Sudan University of Science and Technology

College of Graduate Studies

Molecular and Serological Studies on Cystic Hydatid Infection in Man and Camels in Sudan

دراسة الاختبارات المصلية والجزيئية فى كشف الاصابة بالاكياس العدارية عند الانسان والحيوان بالسودان

A thesis submitted in fulfillment for the requirements of the degree of PhD in Medical Laboratory Parasitology and Medical Entomology

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Dedication

- *To the soul of my father who has dreamed a lot to see the day I get this title*

- *To my mother who has supported me and gave me her blessing to overcome all the difficulties I faced during my work*

- *To my brothers and sisters*

- *To my wife the one who was always behind me providing all the support encouragement, power and strength*

- *To my sons and daughters the beautiful smile which shone and enlightened my life during the last period of my work*

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Abstract

This study was carried out at Tambool area during the period 2011- 2013 where camels are slaughtered on Saturday and Tuesday every week. The study covered 200 camels. Aspiratations from 14 human that had or were suspected to have hydatidosis, were performed in Khartoum hospitals.

Camel: Macroscopic examination of the cyst was carried out after removing the tissue around the cyst by scalpel and looking for local site, and size of the cyst. Microscopic examination was performed for the fertility and viability of the cyst. The highest rate of infection in camels was found to be 26.5%, with a fertility rate of 68.0%. The high rate of infection was reported in the lung (90.0%). The fluid of hydatid cyst was extracated and treated as antigen and used for the serological tests.

Human: This study was carried out in patients who attended Khartoum state teaching hospitals. Specimen were aspirated from internal organs (lung, liver bone, spleen, cerebrospinal, and eye), from patients for diagnosis. To confirmed the serological investigations which were carried out in 14 patients showed four samples positive.

Histopathology: Microscopically, the hydatid cyst in most cases showed the typical structure of a common *Echinococcus* cyst. They consisted of germinal layer, cuticular membrane; fibrous capsule consisted of thick connective tissue layer which was infiltrated with aggregates of lymphocytes and plasma cells.

Polymerase chain reaction (PCR): PCR was carried out to show the gene and strain of *Echinococc*us *granulosus* and Gene 6 was the infectious gene for camel and human by PCR and RFLP.

The use of the serological tests in diagnosis of hydatidosis in man: The sensitivity of LA, IHA, ELISA, CIE and AGID were 80%, 80%, 85.7%, 66.7% and 57.1% respectively, and the specificity was 77.8%, 88.9%, and 71.4%, 87.5% and 71.4% respectively.

The use of the serological tests in diagnosis of hydatidosis in camel: The sensitivity of IHA, ELISA, CIE and AGID were 88.3%, 57%, 86.9% and 89.8% respectively, and the specificity was 90.5%, 86.9%, aand 44.4%, 78.0% and 82.0% respectively.

ملخص الدراسة

اجريت الدراسة بمدينة تمبول شرق الجزيرة و على الابل التى تنحر فى يومى السبت و الثلاثاء من كل اسبوع خلال الفترة من2011 الى 2013 . اجريت الدراسة على الاعضاء (كالكبد و الرئة). ازيلت جميع الانسجة المحيطة بالكيس العدارى باسخدام الملقط, المقص و احيانا المشرط. اتضح من خلال الفحص العينى و المجهرى بالاختباراتالمصلية كالاتى:

- 200 راس من الابل اجريت لها الفحوصات و كانت النتيجة 53 راس ابل مصاب بالاكياس العدارية بنسبة 26.5%.
- حيث ان فحص الاكياس العدارية اوضح مكان وجود و مدى خصوبة الكيس ان الاكياس الموجودة بالرئة اكثر خصوبة من الاكياس الموجودة بالكبد و علية نسبة الرئة المصابة 47 رئة اى 90% و الكبد6 مصابة اى 10%.
- اوضحت الدراسة ان الرئة هى المنطقة المفضلة للاكياس العدارية فى الابل و علية النتائج عكست اهمية الابل كعائل وسيط رئيسى للمرض الحيوانى المنشاء فى تمبول.
- استخلص السائل الموجود بالكيس وحضر كمصل بعد اجراء المعالجة الخاصة و استعمل فى اجراء الفحوصات المصلية التالية:
- الاختبار المصلى المباشر
- الاختبار المصلى الغير مباشر
- اختبارىى الرحلان المناعى و يظهر فية قوس
- فحص المناعة الامتصاصى الانزيمى و من المفضل اجراء نوعين من التفاعلات (الاليزاء). اثبت فحص المناعة الامتصاصى الانزيمى بالنسبة للابل ضعيف الحساسية اما بالنسبة للانسان فهو ذو حساسية عالية.
- فحص الانسجة المريضة اوضح العينات الاجابية وجود الاجنة و رووس الديدان المستقبلية و كذلك الفرق بين الاكياس العدارية و الاكياس المصابة بالالتهاب البكتيرى و التى بها قيح او صديد و الكياس السرطانية.

- تفاعل البلمرة المتسلسل و تقانة الانزيمات القاطعة لتحديد الانماط الجينية. اجرى هذاالفحص لتاكد من الجينات من طفيل الاكياس العدارية من الابل و الانسان و تم تحديد الجين (G6). هو الجين المسئول عن الاصابة بالمرض للابل و الانسان.
- كما ان هنالك فحوصات تساعد على التشخيص مثل: الاشعة, الاشعة المقطعية, الموجات الصوتية و الرنين المغنطيسى.
- الانسان: اجريت هذة الدراسة بمستشفيات ولاية الخرطوم على المرضى و المشتبة فيهم بداء الاكياس العدارية و جمعت العينات من السائل المسحوب من الطحال و الكبد و اجريت الفحوصات المصلية, الانسجة المريضة و تفاعل البلمرة المتسلسل وتقانة الانزيمات القاطعةعلى اربعة عشر عينة و اوضحت النتايج بان هنالك اربعة عينات ايجابية. كما اوضح تفاعل البلمرة المتسلسل وتقانة الانزيمات القاطعة و تفاعل البلمرة المتسلسل للجينات لتحديد الانماط الجينية و تم تحديد جين (G6).
- وعلية اتضح من هذة الدراسة ان داء الاكياس العدارية اكثر انتشارا فى الابل و ان الرئة بالابل اكثر ممنطقة مفضلة لنمو الطفيل من غيرة من الاعضاء الاخرى. كما اثبت ان G6 هو الجين الخاص بالابل و الانسان بفحص البلمرة المتسلسل وتقانة الانزيمات القاطعة و تفاعل البلمرة المتسلسل للجينات لتحديد الانماط الجينية.

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List of abbreviations

Abbreviation	Full name
Ab	Antibody
Ag	Antigen
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline with Tween- 20
PH	Hydrogen ion concentration
xg	Times of gravity
Rpm	Revolutions per minute
CE	Cystic echinococcus
LAT	Latex agglutination test
IHA	Indirect haemagglutination test
CCIEP	Counter current immunoelectrophoresis
AGID	Agar gel immunodiffusion
ELISA	Enzyme linked imunosorbent assay
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
G	Genotype
RFLP	Restriction Fragment Length polymorphism
EgG6	Echinococcus granulosus gene 6
Hph1	Haemophlus parahaemolysus 1
bp	Base pair
IEP	Immunoelectrophoresis test
CFT	Complement fixation test
RIA	Radio-immunoassay
BF	Bentonite flocculation
IFA	Indirect fluorescent antibody test
IED	Immunoelectrodiffusion

X-RAY	Standard radiology
US	Ultrasonography
C.T.scan	Computerized tomographic scan
MRI	Magnetic resonance imaging

CHAPTER ONE

INTRODUCTION

Chapter 1

1: Introduction:

Echinococcosis is a disease caused by the adults and larval (metacestode) stages of taeniid cestodes in the genus Echinococcus. Four species are recognized in this genus viz E. *granulosus, E*. multilocularis, *E*. oligarthrus and *E*. vogeli. *Echinococcus granulosus* has a cosmopolitan distribution and occurs in almost all ecological zones (Schantz et al., 1995). The other two species are prevailing in cool climates of the northern hemisphere and South–Central America. Transmission of *E*. *granulosus* occurs predominantly in synanthropic cycles where dogs act as the definitive host of *E*. *granulosus* shows high fertility in sheep and is mainly transmitted in a sheep–dog cycle (Eckert, et al., 2001).Many livestock-rearing areas of northern and eastern Africa have high prevalence of *Echinococcosis*, both in the human populations and farm animals (Macpherson, et al1997, Eckert et al., 2001). The genus *Echinococcus* (Family: Taeniidae) has been the subject of several taxonomic revisions since the 1960s.

All those species of *Echinococcus* known to cause CE in the intermediate host may be referred to as *E*. *granulosus* sensu lato (s.l.), whereas strains G1–G3 (which are closely related) are now referred to as *E*. *granulosus* sensu strictu (s.s.) (Nakao et al., 2013; Romig et al., 2015, this issue). The global public health impact of human CE is significant and is caused primarily by the G1 genotype (Budke et al., 2006). Other zoonotic species of *E*. *granulosus* s.l. include *Echinococcus* ortleppi (G5) and *Echinococcus* canadensis (G6–9) (Alvares Rojas et al., 2014); the zoonotic status of *Echinococcus* equinus (G4) appears unlikely (McManus and Thompson, 2003), Corresponding author. E-mail address: P.S.Craig@salford.ac.uk (P. Craig).and that of *Echinococcus* felidis remains unknown (Huttner et al., 2008). In central part of Sudan, ruminants and dogs are frequently affected (Saad et al., 1986). This diversity was confirmed by genetic studies which lead to recognition of a minimum of 10 strain-types G1–G10 (Thompsonو et al; 1995, Lavikainenو et al 2003.

Diagnosis of *Echinococcosis* in animals is primarily concerned with infections in dog and sheep hosts. The domestic dog is the key definitive host for *E*. *granulosus* s.s. and thus the

main source of human CE worldwide. Dogs also appear to be highly susceptible to all genotypes of *E*. *granulosus,* and may exhibit different pre-patentperiods (Carmena and Cardona, 2013). Wild canids (e.g. Canis lupus,Canis aureus, Vulpes vulpes) also show a range of susceptibilities(Jenkins and Macpherson, 2003; Rausch, 2003; Lahmar et al., 2009).Sheep (and goats) are the most important domestic intermediate host for *E. granulosus* s.s. G1, and this genotype itself may also infect other herbivore hosts (e.g. cattle, camels, donkeys and macropods) (Jenkins, 2006; Boufana et al., 2014). Small ruminants are also susceptible to other *Echinococcus* species or genotypes, for example *E.* canadensis (G6) in goats (Soriano et al., 2010).

1.1: Classification of *E. granulosus:*

According to Rausch (1994a), the systematic arrangement of *Echinococcus granulosus* was accepted as follows:

Kingdom: Animalia
Phylum: Platyhelminthes
Class: Cestoda
Order: Cyclophyllidea
Family: Taeniidae
Genus: *Echinococcus*
Species*:* *E.* granulosus
Binomial name: *Echinococcus granulosus* also called the hydatid worm or hyper tape-worm or dog tapeworm

Objectives

General objective

- To study the frequency of molecular and serological studies on Cystic Hydatid Infection in Man and Camels in Sudan

Specific objectives:

- From cysts fluid collected from camels for preparing antigen to study the sensitivity and specificity of IHA and ELISA tests.
- To investigate the occurrence of hydatid cysts in camels in Tambool area and in man in different surgical theatres.
- To characterize cysts derived from camels and man as well using the PCR technique.
- To study the histopathological changes of hydatid disease in lungs and livers of infected camels and man.

CHAPTER TWO

LITERATURE REVIEW

Chapter 2

2.: *Genus: Echinococcus:*

Cystic hydatid disease (CHD) is caused by the larval stages of the cestode and affects humans and domestic animals worldwide. Protoscoleces (PSCs) are one component of the larval stages that can interact with both definitive and intermediate hosts. Previous genomic and transcriptomic data have provided an overall snapshot of the genomics of the growth and development of this parasite. Currently there are six species recognized within the genus *Echinococcus* (Jenkins et al., 2005), these are E. *granulosus,* E. multilocularis, E. vogeli, E. oligarthus, E. ortleppi and E. equinus. Seventh species two relatively new species have been identified: *Echinococcus shiquicus* in small mammals from the Tibetan plateau and *Echinococcus felidis* in African lions (Xiao et al., 2005, Hüttner, et al 2008).Based on morphology, host specificity and molecular characteristics (Pearson et al., 2002, McManus and Thompson, 2003). The *E. granulosus* complex is divided into three species and eight defined strains. The present recognition of *Echinococcus* species reflects a series of largely host adapted species that are maintained in distinct cycles of transmission (Thompson, 2001, Thompson and McManus, 2002).These are characterized by the principal intermediate hosts which are; sheep, horses, cattle, camels and different species of rodents. Although these cycles of transmission may overlap in some geographical areas, the parasites involved have been shown to maintain their genetic identity (Thompson et al, 1995, Thompson and McManus 2001&2002., McManus and Thompson, 2003, Haag et al, 2004).ten distinct genetic types (G1-G10) of *E. granulosus* have been identified and divided into the following groups: *E. granulosus* sensu stricto (G1; sheep strain, G2; into the Tasmanian sheep strain, G3; buffalo strain), *E.* equinis (G4; horse strain*), E.* ortleppi (G5; cattle strain) *E.* canadensis (G6; camel strain, G7; pig strain, G8; cervid strain, G9; human strain, G10; Fennoscandian cervid strain) (Nakao, *et al.;* 2007).

2.1: Biology of *E. granulosus:*

Echinococcus granulosus is found in the small intestine of carnivores (particularly the dog) and the metacestode (hydatid cyst) is found in a wide variety of ungulates and man. The parasite has a cosmopolitan distribution. Adults are 2-7mm long and usually possess

three or four proglottids (rarely up to six). The penultimate proglottids are mature and the terminal proglottid is gravid and is usually about half the length of the worm. The rostellum has two rows of hooks. The ovary is kidney –shaped. Gential pores alternate irregularly and normally open in the posterior half of the mature and gravid proglottids. The uterus of the gravid proglottid has well-developed diverticulitis. The gravid proglottid usually disintegrates in the intestine so that only eggs and not proglottids are found in the faeces. The eggs are typical taeniid eggs and measure 32-36 by 25-30µm (Soulsby, 1986).

2.2: Geographical distribution

Echinococcosis/ Hydatidosis have a world-wide geographic distribution and occur in all continents. High parasite prevalence is found in parts of Eurasia (for example Mediterranean region, Russian Federation and adjacent independent states, the People's Republic of China), Africa (northern and eastern regions), Australia and South America. A few islands are now free of *E. granulosus* (Iceland, Greenland) or 'provisionally free' (New Zealand, Tasmania, southern Cyprus). The occurrence of *E. granulosusis* sporadic or has not been reported from other regions, including countries in northern and central Europe, in the Pacific Region, and in the Caribbean. The synanthropic cycle with domestic dogs as final hosts and sheep or other livestock animals as intermediate hosts predominates as an infection source for humans world-wide, (Eckert, et al, 2002). *Echinococcosis*/hydatidosis is one of the most important zoonotic diseases in the world. High parasite prevalence are found in the Middle East as well as Arabic North Africa (Anderson,et al1997, Romig, T., 2003)

2.2.1: The disease in Europe:

In Europe, zoonotic members of the *E.granulosus* complex have been reported in every country with the exception of Ireland, Iceland and Denmark. They are most intensely endemic in the Mediterranean areas and parts of Eastern Europe such as Bulgaria (Torgerson and Budke, 2003). In the UK, the parasite has a restricted distribution, being found mainly in mid and southern Wales.

2.2.2: The disease in the Americas: -

In the USA, most infections are diagnosed in immigrants from countries in which *Echinococcosis* disease is highly endemic. Sporadic autochthonous transmission is currently recognized in Alaska, California, Utah, Arizona, and New Mexico. *E.* granulosus infection has re-emerged in certain areas where it was once believed to be controlled. In Bulgaria the incidence of cystic *Echinococcosis* in children increased from 0.7 to 5.4/100 000 between the 1970s and the mid-1990s, following the collapse of control efforts, and in Wales the prevalence of infected dogs has more than doubled between 1993 (3.4%) and 2002 (8.1%), following policy changes favoring health education over weekly dosing of dogs with praziquantel. Romig, et al (2006).

2.2.3: The disease in Asia:

Echinococcosis is also unusual in northern Europe. The endemic areas are the Mediterranean countries, the Middle East, the southern part of South America, Iceland, Australia, New Zealand, and southern parts of Africa; the latter 5 are intensive endemic areas. Central Asia, particularly China, is also an endemic area. (Wang, et al 2013, Torgerson, et al 2013). In Asia, the parasite is endemic in large parts of China and is an important reemerging zoonosis in the former Soviet Republics in Central Asia (Torgerson et al., 2002a, b). Furthermore, human cases are regularly reported from medical centers in different parts of Iran. Population studies using serologic and ultrasonography has been used to determine the prevalence of *Echinococcosis* in human populations (Sadjjadi, et al 2001)

2.2.3.1: China

It is difficult to determine if cystic hydatidosis is increasing in China. Increasing numbers of people are treated annually, but this may just reflect improved diagnostic methods and improved outreach programs contacting communities hitherto poorly served with medicalfacilities. For a detailed description of the Chinese situation, the reader is referred to the review of (Craig (2004).

2.2. 4: The disease in Africa:

In Africa, cystic *Echinococcosis* (CE) is endemic in all North African countries including Morocco, Algeria, Tunisia and Libya. The disease has been also recorded from most of

Sub-Saharan states such as Sudan, Ethiopia, Kenya and Uganda, and most of Western, Central and Southern Africa (Torgerson and Budke, 2003; Cardona and Carmena, 2013). CE is a major public health problem among transhumant pastoralists of eastern Africa (Karamajong, Maasai, Nyangatom, Toposa and Turkana peoples) with the highest worldwide incidence in man in the Turkana communities in the north west of Kenya (Casulli etal.,2010).

. In a study on hydatid cysts collected in Algeria, sheep strain has been found in the human, sheep and cattle samples, Bart, et al (2004). Studies in Libya from each of 30 protoscoleces samples (12from cattle, 3 from humans, 5 from camels and 10 from sheep) and sequencing revealed that all isolates are identical to that published for the common sheep strain of *E. granulosus*, Tashani, et al (2002).

The study implies that human cases in Egypt are of the camel/dog strain, and camels are important hosts for the transmission of human hydatidosis, Azab, et al, (2004). Molecular studies in Tunisia on cyst material from camels demonstrated that the sheep strain of *E. granulosus.* So, it confirms that camels in Tunisia are infected with the sheep strain (G1) of *E*. granulosus in Libya, Tashani, et al (2002). In the current study, camels were infected with fertile cysts, which suggest that camels are suitable hosts for the sheep strain Lahmar, et al, (2004).

2.2.5: The disease in Sudan:

In the Sudan, the basic data on the prevalence of E. *granulosus* in dogs is still lacking. However, Saad and Magzoub (1986) reported that 25 dogs out of 49 (51%) were infected with *E*. *granulosus* in the Tamboul area in central Sudan. In another necropsy study conducted in 2001, 54.2% of the dogs in southern Sudan were recorded positive for the presence of *E*. *granulosus* infection indicating that the prevalence of CE in this area may be higher than in other parts of the world (Njoroge et al., 2001).

Studies conducted in Southern Sudan, infection rates of 7.1%, 2.7% and 4.3-7.1% were found in cattle, sheep and goats respectively (Njoroge et al., 2000; Omer et al., 2010). In another necropsy study conducted in 2001, 54.2% of the dogs in southern Sudan were recorded positive for the presence of *E. granulosus* infection indicating that the prevalence of CE in this area may be higher than in other parts of the world (Njoroge et al., 2001).

Reported a prevalence of 2% (132/6728) in mass population survey of seven villages in Southern Sudan **(**Elmahdi, 2003).

In central Sudan, a recent ultrasound survey with 300 and 651 people in two different areas showed prevalence in humans between 0.3% and 0.8%, respectively (Elmahdi, et al (2004). In 2005, an ultrasound survey by Elamin et al in Tambool area, central eastern Sudan, showed a prevalence of 0.92% of hydatid cysts in humans (M Elamin Ahmed et al, unpublished data). A more recent and extensive survey in the same area revealed a percentage of (1.04%) of patients screened having features of liver hydatid cysts on abdominal ultrasonography, M Elamin, Ahmed. (2010). Where the disorder does occur in Sudan, pulmonary presentations are common, accounting for 17 of 38 cases in one series from Khartoum, (Ahmed, ME, 2007)

2.3: *Biology of E. granulosus:*

Adult Worm*:* Mature worms are only found in the small intestine of the definitive hosts, which are carnivores (dogs and other canine). Adult worms are 3 to 7 mm long with only 3 proglottids. The body consists of a typically Taeniid scolex, a short neck and the proglottids; the mature segment is in the middle and the gravid is the last segment (Ghaffar, A., & Brower, G. 2010, Moro, P., & Schantz, P. M. 2009, Garcia,et al 2007, Badley, et al 2001).

Eggs*:* Eggs are found in the feces of the definitive infected hosts. Eggs are spherical with a diameter of 30-50 μm. The embryo, or oncosphere, is protected by the embryophore, which consists of keratin-like material. The embryophore is thick and impermeable, thus making the eggs extremely resistant.

Larvae form*:* When intermediary hosts (farm animals) or humans (accidental host) ingest the eggs, the oncospheres hatch and become activated and transported by the blood stream to the liver or other organs. Once the oncosphere reaches its final destination, it develops into a unilocular hydatid cyst which enlarges and produces protoscolices or daughter cysts within the hydatid cyst interior the oncospheres develop in the liver but some are swept to the lungs and more rarely to the long bones and central nervous system. Those which

develop in the liver migrate from the capillaries to the liver parynchyma, and in four days increase in size to about 250µ. After three months the cyst has reached 4-5 mm in diameter and an inner layer of epithelial cells which form the germinative membrane is visible. The cyst is surrounded by a layer of damaged liver cells, which as the cyst grows becomes replaced by a fibrous tissue capsule. The cyst continues to grow and after six months, it reaches 20 mm in diameter. Brood capsules which are formed from the germinal membrane are budded off, and float in the vesicular fluid forming hydatid sand. When 5-6 months old, the cyst is capable of producing infection in the dog in which the tapeworm takes six to seven weeks to reach maturity (Saad, 1985).

2.3.1: Definitive hosts:

The domestic dog is the principal definitive host of *E. granulosus*, but in certain regions, wild canids may be involved in the life cycle of the parasite. Although *E. granulosus* penetrates deep between the villi of the small intestine of a definitive host, there are no pathogenic effects even in animals with a heavy infection (Eckert, et al. 2001).

2.3.2: Intermediate hosts:

Infections with *E. granulosus* cysts in the intermediate hosts include herbivores, sheep, cattle, goats, pigs, horses, kangaroos, wallabies and camels. Worldwide, sheep are the major intermediate hosts. Sheep eat the worm eggs from pasture contaminated with dog faeces. These hatch inside the sheep, forming cysts. The life cycle is completed when dogs are infected through eating the offal of infected livestock or wild animals (Eckert, et.al 2001).In Sudan, sheep do not play any role in the transmission cycle (Saad and Magzoub, 1989). Camels proved to be the main intermediate host in central Sudan. Fertility rate of cysts in camels is 42.4%, and cattle 29% (Saad et. al.1983).

2.3.3: Transmission:-

Transmission to humans can occur through the fecal-oral route, ingestion of food or water contaminated with *E. granulosus* eggs released in the feces of final hosts such as dogs, or through hands contaminated with egg-containing soil, sand or hairs of infected dogs

(Moro, P., & Schantz, P. M. (2009).. Final hosts such as dogs acquire infection through ingestion of cysts, for e.g., slaughtered sheep carrying infectious cysts (Krauss, et al 2003)

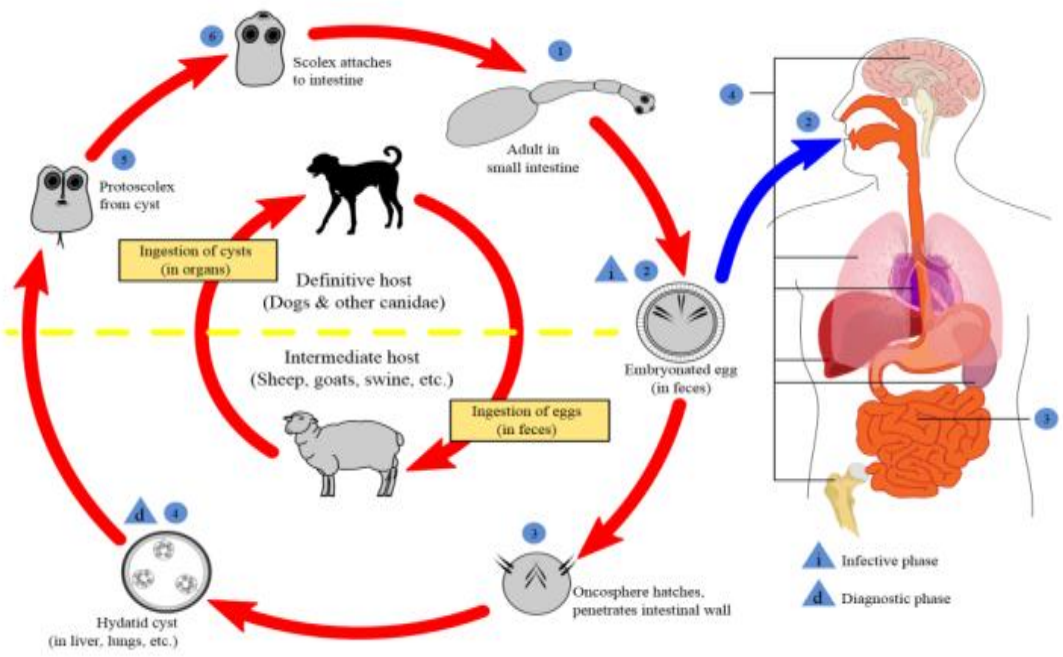


Figure 1: The life cycle of *Echinococcus* granulosus

(Centers for Disease Control and Prevention CDCP).

2.4: Experimental transmission of hydatid infection from camels and cattle to dogs

Hydatidosis/ e*chinococcus* in the Sudan were investigated by Saad and Magzoub (1986). They found the disease to be highly prevalent in camels (48.7%) and in cattle (3.8%). The reported fertility rates of cysts in camels and cattle were 49% and 29% respectively. In experimental infection studies of *Echinococcus granulosus*, by viable protoscoleces obtained from the lungs of naturally infected camels, Derbala and Al-Massry, (1999) stated that camel protoscolices were infective to all puppies and the pre-patent period was 56 days. The results of the present studies confirmed the previous studies, concerning high infectivity rates of the protoscolices removed from camel's lungs,

but it is different in the mean of the pre-patent periods. (Mohamed, 2004, Saad and Magzoub, ,1989a, Slepnev, et al., 1977).

2.5: Histopathology of the disease:

2.5.1: Introduction:

In the Sudan, many reports of cystic *Echinococcosis* have been described in humans and animals (Abdel Malek, et al 1959, Ahmed, et al 2011, Saad, et, al 1983), reported a high prevalence of disease among camels especially in the area where they are slaughtered in large number. The histopathological changes in different organs of camels and human due to the presence of hydatid cysts have not yet been studied in the Sudan. Surgery remains the mainstay in the treatment of hepatic hydatid disease. Cystectomy and pericystectomy offer a good chance for cure and should be undertaken wherever possible. Occasionally, formal hepatic resection will be required. Radical surgery – either pericystectomy or resection – is possible in 50–85% of cases. In the absence of complications this can be achieved with little mortality and an acceptable morbidity. Recently, laparoscopic pericystectomy has been demonstrated to be as safe and effective as open laparotomy in selected cases with hepatic and/or splenic involvement (Seven, et.al 2000).

2.5.2: In the lung:

Microscopically, the hydatid cysts in most cases showed the typical structure of a common *Echinococcus* cyst. They consisted of germinal layer, cuticular membrane, fibrous tissue capsule and cellular infiltration. The fibrous capsule consisted of thick connective tissue (C.T) layer which was infiltrated with aggregates of lymphocytes and plasma cells. In most instances, the fibrous layer was thicker than the inner chitinous layer. In old sterile cysts, the connective tissue capsule showed hyalinization focal necrosis and calcification. These two layers were clearly separated by a serious layer. The cellular infiltration was consisting mainly of lymphocytes and few plasma cells. In fertile cysts, this layer was diffuse and dense while sparse and focal in sterile degenerating ones. The cellular infiltration is usually seen adjacent to the lung tissue, but in some few cases it was in between the serous and fibrous layers. The adjoining lung tissue in most cases

exhibited slight alveolar oedema or at elacctasis and emphysema. In addition to mild or severe congestion. Bronchioles adjacent to the cyst were compressed. Some areas of focal cellular infiltration were observed in the lung tissue and around the bronchioles. Fertile cysts, scolices were found bordering the outer layer capsule or in between the lung tissue and the filtration zone (Saad et al, 1983).

2.5.3: In the liver:

In these organs the cyst had actual germinal layers and brood capsules with scolices, long the fibrous layers, area of dense infiltration were observed. These infiltrations consisted mainly of lymphocytes and plasma cells. The infiltration characteristically divided the fibrous into two layers and this lied between them. Marked infiltrations were lung and then the liver. Cysts in the spleen were found only in two cases. It was notices that lung infection was more common (90%) cases were detected. Hydatid cysts in liver were encountered in (9%) out of the effected cases. Hydatid cysts of liver and lung of the same animal were seen in 57 cases of camels and in 4 cases in human. Cyst in the lung and spleen in the same animals were observed in two cassette prevalence of hydatid cysts in different parts of Sudan. The following rates for the prevalence in lungs, livers. Spleen was observed (Saad, 1985). Uncomplicated of hepatic cysts, are common lesion linked by single layer of epithelium. They could be solitary or multiply, the computed tomography CT appearance of a simple hepatic cyst consists of well-circumscribed, homogeneous mass with no discernible wall. It has a no enhancement after intravenous contrast material administration, (Sayek, &Onat, 2001, Suwan, 1995)

2.5.4: In the spleen:

The histological structure of the hydatid cyst in this organ was principally similar to that seen in the liver. The host capsule consisted of two layers which were separated by focal cellular infiltration mainly lymphocytes. In one case, extensive haemorrhages were near the trabiculae and in the inner fibrous tissue layer (Saad et al, 1983).

2.6: Clinical features:

Many hydatid cysts remain asymptomatic, even into advanced age. The parasite load, the site, and the size of the cysts determine the degree of symptoms. A history of living in or visiting an endemic area must be established. Also, exposure to the parasite through the ingestion of foods or water contaminated by the feces of a definitive host must be determined. Theoretically, *Echinococcosis* can involve any organ. The liver is the most common organ involved, followed by the lungs. These 2 organs account for 90% of cases of *Echinococcosis.* In CE, symptoms can be produced by a mass effect or cyst complications. Symptoms due to the pressure effect of the cyst usually take a long time to manifest, except when they occur in the brain or the eyes. Most symptomatic cysts are larger than 5 cm in diameter. Organs affected by *E granulosus* are the liver (63%), lungs (25%), muscles (5%), bones (3%), kidneys (2%), brain (1%), and spleen (1%). I.S Dandan, (2016).

2.7: Complications:

The symptoms appear with pressure on the adjacent organs and the development of complications. Hydatid cysts can reach to large dimensions (≥10cm) without symptoms in the pediatric age groups since their immune response has not yet fully developed; furthermore, the parenchyma elasticity of the lung and the respiratory capacity is greater compared to adults, Arroud ,et al (2009). Such cysts generally lead to chest pain and coughing, depending on bronchial and/or pleural irritation and to dyspnea, depending on the pressure on the parenchyma, Arroud, et al (2009). Larger cysts may lead to the symptoms due to pressure on the adjacent vital organs, such as the esophagus, heart, trachea, and large veins and may cause mediastinal shift, cardiac malposition and arrhythmia, pressure on the main vascular structures, and Vena Cava Superior Syndrome, Yekeler, E, Karaoğlanoğlu N (2012).

Hemoptysis is a complication frequently observed as a result of the rupture of intraparenchymal cysts. It is reported at a rate of 1.8-8% in different series, Kılıç, et al (2007). Rupture is a predisposing factor for infection, which is the most serious complication. The frequency of this complication is reported between 30-90%. Infected

hydatid cysts can show a thick and opacifying wall structure, similar to lung abscesses, and includes air-liquid level and a pneumonic consolidation area can surround them, García, et al. (2010). When the cyst cavity is involved in the bronchial system, this prepares the environment for bacterial and fungal infections. The pneumonia in the peripheral parenchyma after an infection of the cyst cavity, bronchiectasis in the delayed cases and a destroyed lung are the pathologies encountered. (Kılıç, et al, 2007, Fındıkcıoğlu, et al, 2010, Balcı, et al, 2002)

Case that the cyst is infected, immune complex disease, glomerulonephritis, nephrotic syndrome, and secondary amyloidosis can develop, García, et al. (2010). Pleural involvement in hydatid disease is formed primarily with the hematogen or lymphatic route and direct larval infestation of the pleura or secondarily with the spread of cyst content throughout the pleura as a result of a rupture of an adjacent pulmonary or hepatic cyst, Arıbas, et al (2002). The complications are reported to be associated with the cardiac cysts: rupture, pulmonary and systemic embolism, heart block, cardiac valve obstruction, congestive heart failure, pericarditis, cardiac tamponade, acute myocardial infarction, anaphylaxis, and pulmonary hypertension, (Yekeler, et al, 2012, Laglera, et al, 1997). If the liver dome cysts become a perforated thorax, they may lead to pleural effusion, empyema, or parenchymal destruction. The opening of the liver hydatid cyst to the pleural cavity or bronchus is the cause of high mortality (9-43%) and morbidity, Rando et al, (2008).

2.8: Diagnosis of hydatid disease:

The detection of the characteristic structure and size of the hydatid cyst is visualized by various imaging techniques including:

2.8.1: Ultrasonography (US):

Abdominal ultrasonography has emerged as the most widely used imaging technique for *Echinococcosis* because of its widespread availability and usefulness for defining number, site, dimensions, and vitality of cysts.10 Portable ultrasonography machines have been applied for field surveys with excellent results. (Moro, et al 2005, Macpherson, et al 2003).

\2.8.2: Standard radiology (X-ray):

The radiological appearance of the hydatid disease in bone is not specific and mimics tumours and other inflammatory conditions. Solitary lesions in bone can be mistaken for a plasmacytoma, a simple bone cyst or a brown tumour of hyperparathyroidism. A botryoid configuration of the cysts could resemble a chondromyxoid fibroma or skeletal metastasis. A soft tissue component adjacent to a lytic bone lesion would raise the differential of a chondrosarcoma, osteomyelitis (pyogenic/tuberculous) or a giant cell tumour or an aneurysmal bone cyst **(**Morris, et al 2002**)**.

2.8.3: C.T. (Computerized tomographic) scan:

Several diagnostic methods have been employed, but CT scan has been providing definitive results up to recent years. The exact location, size and number of hydatid cysts in the brain can be determined with a CT scan. However, MRI is becoming more and more widely used as a diagnostic tool, as it can show some details that are not seen on CT. (El-Shamam, et al, 2001).

2.8.4: Exploratory cyst puncture:

Material obtained by biopsy puncture or surgery can be examined for hydatid fluid for *Echinococcus* protoscolices or hooks **(**Bahr and Bell, 1987).

2.8.5: Magnetic resonance imaging (MRI):

MRI gives good structural details of hydatid cyst. It does not provide additional information for the pleuropulmonary and abdominal lesion, and it is not cost-effective compared with US and CT. however MRI is superior in identifying changes in the intr-and extra-hepatic vascular system due to intrinsic contrast of vascular structure. (Buttenschoen,et al 2003, Pawlowski, et al 2001)

2.8.6: **Bronchography:**

Radiological findings change when the cyst ruptures into the bronchus and the air erodes the layers of the cyst. In countries like Turkey where lung cancer and cystic hydatidosis

are common causes of pulmonary diseases, the definitive diagnosis may become more sophisticated with cysts having a solid appearance or atypical localization mimicking bronchial carcinoma (Fidaneret al., 2001; Bozkurt et al., 2004; Yazar et al., 2008).

2.8.7. Angiography:

Pulmonary hydatid cyst embolization is rare complication of cardiac or hepatic echinococcosis. Jorens, et al (2009). It can be mistaken for pulmonary embolism based on the similar clinical manifestations of hemoptysis and acute onset of chest pain. Namn, et al (2013) Both spiral CT and MRI angiography can clearly disclose cystic occlusion of the pulmonary artery and its branches, Bayarogullari, et al (2013),

2.9: Serodiagnosis:

Infection with larval cysts of Echinococcus in humans and intermediate animal hosts results in a specific antibody response, mainly of the IgG class accompanied by detectable IgM, IgA, and IgE antibodies in some patients (Zhang, and McManus, 2006, Zhang, et al 2010, Sbihi, et al1997). In terms of methodology, almost all serological tests developed for immunodiagnosis of human CE cases have incorporated the detection of antibodies. There are considerable differences between the various tests both in specificity and sensitivity. As the sensitivity of a test increases, so generally does the demand for improved antigens in order that sufficient specificity can be achieved to take advantage of the greater sensitivity. An optimum test should be specific with high sensitivity. Insensitive and nonspecific assays including the Cassoni intradermal test, the complement fixation test (CFT), the indirect haemagglutination (IHA) test, and the latex agglutination (LA) test have been replaced by the enzyme-linked immunosorbent assay (ELISA), the indirect immunofluorescence antibody test (IFAT), immunoelectrophorosis (IEP), and immunoblotting (IB) in routine laboratory application. (Nasrieh, et al 2004):

Sensitivity * Specificity * Persistence * Ease of performance * Amount of antigen required.

This antigen mixture is used in several techniques such as the enzyme linked immunosorbent assay (ELISA), the indirect haemagglutination test (IHA), and the immunoblotting (IB). Both the ELISA and the IHA are usually the first line tests for CE

patients, while the IB is used as confirmatory test. As mentioned, the use of HF for the detection of CE specific antibodies is limited by several drawbacks. First, a percentage of CE patients are serological negative against HF. Specifically, the use of HF for the detection of total IgG in ELISA test leads to variable results regarding Se and Sp. In Table 1, a number of recent studies that used IgG-ELISA tests for CE diagnosis are shown. Se reported in these studies varied from 64.8% to 100%. Reasons for false negative results depend on several factors comprising cyst location other than the liver (Kilimcioğlu, et al 2013, Akisu, et al 2006). early (CE1) and inactive (CE4 and CE5) cyst stages

(Wang, et al 2013, Schweiger,et al (2012). Serum collection before treatment Tamarozzi, et al 2013, Hernandez**, (**2012).single and small cysts, (Hernández, et al (2012), and HF antigenic source variability. (Rahimi, et al 2011). Cross-reactivity is quite high in patients with other parasitic diseases, such as alveolar *Echinococcosis* (AE) and cysticercosis, but also schistosomiasis and fascioliasis Zhang,et al 2012, Moro, P. and Schantz, P. M., 2009).For instance, the cross-reactivity of E. granulosus HF with antibodies from AE patients can reach more than 50% (Hernández, et al (2012). The HF has also been shown not to be a good antigen for patients' follow-up during the clinical management of CE. During the follow-up, ELISA-IgG test is difficult to interpret Manterola, et al (2007). And anti-HF IgG antibody reactivity may remain high many years after successful cyst removal Galitza, et al (2006).

2.9.1: Latex agglutination test (LA):

Serological methods currently employed in the diagnosis of CE include the enzyme-linked immunosorbent assay (ELISA), the indirect hemagglutination test (IHA), the latex agglutination tests (LA), and immunoblots (Nunnari, et al 2012).

2.9.2: Indirect haemagglutination (IHA) test:

IHA was performed with sheep RBC that was sensitized by various concentrations of crude antigens and antigen B. The best result was obtained by IHA with applying antigen B (10µg/ml) for 40 min. at 37c° or 60 min. at room temperature. It is suggested that the IHA as serological assay, is valuable method with high diagnostic efficiency for serodiagnosis of hydatid disease, when is performed by purified antigen B. It is a rapid

diagnostic assay with any needs neither expensive instruments nor expert personal so is useful for seroepidemiological studies and field trial in endemic areas (Assmar, et al, 2004).

2.9.3: Enzyme linked immunosorbent assay (ELISA) test:

ELISA was performed in 96-well microtitration plates. The plate was coated with secreted antigens of *Echinococcus* granulosus (diluted in 100mM carbonate buffer PH 9.6) in order to give protein concentration 2.5 µg/ml to detect *E. granulosus* specific antibodies. All the solution were used as 300 µl per well (Hashemi, et al 2009).

2.9.4: Indirect fluorescent antibody (IFA) test:

Serological tests such as immunoelectrophoresis, double diffusion in agar, or indirect hemagglutination are being replaced by more sensitive assay methods such as enzyme-linked immunosorbent assay (ELISA), immunoblot (IB), and indirect immunofluorescent antibody test (IFA) (Virginio et al., 2003).

2.9.5: Immunoelectrophoresis (IEP) test:

Diagnosis was based on imaging techniques including ultrasound examination and computed tomography (CT) as well as immunologic tests, such as the immunoelectphoresis (IEP) and the indirect immunofluorscence assay (IFA); (Biaua, et al 2001, Carmena ,et al 2007).

2.9.6: Radio-immunoassay (RIA)

Radial immunodiffusion (RID) or Mancini method, Mancini immunodiffusion or single radial immunodiffusion assay, is an immunodiffusion technique used in immunology to determine the quantity or concentration of an antigen in a sample. Antibody is incorporated into a medium such as an agar or agarose gel (Berne, Bernard H 1974. Stanley, Jacqueline 2002). The antigen is then placed in a well that is punched out of the medium while the medium is on a microscope slide or in an open container, such as a Petri dish. The slide or container is then covered or closed to prevent evaporation(Stanley, Jacqueline 2002, Lsumc, 2002). The hydatid anti-based serological tests include indirect haemagglutination (IHA), indirect immunofluorescence (IFA),

immunoelectrophoresis, countercurrentimmunoelectrophoresis (CIEP), radio-immunoassay (RIA) and ELISA, (Ortona, et al (2000).

Immunoelectrodiffusion (IED):

Enzyme-linked immunoelectrotransfer blots (EITB), enzyme-linked immunoelectrodiffusion assay (ELIEDA), time-resolved fluoroimmunoassay (TR-FLA), and immunoblot assay have been developed for detection of anti-hydatid cyst antibodies (Aceti, et al**.** 1991. Ortona, et al 2000)

2.10: DNA markers:

Methods which examine the parasite genome directly are not confounded by variability induced by environment or hosts, or associated with the life cycle stage or posttranslational modification. Genetic variation can be investigated in either the mitochondria or nuclear related organisms because of its relatively rapid rate of evolution. (Elmahdi, 2003).

2.10.1: Restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR):

RFLP procedure has now been greatly simplified, without loss of resolution and accuracy, by linking rDNA RFLP analysis with the polymerase chain reaction (PCR) (Bowles and McManus, 1993). During the PCR, a fragment of DNA, defined by oligonucleotides primers at either end, is amplified several million fold using a thermo stable Tag polymerase (Saiki, et al, 1985). Ribosomal RNA genes are organized into rDNA units with the very highly conserved coding regions separated by relatively poorly conserved non-coding spacer region. One of the advantages of this method is its sensitivity, its possibility to type a single fresh protoscoleces using this technology. Furthermore, the approach is ideal for strain identification and the investigation of transmission pattern (Elmahdi, 2003).

2.10.2: PCR system for identification of *Echinococcus* species and genotypes:

Specific and sensitive PCR /semi nested PCR system for rapid diagnosis of *E. granulosus* genotypes G6/7 and *E. ortleppi* (G5) was developed by Dinckle et al. (2003). This system

includes a simple PCR specific for G1 as well as a PCR for diagnosis of G5/6/7. For subsequent discrimination between *E. ortleppi* and G6/7 two semi nested PCRs were used. Target sequence for amplification is part of the mitochondrial 12S rRNA gene. Specificity of the PCRs was 100% when evaluated with isolates of 16 cestode species including *E. multilocularis, E. equinus, E. ortleppi* and 3 strains of *E. granulosus* (G1, G6 and G7). Sensitivity threshold was 0.25 pg DNA. Additionally, two internal DNA probes were developed, one hybridizing only with G1, the other with G5, G6 G7 amplification products. The system could also be used for strain specific diagnosis of *Echinococcus* spp. eggs contained in fecal samples of final hosts. The sensitivity of a G1 specific coprodiagnostic PCR was sufficient to give a positive result with a single egg (Dinckle et al 2003, Elmahdi, 2003).

2.11: Diagnosis of *Echinococcosis* in the definitive host:

Diagnosis of echinococcosis in animals is primarily concerned with infections in dog and sheep hosts. The domestic dog is the key definitive host for E. granulosus s.s. and thus the main source of human CE worldwide. Dogs also appear to be highly susceptible to all genotypes of E. granulosus, and may exhibit different pre-patent periods (Carmena and Cardona, 2013). Wild canids (e.g. Canis lupus, Canis aureus, Vulpes vulpes) also show a range of susceptibilities (Jenkins and Macpherson, 2003; Rausch, 2003; Lahmar et al., 2009). Sheep (and goats) are the most important domestic intermediate host for E. granulosus s.s. G1, and this genotype itself may also infect other herbivore hosts (e.g. cattle, camels, donkeys and macropods) (Jenkins, 2006; Boufana et al., 2013). Small ruminants are also susceptible to other Echinococcus species or genotypes, for example E. canadensis (G6) in goats (Soriano et al., 2010). A wide range of other domestic livestock hosts are susceptible to CE and/or involved in transmission of E. granulosus s.l. and include cattle, yak, buffalo, camelids, pigs and equids (Eckert et al., 2001).

CHAPETAR THREE

MATERIALS

AND

METHOD

Chapter 3

Materials and methods

3: Study design: It is an observational, cross sectional study.

3.1: Study area:

3.1.1: Tambool study area (figure 2):

The study was carried out at Tambool town market (Central Eastern Sudan) which is located 150Km South East of Khartoum. Al butane area is part of the Central rain lands that provides good grazing for camels, sheep, goats, and cattle; stretches from the Ethiopian border in the East to Gezira State in the West roughly occupying the area between isohyets 400 and 700mm. It comprises 120,000 square kilometers and lies between latitude 13.5∘–17.5∘ N and longitude 32.4∘–36.0∘ E. It is situated in the rich savanna environment.

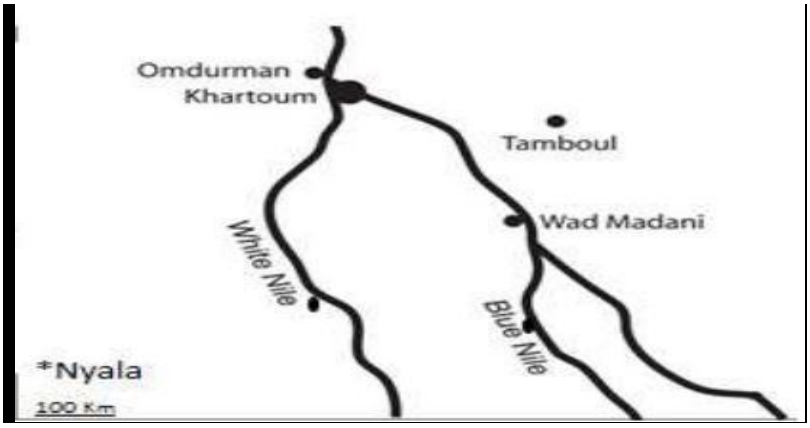


Figure 2: Map showing Echinococcosis: Epidemiology and Genotyping of Echinococcus Speices in Sudan, Ibrahim Elhag 2013)

3.1.2: Khartoum state study area:

The study was carried out at hospitals in Khartoum state (Alshaab hospital, Ibn seni hospital, Omdurman Asia hospital), and other private clinics. Specimens were taken from patients who have been already had or have been to have hydatidosis.

3.2.: Ethical consideration:

Ethical approval for the study was obtained from the Ethical Committee of the federal ministry of health, and permission was provided from all hospitals where investigation was conducted.

3.3: Sampling

3.3.1: Sample size:

The sample size was calculated according to the following formula:

$$N= \frac{z2\times pq}{d2}= \frac{(1.95)2\times 0.26\times 0.74}{(0.05)\,2}= \frac{3.8\times 0.19}{0.0025}= \frac{0.722}{0.0025}= 288.8= 289$$

Where:

N: required sample size

Z: confidence level at 95% (standard value of (1.95)

P: estimated incidence of *Echinococcus* in project area (0.5)

D: margin of error at 5% (standard value of 0.05)

N= **N=** 3.8×0.19 =0.722÷0.0025= 289. For camel.

N= 3.8×0.16 =0.608÷0.0025= 243.2.For man.

3.3.2: Study population and samples collection:

3.3.2.1: Human study group:

The study was conducted on patients' attending Khartoum, Ibn seni and Omdurman Asia hospitals. They had or have been to have hydatidosis at different hospitals in Khartoum state.

3.3.2.1.1: Aspiration of fluid:

Fourteen aspirations were taken from hydatid cysts patients from different hospitals

3.3.2.1.2: Serum collection:

Seventy eight serum samples were obtained from surgically or pathologically and confirmed by CT scan.

3.4: Histopathology:

Samples from the hydatid cyst were preserved in 10% formal saline for histopathology.

3.5: The PCR:

Fluid was aspirated from an individual hydatid cyst. Then, protoscoleces were rinsed in physiological saline solution fixed in 70% (v/v) ethanol and stored at -20 °C. For the genomic DNA (g DNA) extraction, the protoscoleces were rinsed several times with sterile distilled water to remove the ethanol prior to DNA extraction.

3.6: The healthy subjects:

Fivity blood samples were collected from healthy donors as the negative control negative. And also 86 specimens from patients with different diseases e.g. *leishmainiasis, filariasis, salmonallesis, malaria*, malignance tumor for cross reaction.

3.7: Camels study group:

The study was conducted on 200 camels slaughtered at Tambool market. Cysts were collected from livers and lungs of slaughtered camels.

3.7.1: Examination of hydatid cysts:

Cysts when found were collected and categorized according to WHO (2001) as follows:

A Active cysts with germinative layer and with living protoscoleces (examined the hydatid fluid for viability and protoscoleces staining by microscope) of there were normally round or oval with variable sizes ranging from 5cm to 10cm.

B. Inactive cysts (do not contain living protoscoleces).

1. Sterile cysts without germinative layer. It contains fluid that does not contain living protoscoleces. They were normally round but may be oval with variable sizes 5-10cm.
2. Calcified cyst which is characterized by thick calcified wall. Degree of calcification varies from partial to complete. It is round but may be oval with variable sizes 5-10cm.
3. Pyogenic cyst which contains few to uncountable pus cells. It is round but may be oval with variable sizes 5-10cm.

3.7.2: Macroscopic characteristics:

The hydatid cysts were having different sizes (small, medium, and large). The cysts washed by normal saline, into 50 ml tube transferred the cysts fluid and centrifuged at 500rpm for 5 min.

3.7.3: Microscopic examination:

The 4 positive cases were fertile and viable for protoscoleces. Fertility of the cyst was assessed by examining the cysts fluid for the presence of protoscoleces which were determined using staining with an aqueous solution of 0.1% eosin and examination under a light microscope. Live protoscoleces did not take the dye up, whereas the dead ones did (figure 5).

3.8.: Preparation of crude antigen:

The antigen was prepared in national public health laboratory at the department of parasitology using hydatid cystic fluid (HCF) of *Echinococcus* granulosus. The fluid was collected from cysts recovered from the livers and the lungs of camel. To remove the protoscoleces and large particles, HCF was centrifuged in cooled centrifuge (4°C) at 1000g for 30 min. Protein content of the sample was determined by protein biuret method.

3.8.1: Preparation of purified antigen

100 ml of hydatid cyst fluid (HCF) were dialyzed overnight against 5mM acetate buffer PH 5 at 4°C. The samples were centrifuged at 50.000g for 30 min. to remove the albumin. The supernatant was removed and the pellet was dissolved in 0.2M phosphate buffer PH 8. Saturated ammonium sulfate was used to remove the globulin from the sample. Finally the sample was boiled in a water bath for 15 min. and centrifuged at 50.000 for 60 min. to isolate purified antigen. Unlike crude antigen, purified antigen is heat stable.

3.9: Serological diagnosis:

3.9.1: Latex agglutination (L.A) test:

A drop of serum and a drop of antigen were mixed and rotated for two minutes. Agglutination indicates positive result. No agglutination indicates negative result.

3.9.2: Sheep erythrocytes:

3.9.2.1.: Method of agglutination of antigen-coated erythrocytes:

Fresh sheep blood was collected aseptically by bleeding into a flask containing equal volume of Elsevier's solution (Solution1) and the flask was gently shacked. The suspension was kept at least 3 days at 4 °C. 1gm of tannic acid was added to 0.35 ml of phosphate buffer saline and mixed with 50 ml of 4% SRBC suspension, then incubated at 37 °C for15 minutes. One aliquot was used for antigen coating and the other as control cells. Aliquot of cells were resuspended in 50 ml phosphate-saline buffer and 50 ml of HAS solution (2mg/ml- initial concentration) was added. Then the mixtures was incubated at 37 °C for 30 minutes and washed in phosphate saline buffer by gentle centrifugation and re-suspended in 100ml of borate- succinate buffer (Solution 2).

The aliquot of cells was resuspended in 100 ml of borate- succinate buffer.. 10 ml of 40% formalin was added to both cell suspensions while stirring. The formalin was added drop wise during 20 – 30 min and left overnight at 4 °C. Further 10 ml of formalin was added to both suspensions. The cells were left to settle (24hour) and washed again by sedimentation in borate-succinate buffer. Both cells suspension were adjusted to v/v and then 0.2% formalin (final concentration) was added as a preservative. Two packed tanned

RBC were also sensitized by adding 1:10 v/v of 5, 10, 15, 20µg/ml protein concentration of crude antigen and purified antigen separately. Mixture was suspended in PBS PH 7.2 and kept overnight at 4°C. Sensitized RBCs were packed and washed 5 times with PBS and adjusted to a 2-2.5 v/v suspension with normal rabbit serum (1:100 with PBS15mol.PH 7.2).

3.9.2.1.2: Assessment of results:

The microtitre plate was read either on the white surface or using a magnifying mirror. A typical pattern of agglutination is seen when the cells form a continuous carpet on the base of the cup. If no agglutination has occurred the cells fall as a tight button to the bottom of the V shaped plate (Solution 3).

3.9.2.1..3: Indirect haemoagglutination test:

The comparative sensitivity and specificity of the indirect haemagglutination test (IHA) for hydatidosis with tannic acid, and formal treated cells, the same pool of hydatid cyst fluid and sera from hydatid and non-hydatid persons were studied. The number of reactors in sera and the degree of reactivity of each serum sample varied with the IHA test. The sensitivity and specificity of the technique was related to the criterion on which IHA test positivity was based and to the group of sera examined. Of employing tannic acid was considered as the choice IHA test for hydatid immunodiagnostics.

3.9.2.1.4.: Method:

From PBS containing BSA 0.1% twenty five microliter were dispensed to each well of V bottomed micro titration tray, and then 25µl of serum was added to the first well of the appropriate row. The sera were then serially diluted up to the eleventh well, leaving the last well as serum free control. 25 µl volume of SRBC sensitized with the optimum sensitizing dose of antigen were added to each well. The plate was gently agitated for 2 minutes and incubated at room temperature for 30, 45, 60, 120 followed by incubation overnight at 4°C. Controls consisting of known positive and unsensitzed SRBC were included with each test.

3.10. Enzyme linked immunsorbent assay (ELISA):

ELISA was carried out in flat bottom 96 well micro plates. The plates were coated with 5 µg/ml of purified antigen (100ul/well) in coating buffer carbonate-bicarbonate, (Solution 4). Excess antigen was removed by washing the plate five times in phosphate buffered saline –Tween 20 (Solution 5) Blocking was done with 3% skimmed milk in PBST for 2 hours. The wells were washed and 100µl of serum samples (1/50 dilution in PBST) from surgically confirmed cystic *echinococcosis* patients with samples from healthy subjects as negative control and sera from patients with *leishmainiasis, filariasis, salmonellosis, malaria,* malignance tumor, were applied to the plate and incubated for 90 min, then the plate was washed as before and 100µl of conjugate was added and incubated for 60 min. at room temperature. After washing as before, the stop solution or blocking solution was added after 3o min. The absorbance at 450 nm was monitored with micro plate reader. The cut off was set as 2SD above the mean of control samples. ELISA was performed essentially as described by Barbieri (1998). Cut-off was calculated by the main absorbance reading in duplicated wells of seropositive and seronegative controls. IgG level in sample sera were determined by standard Serum (bohring) (Vatankhah, et al, 2004).

3.11: Countercurrent immunoelectrophoresis (CCIE):

3.11.1: Method:

1.5 gm agar powder **(**Solution 6) was dissolved in 100ml of distilled water. 0.9 gm was mixed with sodium chloride and autoclaved at 121°C for 15 minutes. Poured in microscopic slides and left to dry. By Cork borer, two wells were punched in the slide (4mm diameter). The wells were filled with patient's sera (30 µl of undiluted serum) and cathodes wells with 30µl of antigen of hydatid cyst fluid having 50µg/ml proteins. 250ml of buffer (Solution 7) were added to each side of chamber of electrophoresis tank, then run at 40mA in refrigerator for 2 hours. Then the slide was incubated in washing buffer (Solution 8) overnight. The slide was separated in single and the gel was left to dry at room temperature. The slides were stained by naphthalene blue (Staining 9) for 15 minutes after that

differentiated under differentiator solution (Solution 10) to remove any access stain.

3.12: Agar Gel Immunodiffusion (AGID) Test:

3.12.1: Gel Preparation:

1.5gm of agar powder was dissolved in 100ml of distilled water with 0.9 gm of sodium chloride and autoclaved at 121°C for 15 minutes. The mixture was cooled to about 60-80°C and dispensed in petri dish (85mm diameter) and let to dry on same day, By Cork borer five wells were punched in petri dishes (4mm diameter). Wells 5.3 mm in diameter, Agar 2.8 mm thick.

3.12. 2: Method:

The wells were filled with patient's sera (30 µl of undiluted serum) and 30µl of antigen of hydatid cyst fluid having 1.5 g/dl proteins in the center of the wells. After that the petri dish was incubated for7 days at 26°**C.** The buffer was washed overnight. The gel was cut and separated from the petri dish in single microscopic slide. The slide gel was covered by wet filter paper and left to dry at room temperature. Staining for 15 minutes after that differentiated under differentiator solution to remove any access stain.

3.12.3: Reading under view light

The slide was read under view light. Appearance of a line of precipitation between antigen and antibody wells was considered as a positive result.

3.13: Histopathology:

The technique was carried out on formalin fixed paraffin-embedded tissues (FFPT) from patients with histologically confirmed *echinococcosis* 2 sections (5 µm) were prepared from tissue blocks and excess paraffin was removed. Sections were placed in oven for dewaxen for 10 min at 37 °C. After dewaxen rehydration in 100%, 90%, 80% and 70% ethanol was followed by 70% ethanol for 3 min, then, water for 5 min. then stained in Mayer haematoxyline for 5 min. and bluing under tape water for 10 min and 3 minutes in eosin then rinsed 3 times in ascending alcohol and let to dry then mounted by DPX media.

3.14: Molecular diagnosis of *Echinococcus granulosus*:

3.14.1: Detection of *Echinococcus granulosus* by polymerase chain reaction:

Polymerase chain reaction (PCR) technique was used to detect *Echinococcus granulosus* in seven samples as confirmatory test. Using specific primers followed to amplify gene 6 of *Echinococcus granulosus* (table 1). The PCR followed by restriction fragment length polymorphism (RFLP) for detection of the sequence specific of EgG6 (table 2).

3.14.2: DNA extraction:

DNA isolation was carried out on cyst samples using DNA extraction. Kit (Germany) according to manufacturer's instruction (Fermentas). Approximately, 10 µl 0.02 N NaOH (try to transfer in less than 1 µl) protoscolices was mechanically grinded and incubated at 55 °C for 1-3 hours and terminated with 10 min incubation at 95 °C . The pure DNA was eluted in Tris-HCl buffer by effective washing and stored at -20 °C. Conc. of DNA 100-300 µg/ml (or 1 µl protoscoleces lysate or 1 µl of PCR product).

3.14.3: Polymerase chain reaction (PCR):

3.14.3.1: PCR reagents:

Reagents and materials required for PCR are listed in appendix15 Deoxynucleatide triphosphate (dNTPs) solutions were supplied either as 100mM stock solution diluting to 10 mM working solution by mixing 10 µl from each DATP, dCTP, dGTP and dTTP (100mM) and the volume completed to 100 µl with distilled water and stored at -20 °C or as ready dNTPs stock solution for direct use. Primers used for amplifying different genes (table 1) were supplied by company according to the required sequence of the primers were diluted to 10 µm (100 xs) according to manufacture instructor and stored at -20 °C.

Table 1: PCR of the nad1 gene:

Spp.	**primer**	Primer sequence	**Ta[a] °C**	bp[b]	Reference
E.gran.	Forword Primer nadB	**5' CAG TTC GGT GTG CTT TTG GGT CTG 3**	1073-1078 bp-long	55	**Hüttner et al (2008,2009).**
	Reverse Primer nadD	**5' GAG TAC GAT TAG TCT CAC ACA GCA 3'**			

Ta[a] °C: annealing at 55 **°C**

Product bp[b]: 1073–1078 bp-long Target gene: *nad1*, length of resulting fragment ~ 1075bp

3.14.3.2: PCR buffer and enzyme:

PCR buffer was supplied by the manufacture at10x concentrate solution (20 µl mix containing 1xPCR buffer (1.5mM Mgcl2, Kcl2, 10mM Tris-Hcl, PH8.3). and stored at -20°C. 200µM of each dNTP, 12.5 Pmol of each primer. And 1.25µl tag DNA polymerase enzyme.

3.14.3.3: General PCR protocol:

For PCR test, 2 primers were used to increase the sensitivity of detection. PCR reagent were pre mixed in sterile 0.5 µl microfuge tubes, labelled PCR tubes for the primary amplification reaction, was prepared in a 1.5ml sterile tube according to the number of samples. Then the aliquot was mixed in 0.5ml PCR sterile tube and1µl of extracted DNA was added to each corresponding labelled tube. Both positive and negative controls were included in each PCR run. Negative control tube containing PCR mixture without DNA was added to identify contamination of the PCR reagents with extraneous DNA. The positive control tube contained genomic DNA extracted from protoscoleces of hydatid cyst. This assured that all reagents were added and that correct cycling parameters were used. The following cycling condition were used for the reaction, primary denaturant 94°C for 5 min. 94°C for 30 seconds denaturing at55°C for 30 seconds annealing for55°C for 30 seconds, elongation for 72°C for 35 cycles, and final elongation for 72 °C for 5 min, and hold a temperature at 4°C (Solution 11) .

3.14.3.4: E.g.G6 specific PCR:

For the G6 specific PCR, the primer pair *E. granulosus* camel strain1 foreword Primer (E.g.nadB for) 5' CAG TTC GGT GTG CTT TTG GGT CTG 3 and *E. granulosus* camel strain 1 reverse (E.g. nad D rev) 5' GAG TAC GAT TAG TCT CAC ACA GCA 3'were used . The 100 µl reaction mixture consisted of 10 mM tris-Hcl(PH8.3), 50 mM KCl, 2 mM MgCl2 200µM of each dNTP, 25 pmol of each primer and 1.25 units Ampli-Taq Polymerase (Perkin Elmer Biosystems).

3.14.3.5: Analysis of PCR production (table 2):

Following PCR, agarose gel electrophoresis was used for detection of presence of the target DNA sequence and the variation within it.

Preparation of 1.5% agarase, a weight of 1.5 gm agarase powder were dissolved in 100 ml of 1xTBE, the solution was then heated until the agarose completely dissolved, the gel was then allowed to cool to about 60% , to this 5 µl of ethidium bromide solution (0.5 µg/ml) were added. The solution was then poured onto the gel tank with the suitable of combs, and allowed to set. The combs were removed and 1xTBE solution (0.09boric acid, 0.09 M Trise base, 0.002M EDTA) was poured to cover the gel.

The following PCR amplification 5 µl of PCR product was mixed with 2 µl loading gel dye. The molecular weight marker buffer containing (5mg/ml) visualize the DNA 1075Pb ladder. The gel was run for about 30 min. In 1XTBE at 50–100 volts. The gel was examined using gel documentation system. The PCR control and samples were examined and using the UVI images software package.

Table 2: Analysis of PCR production:

Species	Enzyme	Primer sequence	Ta[a] °C	Product (bp[b])	Reference
E. granulosu	Hph 1	Forward: 5ATTTTTAAAATTCGTCCTG-3	55	254 bp-long	Hüttner et al 2008,2009
		Reversed: 5CTAAATAATATCAATTACAAC-3			

3.15: The determination of the sensitivity and specificity of serological tests:

The sensitivity and the specificity for serological test were more determined by the use of the fourfold classification (table 3) according to Schanz, et al (1986)

Table 3: determination of sensitivity and specificity for serology test

Screening	True diagnosis		Total
Test	+ ve	-ve	
Positive	A	B	a + b
Negative	C	D	c + d
Total	a +c	B + d	a+ b+c+d

The sensitivity and specificity is calculated by $\frac{a}{a+c}\ x100$.

This is the percentage of camels and human with hydatidosis who were detected by the test.

Specificity is calculated by $\frac{d}{b+d}\ x100$.

This is percentage of non diseased camels and human who were negative to the test.

The false positive are calculated by $\frac{b}{b+d}\ x100$.

The false negative were calculated by $\frac{c}{a+c}\ x100$.

3.16: Statistical Analysis:

Epidemiological data were analyzed by using the Statistical Package for Social Science (SPSS). For genotyping bands pattern obtained were compared with published band pattern reported by Huettner *et al.,* (2009) for RFLP PCR and by Dinkle *et al.,* (2004) for specific G5/6/7 PCR (specific G5 PCR and specific G6/7 PCR

CHAPTER FOUR

RESULTS

CHAPTER 4

Results

4: Field investigation on cystic hydatid Infection in camel and human

4.1: Man:

Fourteen cases were aspirated (table 4); (7 livers, 5 lungs, one abdominal and one spleen). Out of the 8 male samples, 25% were found positive while out of 6 female samples, 33.3%% were found positive. The 2 positive females aged 16 years with a large cyst (9.8x9.6x8.9cm) in the right liver lobe. This was detected by CT scan. The other female was 42 years old with a lung hydatid cyst. The age group of the other patients ranged from <15 to > 60 (table 5).

From 8 patients in Khartoum state there were 2 patients positive for hydatid disease. One of the patients52 years from Elgedarif state had hydatid cyst in the liver while other 14 year old boy from Elgezira state had hydatid cyst in the lung. (Table 6) on the other hand the comparison of sensitivity and specificity of serological tests detection for hydatidosis in human and the results of serological tests for patients, healthy subject and other parasitic infection are shown in tables 7 and 8.

Table 4: Aspiration for hydatid cyst among human

Location	Lung	Liver	Spleen	Abdominal	Total
Positive	**2**	**2**	**0**	**0**	**4**
Negative	**3**	**5**	**1**	**1**	**10**
Total	**5**	**7**	**1**	**1**	**14**

Table 5: The location of hydatid cyst against age group

Location	<15	16– 30	31 – 45	46 -60	>60	Total
Liver	0	1	4	2	3	10
Lung	1	0	1	0	0	2
Spleen	0	0	1	0	0	1
Abdomi.	0	0	0	1	0	1
Total	1	1	6	3	3	14

Table 6: The location of hydatid cyst in different organs against sex

Gender	Liver	Lung	Abdominal	Spleen	Total
Male	5	1	1	1	8
Female	5	1	0	0	6
Total	10	2	1	1	14

4.2. 1: Reading under view light

Reading the slide under view light revered the appearance of one line or more of precipitation between antigen and antibody wells was considered as a positive result.

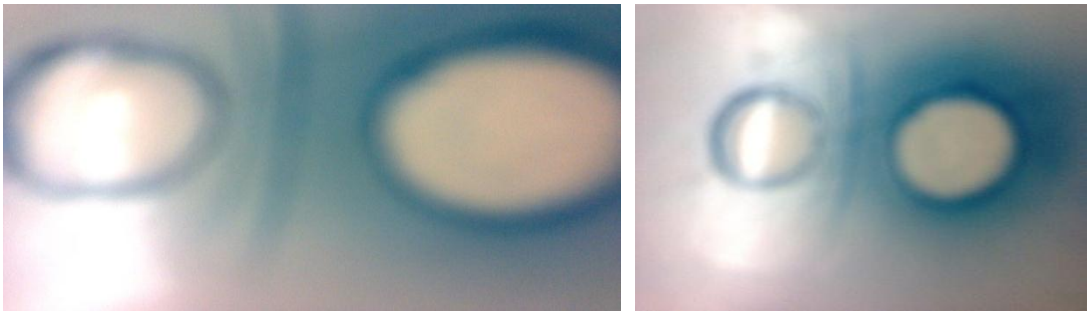


Figure3: show the precipitated line when positive by CIE

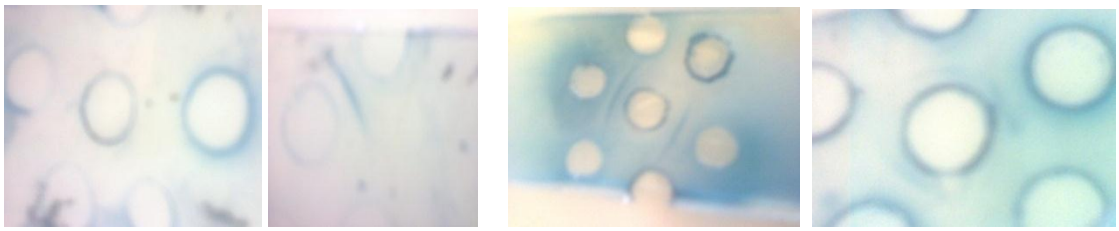


Figure 4: shows the precipitated one line or more when positive by AGID.

Figure 1: Negative **fig.6-.2**: one line precipitated **fig.6-3:** two lines precipitated **fig.6-4:** three lines precipitated by AGID

Table 7: comparison of sensitivity and specificity of serological tests in detection of human hydatidosis.

Types of test	LA	IHA	ELISA	CIEP	AGID
Positive	4	4	4	4	4
Negative	10	10	10	10	10
Sensitivity	80.0%	80.0%	85.7%	66.7%	57.1%
Specificity	77.8%	88.9%	71.4%	87.5%	71.4%

Table 8: Results of IHA, ELISA and CIE tests in patients with hydatidosis, healthy individuals and others parasitic infections

Factor		Positive reaction			Negative reaction			Sensitivity			Specificity		
Type serum	No. serum	IHA	LISA	CIE	IHA	ELISA	CIE	IHA	ELISA	CIE	IHA	ELISA	CIE
Hydatidos	14	4	4	4	10	10	10	80.0	85.7	66.7	88.9	71.4	87.5
Serum patients	78	4	4	4	74	74	74	0	0	0	0	0	0
Healthy	50	0	0	0	50	50	50	0	0	0			
Other diseases	86	0	0	0	84	84	84	0	0	0	0	0	0
Total	228	4	4	4	144	1	144	0	0	0	0	0	

Table 9: The use of the serological tests in diagnosis of hydatidosis in human

Test	Sensitivity	Specificity
Latex agglutination (L A)	80.0%	77.0%
indirect haemagglutination test	80.0%	88.9%
Enzyme Linked immunosorbent assay ELISA)	85.7%	71.4%
Countercurrent immunoelectrophoresis (CCIEP	66.7%	87.5%
Agar gel Immunodiffusion (AGID) test	57.1%	71.4%

4.3: camel:

4.3.1: Hydatidosis in slaughter house:

The infection rate of hydatid cyst in camels was found to be (26.5%). lungs were found to be the main predilection site of camel cysts (table 9). About 5% of camel cysts were 2 cm in diameter and 52% were 2 - 6 cm in diameter, 37% of camel cysts were 7 – 10 cm in diameter and only 5% were more than 10 cm in diameter (table 10).

Table 10: The infection rate of hydatid cyst in camels slaughtered at Tambool during 2011 - 2013

Animal species	No. of animal examined	No. of Infected animal	% rate of infection	Total no. of cysts counted	Location of cysts	
					Lung	Liver
Camels	200	53	26.5	99	90 90%	9 9%

Table11: Frequency distribution size and type of hydatid cysts collected during inspection of camel at Tambool slaughter house in the central of Sudan

nimal	No. of cysts	Size of cysts (cm)				Frequency of cysts			Type of cyst		
		< 2	2– 6	6– 10	>10	2-5	5-10	>10	Fertile	Steril	Calcified
200	99	5	52	37	5	46	48	5	68	6	25

4.3.2: The fertility rate of the cyst:

The total number of cysts encountered was 99 cysts. The majority of the cysts were fertile, the number of fertile cysts in the lungs (64 fertile cysts) was approximately 15 times greater than in the liver (4 fertile cysts), and the majority of the sterile cysts (5) were in the lungs then in the liver (1), and calcified cysts (25) were in the lungs then the liver, The highest and lowest rate of fertility was observed in hepatic cysts, (table 11, and figure 5).

Table12: fertility rate of hydatid cysts and viability of protoscolices of fertile cyst recovered from different organs of camel at Tambool slaughter house.

Location	Fertile	Sterile	Calcified	Viability of protoscolices in fertile cyst	Total
Lung	64	5	21	64.6	90
Liver	4	1	4	9.1	9
Total	68	6	25		99

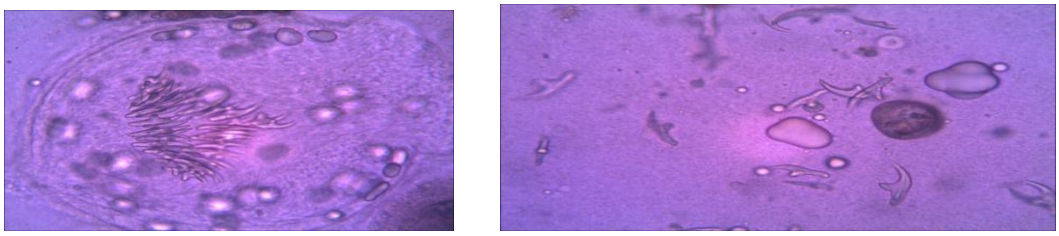


Figure 5: shows the viable protoscoleces and hook let stain by staining with an aqueous solution of 0.1% eosin.

Table 13: comparison of sensitivity and specificity of serological tests in detection of camel hydatidosis.

Types of test	LA	IHA	ELISA	CIEP	AGID
Positive	53	53	53	53	53
Negative	147	147	147	147	147
Sensitivity	93.0%	88.3%	57%	86.9%	89.8%
Specificity	90.5%	86.9%	44.4%	78.0%	82.0%

Table14: **The use of the serological tests in diagnosis of hydatidosis in camels**

Test	**Sensitivity**	**Specificity**
Latex agglutination (L A)	93.0%	90.5%
indirect haemagglutination test (IHA)	88.3%	86.9%
Enzyme Linked immunosorbent assay ELISA)	57.0%	44.4%
Countercurrent immunoelectrophoresis (CCIEP	86.9%	78.0%
Agar gel Immunodiffusion (AGID) test	89.8% %	82.0%

4. 4: Histopathology of the disease:

4. 4.1: Histopathology:

The main microscopic changes were characterized by proliferation of connective tissue, infiltration of mononuclear cells and presence of atrophy of the host tissue around the cyst in both cysts of camels and human. The cyst showed the characteristic changes similar to these resulting from chronic inflammation. Section of the specimens (figure 6) Section of the specimens show cyst wall composed of fibrous laminated layer with inner germinal layer with broad capsule and scolices surrounded by fibrous capsule.

4.4.2: Result of histopathology:

1- **Specimens (1,4 AND 5)**

Specimen (1): liver tissue

Specimens (4 and 5): lung tissue

Diagnosis: hydatid cysts

Microscopy: Section of the specimens show cyst wall composed of fibrous laminated layer with inner germinal layer with broad capsule and scolices surrounded by fibrous capsule.

2. **Specimen (2):** Lung tissue

Diagnosis: pyogenic cavity

Microscopy: section show lung tissue with cyst wall composed of inflammatory granulation tissue with suppuration. No hydatid cyst.

Specimen (3): Fibrovascular tissue with inflammatory granulation tissue. No hydatid cyst.

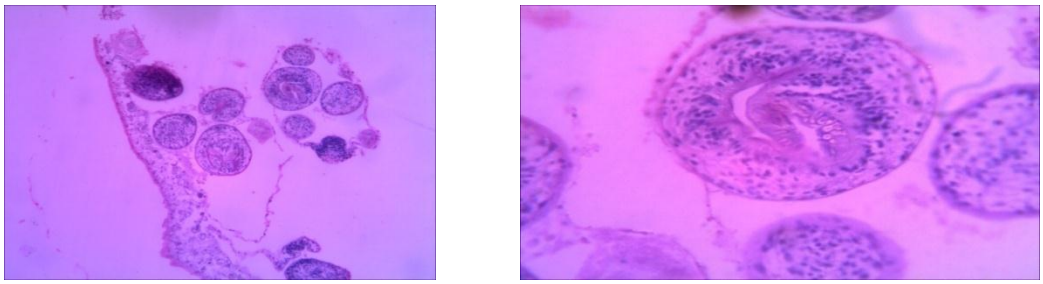


Figure.6: Section of the specimens show cyst wall composed of fibrous laminated layer with inner germinal layer with broad capsule and scolices surrounded by fibrous capsule.

4.5: Molecular diagnosis of *Echinococcus* granulosus:

4. 5.1: Detection of *Echinococcus* granulosus by polymerase chain reaction

4. 5.2: Strain characterization of *Echinococcus granulosus*

In this study a total of 57 cysts *Echinococcus granulosus* (4men and 53 camels) were genotyped by PCR-RFLP, gene sequencing (G6) specific PCR. The isolates were categorized into distinct and uniform genotypic grouping. The data indicated that the man and camel strain (G6) occur in central Sudan (figure 7).

4. 5.3: PCR-RFLP:

Represents PCR amplified NAD1 fragments of representative isolates. Only one fragment with a molecular weight of 1 kb was produced by *E. granulosus* isolates examined. Out of 57 specimens' isolates of human and camel originated from Sudan, 5 were detected as G6 genotype with PCR-RFLP but two isolates could not be amplified giving sensitivity the PCR-RFLP. Although of diverse geographical origin and intermediate hosts of camels and human isolates they gave similar RFLPs pattern in NAD1Hph1 restriction enzymes.

Restriction with Hph1 endonuclease revealed 5 major bands in *E. granulosus* as illustrated in the figure 8. These bands had a molecular weight of 700,390, 250, 210 and

180bp.whereas human *E. granulosus* isolates had only two bands pattern with a molecular weight of 300 and 700bp**.**

4.5.4: G6 specific PCR:

This PCR amplified the target sequence of 12S rRNA gene. The G6 specific PCR selectively amplify G6 genotype of *E. granulosus* with characteristic band of 254bp. All Sudanese *E. granulosus* isolates (human and camel) gave amplification bands with 254bp. (Figure 7).

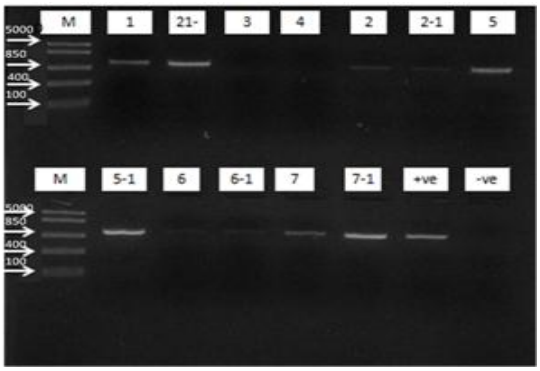
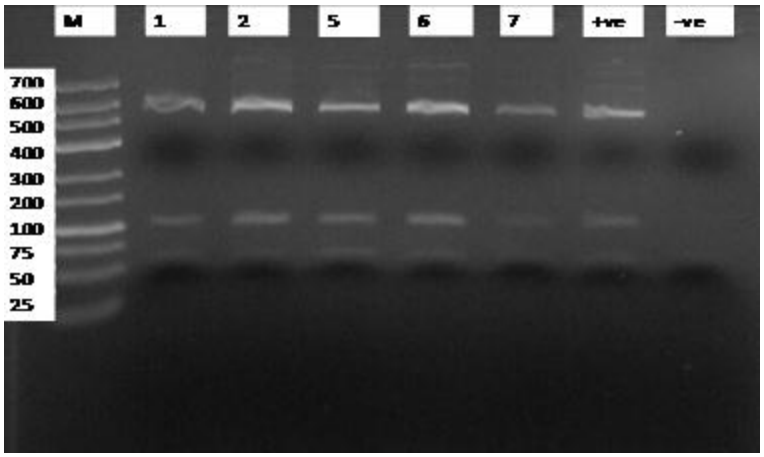


Figure7: PCR of nad 1 gene.

Demonstrated the PCR result for the diagnosis of EgG6 among samples collected from lung and liver of camels and human in Tambool slaughter house and hospitals of Khartoum state by PCR from left to right 1,1.2.2,2-1,3, .4, .5,5-1.6,6-1.7,7-1. M (molecular marker 1075 bp).



Figar 8: RFLP-PCR of the nad1 gene with restriction enzyme Hph1

This fragment was digested by restriction enzyme Hph1 yield 200 and 650 bp fragment M. Marker .1: camel, 2, 3, 4, and 5 human 6: camel and 7 human. Positive control, negative control.

4.6: Healthy subject and cross reaction samples

Carried out on 50 blood samples collected from healthy subject for negative control, and also 86 blood samples collected from patients with *leismainaisis* (26 samples), *salmonellosis* (16 samples), malaria (4 samples), *Onchocercia volvulus* (20 samples), and 20 serum samples for malignant tumor for cross reaction.

Table 13: show the serological tests among donor control and patients for cross reaction

Test	Positive	Negative
Latex agglutination (L A)	0	0
indirect haemagglutination test (IHA)	0	0
Enzyme Linked immunosorbent assay ELISA)	0	0
Countercurrent immunoelectrophoresis (CCIEP	0	0
Agar gel Immunodiffusion (AGID) test	0	0

CHAPTER FIVE

DISCUSSION

Chapter5

Discussion

The diagnosis of CE mainly depends on radiological and immunological procedures. Imaging methods are sometimes limited by small size of the lesion and atypical images which are not easy to be distinguished from abscesses or neoplasm. Routine laboratory diagnosis of CE is dependent on detection of specific antibody response. Serum is generally used for detection of specific antibody although some studies show the detection of antibody in urine might also be a good alternative (Sunita et al 2007). Hydatid cyst fluid (HCF) is a complex mixture of glycol-and lipoprotein, carbohydrates and salts. Some of its components derive from the host (mainly albumin and immunoglobulin`s), while the remaining are the products of the metacestode. HCF is considered the main antigenic source for immunodiagnostic of human CE. For clinical practice, crude HCF has a high sensitivity, ranging typically from 75% to 95% (Pawlowski et al, 2001). However its specificity is often unsatisfactory and cross-reactivity with sera from patients infected with other cestode, nematode and trematode species is commonly reported (Eckert and Depalez`z 2004). The results of the serological tests including LA, IHA, ELISA CCIP and AGID indicated that the crude and purified hydatid fluid can be usefully applied in serological diagnosis, as an antigen. They have shown that the antigen gave a sensitivity of 80.0%, 80.0%, 85.7%, 66.0% and 57.1% respectively, which is in agreement with other reports (85.0% - 92.0%) (lui, 1992, Njeruh, 1989, Navarrete, 1995). The specificity 77.0%, 88.9%, 71.4%, 87.5, and71.4%, respectively is similar as previous studies (Bombardieri & Ggiordano 1974, Kaddah et al 1992). The serum of all normal persons gave negative result in LA, IHA, ELISA, CCIP and AGID and cross reaction also is the similar result. (Babba, and Messedi, 1994, and Rickard, 1979).

Malignant tumor shows a more serious clinical picture and the imagistic computed tomography, ultrasonography and imaging through magnetic resonance makes the difference diagnosis. Polycystic kidney disease is always bilateral and the renal function and appearance of arterial hypertension are almost present. Renal abscess comes from skin infection and it ultrasonographically reveals a hipoechogen content of this (Krige and Beckingham, (2001). Demonstrated that the ELISA was able to detect hydatid antibodies either by purified or crude hydatid fluid antigens. For screening and epidemiological study

of hydatid disease the sensitivity and specificity of ELISA comparable to the AGID test (Bombardieri & Ggiordano 1974, Navarrete, et al 1995).

Since the ELISA has not routinely carried out in all laboratories, because of its equipment, and a reliable test has not established for definitive *echinococcal* antibodies in hydatidosis. In conclusion, according to this and other comparative studies, the IHA and AGID are suggested for diagnosis of hydatid disease and screening of serum samples in epidemiological studies in high risk population.

The lethality of CE is considerably high up to 60% in patients without surgical intervention (Schantz, 1997). The prevalence of CE in human in areas of extensive farming reaches up to 5 % (Schantz et al, 1995). Therefore, (CE) is particularly important in developing countries, where many nomadic rural inhabitants live under poor sanitary conditions without adequate supplies of clean water and in close proximity to their domestic animals (Anderson, 1997).

In Sudan several studies documented the endemicity of cystic *Echinococcosis* in different part of the country (Saad and Magzoub 1989; Elmahdi *et al*; 2004; Osman *et al*., 2007). The result from this survey is lower than those observed, in the same region, by Elmahdi *et al., (*2004). Who reported prevalence of 44.6%, 6.9% and 3.0% in camel, sheep and cattle, respectively, the infection rate reported (26.5%) is likely reflecting the true epidemiological situation in camel since only one camel infected in age group less than three years. However, the prevalence of *E. granulosus* is known to be positively correlated with age in sheep (Lahmar *et al.,* 1999, Dueger and Gillman 2001). Specific PCR for identification of G6/7 and G5 described previously by Dinkle, is proved to be useful for epidemiological studies (Dinkle, *et al*., 2004). Epidemiological studies indicated that the camel strain (G6) rep-resents the most prevalent genotype circulating in Sudan[(Ahmed and Aradaib; 2006, Ibrahim,et al 2011).

As indicated in this study, 81 *Echinococcus granulosus* isolates genotyped by both RFLP-PCR and specific G5/6/7 PCR were identified as G6 genotypes. However, in most areas where CE is a major public health problem, it is the G1 genotype (which is highly fertile in sheep) that predominates. Dinkel *et al.,* (2004) previously detected G6 (camel strain) in 44 of the 46 *Echinococcus* isolates (35 from camels, eight from cattle and three from sheep) collected from the same area. It was recorded from this study that G1 genotype,

which is highly infective to human was not reported in this study. However, relative rarity of human CE in Sudan may result from the absence or rarity of G1 genotype. Human infections with genotype G6 (camel strain) have also been reported. *Echinococcus* had genotyped isolates from different areas confirmed the predominance of G6 genotypes with only two isolates reported as G5 genotypes G1 genotypes were reported by Omer, et al (2010) in few isolates from human from Nuba Mountains bordering Southern Sudan State. G6 genotypes are also reported by Elmahdi, (2006) in dogs. Recent epidemiological studies in Sudan indicated that the camel genotype (G6) was reported to be the most prevalent strain (Ahmed and Aradaib; 2006, Dinkle, et al 2004, Omer, et al 2004).

The camel strain (G6) of *E. granulosus* was detected for the first time in a human patient from eastern Africa. This strain appears to have limited pathogenicity to man with few previous records from humans. Molecular investigations of human cystic hydatid cases from Argentina (Rozenxvit *etal*., 1999) had shown for the first time an involvement of G6, and in Nepal two human isolates have been identified as G6 (Zhang *et al*., 2000). Additionally a study carried out in Mauritania had also identified this genotype in two samples of human origin based on mitochondrial cox1 and nad1gene sequencing (Bardonnet *et al*., 2001). In Kenya, also a single human infection with G6 contrasts with 177 human cases of G1 infection, despite the fact that G6 is wide spread and frequent in animal hosts. The identification of *Echinococcus* infections in dogs and other final hosts is of high priority in epidemiological studies.

The other work of this study includes the collection of samples from the camels at slaughter house at Tambool area for serological tests. The sensitivity of LA, IHA, ELISA, CIE, and AGID were 93%, 88.3%, 57%, 86.9% and 89.8%, respectively, whereas the specificity were 90.5%, 86.9% 44.4%, 78% and 82.0% respectively.

To an understanding the problem of hydatidosis/ Echinococcus in the Sudan, describing and evaluating data from different parts of the Sudan might be of help in an effective control programme. The results of our slaughterhouse survey are with previous survey in the same region. El-khawad et al (1979), reported infection rates of 4%, 8%, 3% and 35% in cattle, sheep, goats and camels, respectively. Saad and Magzoub (1989a &1989b) reported prevalence rate of 49% in camels. Given the fact that in our study, only (68 out of

99 of cysts from camels were found to be fertile (compared to 22% and 24% of cysts from cattle and camels respectively), the principle transmission in this region seems to be based on camels and secondarily on cattle, with sheep only playing a marginal role in the life cycles. This finding was previously documented by Saad and Magzoub (1989a & 1989b) who encountered only calcified or under calcified cysts in sheep. They reported a fertility rate of 24.4% and 29% in camel and cattle cysts; respectively this is in contrast with other regions of Africa including parts of southern Sudan, Kenya and countries of Maghreb, where sheep are heavily involved in the transmission of *E. granulosus* (Macpherson, et al 1987).

Cystic *echinococcus* (CE) occurs in all continents including circumpolar, temperate, tropical and subtropical and zones ranks in some areas as the leading disease of public health significance (Schantz et al, 1995). Camel appear to play the major role for the maintenance of *E. granulosus* in Central Sudan, for about 26.5% of camels examined in this study were found to be infected with cystic *echinococcus* with high fertility rate (68%). Moreover, molecular characterization indicated that camel strain (G6) is more prevalent in this area. Since it had been found in camel, however, in all countries where camel has been reported as an intermediate host, it was too important for the local maintenance of the life cycle (Schantz, 1995).

DNA techniques are now available that allow the identification of *Echinococcus* species and of *E. granulosus* strains by using metacestode material from intermediate hosts (*Thompson, et al 2001*). *Echinococcus* species and genetically distinct strains of *Echinococcus granulosus* can be rapidly and reliably identified by using RFLP- PCR method, which surveys the sequence of a rapidly evolving region of ribosomal DNA (rDNA) (Bowles and McManus, 1993). Mitochondrial DNA has a relative rate of evolution and is therefore useful for the discrimination of closely related organisms (Eckert and Thompson, 1997). In this, study, using RFLP-PCR, Hph1 sequencing and specific PCR approach we confirmed the occurrence- of camel and human strain (G6). Demonstrated characteristics bands pattern previously referred to as camel strain (G6) by Bowles and McManus (1993a). We recognized considerable problems in sensitivity since we obtained amplification products suitable for RFLP from only seven samples from

human and Sudanese camel *E. granulosus* isolates, giving a sensitivity of 75.0% .A specific and sensitive PCR/semi nested PCR system for rapid diagnosis of *E. granulosus* genotypes G6was developed by Dinckle et al (2003)

Most of *E. granulosus* materials obtained from human patients by surgery confirmed the presence of sheep strain (G1) (Bowels and McManus, 1993 a&c). In one case of human cystic *echinococcosis* in the Netherlands the Argentina molecular analysis of *Echinococcus granulosus* isolates encountered from man intermediate host showed for the first time that the camel strain genotype (G6) is infective for human (Rozenxvit et al.. 1999). Another study carried out in Mauritania identified the camels strain genotype (G6) in two human samples.

CHAPTER SEX

CONCLUSIONS

AND

RECOMMENDATION

Chapter 6

Conclusions

Serological test, histopathology, PCR and others comparative studies, such as radiography, ultrasound, CT scanning, MRI may confirm the diagnosis in most cases. The most sensitive test was ELISA (100%) from the all patients together followed by LA (80.0%), IHA (80.0%), CCIEP (66.7) and AGID (57.1). IHA and AGID were suggested for the detection of *echinococcal* antibodies and were suitable tests for diagnosis of hydatid disease and screening of serum samples in the epidemiological studies in high risk population.

1. The results obtained showed that serologic examinations allow active identifying of people with hydatidosis, in the early phases, undetectable by imagistic examinations, which represents a premise of premature hydatidosis diagnosis and permits the establishment of drug therapy and avoidance of complicated forms or inter/postoperative complications.
2. ELISA IgG anti*echinococcus* technique must be associated with other diagnostic methods to confirm results. Screening, where corrections of the results can be made through statistical calculation.
3. Molecular characterization of the studies of *E. granulosus* indicates that camel strain (G6 genocides) is more prevalent in the Central Sudan.

4. Camels were found to be the most suitable intermediate hosts that maintains hydatidosis among man and animals in Sudan

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Recommendations

1. Preventive measures should be taken to control the disease in domestic livestock.
2. CT. scan examination requires the application of serodiagnosis tests to confirm the result.
3. A future CT scan survey on human in Tambool area is highly needed.
4. General education of the public about the seriousness of this zoontic disease.
5. Proper inspection of carcasses in slaughter houses by highly skilled and trained staff.
6. Proper disposal of infected offal.
7. Treatment of infected dogs.
8. Campaign should be directed towards the eradication of stray dogs especially in areas where camels are slaughtered in large numbers.
9. More comprehensive studies to genotype *E. granulosus* isolates from human patients are required

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Appendices:

Solution 1: The composition of Elsevier solution:

Dextrose	2.05 gm
Sodium citrate	0.8gm
Sodium chloride	0.42gm
Distilled water	100ml

Solution 2: Materials:

1. Phosphate buffer saline
2. Borate-succinate
3. Buffer
4. Saline
5. Tannic acid
6. Human serum albumin
7. 40% aqueous formaldehyde
8. Sheep erythrocytes (SRCB) in Elsevier's solution

Solution 3: Materials and equipments of haemagglutination test:

1. Anti-sheep erythrocytes sera, (anti-SRBC).
2. 2 Normal serum as control
3. SRBC in phosphate buffer saline, PBS 2% v/v
4. M2-mercaptoethanol in PBS
5. Microtitre plate 96 wells (V-shape)
6. Eppendofi pipette unadjusted or multichannel automatic pipette.
1. Sealer strip.

Solution 4: ELISA: carbonate-bicarbonate buffer:

0.05M carbonate-bicarbonate buffer. PH 9.6 and incubated at 4 °C overnight.

Solution 5: Buffer PBST -20:

PBST PH 7.4 containing 0.05% Tween 20

Solution 6: Agar of countercurrent immunoelectrophoresis (CCIE)

Oxoid agar no.1 = 1.5g

Sodium chloride = 0.9gm

Distilled water = 100ml

Mix and autoclave at 121°C for 15 minutes

Solution 7: Buffer:

Sodium acetate = 2.5gm

Barbitone sodium = 2.7gm

1/10N HCL = 29.9ml

Distilled water (up to 500ml) = 470.1m

Solution 8: Washing buffer:

Sodium tetraborate = 4gm

Sodium chloride = 4gm

Distilled water = 1 liter

(Dissolve and put in clean tank)

Solution 9: Staining:

Naphthalene black = 0.5gm

Methanol = 500ml

Glacial acetic acid = 100ml

Distilled water = 400ml

Solution 10: Differentiator:

Methanol 500ml

Glacial acidic acid 100ml

Distilled water 400ml

To removed any excess of stain

Solution 11: 50 µl PCR mixture:

Polymerase chain reaction

. no.	**1**	**1-1**	**2**	**2-1**	**3**	**3-1**	**4**	**4-1**	**5**	**5-1**	**6**	**6-1**	**7**	**7-1**	**C+ve**	**C-ve**
Mix	**15.25**	**15.25**	**15.25**	**15.25**	**15.25**	**15.25**	**15.25**	**15.25**	**15.25**	**15.25**	**15.25**	**15.25**	**15.25**	**15.25**	**.25**	**15.25**
DNA	**2** µl	**2** µl	2µl	**2** µl	**2** µl	**2** µl	**2** µl	**2** µl	**2** µl	**2** µl	**2** µl	**2** µl	**2** µl	**2** µl	µl	**0**
PCR water	**32.75** µl	**32.75** µl	**32.75** µl	**32.75** µl	**32.75** µl	**32.75** µl	**32.75** µl	**32.75** µl	**32.75** µl	**32.75** µl	**32.75** µl	**32.75** µl	**32.75** µl	**32.75** µl	**32.75** µl	**34.75** µl