sensitivity test

The PCR based assay was able to detect *Echinococcus granulosus-* complex DNAs amount as little as 100 pg (Figure 79).

3.2.5 Nested Polymerase Chain Reaction (nPCR)

Using the nested primers (EGL3 and EGR4), all examined samples resulted in amplification of a 276-bp PCR products specific for the camel genotype (G6) (Figure 80).
Figure 77: Visualization of the specific 435-bp PCR products from 100 ng DNA extracted from different genotypes of *E. granulosus* hydatid cysts using primers (EGL1 and EGR2).

MW: molecular weight marker; Lane 1-6: samples collected from naturally infected animals; Lane 7: negative control.
Figure 78: Specificity of the PCR for detection of *E. granulosus*-complex using the outer pair of primers (EGL1 and EGR2). MW: Molecular weight marker; Lane 1: 1.0 ng DNA extracted from hydatid cyst of *E. granulosus*; Lane 2: DNA extracted from *Cysticercus taenicholis*; Lane 3: DNA extracted from *Coenurus cerebralis*; Lane 4: DNA-free sample (negative control).
Figure 79: Sensitivity of the PCR for detection of *E. granulosus*-complex using the outer pair of primers (EGL1 and EGR2).

MW: Molecular weight marker; Lane (1-6): DNA extracted from hydatid cyst camel strain (G6) at concentrations of 1.0 µg, 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, respectively; Lane 7: negative control sample.
Figure 80: Visualization of the nested 276-bp specific PCR product from DNA extracted from hydatid cysts of camel strain (G6) of *E. granulosus*. MW: molecular weight marker; Lane (1-6): DNA extracted from different hydatid cysts of the camel strain (G6); Lane 7: negative control.
Cystic echinococcosis (Hydatidosis) is a cyclo-zoonic disease of worldwide importance caused by larval (metacestode) stage of cestode belonging to the genus *Echinococcus granulosus* of the family *Taeniidae* (FAO/UNEP/WHO, 1981; Soulsby, 1982; Thompson and McManus, 2001).

Adult parasites are usually attached to the mucosa of the anterior part of the small intestine of definitive hosts. The larval stages are found in the internal organs of a wide variety of domestic and wild intermediate hosts including cattle, camels, goats and man (Thompson and Lymbery, 1988; Thompson, 1995).

Usually the intermediate host gets the infection after ingestion of infective eggs in grass, feed or in water (Horton, 2003), and hatch into oncospheres (larvae) inside the stomach and intestines. The liberated larvae penetrate the small intestine and reach their final localization passing through vascular and lymphatic systems to the liver and lungs (Soulsby, 1982).

The disease is widely spread in sheep-rearing areas and where definitive hosts occur, so sheep were found to be the major intermediate hosts in continuity of life cycle of the disease worldwide (Torgerson and Health, 2003). However, in Sudan camels were reported to be the major intermediate host of *Echinococcus granulosus* (El Khwad et al., 1979; Saad et al., 1989; Adam, 1997).

In previous reports, the highest prevalence rates of hydatidosis were in camels. High rates of 35.3%, 45.41%, 56.4%, 67.74%, 48.69%, 37% and 79.51% were reported by El Khwad et al., (1979), Saad and Magzoub, (1986), Tola, (1987), Saad et al., (1989), Saad and Magzoub, (1989 a), El Hussein and Ali, (1990) and Adam, (1997), respectively. Infectivity rates of 25%, 6.2%, 24.8%, 8.16%, 2.1%, 3.84% and 6.42% were reported in cattle by Eisa et al., (1962), El Khawad et al, (1976), El Khwad et al., (1978), El Khwad et al., (1979), Tola, (1987), Saad and Magzoub, (1989 a), and Adam, (1997), respectively. In sheep, prevalence rates of 19.4%, 12.2%, 4.28%, 2%,
12.9% and 8.9% were reported by Eisa et al., (1962), El Khwad et al., (1978), El Khwad et al., (1979), Tola, (1987), Saad and Magzoub, (1989 b) and El Sawi, (1994), respectively.

In this study, infectivity rates were 58.06%, 1.67 and 0.4% in camels, cattle and sheep, respectively. It is obvious that camels have the highest prevalence rate and that is in consistency with the previous reports. Infectivity rates in cattle and sheep are low when compared with former reports and that could be due to routine administration of anthelmintic drugs by owners.

Lungs are reported to be the preferable site of hydatid cysts in camel, while the liver is the predilection site in cattle and sheep (Saad and Magzoub, 1989 a; Adam, 1997).

In the present study, 72.22% of the cysts were in the lungs and 27.78% were in the liver in camels. In cattle, all hydatid cysts (100%) were in lungs, while no cyst was found in the liver, but in sheep, 100% hydatid cysts were in the liver; and non observed in the lungs. Based on these result, lungs seem to be the predilection site of hydatid cysts in camels and cattle, whereas liver is the most affected side in sheep and this agrees with Saad and Magzoub, (1989 a &b) and Adam, (1997) that most cysts in camels usually found in the lungs, but disagreed with Saad and Magzoub, (1989 a) and Adam, (1997) that lungs is the preferable affected site in cattle.

Adam, (1997) reported that 71.43 of hydatid cysts are medium (2 – 6 cm) and 28.57% are small in size (< 2 cm) in cattle and 26.62% of the cysts are small, 72.4% were medium and 1.04% were large (7-10 cm) in camels. In sheep all cyst were small (Saad and Magzoub, 1989 b). Fluid volumes ranged from 2 ml to 41 ml in cattle and from 0.3 to 124 ml in camels (Adam, 1997).

In the current work, 72.22% of the camel cysts were small (1- 5 cm) and 27.78% were medium in size (6- 10 cm). Most of small cysts were in the liver. In cattle, 73.33% cysts were small and 26.67% were medium in size. All cysts were found to be small in sizes in sheep. Fluid volumes ranged from 3.5 ml to 25 ml in camels, 0.5 ml to 25 ml in cattle and from 0.3 ml to 2.7 ml in sheep.

Despite the difference in size measuring criteria in this study and that used by Adam, (1997), most cyst lied between 2cm to 6 cm in camels and cattle in both studies. Also the obtained results concerning cysts sizes in
sheep agree with those reported by Saad and Magzoub, (1989 b). Fluid amounts measured in this work were less than that reported by Adam, (1997).

In the current study, the fertility rates were 50% in camel hydatid cysts, 66.67% in cattle hydatid cysts, while no sheep origin hydatid cyst was found fertile. 11.11%, 6.67% and 30% were the percentage of cysts showing calcification in camels, cattle and sheep, respectively.

Previous studies reported fertility rates of 42.4%, 29% and 0.81% for camels, cattle and sheep, respectively. (Saad and Magzoub, 1989 a & b). Calcification was predominant feature in sheep hydatid cysts (Saad and Magzoub, 1989 b).

Generally, the results show that the cattle cysts appeared more fertile than cysts collected from camels and this disagrees with results obtained in the former studies. Most camel liver cysts were sterile and this decreases the overall fertility rate in camels. Liver hydatid cysts in sheep and camels were mostly observed to be small in size, sterile or undergoing calcification; however, the liver may have a kind of resistance to development of these cysts.

Hydatid cyst fluid (HCF) is a complex mixture of parasite-derived and host-derived molecules. It contains several components derived from the metabolism of the parasite together with many components from the host (Rickard and Lightowlers, 1986). The quantitative differences in the metabolism of E. granulosus and variation in the biochemical composition of hydatid fluids differ within intermediate hosts (Shaafie et al., 1999; Thompson and Lymbery, 1995; McManus and Macpherson, 1984).

In this study, total proteins concentrations averaged 1.13 g/l, 1.74 g/dl and 2.23 g/dl in camel, cattle and sheep hydatid fluids, respectively.

Shaafie et al., (1999) reported that levels of proteins were significantly lower in the cyst fluids of camels, cattle and humans compared with sheep.

It is obvious that hydatid fluids of sheep origin have high total proteins concentrations, and that is in consistency with Shaafie et al., (1999) and Kadir, (2006). Great concentrations of total proteins were marked in hydatid fluids with thick consistency and caseated cysts in camels and cattle. This may indicate that cysts proteins increase during degenerative process, ie degradation of parasitic membranes or protoscolices.
While different observations of comparatively low levels of cholesterol in sheep and human hydatid fluids were reported by (Sheriff and Ghwarsha, 1985). (Sheriff et al., 1989) suggested that cholesterol levels increase in degenerated hydatid cysts.

In this work, the average cholesterol levels were 0.75 mmol/l, 0.95 mmol/l and 1.28 mmol/l in camels, cattle and sheep, respectively. Sheep were also having the highest concentrations of cholesterol. This disagrees with Sheriff and Ghwarsha, (1985) who observed low cholesterol levels in sheep and human hydatid fluids. Saad and Magzoub, (1989 b) reports that sheep may have natural resistance to hydatid cysts, and because of this, most cysts in sheep are rudimentary, degenerated or calcified. Based on this observation, increases in cholesterol levels in sheep hydatid fluids may be related to the degenerative changes happening in these cysts. Cholesterol was also seen in higher concentrations in thick or casiated hydatid fluids of camels and cattle origin. These cysts, as mentioned above, could be undergoing degenerative process and this agrees with Sheriff et al., (1989).

However, higher quantities of cholesterol in human and animals hydatid cyst fluids were found by Shaafie et al., (1999)

Anwar, (1994) showed that sodium contents were significantly higher in fluid of sterile hydatid cysts compared to fertile ones in bovine livers. Potassium levels in both cattle and camel cysts were higher than that of sheep and goats (Radfar and Iranyar, 2004).

In the present study, averages of sodium contents in hydatid fluids were 53.11 mmol/l, 38.61 mmol/l and 72.94 mmol/l in camels, cattle and sheep, respectively, while averages of potassium contents were 1.49mmol/l, 1.47mmol/l and 5.95 mmol/l in camels, cattle and sheep respectively. Since all hydatid cysts in sheep were in the liver and were sterile, these results are supported by the findings of Anwar, (1994) previously stated. The results obtain in this study also revealed higher potassium contents in sheep hydatid fluids as compared to those of camels and cattle, which disagrees with Sheriff et al., (1989).

In cattle, phosphorous levels were found significantly higher in concentration in the fluid of fertile liver and lungs hydatid cysts as compared with the sterile ones (Anwar, 1994), while camel origin hydatid cysts were
found to have significantly large amounts of calcium than those of other intermediate hosts (Radfar and Iranyar, 2004).

This study revealed that averages of phosphorus levels were 1.31mg/100ml, 2.05 mg/100ml and 1.02 mg/100ml in camels, cattle and sheep cystic fluids, respectively; averages of calcium were 64.07mg/100ml, 39.67 mg/100ml and 66.96 mg/100ml in camels, cattle and sheep hydatid fluids, respectively. The high amounts of phosphorus in cattle in this study may be due to high fertility rate obtained here. Therefore this result could match with that reported by Anwar, (1994). The high calcium levels reported in both camels and sheep cystic fluids, may agree with the findings of Radfar and Iranyar, (2004) in camels cysts, but the increment in calcium levels in sheep cystic fluids could be due to the relatively high calcium levels in sheep serum or due to the degenerative changes and calcification occurring in the cysts.

Several histopathological studies on hydatid disease were done. Adam, (1997) carried out a study on four hydatid cysts, two cysts of camel origin and the rest were of cattle origin comprising lungs and liver. The changes observed in the lungs were found to be cellular infiltration lymphocytes and plasma cells, alveolar edema, atelectasis mild congestion and compression of bronchioles adjacent the cyst wall. In the liver the changes were atrophy of the liver tissue around the capsule, hyperplasia of the bile ducts and dilatation in the sinusoids. Saad, (1985) also reported a cellular reaction and he suggested that the immunological response of the host to the infection to be a delayed hypersensitivity type of reaction. Formation of fibrotic capsules around biliary tracts and portal veins, pre-malignant changes around the biliary tracts and portal veins in sheep liver were reported by Rashed et al., (2004). In early infection, an increased leukocytosis, mainly of eosinophils, lymphocytes, and macrophages were seen around the developing onchspheres (Petrova, 1968). In already established cysts, the cellular infiltrations included eosinophils, neutrophils, macrophages, and fibrocytes (Archer et al., 1977; Slais and Vanek, 1980; Richards et al., 1983; Riley et al., 1985 and Rigano et al., 1996). However, this generally does not result in a severe inflammatory response and aged cysts tend to become surrounded by a fibrous layer that separates the
laminated layer from host tissue. Eosinophilia is the common consequence of parasitic infection (Bell, 1996).

In this work, lung sections showed fibrous tissue reaction, capsules formation, cellular reaction, necrosis and collapsed lung tissue neighboring the cyst wall, while liver sections showed fibrous tissue reaction, cellular infiltration atrophy, necrosis and hepatocytes damage neighbouring the cyst wall. Parasitic membranes (laminated membranes and germinal layers) were obvious in most examined sections, some were continuous and intact containing brood capsules and protoscolices and others were disrupted. Some sections showed remnants of these membranes in both lung and liver sections. Laminated membranes were either moderate or large in thickness and multi-laminated (multi-layers). Parasitic sections (scolices) were seen in lung tissue in most sections in different numbers, whereas such feature was not observed in liver sections. Cellular infiltrations were in different manners, intensive (diffused) slight (scanty), but occasionally some sections showed focal cellular infiltrations as kind of granulomas and some showed tendency for giant cells formation. Types of infiltrating inflammatory cells were mainly mononuclear cells, macrophages, lymphocytes, plasma cells and neutrophils.

The fibrous tissue capsules are considered as one of host's defense mechanisms to restrict parasitic development. These capsules were seen in both lung and liver sections. In the former the changes neighbouring the cyst wall were cellular reaction, necrosis and collapsed lung tissue, while in the later, cellular infiltration, atrophy and necrosis of hepatocytes. This seems agree with former reports.

Cellular infiltrations were in different cell types and manners. Mononuclear cells (macrophages, lymphocytes, plasma cells) and neutrophils were the common cells. Eosinophils could not be seen. These results may agree with Adam, (1997), but disagree with (Petrova, 1968; Archer et al, 1977; Slais and Vanek, 1980; Richards et al., 1983; Riley et al., 1985; Bell, 1996 and Rigano et al., 1996) who reported presence of eosinophils. Early reports described no severe inflammatory response, but here we observed highly cellular infiltrations in and around the fibrous tissue capsules and sometimes extending to the parasitic membranes especially in caseated cysts. In such cases neutrophils were the predominant cells. This indicates that bacterial
contamination might have reached the cyst and induced that severe inflammatory response. Scanty cellular reaction also denotes mild host response. Chronic reaction was also marked in some cysts by presence of assemblages of macrophages (granulomas) in addition to presence of giant cells.

Parasitic membranes (laminated membranes and germinal layers) were seen in most sections. Some sections showed disrupted parasitic membranes. This disruption may happen during slides preparation or may indicate degenerative process.

Parasitic sections (scolices) were obviously marked in most lung sections in different forms.

In camel lung sections, the fibrous tissue capsules were moderate in thickness and in most sections the cellular reaction was minimal except in casiated cysts where cellular infiltrations were found to be severe invading the inner wall of the cyst. In such cases neutrophils were the predominant cell type and parts of them were degenerated or dead forming pus in the cyst cavity. Laminated membranes were mostly moderate, but were very wide and multi-laminated in casiated cysts. Most of the sections showed parasitic sections (scolices) spread in lung tissue in different numbers and one section was showed big cyst neighboured daughter cyst. Proliferation of the bronchial epithelium cells were marked in some sections, while some slides showed calcification, fibrosis in lung tissue and alveolar congestion.

Moderate fibrous tissue reactions and slight cellular reaction with presence of scolices in large numbers may reflect a mild inflammatory response. This may be due to camel's susceptibility to infection with *Echinococcus granulosus* with the as predilection site. Marked proliferation of bronchial epithelial cells could be as a sort of compensatory hyperplasia to compensate for the compressed bronchioles.

In camel liver sections, fibrous tissue reaction was also moderate as well as the cellular reaction, but one section showed heavy cellular reaction. Laminated membranes were disrupted in most sections and vacuolated in some. All sections showed atrophy, degenerative changes and necrosis in hepatocytes nearby the cyst wall. Sinusoidal dilatation, proliferation of small
capillaries, young fibroblast, fibrosis in portal areas, hemorrhages and fatty change were occasionally seen, but some calcifications were marked.

As in lung sections, fibrous tissue reaction and cellular reaction were moderate and that may indicate mild inflammatory response, but disruption in parasitic membranes and calcifications in most sections may indicate degeneration and necrosis in these cyst. These findings in addition to above mentioned results in cysts size and sterility of most liver cyst could give an idea that the liver is not a preferable site of hydatid.

In lung sections of cattle origin hydatid cysts, fibrous tissue capsules were broad, well developed and were supplied with a number of small blood vessels. Vacuolated round structures containing cells were observed in the most inner end of the fibrous tissue capsule. Cellular infiltrations were rather intensive or diffused, but some sections showed focal mononuclear cells infiltration in the form of granulomas. Cellular infiltrations were seen in most sections in the most inner side of fibrous tissue capsules before the laminated membranes. Neutrophils were seen invading the laminated membrane particularly in caseated cysts. Cellular structures resembling giant cells were seen in most sections at the inner part of fibrous tissue capsules just before laminated membranes. Cyst walls were observed to be irregular in most sections. As in camel lung cysts, laminated membranes were found to be broad and multi-laminated in the caseated cysts.

Cattle reaction to lung cysts was different from that in camels. Host fibrous tissue capsule were very broad and much developed than those of camels. Severity of cellular infiltrations also more than that found in camels. These findings could reflect some sort of immunological resistance to infection with *Echinococcus granulosus* in cattle, would explain low infectivity rate. In other point -at the molecular level- the camel genotype (G6) is the most prevalent stain of *Echinococcus granulosus* in Sudan (Dinkel et al., 2004; Omer et al., 2004), therefore, this severe immunological response may be a reaction against camel genotype (G6) of *Echinococcus granulosus*.

Most of cellular infiltrations were inbetween fibrous tissue capsule and laminated membrane. That could be as a result of the presence of many small blood vessels in fibrous tissue capsule, which may increase the chance to leukocytes to reach this area. Granulomas, presence of giant cells and
interlobular fibrosis were marked as features of chronic reaction. Vacuolated round structures were seen in the inner ends of fibrous tissue capsules in cattle, while that was not observed in camels. These structures seem to be giant cells.

In sheep origin hydatid cysts, all liver sections showed very wide zone of fibrous tissue capsules. Cellular infiltrations were diffused mostly, but number of sections viewed scanty cellular infiltration and others showed mononuclear cells in a manner of granulomas. Macrophages were predominant type of cells in addition to presence of Langhans giant cells. The parasitic membranes were not clearly seen, but some debris were obvious in cyst cavity. Perivascular cellular infiltration composed mainly of mononuclear cells and neutrophils was observed. Calcifications were marked in most sections. Neighbouring the cyst wall, many degenerated, necrotic hepatocytes with nuclear pyknosis, some mitotic figures and loss of hepatic structure were seen. Sinusoidal dilatations were occasionally marked as well as strangulated hepatic tissue surrounded by the fibrous tissue reaction. Some sections showed fibroplasia surrounding necrotic liver parenchyma, epithelial hyperplasia in bile ducts, bile ductules hyperplasia, vacuolar degeneration (regular & irregular), presence of large number of leukocytes in the blood vessels, and fibrosis in portal areas.

As in cattle, sheep also have broad fibrous tissue reaction capsules and diffused cellular infiltrations. This severe reaction also may reflect the modest infectivity rate, small cyst sizes, high percentage of sterile cyst and increased number of cysts undergoing calcification. Garnulomas and giant cells were also seen as indicator to chronic inflammation.

Necrotic changes in hepatocytes adjacent to cysts wall were marked and degenerated cells were recognized by nuclear pyknosis. Presence of mitotic figures could be considered as a sign of cellular division.

The acellular laminar layer is a carbohydrate-protein complex with galactose, galactosamine and glucosamine as the principal component of the polysaccharide portion (Kilejian and Schwabe, 1971). The germinal layer consists of distal cytoplasmic syncytium and a perinuclear layer containing tegument, muscle, glycogen, undifferentiated cells (Lascano et al., 1975; Thompson, 1995).
Histochemical studies were conducted in hydatid cyst wall. Since the laminated layer of the hydatid cyst is an acellular, polysaccharide protein complex, it stains strongly by periodic acid Schiff's reagent (PAS) and that provides a useful diagnostic marker in histological studies (Kilejian et al., 1962). Rashed et al., (2004) did comparative histochemical studies using periodic acid Schiff (PAS) and Mossaon's trichrome stains. The study revealed that the cystic wall consisted of three layers which are germinal, laminated and fibrous layer, respectively. The thickness varied with each stain. The glycogen and mucopolysaccharide content increased in infected sheep. Histochemical staining on human origin hydatid cyst was done (Handa et al., 2005) Masson’s trichrome stain. Laminated membrane appeared as fragments of acellular material with delicate parallel striations staining deep blue. Many scolices which were round to oval structures are about 100µm in diameter.

In this work, generally, laminated membranes, germinal layers, brood capsules and scolices in lung tissue have taken very deep positive PAS magenta color. This result is agreed with previous studies that the parasitic membranes are rich with mucopolysaccharides compounds. Parasitic membranes were intact, interrupted or as remnants in camels and cattle, while these membranes could not be seen in sheep origin hydatid cysts. Laminated membranes have variety of sizes. Filamentous rods on laminated membrane may probably be mycotic hyphae. Centric glycogen accumulation was seen in liver sections of sheep and this agrees with Rashed et al., (2004) who mentioned that glycogen contents increased in infected sheep.

Generally, Masson’s trichrome stain confirmed fibrous tissue reaction capsules, laminated membranes and germinal layers. Fibrous tissue reaction capsules were green in color, laminated membranes appeared as a cellular layers with slightly gray to green color, whereas germinal layers were nucleated and dark purplish in color. Brood capsules and scolices in lung tissue were also taking slight green color. Size variations of laminated membrane were also confirmed. Interlobular fibrosis in cattle lung sections was obvious. Fibrosis in portal areas in camels and sheep livers was quite remarkable. Irregular fibrosis with connective tissue fibers arranged in
different directions were observed in sheep liver sections and that may be a beginning of neoplastic changes.

*Echinococcus granulosus* exhibits substantial genetic diversity that has important implications for the design and development of vaccines, diagnostic reagents and drugs effective against this parasite. DNA approaches have been used for accurate identification of these genetic variants and has been applied in molecular epidemiological surveys of cystic echinococcosis in different geographical settings and host assemblages (McManus and Thompson, 2003). The recent publication of the complete sequences of the mitochondrial (mt) genomes of the horse and sheep strains of *E. granulosus*, and the availability of mt DNA sequences for a number of other *E. granulosus* genotypes, has provided additional genetic information that can be used for more in depth strain characterization and taxonomic studies of these parasites. (McManus and Thompson, 2003). A number of molecular techniques have been described previously in identification of *Echinococcus granulosus* worldwide (Bowles *et al.*, 1992; Bowles and McManus, 1993a; Gasser and Chilton, 1995; Bowles and McManus, 1993b; Scott and McManus, 1994; Siles-Lucas *et al.*, 1994; Dinkel, *et al.*, 2004). Utility of the PCR would be more accurate in diagnosis of echinococcosis even in atypical cases (Georges *et al.*, 2004). Identification of *Echinococcus granulosus* strains in Sudan is still limited in two reports (Dinkel *et al.*, 2004; Omer *et al.*, 2004).

The molecular biological study on 79 hydatid cysts collected from different anatomical locations of naturally infected camels, cattle and sheep was able to afford specific primary PCR products to all genotypes of *Echinococcus granulosus*- complex even in small amounts as little as 100 pg. The nested PCR amplification resulted in amplification of camel strain (G6) only in all 79 samples. Therefore, it is obvious that results obtained in this study agree with previous reports in high epidemicity of camel strain (G6) of *Echinococcus granulosus* in Sudan. Using the primary PCR amplification and the nested amplification, rapid, specific and sensitive detection of *Echinococcus granulosus*- complex and specific identification of camel strain (G6) could be afforded. The second amplification step using the nested primers EGL3and
EGR4 is necessary to confirm the specificity of the camel genotype and to increase the sensitivity of the PCR-based assay by at least 1000 times (Osman et al., 2007). The nested PCR does not require hybridization assay and this removes the hazardous and cumbersome radioactive laboratory procedures of working with 32P or 33P in hybridization assays. Sample preparation and DNA extraction using QIAamp extraction kit was a simple procedure, which takes one hour. The thermal cycling profiles for production of the primary and the nPCR products were consistently 4 hours. Running of the agarose gel and electrophoresis usually takes one hour. Thus, confirmatory diagnosis with camel strain using the described nPCR assay could be made within the same working day. Application of this nested PCR on a practical scale to determine the prevalence of camel genotypes of EG in infected animals in different states of Sudan is in progress. Based on these sensitivity and specificity results of this study, this PCR-based assay would be useful as a rapid test of further molecular surveys on camel strain (G6) of *Echinococcus granulosus*. 
Conclusion

From this study we can conclude the following:

- Camels have the highest prevalence rate of hydatidosis in Sudan.
- Prevalence rates of hydatid disease in cattle and sheep are lower than camels.
- Camels are important intermediate host of hydatid disease in Sudan.
- Lungs are predilection site of hydatid cysts in camels and cattle, while the liver is preferable site in sheep.
- Most cyst sizes approximately lie between 2 cm to 6 cm in camels and cattle and less than 2 cm in sheep.
- Cysts in camels and cattle have large amounts of hydatid fluid.
- Cattle origin hydatid cysts are more fertile than those of camels and sheep.
- Cysts under calcification are more in sheep origin cysts.
- Most of liver cysts are small in size, sterile and under calcification.
- Hydatid fluids of sheep origin have great total proteins and cholesterol concentrations than those of camels and cattle.
- Concentrations of total proteins and cholesterol are higher in degenerated or casiated cysts.
- Sodium and potassium contents in sheep cystic fluid were greater than those of camels and cattle.
- Phosphorous levels were higher in cattle cystic fluid.
- Calcium levels are higher in both camels and sheep cystic fluid, but not in cattle cystic fluid.
- Camels reaction to hydatid cyst is moderate, therefore, camels are highly susceptible to infection with *Echinococcus granulosus*.
- Cattle and sheep have severe immunological response to hydatid disease.
- Parasitic sections (scolices) could be seen in lung tissue in many numbers.
- Casiated cysts may be infected with bacteria or fungi.
- Parasitic membranes (laminated membrane and germinal layers) and protoscolices are many composed of mucopolysaccharide.
- Laminated membranes are a cellular and are composed of parallel striations and could be found in different sizes.
- Germinal layers are nucleated and are composed of many cells.
- The PCR-based assay using primers EGL1 and EGR2 could afford rapid, reliable, specific and sensitive identification of *Echinococcus granulosus*- complex.
- The nested PCR using the nested primers EGL3 and EGR4 could easily detect camel strain (G6) of *Echinococcus granulosus*.
- Camel strain (G6) is the most prevalent genotype of *Echinococcus granulosus* in Sudan.
- Described nested PCR assay could be used as a reliable diagnostic test for molecular epidemiological surveys of camel strain (G6) of *Echinococcus granulosus*. 
RECOMMENDATIONS

- Conduction of pathological study for specific genotype of *Echinococcus granulosus* in different intermediate hosts. For example pathological study on camel strain (G6) in camels, cattle and sheep.
- Conduction of microbiological examinations in casiated hydatid cysts.
- Conduction of further molecular surveys of *Echinococcus granulosus* to know more about the situation of hydatid disease in Sudan.
- Application of described nested PCR-based assay in molecular epidemiological surveys of camel strain (G6) of *Echinococcus granulosus*.
- Development of nested PCR-based assay for specific identification of remaining genotypes of *Echinococcus granulosus*. 
REFERENCES


