Molecular Characterization of *Echinococcosis granulosus* Isolated from Camel, South Darfur and Gezira States- Sudan (2017-2018).

Arafa Aballah Babiker Abdallah

B.Sc in Medical Laboratory Sciences, University of Gezira (2011)

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Faculty of Medical Laboratory Sciences

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Molecular Characterization Of Cystic Echinococcosis (CE) Isolated From Camel, South Darfur And Gezira States-Sudan (2017-2018).

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Date: //2018
Molecular Characterization Of Cystic Echinococcosis (CE) Isolated From Camel, South Darfur And Gezira States-Sudan (2017-2018).

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Declaration

I authorized that my dissertation “Molecular Characterization Of Cystic Echinococcosis (CE) Isolated From Camel, South Darfur And Gezira States- Sudan (2017-2018)” submitted by me, under the supervision of Dr. Albadawi Abdelbagi Talha and Prof. Adam Dawoud Abakar salim for the partial fulfillment for the award of Master degree in Medical Laboratory Sciences in Medical Parasitology. University of Gezira Faculty of Medical Laboratory Sciences Department of Medical Parasitology; Wad-Medani, Sudan and this is original and it was not submitted in part or in full, in any printed or electronic means, and is not being considered elsewhere for publication.

Name and Signature of Candidate:

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Date: / /2017
Dedication

To my parents; thanks for support; Thanks for giving me a chance to prove and improve myself through all my walks of life.

To my husband, thanks for believing me, for allowing me to further studies.

To my brothers; hoping that with this research I have proven to you that there is no mountain higher on our side.

To my friends and all people support me.

If I forgot anyone, please don’t ever doubt my dedication and love for ALL.
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Abstract

Cestodes of the genus *Echinococcus* species are causative agents of various forms of zoonotic echinococcosis. The adult tapeworms are only 2–7 mm in length and inhabit the small intestine of carnivorous definitive hosts belonging to the families Canidae, Felidae and Hyaenidae. This study aimed to Molecular Characterization of cystic Echinococcosis (*CE*) isolated from camel, south Darfur and Gezira states- Sudan (2017-2018). 63 tissue Samples of protoscoleces or hydatid materials (42) 66.7% from Central and (21) 33.3% from Western Sudan. were preserved in 70% ethanol and stored at room temperature for genetic characterization. Genomic DNA was extracted from protoscoleces preserved in 70% ethanol samples. The product was used as extracted DNA template for PCR. the PCR amplification of the nad1 gene, RFLP-PCR of the nad1 gene and PCR specific for *E. ortleppi* (G5) and *E. granulosus* G6/7 and LAMP Technique for G6 was done. Data was collected and analysis The result show Amplification of 63 isolates of protoscolices of camels' origin of the samples identified by PCR belonged to G5, G6/7 extracted from the worm suspension were characterized as camel strain of *E. granulosus* G6/7. 54 isolate positive (85.7%),38(90.5%)central and 16 (76.2%) (out of 16 which positive by PCR) all were positive by LAMP technique to west sudan samples. more molecular genotyping research in *E.granulosus* LAMP and PCR Techniques should be implemented in endemic area as diagnostic tools. similar studies are needed to clarify the role of wild host in transmission of *Echinococcus granulosus*. 
التشخيص الجزيئي لمرض المشوكة الكيسي (CE) الذي يؤثر على الإبل في وسط وغرب السودان في المختبر الجزيئي لكلية المختبرات الطبية بجامعة الجزيرة في الفترة من (2017-2018)

عرفة عبد الله بابكر عبد الله

ملخص الدراسة

الدودة المشوكة من جنس أنواع الديدان الشريطية هي من العوامل المسببة لأشكال مختلفة من داء المشوكة حيواني النشأة. يبلغ طول الديدان المشوكة البالغة 2-7 مم فقط وتعيش في الأمعاء الدقيقة للمضيفين النهائيين الذين ينتمون إلى عائلات كاتيبي وأيبيدي و هاينيديز. هدفت هذه الدراسة إلى التشخيص الجزيئي لمرض المشوكة الكيسي الذي يؤثر على الإبل والماشية التي يتم تربيتها في وسط وغرب السودان في المختبر الجزيئي لكلية المختبرات الطبية بجامعة الجزيرة في الفترة من 2017-2018. تم عزله من أنسجة الدم (42٪) من الوسط و (21٪) من غرب السودان. تم حفظ عينات الأجسام البديلة في الإيثانول بنسبة 70٪ وتخزينها في درجة حرارة الغرفة للتشخيص في الدودة. تم استخراج الحمض النووي الجيني من الروس البديل المحفوظ في الإيثانول 70٪. تم استخدام الحمض النووي المستخلص لتفاعل الثرثرة المتسلسل G6 و تقنية الاكتثار العقدي لمتشابك لمرشح الفطر素养ي لـ G6. تم جمع النتائج وتحليلها. أظهرت النتائج أن اكتثار 63 عزلة من الروس من أصل الإبل G6 المستخرج من العينات التي تم تخزينها بواسطة تفاعل البلمرة المستخلص وتنتمي إلى G5 و G6. تم وصف 17 عزلة G6 من عزلة الدودة في إبادة النسبية 85.7٪ و 16 (من أصل 16 عينة إيجابية) بواسطة تفاعل البلمرة المستخلص. كانت إيجابية عن طريق تقنية الاكتثار العقدي لمرشح الفطر素养ي للدودة مشكلة في مجال نمط المثير الجيني لكن الحمض النووي البديل من G6 المستخلص لمترشح الفطر素养ي لـ G6. تم توثيق العديد من الدراسات المختلفة للتشخيص الجزيئي في المناطق الموبوءة كأدوات للتشخيص، ولكن هذه الدراسة توضح دور بعض المضيفات البرية في نقل المشوكة الحبيبية.
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<td>Cystic echinococcosis</td>
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<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<td>PAIR</td>
<td>puncture-aspiration-injection-reaspiration</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
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<td>LAMP</td>
<td>loop mediated isothermal amplification</td>
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<tr>
<td>PTA</td>
<td>percutaneous thermal ablation</td>
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<td>DNA</td>
<td>deoxy ribo nucleic acid</td>
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CHAPTER ONE

INTRODUCTION

1.1 Introduction:

Cestodes of the genus *Echinococcus* species are causative agents of various forms of zoonotic echinococcosis. The adult tapeworms are only 2–7 mm in length and inhabit the small intestine of carnivorous definitive hosts belonging to the families Canidae, Felidae and Hyaenidae (Nelson et al. 1965; Rausch and D’Alessandro, 2002; Eckert and Deplazes, 2004). The gravid segments are released into the environment with the feces of these carnivores. Herbivorous intermediate hosts including humans become infected with the larvae of *Echinococcus spp.* following ingestion of the eggs. The development of massive hydatid cysts, mainly in the liver and lungs, cause severe pathological effects in the intermediate hosts. In spite of the medical and veterinary importance of these cestodes (Budke et al., 2006) their taxonomic classification is still controversial, particularly concerning the cryptic species complex of *Echinococcus granulosus sensu lato* (Bowles et al., 1995). Recently, a robust molecular phylogeny of *Echinococcus spp.* was reconstructed from their complete mitochondrial genomes (Ito et al., 2007; Nakao et al., 2007), and *E. granulosus sensu lato* was split into *E. granulosus sensu strict* (genotypes G1–G3), *Echinococcus equines* (genotype G4), *Echinococcus ortleppi* (genotype G5) and *Echinococcus canadensis* (genotypes G6–G10) (Thompson and McManus, 2002). The susceptibility of different animal species (including humans) to echinococcosis differs with the *Echinococcus* strain or species involved, leading to a variety of transmission cycles in different parts of the world. In most regions where human CE is common, the ‘sheep strain’ of *E. granulosus*, which shows high fertility in sheep and is mainly transmitted in a sheep – dog cycle, predominates (Eckert et al., 2001). Many livestock-rearing areas of northern and eastern Africa have particularly high prevalences of echinococcosis, both in the human populations and livestock (Macpherson and Wachira, 1997; Eckert et al., 2001). In central Sudan, domestic livestock and dogs are frequently affected (El-Khawad et al., 1979; Saad and Magzoub, 1986). In this area, the traditional methods of animal husbandry, the home slaughtering of livestock, the large numbers of guard dogs, and the frequent absence of appropriate anthelmintic care all probably favour the transmission of *Echinococcus*. Preliminary observations, based on poor hospital
records, indicate that CE occurs rather sporadically in the human population and at incidences that are markedly lower than those seen in southern Sudan or neighbouring Kenya (Macpherson et al., 1989; Magambo et al., 1996). Concurrent descriptions of species, strains and genotypes have been used to record the biodiversity of *Echinococcus* in the context of medical and veterinary criteria for diagnosing and controlling echinococcosis in various settings (McManus and Thompson, 2003)

1.2 Justification:

*Echinococcus granulosus* and *ortleppi* are a parasitic cestode of human and mammals with an important role of causing disease (Cystic Echinococcosis). Camel were highly infected group (44.6%) (Elmahdi et al., 2004). Molecular diagnosis become most sensitive and specific for parasite diagnosis and genotyping. This study was done to introduce specific diagnostic tools for Echinococcosis diagnosis.

1.3 Objectives:

1.3.1 General objective:

To molecular characterization of Cystic *Echinococcosis* (*CE*) isolated from camel, south Darfur and Gezira States- Sudan (2017-2018).

1.3.2 Specific objectives:

To perform polymerase chain reaction (PCR).

To discrimination of *Echinococcus genotypes* (G5,G6/7)

To differentiation the genotypic of *E. granulosus* and *Echinococcus ortleppi*.

To perform loop mediated isothermal amplification (LAMP) for *E. granulosus*.

To Compare the diagnostic power of polymerase chain reaction (PCR) with loop mediated isothermal amplification (LAMP).
CHAPTER TWO
LITERATURE REVIEW

2.1 History and Background:

Hydatid disease, also called Hydatidosis or Echinococcosis, is a cyst-forming disease resulting from an infection with the metacestode, or larval form, of parasitic dog tapeworms from the genus *Echinococcus*. There are four species of *Echinococcus* spp have been characterized are recognized *Echinococcus granulosus* (unilocular hydatid Cyst), *Echinococcus multilocularis* (multilocular (alveolar) hydatid Cyst), *Echinococcus Vogeli* (polycystic hydatid Cyst), *Echinococcus oligarthrus* (unicystic echinococcosis). Recently, two species have been discovered. *E. shiquicus* and *E. felidis*. The vast majority of human diseases are from *Echinococcus granulosus* and *Echinococcus multilocularis* which cause cystic echinococcosis and alveolar echinococcosis, respectively. Millions of people worldwide are affected by human hydatid disease and as a result, the diagnosis, treatment and prevention of the disease (Jet et al., 2009). Hydatidosis is a disease with an extremely long history as it has been known since the time of Hippocrates, and it is caused by the tapeworm *E. granulosus*. According to (Belding, 1965) *Echinococcus granulosus* has been known since ancient times, for example, Aretaeus and Galen were familiar with hydatid cysts. The generic name *Echinococcus* spp is from the Greek words “echinos”, which means hedgehog and “kokkos” which means berry, while the species term *granulosus* is from the Latin word “granulum” which means little grain. The term hydatid is from the Greek word “hydatis” which means a drop of water (Belding, 1965; Beaver et al., 1984). At present *Echinococcus granulosus*, the causative agent of hydatidosis is almost ubiquitous. It was Redi in 1684, Hartmann in 1685, and Tyson in 1691 who first suspected their animal and the bladder were really worms. In 1766, Pallas first mentioned the similarity of hydatids in man and other mammals and Goeze in 1782 first studied the protoscoleces of the metacestoda and recognized their relationship to those of taenial origin and differentiated the hydatid cyst from the cysticercus. In 1695, Hartman first observed the adult worms in the dog’s intestine, and later Rudolphi in 1808 studied details of the adult worms. Then Von Siebold in 1852 followed by Haubner, Leuckart, Kuchenmeister and Nettleship fed protoscoleces of cysts on domestic animals to dogs and observed the development of the adult worms.
in the intestines of the hosts. Later Naunyn, 1863 in Germany, Krabbe, 1863 in Iceland, and Thomas, 1885 in Australia obtained adults worms in dogs from protoscoleces of human origin (Beaver et al., 1984).

2.2 Classification of Echinococcus Granulosus:

According to Soulsby (1982), the systematic arrangement of E. granulosus was accepted as follows:

Kingdom: Animalia

Sub-kingdom: Metazoa

Phylum: Platyhelminthes

Class: Eucestoda (Southwell, 1930)

Order: Cyclophyllidea (Wardle, McLeod and Radinovsky, 1974)

Family: Taeniidae (Ludwig, 1886)

Genus: Echinococcus (Rudolphi, 1801)

Species: E. granulosus (Batsch, 1786)

Species: E. multilocularis (Leukart, 1863)

Species: E. oligarthus (Diesing, 1863)

Species: E. vogeli (Rausch and Bernstein, 1972)


2.3 Taxonomy:

The taxonomy of Echinococcus has been a controversial issue for decades, but the outcome of recent molecular epidemiological studies has served not only to reinforce the need to revise the taxonomy of Echinococcus, but also to recognize the contribution of early taxonomists. This is because many of the species described over 50 years ago and subsequently invalidated, have now been shown to be valid as a result of extensive molecular epidemiological studies throughout the world.
(Thompson; McManus, 2002). The situation was exacerbated by a lack of appreciation of the extent and significance of variability in *Echinococcus*. As a result, certain features that characterized a particular population were overlooked because of uncertainty regarding its taxonomic status. During the past 40 years, observations in the laboratory and the field have revealed considerable phenotypic variability between isolates of *Echinococcus*. This variation has largely been observed in *E. granulosus* and between isolates of the parasite from different species of intermediate host in different geographical areas. Given the epidemiological significance of intra-specific variation in *E. granulosus* and the international efforts to establish control programmes in different endemic regions, an informal nomenclature was needed to reflect the phenotypic variability evident between host-derived populations of *E. granulosus* in different geographical areas. Thus, the concept of a ‘strain’ was developed and defined as variants ‘which differ statistically from other groups of the same species in gene frequencies, and in one or more characters of actual or potential significance to the epidemiology and control of echinococcosis’ (Thompson; Lymbery, 1988).

### 2.3.1 *Echinococcus granulosus*:

The genus *Echinococcus* was established for the larval cestoda which was earlier designated *Taenia visceralis socialis granulosus* by Goeze in 1782 obtained from the liver of sheep. In 1786, Batsch adapted the binary system of nomenclature of Linnaeus and gave the name *Hydatigene granulosus* to the taxon described by Goeze (Thompson 1986). In 1963, Rausch and Nelson reviewed the species of *Echinococcus* and concluded that the status of *E. felidis Ortlepp*, 1937 and *E. patagonicus Szidat*, 1960 was uncertain, but that both were possibly co-specific with *E. granulosus*. In addition, *E. cameroni Ortlepp*, 1934; *E. intermedius Lopez-Neyra and Soler*, 1943; *E. longimanubrius Cameron*, 1926; *E. lycaontis Ortlepp*, 1934; *E. minimus Cameron*, 1926, and *E. Ortlepp Lopez-Neyra and Soler* 1943 were considered synonyms of *E. granulosus* Rausch and Nelson 1963. *Echinococcus granulosus* has been divided into several strains according to the host (Thompson, 1995). *Echinococcus granulosus* has been researched extensively and discussed in detail as it is the species that is most widely distributed throughout the world. The taxonomy of *Echinococcus granulosus* has been separated into ten different genotypes, named G1 to G10. For example, it
has six strains which have been identified as G1 genotype (common sheep strain), G2 genotype (Tasmanian sheep strain), G4 genotype (horse strain), G3, G5 genotypes (cattle strain), G6 genotype (camel strain), G7 genotype (pig strain) (Rosenzvit et al., 1999; Lymbery, 1995). In addition, recorded five potential hybrid genotypes between cattle and sheep strains found only in Southern Brazil (Haag et al., 1999).

Mitochondrial DNA (mtDNA) has more power than nuclear DNA in reconstructing phylogenetic relationships among closely related species because of the rapid sequence evolution (Brown et al., 1979), and large data sets derived from mitochondrial genomes have the potential for resolving problematic issues in taxonomy (Mueller et al., 2004) We describe the development of a specific and sensitive PCR/semi-nested PCR system for the rapid diagnosis of *Echinococcus granulosus* genotype G1, *E. granulosus* genotype G6/7, and *Echinococcus ortleppi* (G5). Diagnosis of G1 and the group G5/6/7 is performed by a simple PCR, while discrimination between *E. ortleppi* (G5) and G6/7 involves a subsequent semi-nested PCR step. The target sequence for amplification is part of the mitochondrial 12S rRNA gene. Specificity of the PCRs was 100% when evaluated with isolates of 16 species of cestodes, including *Echinococcus multilocularis, Echinococcus equinus, E. ortleppi* and three strains of *E. granulosus* (G1, G6 and G7). Sensitivity threshold was 0.25 pg of DNA. This new approach was compared with published protocols of restriction fragment length polymorphism-PCR and sequencing of mitochondrial cytochrome c oxidase subunit 1 and NADH dehydrogenase 1 genes using *Echinococcus* isolates of human, sheep, goat, camel, cattle and pig origin from Kenya and Sudan. Additionally, two internal DNA probes were developed, one hybridising only with G1, the other with G5, G6 and G7 amplification products. Preliminary epidemiological results obtained with this PCR approach include the detection of a camel strain (G6) infection for the first time in a human patient from eastern Africa, and the first reports of *E. ortleppi* (G5) in livestock from Kenya and the Sudan. Anke Dinkel etal(2003). The LAMP is characterized by the use of six different primers specifically designed to recognize eight distinct regions on the target gene . The amplification proceeds at a constant temperature using strand displacement reaction. Amplification and detection of a gene can be completed in a single step, by incubating the mixture of samples, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature (about 63C) [Notomi ,etal.200]. Compared to PCR and real-time PCR, LAM P has the advantages of reaction simplicity and higher
amplification efficiency. The LAMP reaction also yields large amounts of by-product, pyrophosphate ion, leading to a white precipitate of magnesium pyrophosphate in the reaction mixture. Since the increase in turbidity of the reaction mixture according to the production of precipitate correlates with the amount of DNA synthesized, real-time monitoring of the LAMP reaction can be achieved by real-time measurement of turbidity (Mori et al., 2001). The phylogeny of the genus *Echinococcus* was clarified by using the complete mitochondrial genomes of representative taxa in order to improve the consistency between species status and traditional nomenclatures. The concatenated data sets of nucleotide and amino acid sequences derived from all mitochondrial genes were analysed with an array of phylogenetic methods including maximum likelihood and partitioned Bayesian analyses. In particular, the latter is the only method that can be used to analyse a large data set by incorporating complex substitution models into the statistical process (Ronquist and Huelsenbeck, 2003; Nylander et al., 2004). Also, the ancestral origin of *Echinococcus* was inferred, based on the phylogeny of *Echinococcus* and the evolutionary history of mammalian host animals. Published sequences were available for mitochondrial genomes of *E. granulosus sensu stricto*, *E. equinus*, *E. multilocularis* and *T. solium* (Le et al., 2002; Nakao et al., 2003). The *E. granulosus* genotypes corresponding to the camel, pig, and cervid strains were monophyletic, and their high level of genetic similarity supported taxonomic species unification of these genotypes into *E. canadensis*. Sister species relationships were confirmed between *E. ortleppi* and *E. canadensis*, and between *E. multilocularis* and *E. shiquicus*, regardless of the analytical approach employed. The basal positions of the phylogenetic tree were occupied by the neotropical endemic species, *E. oligarthrus* and *E. vogeli*, whose definitive hosts are derived from carnivores that immigrated from North America after the formation of the Panamanian land bridge. Host-parasite co-evolution comparisons suggest that the ancestral homeland of *Echinococcus* was North America or Asia, depending on whether the ancestral definitive hosts were canids or felids (Nakao et al., 2006).
2.3.2 *Echinococcus ortleppi*:

In many parts of the world, cattle are an accidental host for *E. granulosus* of sheep origin, and resultant cysts in cattle develop poorly and are rarely fertile (Thompson et al., 1984 and 1995). In contrast, in parts of Europe, South America and Africa, a form of *Echinococcus* which appears to be adapted to cattle as its principal, or sole, intermediate host has now been well characterized (Thompson; McManus, 2001; 2002). According to morphological and genetic analyses, the cattle strain occurs throughout Europe as well as parts of Africa, Asia and South America. The South African bovine form was initially described as the species *E. ortleppi* by Lopez-Neyra and Soler Planas in 1943 based on adult worms originally described by Ortlepp in 1934 from the type locality in South Africa and which was considered to be of cattle origin (Verster, 1965; Kumaratilake; Thompson, 1982; Thompson et al. 1984). Once again, the taxonomic status of this form was questioned (Rausch and Nelson, 1963) in relation to, it is now clear, a limited appraisal of the morphological features which characterize this form of *Echinococcus*. It was not until 40 years later when the results of experimental infections and molecular characterization revealed that yet again, the early taxonomists were correct in their proposals. There is indeed a form of *Echinococcus* which utilizes cattle as its intermediate host. It is quite distinct from *E. granulosus* of sheep and horse origin in morphology and developmental characteristics, and moreover it is genetically distinct (Thompson et al., 1984; Bowles et al., 1995; Thompson and McManus, 2001 and 2002). Lopez-Neyra and Soler Planas (1943) proposed that it be called *E. ortleppi*. Thus *E. ortleppi* is now widely accepted as the most appropriate species name for the cattle strain (Lavikainen et al., 2003; Obwaller et al., 2004; Romig et al., 2006; Jenkins et al., 2005; Nakao et al, 2007; Moks et al., 2008).

2.4 Epidemiology of *E. granulosus*:

The disease occurs in most areas of the world and currently affects about one million people. In some areas of South America, Africa, and Asia up to 10% of the certain populations are affected (WHO, 2014). *E. granulosus* is primarily found in areas in which sheep or other herbivores are raised and are in close contact with dogs or wild canines. Other criteria for areas at risk include those in which close
contact between canines and humans occurs. These areas include Great Britain, parts of South America, Australia, parts of Africa, Asia, and China, and select portions of the Middle East. There have been several cases reported in the United States, particularly in Alaska, as well as in the West and Southwest. In central Sudan, domestic livestock and dogs are frequently affected (El-Khawad et al., 1979; Saad and Magzoub, 1986). In this area, the traditional methods of animal husbandry, the home slaughtering of livestock, the large numbers of guard dogs, and the frequent absence of appropriate antihelmintic care all probably favour the transmission of *Echinococcus*. Preliminary observations, based on poor hospital records, indicate that CE occurs rather sporadically in the human population and at incidences that are markedly lower than those seen in southern Sudan or neighbouring Kenya (Macpherson et al., 1989; Magambo et al., 1996). In livestock found in Sudan, the highest prevalence of infection was found in the camels (44.6% of 242 infected), followed by the sheep (6.9% of 5595) and cattle (3.0% of 2368) (Elmahdi et al., 2004). Information about strain differentiation is only available from Kenya and Sudan. After characterisation of *Echinococcus* isolates, from different livestock (camel, cattle, goats and sheep) and humans and from different localities using genotype s—specific PCR, PCR-RFLP and CO1 sequencing the results obtained shows that camel strain (G6) of *E. granulosus* is endemic in camel s, goats, cattle and human s while *E. ortleppi* (or cattle strain G5 of *E. granulosus*), was only found in cattle in Sudan [Dinkel et al., 2004]. In contrast, the common sheep strain (G1) which is suspected to be the principal genotype, affecting humans in sub-Saharan Africa, was not found and therefore appears to be rare or even absent in the central Sudan. In another study CE isolates from five human patients from western and southern Sudan were characterized using genotype specific PCR and sequencing and confirmed that camel strain (G6) infects humans in Sudan [Omer et al., 2004]. No available data records for the prevalence of differ among human in Sudan.
2.5 Life Cycle and Morphology of Species *E. granulosus*:

**Figure (2.1):** The life cycle of *E. granulosus* (CDC, 2012).

Hydatid disease is caused by infection with the larval form of *E. granulosus* (and/or *E. multilocularis*) and results in the formation of cysts within various host tissues. The complete life cycle of *Echinococcus granulosus* requires two hosts. Domestic dogs act as the primary definitive host of the mature adult worms and a single infected dog may harbor millions of adult worms within its intestines. Other canines such as wild dogs, wolves, coyotes, foxes and jackals may also act as a definitive host harboring the adult tapeworms. Intermediate hosts become infected with the larval form of the parasite and include a wide range of herbivorous animals primarily sheep, cattle, pigs, goats and horses (Moro and Schantz, 2009; Torgerson and Heath, 2003). The life cycle is completed by the ingestion of one or more cysts and its contents by the canine host through the consumption of infected viscera of sheep and and/or other livestock. Protoscoleces released in the small intestine attach to the intestinal wall through the action of four suckers and a row of hooks and within two months mature into adult worms capable of producing infective eggs (Moro *et al.*, 1997). Humans may become infected though the ingestion of food and/or water contaminated with infective eggs.

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released in the feces of dogs harboring the adult tapeworm(s). The hatching process uses the disaggregation of the keratin-like blocks of the embryophore by pepsin, pancreatic enzymes, etc., followed by the activation of the contained oncospheres (Lethbridge, 1980). The eggs release oncospheres capable of actively penetrating the intestinal mucosa. These oncospheres gain access to the blood stream via the hepatic portal vein and migrate to various internal organs where they develop into cysts if not destroyed by phagocytic cells (Belding, 1965). The factors that determine the final localization of the metacestode of *Echinococcus* are not clear, but probably include anatomical and physiological characteristics of the host, as well as the species and strain of parasite (Stephen and Richard, 2001). Hydatid cysts most often localize within the liver and the lungs (McManus *et al*., 2003); however, cysts may also form in the bones, brain, skeletal muscles, kidney and spleen. The clinical manifestations of hydatid disease vary depending on a variety of factors including the location, size and number of cysts present within the infected (Abhay *et al*., 2009). Tape-worms form three different developmental stages: eggs; larvae; and adults.

**Adult worm:**

![Adult worm of E. granulosus](webpathology, 2017)

**Figure (2.2):** Adult worm of *E. granulosus* (webpathology, 2017).

Adult *E. granulosus* worms are small (2-6mm long) and have a scolex with only three attached segments. The scolex has four lateral suckers and the rostellum is non-retractable and armed with a double crown of 28-50 recurved hooks. The anterior
segment is immature, the middle segment is mature with functional testes and ovaries, and the posterior segment is gravid with the uterus filled with eggs (Belding, 1965).

**Egg:**

![Egg of E. granulosus](image)

**Figure (2.3)**: Egg of *E. granulosus* (Rahman *et al*., 2015).

The eggs passed in the faeces of definitive hosts are identical to those of *Taenia* spp. The eggs are infective to various animals including man. Sheep are the most suitable intermediate host for this parasite. Inside the intermediate host, it slowly develops into a hydatid cyst containing ++numerous protoscoleces infective to the definitive hosts (John *et al*., 2006).
Larval stage (hydatid Cysts):

Figure (2.4): Hydatid cyst in the lung

Figure (2.5): Protoscoleces of *E. granulosus* (webpathology, 2017).
The hydatid cyst larval stage of *E. granulosus*, found in human tissue, consists of several structures. These structures overlap somewhat in their definitions, resulting in a confusion of the terms and making a clear and concise description of them a challenge. The following description should be clear. The entire structure is termed a hydatid cyst. Within the cyst, miniaturizations of the entire hydatid cyst may occur; these are referred to as daughter cysts. Both types of cysts are surrounded by a protective cyst wall and laminated layers of germinal tissue. In addition, brood capsules, which lack a protective cyst wall, form from the inner germinal layer. Developing scolexes are found within these structures. Each scolex, once fully developed, has the capability of developing into an adult worm when present in the definitive host. A hydatid sand, defined as components found in the fluid of older *E. granulosus* cysts that typically include daughter cysts, free scolexes, hooklets, and miscellaneous nondescript material may evolve. Some hydatid cysts fail to produce all or some of these structures. In this case, some hydatid cysts may start out by developing such structures but, because of the onset of secondary bacterial infections, may result in sterility or death of the cyst, with subsequent calcification (John *et al.*, 2006; Mandell *et al.*, 2010).

### 2.6 Transmission:

The disease is spread when food or water that contains the eggs of the parasite is eaten or by close contact with an infected animal (WHO, 2014). Animals to become infected by eating the organs of an animal that contains the cysts such as sheep or rodents (CDC, 2014). Humans are accidental intermediate hosts that become infected by handling soil, dirt or animal hair that contains eggs (Eckert and Deplazes, 2004). Coprophagic flies, carrion birds and arthropods can act as mechanical vectors for the eggs (MSDS, 2001).

### 2.7 Pathogenesis

Evolution of Hydatid Cyst at the site of deposition, the embryo slowly develops into a hollow bladder or cyst filled with fluid. This becomes the hydatid cyst (Greek +about 6 months. The growing cyst evokes host tissue reaction leading to the deposition of fibrous capsule around it. The cyst wall secreted by the embryo consists of 3 indistinguishable layers.
**Pericyst** is the outer host inflammatory reaction consisting of fibroblastic proliferation, mononuclear cells, eosinophils, and giants cells, eventually developing into dense fibrous capsule which may even calcify.

**Ectocyst** is the intermediate layer composed of characteristic a cellular, chitinous, laminated hyaline material. It has the appearance of the white of a hardboiled egg.

**Endocyst** is the inner germinal layer which is cellular and consists of number of nuclei embedded in a protoplasmic mass and is extremely thin (22–25 mm). The germinal layer is the vital layer of the cyst and is the site of asexual reproduction giving rise to brood capsules with scolices. It also secretes hydatid fluid, which fills the cyst. (Pedrosal *et al* 2000, Li *et al* 2006)

**Hydatid fluid**: The interior of the cyst is filled with a clear colorless or pale yellow fluid called as hydatid fluid. pH of the fluid is 6.7 (acidic). It contains salts (sodium chloride 0.5%, sodium sulphate, sodium phosphate, and salts of succinic acid) and proteins It is antigenic and highly toxic so that its liberation into circulation gives rise to pronounced eosinophilia or may even cause anaphylaxis. The fluid was used as the antigen for Casoni’s intradermal test. A granular deposit or hydatid sand is found at the bottom of the cyst, consisting of free brood capsules and proto scolices and loose hooklets (Jayaram and Sougata, 2013).

**Brood capsules:**

From the germinal layer, small knob -like excrescencesor gemmules protrude into the lumen of the cyst. These enlarge, become vacuolated, and are filled with fluid. These are called as brood capsules. They are initially attached to the germinal layer by a stalk, but later escape free into the fluid-filled cyst cavity. From the inner wall of the brood capsules, protoscolices (new larvae) develop, which represent the head of the potential worm, complete with invaginated scolex, bearing suckers and hooklets. Several thousands of protoscolices develop into armature hydatid cyst, so that this represents an asexual reproduction of great magnitude. Inside mature hydatid cysts, further generation of cyst, daughter cysts and grand-daughter cysts may develop. The cyst grows slowly often taking 20 years or more to become big enough to cause clinical illness and is therefore, particularly seen in man (Jayaram and Sougata, 2013).
Acephalocysts

Some cysts are sterile and may never produce brood capsules, while some brood capsule may not produce scolices. These are called acephalocysts. Fate of hydatid cysts: The cyst may get calcified or spontaneously evacuated following inflammatory reaction. Hydatid cyst of liver may rupture into lung or other body cavity producing disseminated hydatid lesions (Jayaram and Sougata, 2013).

2.8 Signs and Symptoms:

*Echinococcus granulosus* and *Echinococcus multilocularis* are the two species most often identified in human hydatid disease. Cystic echinococcosis, caused by *E. granulosus*, is the most common and accounts for approximately 95% of all global cases. Cystic echinococcosis may affect people of all ages, but hydatid cysts are most often present in patients between 15-35 years of age. Infection with *E. granulosus* results in the rapid growth of large, unilocular cysts filled with fluid. Most cysts develop within the tissues of the liver and lung with 55-75% of cysts found in the liver and 10-30% of cysts found in the lungs. Cysts may survive in the liver for several years and often do not cause any symptoms in the infected host (Kemp and Roberts, 2001). Symptoms arise when the cysts become large enough to be palpable and/or cause visual abdominal swelling and pressure. Patients frequently experience abdominal pain in the right upper quadrant, often accompanied by nausea and vomiting. The rupture or leakage of cysts within the tissue can result in anaphylactic shock and facilitate the spread of secondary cysts through the release and dissemination of germinal elements. Biliary tract disease and portal hypertension may complicate liver involvement and post obstructive infection due to erosion of cysts into the biliary tract may further complicate echinococcal infection. Pulmonary cystic echinococcosis is acquired early during childhood, but the clinical manifestations associated with the disease do not typically appear until the third or fourth decade of life. Cysts residing within the lung tissue often remain silent producing little to no symptoms. Problems arise when cysts grow large enough to obstruct or erode a bronchus, often causing the rupture of cysts and the dissemination of cystic fluids. Patients infected with pulmonary cysts frequently experience chronic dry cough, chest pain and hemoptysis often accompanied by headache, sweating, fever and malaise. Complications (Bitton et al., 1992). All the usual complications related to the surgical
procedure and anesthesia apply. Complications related to the parasite include the following: Recurrence, Metastasis, Infection, Spillage and seeding (secondary echinococcosis) - Allergic reaction or anaphylactic shock. Those related to the medical treatment include the following: (Hepatotoxicity, Anemia, Thrombocytopenia, Alopecia, Embryotoxicity, Teratogenicity) Spillage and seeding (secondary echinococcosis). Complications related to puncture-aspiration-injection-reaspiration (PAIR) include the following: Hemorrhage, Mechanical damage to other tissues, Infections, Allergic reaction or anaphylactic shock, Persistence of daughter cysts, Sudden intracystic decompression leading to biliary fistulas. Those related to scolicidal agents includes chemical sclerosing cholangitis (Imad 2016).

2.9 Diagnosis:

A formal diagnose of any type of echinococcosis requires a combination of tools that involve imaging techniques, histopathology, or nucleic acid detection and serology. For cystic echinococcosis diagnosis, imaging is the main method—while serology tests (such as indirect hemogglutination, ELISA (enzyme linked immunosororbent assay), immunoblots or latex agglutination) that use antigens specific for *E. granulosus* verify the imaging results. The imaging technique of choice for cystic echinococcosis is ultrasonography, since it is not only able to visualize the cysts in the body's organs, (Brunetti et al., 2010) but it is also inexpensive, non-invasive and gives instant results (Macpherson and Milner, 2003). In addition to ultrasonography, both MRI and CT scans can and are often used although an MRI is often preferred to CT scans when diagnosing cystic echinococcosis since it gives better visualization of liquid areas within the tissue (Brunetti et al., 2010). Currently, there is no suitably sensitive and specific serological test available for individual diagnosis in livestock species (Eckert et al., 2001). Nevertheless, serum antibody activity is used for detecting infection at the herd or flock level. This may be useful in hydatid screening programmers, particularly, when cysts may be too small to easily identify at necropsy. Antibodies that react against hydatid cyst fluid antigen can be detected from 4 weeks after exposure, and greater than 90% sensitivity using antigen B enriched hydatid fluid extracts have been recorded. On the other hand, DNA identification of taeniid cestodes has used specific sequences (PCR techniques) from both the nuclear and mitochondrial genomes such as nicotinamide dehydrogenase complex (nad1-6 and
nad4L subunits), cytochrome c oxidase complex (cox1-3 subunits), cytochrome b (cob) and adenosine triphosphatase subunit 6 (atp6), beside two genes encoding ribosomal RNA subunits are present: the large subunit (rrnL or 16S) and small subunit (rrnS or 12S). The mitochondrial genome is located in mitochondria and the much larger nuclear genome distributed on the chromosomes in the nucleus, where mitochondrial genomes are small (generally less than 20,000 bp in metazoans). Mitochondrial DNA is useful for the discrimination of closely related organisms because of its relatively rapid rate of evolution. Furthermore, as mtDNA is haploid, allele haplotypes can be determined unambiguously. In addition to that, mitochondrial DNA is maternally inherited and does not recombine, thus simplifying analysis. Several molecular techniques are now available which would quite easily allow the identification of certain *E. granulosus* strains using genetic markers.

### 2.10 Treatment:

For simple cases of cystic echinococcosis, the most common form of treatment is open surgical removal of the cysts combined with chemotherapy using albendazole and/or mebendazole before and after surgery. However, if there are cysts in multiple organs or tissues, or the cysts are in risky locations, surgery becomes impractical. For inoperable cases such as these, chemotherapy and/or PAIR (puncture-aspiration-injection-reaspiration) become alternative options of treatment (Eckert and Deplazes, 2004). In the case of alternative treatment using just chemotherapy, albendazole is preferred twice a day for 1–5 months (CDC, 2009). An alternative to albendazole is mebendazole for at least 3 to 6 months. The other alternative to surgery is PAIR with chemotherapy. PAIR is a minimally invasive procedure that involves three steps: puncture and needle aspiration of the cyst, injection of a scolicidal solution for 20–30 min, and cyst-reaspiration and final irrigation. Patients who undergo PAIR typically take albendazole or mebendazole from 7 days before the procedure until 28 days after the procedure. While open surgery still remains as the standard for cystic echinococcosis treatment, there have been a number of studies that suggest that PAIR with chemotherapy is more effective than surgery in terms of disease recurrence, and morbidity and mortality (Park et al., 2009). In addition to the three above mentioned treatments, there is currently research and studies looking at new treatment involving percutaneous thermal ablation (PTA) of the germinal layer in the cyst by means of a radiofrequency ablation device. This form of treatment is still relatively new and
requires much more testing before being widely used (Eckert and Deplazes, 2004). An alternative to open surgery is laparoscopic surgery, which provides excellent cure rates with minimal morbidity and mortality (Jani, 2014).

2.11 Prevention and Prophylaxis:

The most effective means to control hydatid disease in humans and eliminate the consequences of *Echinococcus* infections in livestock is through the broad range education of people living in endemic regions. Education to prevent the feeding of infected viscera to dogs is essential for controlling the spread of infection from livestock to dogs. Most human infections are due to close contact with infected dogs. Deliberate actions aimed at reducing the rate of dog infection in endemic regions will undoubtedly reduce the number of human infections. In addition, the reduction and removal of stray and unwanted dogs, as well as the regular treatment of dogs with anthelminthic drugs, will facilitate the widespread efforts geared towards controlling disease transmission. The development of vaccines designed to prevent infection of either or both the definitive and intermediate host(s) offers the greatest possibility of success in the control and eradication of hydatid disease in both the livestock and human populations. EG95 is a 16.5 kDa recombinant GST fusion protein derived from *E. granulosus* oncospheres and functions as a highly effective vaccine for grazing livestock. EG95, which induces immunity through complement-fixing antibodies, has been shown to induce high levels of protection (96-98%) against the development of hydatid cysts. In order to prevent transmission to dogs from intermediate hosts, dogs can be given anthelminthic vaccinations. In the case of intermediate hosts, especially sheep, these anthelminthic vaccinations do cause an antigenic response—meaning the body produces antibody avinash response—however it does not prevent infection in the host (Moro and Schantz, 2009; Craig et al., 2007).

Clean slaughter and high surveillance of potential intermediate host during slaughter is key in preventing the spread this cestode to its definitive host. It is vital to keep dogs and potential intermediate host as separated as possible to avoid perpetuating infection (Moro and Schantz, 2009; Li et al., 2014). According to mathematical modeling, vaccination of intermediate hosts, coupled with dosing definitive hosts with anthelminths is the most effect method for intervening with infection rates (Moro and Schantz, 2009). Proper disposal of carcasses and offal after home slaughter is difficult
in poor and remote communities and therefore dogs readily have access to offal from livestock, thus completing the parasite cycle of *Echinococcus granulosus* and putting communities at risk of cystic echinococcosis. Boiling livers and lungs which contain hydatid cysts for 30 minutes has been proposed as a simple, efficient and energy- and time-saving way to kill the infectious larvae (Li *et al.*, 2014).
CHAPTER THREE
MATERIAL AND METHODS

3.1 Study design:

This was prospectively cross sectional study

3.2 Study period:

The study was done in March(2017) to August(2018).

3.3 Study area:

Surveys were done in Tamboul town (western, Sudan), and Addein, Rehed al Birdi and Kass areas (Western Sudan) (Fig 3.1).

Fig.3.1. Study area, Tambool town (Central Eastern, Sudan) and Addein, Rehed al Birdi and Kass areas (Western Sudan).
3.4 Study population:

Study included Camels at home slaughtering.

3.5 Sample size:

63 tissue samples were collected (liver, lung, spleen, heart and kidneys)

3.6 Ethical approval:

The field work of the study for animal investigation has been conducted at South Darfur State, Faculty of Veterinary Science, Nyala University and Gezira State, Faculty of Health and Environmental Sciences, University of Gezira. The author received an ethical clearance from the Veterinary Ethics Committee (VEC). An approval for conducting this research has been obtained from Ministry of Animal Resource, Fisheries and Ranching, South Darfur and Gezira State.

3.7 Methods:

3.7.1 Collection of samples:

A total of 63 camel carcasses were examined in abattoirs from Tambool, Addein, Rehed al Birdi and Kass, respectively), during routine meat inspection, for presence of cystic echinococcosis according to WHO (1981), where 63 isolates from camel (42 from Tampool and 21 from Addein, Rehed al Birdi and Kass) Samples of protoscoleces or hydatid materials were preserved in 70% ethanol and stored at room temperature for DNA extraction.

3.7.2 Parasitological studies:

Cysts or cyst material were examined microscopically to confirm whether or not they are hydatid cysts. Fertility of the collected hydatid cysts was assured by detection of protoscolices in aspirated fluid samples. Sterile or calcified cysts were considered infertile.
3.7.3 Molecular genotyping:

3.7.3.1 Preparation of hydatid cyst material from intact cysts for DNA extraction:

Each individual cyst was placed in a Petri dish and opened using sterilized scissors and scalpel. Cyst fluid, if present was obtained using a pipette. If the cyst was calcified, part of the cyst wall was taken using a scissors. Scissors and scalpels were sterilized after the opening of each individual cyst using ethanol, flame, and water subsequently.

3.7.3.2 DNA extraction:

Genomic DNA was extracted from protoscoleces preserved in 70% ethanol samples. According to (Nakao et al., 2003) a single protoscolex was lysed in 10µl of 0.02 N, NaOH at 99°C for 10 min. The product was used as extracted DNA template for PCR.

![Figure (3.2): Extracted DNA from protoscoleces](image)

3.7.4 Cestode-specific PCR (cs PCR):

A PCR with the primer pair (forward and reverses) P60.for. and P375.rev. was performed as described (Dinkel et al., 1998; von Nickisch-Rosenegk et al., 1999) with the following modifications: The 100 µl reaction mixture consisted of 10 mM Tris – H(pH 8.3), 50 mM KCl, 2.5 mM MgCl2 each deoxynucleoside triphosphate at a concentration of 200 mM, 40 pmol of each primer and 2.5 units Ampli-Taq Polymerase (Perkin Elmer) for 40 cycles (denaturation for 30 s at 948°C, annealing
for 1 min at 55 °C and elongation for 30 s at 72 °C). After amplification, 10 µl of the amplification products were resolved on a 1.5% ethidium bromide-stained agarose gel.

Figure (3.3) PCR system for diagnosis of *Echinococcus granulosus* G6/7 and *Echinococcus ortleppi*.

3.7.4.1 PCR amplification of the nad1 gene:

This method is based on the amplification of a 1073–1078 bp-long fragment including the complete NADH dehydrogenase subunit 1 (nad1) gene. Thermal reactions were performed according to Hüttner et al (2008, 2009) (Gene bank: EF558355). PCR was performed in a 50 µl vol. containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 200 µM of each dNTP, 12.5 pmol Forward primer nadA: 5’ TGT TTT TGA GAT CAG TTC GGT GTG 3’ and Reverse primer nadC: 5’ CAT AAT CAA ACG GAG TAC GAT TAG 3’, 1.25 U Ampli-Taq Polymerase (Applied Biosystems) and 5 µl of the total DNA. The amplification conditions for 35 cycles were denaturation for 30 s at 94°C, annealing for 30 s at 55 °C and elongation for 60 s at 72 °C. PCR products were separated on a 1.5% ethidium bromide-stained agarose gel.
**Figure (3.4):** 1.5% TBE agarose gel, stained with 2% ethidium bromide and visualized using U.V.

### 3.7.4.2 RFLP-PCR of the nad1 gene:

PCR product of the nad1 gene was digested by restriction enzyme HphI (Hüttner et al. 2008). PCR products were digested for 3 h with the restriction enzyme HphI according to the manufacturer's instruction (Fermentas). Restriction fragments were visualized by gel electrophoreses through a 3% ethidium bromide-stained agarose gel.

### 3.7.3.5 PCR specific for *E. ortleppi* (G5) and *E. granulosus* G6/7:

For the first PCR (G5/6/7) which amplifies 254bp fragment of *E. ortleppi* (G5) and *E. granulosus* G6/7, the primer pair E.g.cs1for. (5’ATT TTT AAA ATG TTC GTC CTG 3’) and E.g.cs1rev. (5’CTA AAT AAT ATC ATA TTA CAA C 3’) was used. The 100μl reaction mixture consisted of 10 mMTris–HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 200 μM of each dNTP, 25 pmol of each primer and 1.25 units Ampli-Taq
Polymerase (Perkin Elmer Biosystems) for 40 cycles (denaturation for 30 sec at 94°C, annealing for 1 min at 53°C and elongation for 40 sec at 72°C) (Dinkelet et al., 2004) (Gene bank: AY462126–AY462129). The system for diagnosis of E. granulosus G6/7 and E. ortleppi is shown in Fig. (4.2) To discriminate between E. granulosus G6/7 and E. ortleppi, semi-nested PCRs specific for G6/7 (g6/7PCR; primer pair E.g. camel. for 5’ ATG GTC CAC CTA TTA TTT CA 3’ and E.g.cs1rev.) and for E. ortleppi (g5 PCR; primer pair E.g. cattle. for 5’ ATG GTC CAC CTA TTA TTT TG 3’ and E.g.cs1rev.) were used in a second step, each amplifying a different fragment of 171 bp. The reaction mixtures of 50 μl contained 1.5 μl of amplification product, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 200 μM of each dNTP, 25 pmol of each primer and 1.25 units AmpliTaq Polymerase (Perkin Elmer Biosystems) for 30 cycles (denaturation for 30 sec at 94°C, annealing for 1 min at 60°C and elongation for 30 sec at 72°C). Amplification products were resolved on a 1.5% ethidium bromide stained agarose gel.

3.7.5 Loop Mediated Isothermal Amplification (LAMP) technique:

3.7.5.1 LAMP primer:

The target gene of the LAMP was mitochondrial NADH dehydrogenase subunit 1 (nad1) gene (Notomi et al., 2000). The binding site of all primer sets are located within the nad1 gene and were designed by using primer explorer software V4 (Eiken chemical Co. Ltd.). The E. granulosus specific primers based on the nad1 sequence of E. ortleppi specific G5 deposited in the database under the Accession No. AB235846 and E. canadensis specific G6/7 deposited in the database under the Accession No AB208063.

3.7.5.2 LAMP assay:

The LAMP assay was designed according to identify E. ortleppi, and E. canadensis. The LAMP-reactions were performed in a 12.5 μl reaction mixture containing 20 mM Tris (pH 8.8), 10 mM KCL, 8 mM MgSO4, 10 mM (NH4)2 SO4, 0.8 M betaine, 1.4 mM dNTPs and 4 U Bst 2.0 polymerase (large fragment New England Biolabs). The primer concentration for E. Canadensis LAMP reaction was 20 pmol of each FIP and BIP primer and 2.5 pmol of each F3 and B3 primer. For the E. ortleppi LAMP reaction the primer concentration was 20 pmol of each
FIP and BIP primer and 5 pmol of each F3 and B3 primer. All components were mixed in a 4°C cooling block. The reaction mixture was incubated for 60 min at 62°C (E. ortleppi) and 66°C (E. canadensis) and subsequently heated up to 95°C for 2 min to inactivate the enzyme. The incubations of the assay were carried out in thermal cyclers at constant temperatures. When thermal cyclers were used the sample tubes were inserted after the heating block had reached amplification temperature. The optimal temperatures and primer concentration were determined using gradients during specificity optimization processes. Three micro liters of the LAMP products were visualized on a 3% agarose gel stained with GelRed™. Gel electrophoresis, although not necessary for detection of a positive signal, visualization was also done by mixing 3 µl LAMP products with GelRed™ in a micro titer plate and viewed under UV light.
CHAPTER FOUR
RESULTS AND DISCUSSION

4.1 Results:

Amplification of 63 isolates of protoscolices of camels' origin, of the sample identified by PCR belonged to G5, G6\7 extracted from the worm suspension were characterized as camel strain of *E. granulosu* In this study 54 isolate positive (85.7 %)

Table (4.1) PCR results:

<table>
<thead>
<tr>
<th>Result</th>
<th>West</th>
<th>central</th>
<th>Percent %</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>16</td>
<td>38</td>
<td>85.7%</td>
<td>54</td>
</tr>
<tr>
<td>Negative</td>
<td>5</td>
<td>4</td>
<td>14.3%</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>21</td>
<td>42</td>
<td>100%</td>
<td>63</td>
</tr>
</tbody>
</table>
**PCR of the nad1 gene:**

Of 63 isolates from camels and worms amplified, 54 isolates (85.7%) revealed similar band pattern of 1073-1078 bp long fragments (Fig. 4.1).

![Fig. 4.1. PCR of nad1 gene. 1. M, marker; 1-3, camel protoscolices; 4-6, worms; 7, negative control](image)

**RFLP-PCR of the nad1 gene:**

A subsequent digestion of 54 isolates with restriction enzyme Hph1 confirmed similar banding pattern for G6 (Fig. 4.2).

![Fig. 4.2. RFLP-PCR of nad1 gene with Hph1. M, marker; 1-4, G6; 5, G6 positive control; 6, G5 positive control; 7, negative control](image)
PCR specific for *E. ortleppi* (G5) and *E. granulosus* G6/7:

A specific and sensitive PCR/semi-nested PCR system for the rapid diagnosis of *Echinococcus granulosus* genotype G6/7 and *Echinococcus ortleppi* (G5) described by Dinkelet al (2004) was proved to be very useful for discrimination of *Echinococcus* genotypes. Thus, of 63 isolates from camels from abattoirs survey amplified, 54 isolates (85.7%) revealed similar band pattern of 373 bp long fragments (Fig. 4.3). The PCR products were determined to species level by genotype 5/6/7 PCR to amplify *E. ortleppi* and G6/G7 genotypes of *E. granulosus* with a characteristic band of 254bp (Fig. 4.4). Using specific G5/6/7 PCR revealed a band of 254 bp (Fig. 4.4). To discriminate between *E. granulosus* G6/7 and *E. ortleppi*, the amplification product underwent different semi-nested PCRs (G5 PCR and G6/7 PCR). The G6/7 PCR amplified only the *E. granulosus* G6/7 genotypes (Fig. 4.5), where the PCRs resulted in a specific product of 171bp. With this system, no discrimination was achieved between *E. granulosus* genotypes G6 and G7.

![Fig. 4.3. Cestode specific PCR. M; marker, 1-5 camel protoscolece, 6; negative control.](image-url)
Lamp Technique:

The loop-mediated isothermal amplification (LAMP) assay of the nad1 gene was designed to identify *E. granulosus*. Thus, LAMP assay for the same 16 (out of 16 which positive by PCR) isolates from camel origin from nyala abattoirs amplified only the *E. granulosus* G6 (100%) genotypes.
**Table (4.2): Lamp technique results:**

<table>
<thead>
<tr>
<th>Frequency</th>
<th>Valid Percent</th>
<th>Cumulative Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent</td>
<td></td>
</tr>
<tr>
<td>Valid</td>
<td>Positive 16</td>
<td>100.0</td>
</tr>
</tbody>
</table>

**Figure (4.6)** Lane: 1-5 isolates ,lane 6 : positive control , Lane 7: Negative control Under U/V.
4.2 Discussion:

Cystic echinococcosis is one of the neglected zoonotic diseases (WHO, 2010). In Sudan, several studies documented the endemicity of cystic echinococcosis in different parts of the country (Saad and Magzoub 1989a; Elmahdi, et al., 2004; Mohammed and Elmalik, 2000 and Osman, et al., 2007).

In the present study, 32.6% of camel was found infected with cystic echinococcosis in Central Sudan and 43.8% in Western Sudan with fertility rate of 45.6%. The result from this survey is in line with that reported by Elmahdi et al. (2013) in the same areas (16.1% and 29.1% in Tambool and Nyala, respectively with fertility rate of 53%) and Shadia and Abdelrahim, (2015) in Central Sudan (22% with high fertility rate 80%). Contrary, than those observed, in the same region, by Omer et al. (2010), they reported prevalence of 61.4% and fertility rate 74% in Western Sudan and 55.6% and fertility rate 75% in Central Sudan. This broad variation observed may be due to development of an earlier natural protective immunity or to the involvement of different strains/genotypes, where the severity of the infection in the intermediate host is largely influenced by the E. granulosus strain/genotype involved. Also, the low prevalence rate in the present study may be due to change in attitude of the people in the study area, where camel lungs become fit for human consumption in comparison with the previous period (Mohammed, 1997).

Similar to that found by Omer et al. (2010) and Elmahdi et al. (2013) in the same areas, the lungs is a main predilection site of camel cysts. In addition to that, older animals had significantly higher number of hydatid cysts. findings confirmed the previous study which stated that, the prevalence of E. granulosus was correlated with age (Ibrahim et al., 2011 and Osman, 2012). However, this study indicated that females had high number of hydatid cysts than males although there was no significant difference in number of hydatid cysts among sex of animals. This could be attributed to the fact that most female animals are slaughtered in old ages. Similar results were found previously in different parts of the Sudan by Elkhawadet al. (1979a), Saad and Magzoub (1989a), Mohammed and Elmalik (2000), Elmahdi et al. (2004), Omer et al. (2010) and Ibrahim et al. (2011).

Therefore, the high fertility rate with high mean volume of cysts (ml) and mean number of protoscolices/ml confirmed the significant role of camel in transmission of echinococcosis not only in the Sudan as described in the previous studies (Elkhawadet al., 1979a; Saad and Magzoub, 1989a; Mohammed and Elmalik, 2000; Elmahdi et al.,
In the present study, 65 Echinococcus isolates collected from camel genotyped by RFLP-PCR, specific G5/6/7 PCR and 16 LAMP were found to be G6 genotype of *E. Canadensis*. This is in agreement with the previous findings by Dinkel et al. (2004), Omer et al. (2010), Ibrahim et al. (2011) and Elmahdi et al., 2013, where all isolates from camel collected from the same area found to be G6 (camel strain). Contrary to that found in most areas, where CE is a major public health problem, the G1 genotype predominates. Also, this is in contrast to that found in the same area (Tambool and Rufaa) by Omer et al. (2013) who have indicated to camel strain’ (G6) in dogs, whereas *E. granulosus sensustricto* (sheep strain) identified in a single case in Rufaa and deposited in GeneBankTM Accession nr HQ 012553. In spite of the fact that most isolates from humans were previously characterized as *E. granulosus* G1 (common sheep strain) few infections with genotype G6 (‘camel strain’) have been reported in different part of the world (Bardonnet, *et al*., 2002; Harandi, *et al*., 2002; Azab, *et al*., 2004 and Dinkel, *et al*., 2004). Whilst, recent study proved that all human isolates in the Sudan found to be G6 genotype (Omer, *et al*., 2010). 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. Similar to that found by Nkouawa et al. (2010), when they have compared LAMP with multiplex PCR in the differential detection of *Taenia* in stool samples from patients with taeniasis, the LAMP technique, with no false positives in this study, demonstrating a high value in detecting molecular echinococcosis (100%) than PCR of the nad1 gene (85.7%) and PCR specific for *E. ortleppi* (G5) and *E. granulosus* G6/7 (76.9%). This in agreement with that reported by Salant, *et al*. (2012), where LAMP showed high species specificity for *E. granulosus* with no cross-amplification of DNA from closely related helminths, including *Echinococcus multilocularis*. Moreover, working at Tamboul and Rufaa, by Omer et al. (2013) who have indicated that to camel strain’ (G6). All the DNA samples extracted from the worms were characterized as camel (G6) strain of *E. granulosus*. This is result reported by (Elhag, A.M. *et al*. 2016), LAMP technique, demonstrating a high value in detecting molecular echinococcosis (100%) than PCR of the nad1 gene (85.7%). This in agreement with that reported by (Salant, *et al*. 2012), where
LAMP showed high species specificity for *E.granulosus* with no cross-amplification of DNA from closely related helminths, including *Echinococcus multilocularis*. However, hydatid disease in the Sudan, has just recently been considered as a recognized zoonoses, but no comprehensive epidemiologic investigation has been done for comparison between livestock and human hydatidosis during a specific period of time.
CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 Conclusion:

LAMP technique is a very sensitive, easy and time efficient method, where takes less time for amplification and detections compared to PCR of the nad1 gene in the detection of *Echinococcus granulosus* isolates collected from camel give (100%) positive result.

LAMP demonstrating a high value in detecting molecular *Echinococcosis granulosus* than PCR of the nad1 gene (85.7%).

5.2. Recommendations:

Depending on the results of this study, study recommend:

1- Special attention to infrastructure of slaughter houses is highly needed to minimize the risk of transmission of the disease.

2- LAMP and PCR techniques should be implemented in endemic area as diagnostic tools.

3- Similar studies are needed to clarify the role of wild hosts in transmission of *E. granulosus* in the study area.

4- Study to examine the internal and external factors that made camel the main intermediate hosts of *E. granulosus* in Sudan is needed.

5- more cheap diagnostic tools like latex kits diagnostic in population at risk Conduct

6 - more molecular genotyping research in *E. granulosus*.
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