## Cystic echinococcosis in humans and domestic animals in central Sudan

*Epidemiology and molecular characterization studies*

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# Cystic Echinococcosis in Humans and Domestic Animals in Central Sudan: Epidemiology and Molecular Characterization Studies

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This Thesis was prepared at the Department of Infectious Disease, amc University of Amsterdam, The Netherland in Fulfillment of the requirements for a Doctoral Degree

**Cystic Echinococcosis in Humans and Domestic Animals in Central Sudan: Epidemiology and Molecular Characterization Studies**

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Faculteit der Geneeskunde

Then older I Get

The more I think

You only get a minute, better live while you're in it

'Cause it's gone in a blink

And the older I get

The truer it is

It's the People you love, not the money and stuff

That makes you rich

And if they found a fountain of youth

I wouldn't drink a drop and that is the truth

Funny how it feels I'm just getting to my best years yet

The older I get, the fewer friends I have

But you don't need a lot when the ones that you got

Have always got your back

And the older I get, the better I am

At knowing when to give, and when to just not give a damn

And if they found a fountain of youth

I wouldn't drink a drop and that is the truth

Funny who it feels I'm just getting to my best years yet

The older I get

The more I think

*(Adam Wright)*

*Alan Jackson Song*

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# Chapter 1

## General introduction

Echinococcosis belongs to the neglected zoonotic diseases, (NZD), as they have been recently classified by the World Health Organization (1). Echinococcosis encompasses a series of clinical entities, such as cystic echinococcosis (CE), alveolar echinococcosis (AE), and the neo tropical forms of poly echinococcosis. The definitive hosts of the tape worm *Echinococcus granulosus*, *E. multilocularis*, *polycystic echinococcosis* (*E. vogeli, E. oligathrus*) are carnivores (dogs, cats, foxes, wolves). The larval form occurs in the intermediate host, namely herbivores and omnivores (sheep, goat, cattle, camel, horse, pig, rodents). Humans get infected incidentally and are not part of the life cycle (2).

Hippocrate was probably the first to describe the disease when he described the liver as being filled with water and bursting into the epiploon, in this case the belly is filled with water and the patient dies (3). Al-Rhazes, an Arabic physician in AD 900, described a hydatid cyst of the liver. The following comment on the Hippocratic aphorism quoted above is translated from Al-Rhazes' original book Al-Hawi in medicine: Watery balloons may form in the liver within its infesting membrane more often than in other organs (2). The etiological agents and its character were delineated during the 17$^{th}$ and 18$^{th}$ centuries, but the complete life cycle was documented not until 1805 by Rudolphi in the 19th century (4). Hydatid is a Greek word meaning watery. In the old nomenclature, *E. granulosus* was also termed *E. hydatidosus*, characterizing its appearance in the intermediate host. Granulosus stays for the granular appearance of the adult tape worm, and is used today.

Cystic echinococcosis is caused by *E. granulosus* and constitutes a medical problem and endemic in many countries worldwide (5). Incidental human infestation with the larval form results in formation of cysts in various parts of the body, with liver and lungs being the most common sites (6). Since the parasite has developed mechanisms to avoid host immunity; the infection is often asymptomatic for many months and years until a mechanical complication occurs, such as rupture (into the biliary tree, bronchial tree, or peritoneum), compression of vital structures, haemorrhage, or tissue failure (particularly with bone involvement).

Cystic echinococcosis (CE) is globally distributed (7). In sub-Saharan Africa, CE is considered to be highly endemic, with variable distributions in different countries (8). United States,

Scandinavia and central Europe, cases only occur sporadically. The most affected regions in Europe are Spain and South Italy incidence is 4-8 per 100000 inhabitant (annually) (8, 9, 10).

In Sudan, the disease was first reported in dogs in 1962 where the prevalence of Echinococcus granulosus was 86.4% (11). In 1987, another study found a prevalence of 12% and 10.3% in sheep and goats, respectively. Saad & Magzoub (1988) stated that the prevalence of hydatid disease in dogs in the Tambool area was 51% (12). In 2001, a survey-based study carried out in southern Sudan reported a prevalence of 2% in humans (14, 13). 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 (14, 15). In southern Sudan the prevalence of CE among Bouya people was 2% (15, 16) and 3.5% among Toposa (16, 17). In 2005, an ultrasound surveyin Tambool area, central eastern Sudan, showed a prevalence of 0.92% of hydatid cysts in human liver, spleen, prostate and eye cases (17). A more recent and extensive survey in the same area by Ahmed et al. revealed a percentage of (1.04%) of patients screened having features of liver CE on abdominal ultrasonography. Five to six percent of camels exported from Sudan to Egypt were found affected by hydatid disease, with camel lungs having been more affected than livers. In Central Eastern Sudan at Tambool abbatoirs, 35 percent of slaughtered camels were found affected with 90 percent of which were affected by lung cysts (18).

Ten genotypes of *Echinococcus granulosus* (EG) designated (G1-G10) are recognized worldwide; namely the sheep strain (G1), Tasmanian sheep strain (G2), buffalo strain (G3), horse strain (G4), cattle strain (G5), camel strain (G6), pig strains (G7/9) and cervid strains (G8/10). The cysts of different strains can be distinguished by molecular methods such as polymerase chain reaction (PCR) and DNA sequencing. Although these PCR-based detection assays proved highly sensitive and specific, they do require individual testing of each submitted sample for the genotype-specific detection and subsequent identification of the genotype using specific primers in PCR amplification (19). This limitation renders these conventional PCR based detection assays rather time consuming, which is not the case for real time PCR, which is rapid and quantitative; however, equipments are more expensive. Recent epidemiological studies in Sudan indicated that the camel genotype (G6) was reported to be the most prevalent strains (19, 20, 21). The G6 strain was found to be endemic in camels, goats, cattle and humans, while E.

ortleppi (or cattle strain G5 of E. granulosus) was found in cattle in Sudan (21). However, a recent PCR and DNA sequencing in samples of hydatid from camels showed also G5(20). On the other hand, the common sheep strain (G1) which is suspected to be the principal genotype, affecting humans in sub-Saharan Africa, which was not found and therefore appears to be rare or even absent in the central Sudan (22). A recent sample extracted from human lung hydatid which has been associated with squamous carcinoma lung at its base came to be G1(23). 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 (24). The sporadic occurrence of this genotype may signify its lower pathogenicity to humans (25). However, this G6 strain of E. canadensis has been previously identified in human patients all over the world (20, 25, 30).

Globally, awareness for EC is important and should lead to better understanding of the natural history in human and animals as well as eradication. Studies have shown high prevalence rates in dogs (50– 70%), camels (35%) and sheep, goats and cattle (10–11%). In total, 0.3–1.0% of humans in Central and South Sudan are infected with the G6 camel strain (28, 31). This strain is almost exclusively the cause of human infections to Echinococcus granulosus.

Cattle known previously to harbour G5 only (8). Further studies by DNA sequencing has confirmed those findings. Using new techniques in molecular not only simple and nested PCR, such as real time PCR where the result was less time consuming and more quantitative has been used in Sudan (9). Developing a rapid, more simple and less cumbersome which can be used in the field has been described, loop mediated lamp also for the first time, which give results instantaneously only with change of colour (LAMP PCR). DNA sequencing is also useful to compare the EC gene which has been detected to other types of EC gener worldwide using Phylogony tree by getting access to EC gene bank, this can allow useful information about molecular epidemiology and distribution of EC.

# Aims and outline

The objective of this thesis is to provide data from various angles, to elucidate the epidemiology and molecular characteristics of cystic echinococcosis in Sudan; data which shall help to improve management of the disease. The other important type of data to inform improvement of disease management is to understand the baseline knowledge, attitudes and practices in the human population in quest.

**Section I-Epidemiological studies**

We conducted a systematic review on cystic echinococcosis in sub-Saharan Africa **(Chapter 2)**. We reviewed the data for cystic echinococcosis available from sub-Saharan Africa, described the present knowledge of the epidemiology of CE in the region, and reviewed options for treatment and prevention from published literature.

In **Chapter 3,** the investigation expands to the existing sequence data generated from EG isolates recovered from camel in the Sudan. It was suggested that sheep and goats have natural resistance to infection with EchinoGran-complex. However, this assumption requires further investigation. To advance beyond the current knowledge of the epidemiology of the disease in camel, attempts were made to better understand the life cycle and molecular epidemiology of EG isolates circulating in different parts of the Sudan. We provided some sequence data and phylogeny of EG isolates recovered from the Sudanese one-humped camel (*Camelus dromedaries*).

**Section II-Molecular epidemiological studies**

The developed LAMP assay would be expected to prove highly significant in epidemiological surveys of CE in developing countries or areas of resource-poor settings for both ease of use and cost. In this study the LAMP assay was evaluated for detection of the Sudanese geno-types of EG-complex hydatid cysts **(Chapter 4)**. In addition to that, the LAMP assay was evaluated for the levels of diagnostic sensitivity and specificity when testing a variety of archived hydatid cysts sampled from human or susceptible animal populations. The performance of the LAMP

assay under isothermal conditions without the need of special apparatus, and visualization of results by the naked eye was investigated as well.

In **Chapter 5,** we investigated the conventional and qPCR assays, the detection of ruptured and calcified cysts was made possible by both. The qPCR assay was preferred for its convenience and minimum sample handling, thus preventing the occurrence of cross-contamination which may decrease the quantitative reliability of the assay. It is well documented that SYBR green-based qPCR assays are less specific than the TaqMan qPCR assays. In addition, the qPCR assay can be implemented in a research laboratory setting for the purpose of rapid diagnosis and epidemiological surveillance of CE in humans and animals in developing countries, such as Sudan. CE is widespread worldwide including the Sudan and identification of the genotype of the cyst would be advantageous for the prevention and control of the disease. **Chapter 5** illustrates that the qPCR offers advantages over the conventional gel-based nested PCR, being less time consuming and preventing cross contaminations.

We investigated the transmission dynamics and the epidemiology of cystic echinococcosis in Sudan (**Chapter 6**). We illustrate in for the first time, an insight of the role of cattle in the transmission of the zoonotic G1 echinococosis by using PCR based genotyping.
To measure the genetic variability, the number of haplo- types was determined using DNASP v5 with insertions and deletions considered as variable sites. We used the median-joining (MJ) network algorithm implemented in NETWORK 4.6 (www.fluxus-engineering.com). The phylogenetic tree revealed the presence of Echinococcus canadenesis genotype 6 (G6) cysts, Echinococcus ortleppi genotype 5 (G5) and Echinococcus granulosus sensu stricto (s.s) genotype 1 (G1). The phylogenetic network analysis revealed genetic variation among the different haplotypes/genotypes.

We investigated the molecular diversity of Echinococcus granulosus isolates collected from human clinical samples removed surgically from lung using mitochondrial gene nad1 in Sudan. Echinococcosis cysts were defined by genetic studies and subsequent phylogenetic analysis.
The molecular characterization was made possible by targeting fragments of the mitochondrial NADH 1 to define the circulating genetic variants in the different part of

Sudan. PCR was performed to amplify fragments of 530 base pair (bp) for NADH dehydrogenase subunit 1(NADH-1) gene. Sequencing for subsequent construction of phylogenetic tree and network analysis was done (**Chapter 7**).

**Section III- Community based cross sectional study on KAP on cystic echinococcosis**

Knowledge, attitudes and practices (KAP) regarding the disease among people living in Central Sudan has been addressed (**Chapter 8**), which has led to useful information regarding future control of disease in human and animals. Seroprevalence was assessed using the locally developed enzyme linked immunosorbent assay (ELISA) which has been used to detect immunoglobulin G (Ig G) antibodies to *Echinococcus granulosus.*

**Section IV-Determinants of cystic echinococcosis among Khartoum state population**

The disease is endemic in many areas of the African continent, including Sudan. However, due to the lack of epidemiological data, problems associated with disease diagnosis and the chronic nature of infection and long-term treatment, it often has a low priority and is therefore part of the group of neglected tropical diseases. Improved surveillance is necessary to optimize control and prevention strategies for CE as an important neglected zoonotic disease among the human population in the study area of Central Sudan. Accordingly, we investigated the risk factors associated with CE seropositivity among humans in Khartoum State, Central Sudan. An in-house enzyme-linked immunosorbent assay was used to detect immunoglobulin G antibodies to *E. granulosus* **(Chapter 9)**. The $\chi^2$ test and logistic regression analysis were used to estimate the prevalence of seropositivity.

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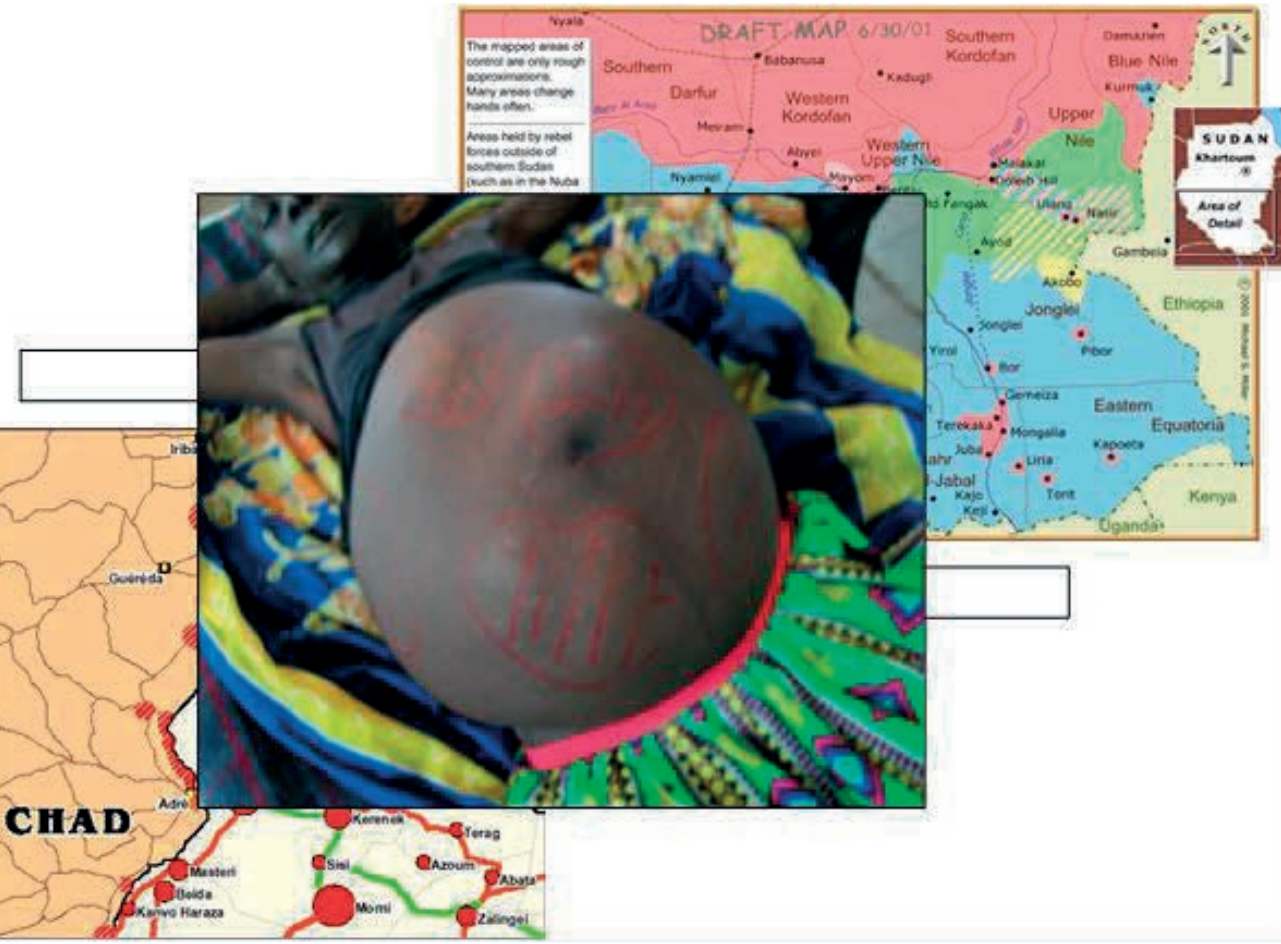
Section

I

# Epidemiological studies


DRAFT MAP 6/30/01
The mapped areas of control are only rough approximations. Many areas change hands often.
Areas held by rebel forces outside of southern Sudan (such as in the Nuba
Nyala
Southern Darfur
Babanusa
Kadugli
Southern Kordofan
Western Kordofan
Meram
Abyei
Western Upper Nile
Nyamlel
Mayom
Blue Nile
Kurmuk
Upper Nile
Malakal
Renk
Fangak
Ulang
Nasir
Ayod
Gambela
SUDAN
Khartoum
Area of Detail
Akobo
Jonglei
Ethiopia
Yirol
Bor
Pibor
Gemeiza
Eastern Equatoria
Terekeka
Mongalla
Juba
Kapoeta
Liria
Jabal
Kajo
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Kejo
Uganda
Guéréda
CHAD
Adré
Kerenek
Terag
Siel
Azoum
Abata
Masteri
Iloida
Kanyo Haraza
Mormi
Zalingei

# Chapter 2

## Cystic echinococcosis in sub-Saharan Africa

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**Abstract**

Cystic echinococcosis is regarded as endemic in sub-Saharan Africa; however, for most countries only scarce data, if any, exist. For most of the continent, information about burden of disease is not available; neither are data for the animal hosts involved in the lifecycle of the parasite, thus making introduction of preventive measures difficult. Available evidence suggests that several species or strains within the Echinococcus granulosus complex are prevalent in sub-Saharan Africa and that these strains might be associated with varying virulence and host preference. Treatment strategies (chemotherapy, percutaneous radiological techniques, but mainly surgery) predominantly target active disease. Prevention strategies encompass anthelmintic treatment of dogs, slaughter hygiene, surveillance, and health educational measures. Existing data are suggestive of unusual clinical presentations of cystic echinococcosis in some parts of the continent, for which the causes are speculative.

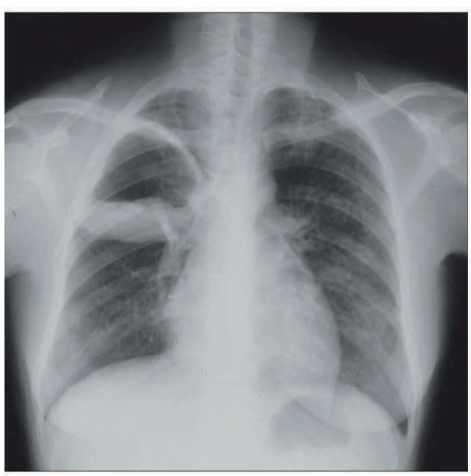
## Introduction

Cystic echinococcosis is a zoonosis caused by cestodes of the *Echinococcus granulosus* complex. Adult tapeworms inhabit the small intestine of carnivores (the definitive hosts) and produce eggs, which are passed with faeces. The intermediate host (including sheep, cattle, donkeys, and camels) is infected by ingestion of eggs. Subsequently, a larval stage (metacestode) develops as a cyst in internal organs of this host. The metacestode produces many protoscolices, each with the potential to develop into an adult tapeworm when ingested by the definitive host. Cysts can be either viable or non-viable. Viable cysts are usually filled with clear fl uid with few calcifications, whereas non-viable cysts are mainly calcified. Viable cysts can be either fertile, containing protoscolices, or sterile, containing only highly antigenic fl uid.1 People can become intermediate hosts after accidental ingestion of eggs; developing cysts cause the morbidity and mortality associated with the disorder. Liver and lungs (figures 1, 2) are the most commonly affected organs·[1,3] conventionally, the causative agent of cystic echinococcosis was regarded as one species, E granulosus. However, researchers have long known that this species is composed of several different taxa, which diff er from each other in adult morphology, host preference, and pathogenicity (including to people).4 To accommodate this diversity, ten different strains (G1–10) were described, with each being attributed to the intermediate host animal that was thought to be most important for transmission (eg, the sheep strain G1, or the camel strain G6). Eventually, some of these strains were reclassified as separate species, on the basis of substantial molecular differences. This reappraisal continues,5 but currently, cystic echinococcosis of people or animals, or both, can be caused by E granulosus (G1–3), E felidis (the so-called lion strain), E equinus (G4), E ortleppi (G5), and E canadensis (G6–10).6 Surveys done up to now generally do not take this differentiation into account and therefor information about distribution, hosts, and the clinical effect of different species and strains is scarce. E granulosus is believed to be the cause of most human cases; E equinus is thought to be the onlyspecies that cannot infect people. Apart from E felidis, all strains use the domestic dog as an intermediate host.[7] Cystic echinococcosis occurs worldwide, and is endemic in several areas, particularly the Mediterranean, central Asia including the Tibetan plateau, northern and eastern Africa, Australia, and southern South America.[8,10] Generally, the highest prevalences of the disorder are in nomadic populations. Nomadic people keep dogs for various reasons, such as herding and guarding, as food, as bed-warmers, and as sanitation animals. The combination of

people and dogs living in close proximity, scarce water resources, and conditions with poor hygiene provide the ideal environment for *Echinococcus* spp.[2] It belongs to the neglected tropical diseases group, which receive little funding for research and treatment relative to the burden of disease.[11,12] The clinical signs and symptoms, diagnosis and management, and prevention and control of cystic echinococcosis, including in sub-Saharan Africa, have been reviewed.[13–15] With regards to the epidemiology of cystic echinococcosis in Africa, the pattern is patchy. Nowadays, researchers generally accept that this disorder is prevalent across the whole continent, with an area of high prevalence in east Africa (especially the Turkana region).[10] The aim of our Review is to summarise the data for cystic echinococcosis available from sub-Saharan Africa, and to best describe present knowledge of the epidemiology of this disorder in the region and options for treatment and prevention**.**

**Treatment and prevention**

In brief, researchers have developed classification systems, based on ultrasound findings, to differentiate early stage disease, active disease with daughter cysts, and end stage cystic echinococcosis.[16,17] Treatment strategies are derived from these classifications, and predominantly target active disease. Treatment options for cystic echinococcosis are chemotherapy, percutaneous radiological techniques, and surgery. Traditionally, surgery was the sole therapeutic option because of its potential to completely remove the parasite. Different surgical approaches have been developed with time, ranging from cystectomy to more aggressive surgery, and ultimately liver transplantation.



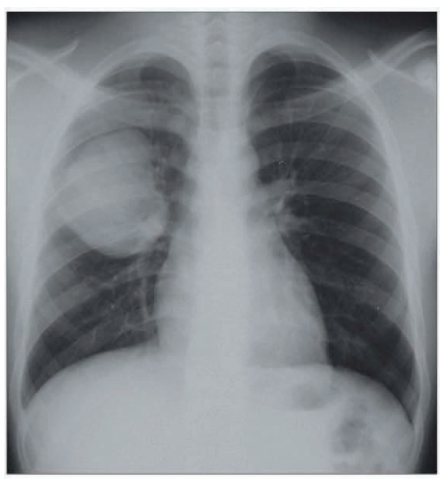


**Figure 1: So-called water lily sign in a patient with pulmonary cystic echinococcosis**

**Figure 2: Pulmonary hydatid cyst**

More invasive surgical techniques are associated with more complications (eg, infection, biliary leakage) but less recurrence of active disease than are conservative approaches.[18] In the past 20 years, ultrasoundguided Percutaneous procedures have partly re placed surgery as the treatment of choice. Puncture, aspiration, injection, and reaspiration (PAIR) and its modified version, percutaneous aspiration of cyst content (PEVAC) are safe and effective alternatives to surgery.[19] Benzoimidazole carbamates (albendazole or mebendazole) are widely established as chemotherapy for cystic echinococcosis. Drug treatment is used alone to suppress early-stage disease, and adjuvant to surgical, PAIR, and PEVAC approaches for large, active hydatid cysts.[13] Despite the possibility of drug treatment, interventional therapy is the mainstay of treatment for cystic echinococcosis in sub-Saharan Africa. This situation arises mainly because the resources needed to provide a reliable supply of expensive medication and laboratory facilities to monitor patients for side-effects are scarce. The nomadic lifestyle of many patients further com plicates the matter.[20] Prevention and control strategies for cystic echinococcosis consist of anthelmintic treatment of dogs, slaughter hygiene, surveillance, and health-focused education about human–dog behaviour. In the future, vaccination of livestock might be possible.[14]

## Cystic echinococcosis in sub-Saharan Africa

The table provides an overview of surveys of human hydatid disease reported from sub-Saharan Africa. The appendix provides detailed information about individual case reports and case series of cystic echinococcosis in people in sub-Saharan Africa, and about surveys of echinococcosis in domestic animals from this region.

### West Africa

Cystic echinococcosis is generally thought to be uncommon in west Africa and epidemiological studies have only been done in Nigeria (people and livestock) and Burkina Faso (livestock only).

However, cases of human cystic echinococcosis are also reported from Senegal, Niger, and Ghana. 33–35 researchers have reported a small case series (n=32) from Niger. As in other parts of Africa, a female predominance was noted (n=20), but by contrast with data from other countries, a predominance of extra hepatic disease was recorded. In a livestock survey, 22% of 513 camels were infected with E granulosus.34 One case of a patient from the Central African Republic has been reported, in which molecular typing identified genotype G6 (camel strain). However, the investigators noted that camels are absent from the Central African Republic and that cystic echinococcosis in sheep (another host of the G6 strain) has not been recorded there up to now.36 Therefore the hosts involved in the lifecycle of Echinococcus spp in the Central African Republic remain to be elucidated. A retrospective survey of cystic echinococcosis in livestock is available from Burkina Faso. Hydatid cysts were found in ten of about a million animals of various species.37 No data for human disease are available, but this disorder is unlikely to be a major health problem in Burkina Faso. No genetic data exist from this country. In Nigeria, cystic echinococcosis has been investigated more extensively. Human hydatid disease is not believed to be common, but is likely to be underdiagnosed.28 A serological survey (complement fixation test) of hospital personnel and patients in the central north and southwest of the country showed the presence of antibodies against echinococcal antigen in 0·53% of 176 individuals,29 but corresponding clinical data for disease were not available. A review 28 of hospital records from three regions in Nigeria identified only one confirmed case of cystic echinococcosis in more than 500 000 records. However, the investigators questioned the accuracy of this finding because four more cases were identified in one region in northern Nigeria during the study period. They argued that low prevalences were the result of poor diagnostic facilities and inefficient record-keeping, rather than representing rue occur rences.28 The highest prevalences of cystic echinococcosis in livestock were reported from the Niger Delta and the north of the country. In the Niger Delta, pigs were the most commonly affected species (55·9% of 320 pigs infected, 21·5% of cysts were fertile), whereas in the north, cystic echinococcosis was mainly recorded in camels (55·5% of 3598 camels infected, 94·5% fertile cysts).38,39 By contrast, in central and eastern parts of the country, cystic echinococcosis was rare, with the exception of the northern central parts, where 11% of 1800 sheep were infected with E granulosus.39 In Ibadan only seven cysts were indentified in 164 sheep examined and none in goats.40 Infection with E granulosus was also common among dogs in the Niger Delta

(63%) and Ibadan.38,40 In Ibadan, dogs from native (12 of 17) and residential neighbourhoods (11 of 18) were most commonly affected.40 By contrast, in the northern, central, and eastern parts of Nigeria prevalences of Echinococcus spp in dogs were low.41 Surprisingly, most human cases of cystic echinococcosis in Nigeria are reported from the northern parts of the country.28,42 One potential explanation for this finding is the difference in lifestyle between different regions of Nigeria; raw off al is commonly offered to dogs in the north and south of the country, whereas in the east it is regarded as a delicacy and not offered to dogs at all.43,44 The differences in infection rates and fertility of cysts suggest that different taxa of Echinococcus spp are present in Nigeria, with varying infectivity to people, explaining the relatively low infection rates in people despite very high infection rates in livestock.

**East Africa**

Cystic echinococcosis has been investigated most extensively in east Africa, particularly in the Turkana and Maasai regions in Kenya (figure 3) and bordering districts in Uganda, Sudan, and Ethiopia. An ultrasound survey in the extreme southeast of Sudan, near the Turkana border, identified an area of high prevalence of human cystic echinococcosis. The prevalence of this disorder was reported to be 2% among the Bouya people and 3·5% among the Toposa.45,46 In the rest of the country human cases seemed to be more sporadic.30,47 Where the disorder does occur in Sudan, pulmonary presentations are common, accounting for 17 of 38 cases in one series from Khartoum.48 In animals, cystic echinococcosis was fi rst reported in dogs in 1962 when the prevalence of E granulosus was 86·4%.49 Data for cystic echinococcosis in livestock are available from central, western, and southern Sudan. From central Sudan, prevalences from 20% (cattle) to 55·6% (camels) were reported.50 In western Sudan, prevalences were highest among camels (61·4% of 565, 74% of cysts were fertile) and sheep (11·9% of 9272, 19% fertile cysts).50 Although the prevalence in cattle was lower (5·2% of 4318), the prevalence of fertile cysts was high (75%). 1·9% of 5523 goats in this area were infected with Echinococcus spp (33% of cysts were fertile).50 In southern Sudan, prevalences in cattle (7·1% of 325), sheep (2·7% of 295), and goats (7·1% of 42) were substantially lower than in the west.50 The camel strain G6 predominates in Sudan. This strain is less infective to people than are others, which

might explain the rather sporadic occurrence of human cystic echinococcosis in most parts of Sudan. 50 Although no data are available in the English scientific literature for strains causing disease in the extreme southeast of Sudan, some investigators have suggested that the sheep strain could be prevalent there, causing the increased prevalence of human hydatid disease. Dogs have been examined only in Tambool in central Sudan, where 25 of 49 dogs were deemed to be heavily infected.51 In Ethiopia, before the introduction of ultrasonography and modern serological tests as routine diagnostic instruments, Fuller and Fuller22 showed that the Dassanetch and Nyangatom people from the southwest of the country had a prevalence of cystic echinococcosis of up to 5% on the basis of findings of clinical examination, and more than 5% when the hydatid skin test was taken into consideration. 22 The Dassanetch and Nyangatom peoples live in the same geographic area as the Turkana people of northwest Kenya, and these populations seem to share customs because they all use dogs for cleaning purposes. By contrast, results of an ultrasound survey of the Hamar people of southwestern Ethiopia showed a much lower prevalence (0·7% of 990 people) than for the Dassanetch and Nyangatom peoples.23 Macpherson and colleagues9 did ultrasound surveys of various ethnic groups in southern Ethiopia and recorded the highest prevalence in the Nyangatom people (2·9% of 1334).9 Case series have been reported from central Ethiopia.52–54 Between 72 and 234 patients were seen over 10–15 years at hospitals in Addis Ababa. By contrast with other countries, researchers did not identify a female pre dominance in these case series

| | Method | Number of individuals | Prevalence |
|---|---|---|---|
| **Ethiopia** | | | |
| Southwest[21] | Physical examination, Casoni skin test | Clinical examination 640<br>Casoni skin test 175 | Clinical examination: Dassanetch 11·3%, Nyangatom 5·8%, Kerre 5%, Hamar 1·6%, Suri 0%<br>Skin test: Dassanetch 39·9% |
| Countrywide[22] | Physical examination, indirect haemagglutination, hydatid skin test | Clinical examination 3408<br>Indirect haemagglutination 1428<br>Hydatid skin test 986 | Clinical examination: overall 2%, Dassanetch highest 5·1% Indirect haemagglutination: overall 1·7%, Dassanetch highest 6·4%<br>Hydatid skin test: overall 15·7%, Dassanetch highest 36% |
| Nyangatom people[9] | Ultrasound survey | 1334 | 2·9% |
| Boran people[9] | Ultrasound survey | 110 | 1·8% |
| South (Hamar people)[23] | Ultrasound survey | 990 | 0·7% |
| Hamar people[9] | Ultrasound survey | 369 | 0·7% |
| Dassanetch people[9] | Ultrasound survey | 267 | 0% |
| **Kenya** | | | |
| Turkana[20] | Retrospective, 21 years of surgical records | Total not available | 710 procedures for hydatid disease in 663 patients |
| Turkana[24] | Patients presenting to hospital over 3 years | Total not available | 355 patients treated for hydatid disease, male:female ratio 1:2 |
| Turkana[25] | Serological survey, indirect microhaemagglutination test | 1190 | North Turkana 9·4%, 85/100 000 per year, South Turkana 2·1%, 25/100 000 per year |
| Turkana[26] | Retrospective review of hospital record | 761 operations | 4·5% for hydatid disease |
| Northeast Turkana[27] | Anti-echinococcus antibody ELISA | 538 | Positive: 16·4%. Strongly positive: 4·1% |
| Turkana (northwest)[9] | Ultrasound survey | 3553 | 5·6% |
| Turkana (northeast)[9] | Ultrasound survey | 3462 | 2·7% |
| Turkana (central)[9] | Ultrasound survey | 1508 | 0·3% |
| Turkana (south)[9] | Ultrasound survey | 1361 | 0·2% |
| Turkana (eastern)[9] | Ultrasound survey | 607 | 0% |
| Pokot people[9] | Ultrasound survey | 2389 | 0·1% |
| Gabbra people[9] | Ultrasound survey | 38 | 0% |
| Somali people[9] | Ultrasound survey | 1252 | 0% |
| Samburu people[9] | Ultrasound survey | 368 | 0% |
| Rendille people[9] | Ultrasound survey | 710 | 0% |
| **Nigeria** | | | |
| Sudan zone | Retrospective analysis of hospital records | 189 861 | 0·0005% |
| Northern Guinea | Retrospective analysis of hospital records | 279 827 | 0% |
| Bauchi plateau[28] | Retrospective analysis of hospital records | 151 007 | 0% |
| Nigerstate, Ogunstate[29] | Serological survey, hydatid complement fi xation test | 176 | 0·53% |
| **Sudan** | | | |
| Central region[30] | Ultrasound survey | 300 | 0·33% |
| Toposa people[9] | Ultrasound survey | 278 | 3·1% |
| **Tanzania** | | | |
| Maasai people[9] | Ultrasound survey | 959 | 1·1% |
| Ngorongoro district[31] | Retrospective analysis of hospital records | Unknown | 10/100 000 per year; 171 cases |
| **Uganda** | | | |
| National[32] | Review of all biopsy and autopsy records from 1967–72 | Unknown | 23 cases |

***Table:*** **Surveys of human cystic echinococcosis in sub-Saharan Africa**

and cases of cystic echinococcosis in lung and liver seemed to be much the same, at about 40%. Regarding livestock, several researchers have investigated cystic echinococcosis in cattle in several parts of Ethiopia, finding regional differences in prevalence and fertility of cysts. The highest prevalences were recorded in central Ethiopia with up to 52·7% of 632 cattle being infected with Echinococcus spp (26·9% of cysts were fertile).[55] The highest prevalence of fertile cysts was recorded in eastern Ethiopia, where 32% of cysts were fertile.[56] The lowest prevalences were recorded in southern parts of central Ethiopia, where 16% of 400 cattle were infected (1·8% fertile cysts).57 Kebede and colleagues58 estimated the financial loss associated with cystic echinococcosis to be US$21 per infected cow. They suggested that the actual loss was even greater because home slaughtering practices were common.58 In Hawassa, an annual loss of about $138 583 was estimated to be attributable to cystic echinococcosis.55 Kebede and colleagues58 argued that in northern Ethiopia, sheep might be the main intermediate host for cystic echinococcosis because they recorded that 10·6% of 380 sheep were infected, with 56·6% of cysts being fertile. By contrast with these findings, Bekele and colleagues49 did not deem sheep to be the main intermediate host in central Ethiopia, where 16·4% of 560 tested positive but only 18·3% of cysts were fertile.[59] In goats, low prevalence was recorded in central Ethiopia (6·7% of 208),60 whereas Sissay and colleagues noted that 65% of 632 goats examined in eastern Ethiopia were infected.[61] Kebede and colleagues58 also investigated dogs for infection with Echinococcus spp in northern Ethiopia where 3 of 18 of eight dogs were infected. In this area, few human cases of cystic echinococcosis were identified. In eastern Ethiopia, Mersie and colleagues56 showed that two of nine dogs were infected with Echinococcus spp. In central Ethiopia, mainly E granulosus G1 has been identified in livestock, whereas in northern Ethiopia (the city of Makale) E granulosus G1, E ortleppi, and E Canadensis G6 and G7 were identified in 21 cysts from cattle.7,62 In a study from central and eastern Ethiopia, E granulosus G1 predominated, but E canadensis G6 was also identified, mainly in camels.[63] In Kenya, cystic echinococcosis occurs in most parts of the country but available data are mostly from Turkana communities in the northwest and from Maasai communities in the south. Both communities are nomadic pastoralists rearing huge herds of livestock (sheep and goats, cattle, donkeys, and in the Turkana also camels). In one serological survey,[27] prevalence of cystic echinococcosis was as high as 16·4% in recently settled communities in the Turkana area. Results of another serological survey showed regional difference within the Turkana district, with prevalence being

9·4% in north Turkana and 2·1% in south Turkana, which was much the same as in a control group from other parts of Kenya where hardly any cases of cystic echinococcosis were identified.[25] Ultrasonography is the most commonly used and most reliable diagnostic technique for surveys nowadays. In such surveys, the prevalence of cystic echinococcosis in the Turkana district was 5·6%.9 Irvin[26] reported that 4·5% of 791 surgical procedures in one hospital were for cystic echinococcosis.26 In clinical cases, a predominance of women has been noted, with women of childbearing age having the highest prevalence.[20,24,64,65] This female predominance was not present in serological surveys.[25,27] Hydatid cysts can occur in all parts of the body; however, in all clinical surveys, hydatid cysts of the liver were most common, followed by abdominal cysts, kidney, spleen, lung, and soft tissue. Because most rural hospitals do not have radiograph facilities, lung disease is likely to be underdiagnosed in these populations.[20,26,65] A domestic lifecycle of Echinococcus spp with dogs as the definitive host and small ruminants, cattle, and camels as intermediate hosts was thought to be most important in the Turkana district. Although jackals were also identified as definitive hosts for Echinococcus spp, they are unlikely to contribute substantially to the maintenance of the parasite‘s life cycle because their access to off al is very limited.[66,67] An independent wildlife cycle has not been described in the Turkana area. Several studies in livestock (ultrasound surveys and abattoir surveys) have been done in Turkana. Prevalences of cystic echinococcosis varied significantly within Turkana, but generally camels and cattle showed the highest prevalences (cattle 19%, camels 61%) with much variability in fertility rates of cysts.[68] Prevalence of infection in dogs in Turkana was reported to be as high as 60%, but great regional differences were identified, with the highest prevalence in the northwest and the lowest in the south and east of Turkana.[62,66,69] Researchers have identified risk factors for Echinococcus spp infestations as: age of dog of less than 5 years, free roaming of dogs, access to raw off al (giving a 12 fold increase in likelihood of infection), frequency of home slaughter, and species of animal slaughtered.69 Human infections in these communities were associated with the amount of time the dog spent in the home, when they were allowed to clean children and scavenge from bowls and skins.70 By contrast with these findings from the Turkana district, much lower prevalences of cystic echinococcosis have been identified in the Maasai area of southern Kenya (0·5%, Zeyhle E, unpublished). Despite high infection rates in their livestock and dogs and a favourable climate for the survival of echinococcal eggs in the environment, infection in people was much lower than in Turkana

(0·5% vs 2·5% in 2010, Zeyhle E, unpublished).[71,72] As in Turkana, sheep and goats seemed to be the most important intermediate hosts, but by contrast with the Turkana area an additional wildlife cycle probably exists.66,73 Although Maasai lead a lifestyle that is much the same as that of the Turkana, they have more water available to them for daily living and they do not rely on dogs for cleaning purposes, therefore their dog–man contact is less close.[71] Many Echinococcus spp isolates from Kenya have been examined genetically, mainly belonging to E granulosus G1 (sheep, goats, cattle, camels, pigs, people, and dogs) and E canadensis G6 and G7 (camels, cattle, goats, people, and dogs), and only one to E ortleppi (pig). Most samples originated from the northwest of the country (Turkana)[74–77]. In the Turkana district, the sheep strain is the predominant taxon in people, sheep, cattle, and goats, whereas the camel strain predominates in camels and partly in goats. Only two isolates of 176 hydatid cyst specimens isolated from people were identified as the camel strain (G6) whereas all remaining isolates belonged to the sheep strain (G1)[75,77].

**Figure 3:** Typical pastoral landscape in Kenya ‘s Maasai region where cystic echinococcosis is highly endemic

From Uganda**,** only one study of cystic echinococcosis in people and cattle is available. Via the national pathology service 23 cases were identified retrospectively over a period of 6 years. A female predominance was noted. Most cases were imported from Sudan (n=12) and only ten cases occurred in Ugandan people; these people were exclusively from the northern and northeastern districts of the country bordering southern Sudan and northern Kenya (Turkana). In the district closest to Turkana (Karamoja), where five of the ten Ugandan cases originated,

20% of cattle were infected with Echinococcus spp. In the two other districts (Acholi and Lango) where human cases were reported, the prevalence in cattle was 1%. In another district (Teso) south of the districts from which human cases were reported, a prevalence of 10·5% in cattle was noted.[32] About two thirds of dogs in the Moroto district were infected with uncharacterised E granulosus.[78] Results of a survey in the Queen Elizabeth National Park in western Uganda showed that a high proportion of the resident lions were infected with E felidis. In warthogs from the same location, cysts of E felidis and E granulosus G1 have been identified.[76] In the late 1980s in Tanzania, Macpherson and colleagues[71] undertook an epidemiological study of cystic echinococcosis, based on surgical records, in the Maasai people. It showed an annual morbidity of 11 cases per 100 000, with women and children being most commonly operated on. The liver was the most commonly affected organ (55% of 159 cases). With ultrasound examination, the prevalence of cystic echinococcosis was 1.1% in 959 people examined.[71] Another retrospective study31 from 1990 to 2003 showed much the same incidence of cystic echinococcosis in the Ngorongoro district. Women and young people were most commonly affected by cystic echinococcosis.[31] Epidemiological data for human disease from other parts of the country are not available. Dogs have also been investigated for echinococcosis in Maasai land. In a small series,[71] five of ten dogs were infected with Echinococcus spp. Ernest and colleagues[79] investigated livestock in the Ngorongoro district for the presence of cystic echinococcosis. In a prospective study, they showed that 63·8% of 105 sheep, 34·7% of 619 goats, and 48·7% of 357 cattle slaughtered at abattoirs or at home were infected with cystic echinococcosis. 61% of cysts were of pulmonary origin and 25·4% were fertile.[79] In the Arusha region 4·2% of cattle and 6·0% of sheep and goats (combined) were infected with Echinococcus spp in an abattoir survey.[80] From Somalia only one case report of human cystic echinococcosis is available.29 Additionally, one isolate from a camel has been genotyped and was allocated to the camel strain G6.[81]

**Southern Africa**

No epidemiological studies of human cystic echinococcosis have been done in southern Africa. however, 162 cases of cystic echinococcosis in South Africa have been described. [82,107]Most researchers focused on unusual presentations and complications of cystic echinococcosis, such

as cysts of the CNS, the spine, heart, or orbital cavity, and these reports are therefore not representative of the epidemiology of this disorder in South Africa. All investigators involved in these reports believed cystic echinococcosis to be common, despite the absence of epidemiological studies. Kayser[105] reported seeing about 20 cases per year at one hospital in the Eastern Cape Province. These case reports provide little information about risk factors associated with human cystic echinococcosis, and therefore which animals are important hosts (definite and intermediate) in the lifecycle of Echinococcus spp in South Africa is unclear.

Only two cases are reported from Zimbabwe, where cystic echinococcosis is believed to be rare.[108] The investigators noted that while most hydatid cysts of bovine origin were fertile, dogs were not easily infected with Echinococcus spp by material of bovine origin, suggesting that people were not at risk of contracting the disease from dogs.[108] In Zimbabwe, at the examination of lungs of cattle at an abattoir, 0·6% of 2000 sets of lungs were infected with Echinococcus spp.[109] By contrast with European findings, most of the cysts were fertile (96·8%).[110] Some data for cystic echinococcosis in animals in South Africa are available. In 1965, Verster and colleagues[111] investigated the prevalence of cystic echinococcosis in livestock at abattoirs nationwide. Prevalences varied greatly between regions and species investigated. For cattle, prevalences ranged between 1·2% and 13·8%, with the highest in the Eastern Cape and the lowest in the Karoo. However, the investigators also noted that prevalences increased with age in cattle, and therefore differences could be attributable to differences in age of animals slaughtered rather than being true variations in prevalence. For sheep, the prevalence ranged from 0·8% in the Karoo to 2·2% in Mpumalanga. For goats, prevalence ranged from 0% in the Western Province to 3·2% in the Eastern Cape but the numbers of slaughtered animals were small. In cattle, infection of lungs predominated, whereas in sheep and goats the liver was the most commonly affected organ. The dog was regarded as the main definitive host, although infected black-backed jackals were identified in the Eastern Cape and Western Transvaal.[111] Only a survey of cystic echinococcosis in cattle is available from Swaziland,[112] where 10·8% of 5886 cattle from different locations had hydatid cysts in their lungs, and 0·3% had cycsts in the liver. The highest prevalence was in cattle originating from a farm in the northeast of the country where wild animals and hyenas were abundant, and the investigators suggested that these animals could be hosts for Echinococcus spp. From Namibia only the identification of a cyst from a zebra as being E ortleppi (G5) is reported.[113] The available data suggest that cystic

echinococcosis is prevalent in southern Africa, but the epidemiology in people and animals remains to be investigated.

**Echinococcus in wild animals**

In addition to people and domestic animals, echinococcal worms and cysts have been identified in many species of wild mammals in sub-Saharan Africa. 76,114 E felidis has been identified in lions in South Africa and Uganda, and in a warthog in Uganda. 67,115 Echinococcus spp recovered from various species of wild carnivores and herbivores have so far not been further characterised. Discussion Despite cystic echinococcosis being described as endemic in sub-Saharan Africa,10 studies have shown large regional differences in the prevalence of this disorder (figure 4) and for many countries (particularly in central Africa) no epidemiological data exist. From some countries only data for cystic echinococcosis in livestock are available, from others only a few case reports of human disease. Comparison of epidemiological data is difficult be cause of an inevitable selection bias. In livestock surveys, mostly abattoirs have been surveyed, where only animals of a particular age are slaughtered. Such surveys might give a skewed measure of prevalence, because the frequency of cystic echinococcosis is closely correlated with host age.[116] Many studies have examined prevalence in dogs, but these reports must be treated with caution because risk of infection with the relatively short-lived worms might vary with season, depends on the age of the dogs, and can vary on small spatial scales. Thus most data only show active transmission in a particular area.[117] In surveys of human cases, incidence and prevalence will diff er depending on whether the survey examines patients presenting to hospitals or individuals who volunteer for the survey at their own initiative. Patients who are symptomatic are more likely to consent than are those without symptoms. Comparison of results from different regions is difficult because of variations in diagnostic instruments used, with several tests, such as the hydatid skin test, now regarded as obsolete because of poor sensitivity and specific city and because of varying case definitions. Data based on erological surveys might be affected by poor specific city and sensitivity of the test, because these factors vary according to the organ affected, and by cross-reactivity with other helminths that are highly prevalent in Africa. In clinical surveys, cases might have been missed or falsely presumed to have cystic echinococcosis because of limited diagnostic facilities available to the

investigators (eg, no ultrasound facilities, no radiograph facilities, diagnosis relying on clinical examination and hydatid skin testing alone) and limitations of the technique used itself (eg, ultrasonography does not detect cases of pulmonary cystic echinococcosis). Therefore, the exact pattern of cystic echinococcosis in sub-Saharan Africa remains to be further elucidated. Even in countries where research has been done, investigators believe that cystic echinococcosis is still underdiagnosed because of lack of knowledge, resources, and record-keeping. Additionally, many countries are faced with epidemics of far greater magnitude (eg. HIV, tuberculosis, and malaria) and they drain the already limited resources. Thus, cystic echinococcosis is rightfully considered a neglected tropical disease. But even in countries such as Kenya, Sudan, Ethiopia, and Nigeria where extensive epidemiological research has been done, many questions are unanswered, and new questions arise from the information available at present (panel). Researchers cannot make the assumption that high numbers of infections in livestock and dogs correspond to high numbers of people affected by cystic echinococcosis and vice versa. As we have discussed, the Maasai and Turkana peoples seem to have much the same exposure to echinococcal eggs, but the Maasai show much lower prevalences of clinical disease.

This difference cannot be explained by sociobehavioural reasons alone. Further confusing the matter is evidence that the camel strain (E canadensis G6), which is thought to be less virulent to people than other strains, is highly prevalent in livestock in the Turkana area of Kenya, yet human infections are mainly attributable to E granulosus G1. Moreover, despite high prevalences of cystic echinococcosis with the G1 strain in Maasailand, the prevalence of human cystic echinococcosis is substantially lower than in the Turkana district. In Nigeria, goats and camels show very high rates of infection and yet human disease is believed to be uncommon. The explanation for this discrepancy is likely to be multifactorial, including sociobehavioural factors and the respective genotype of Echinococcus spp. In most countries, a female predominance in clinical disease has been noted which is not present in serological surveys, thus giving further rise to the suspicion that immunological host factors and immune-suppression in particular have a role in the pathology of cystic echinococcosis, making development of clinical disease more likely after exposure. However, in Ethiopia no female predominance was identified in clinical surveys and by contrast with other countries, no predominance of hepatic disease was identified, with rates of pulmonary and hepatic disease being much the same.

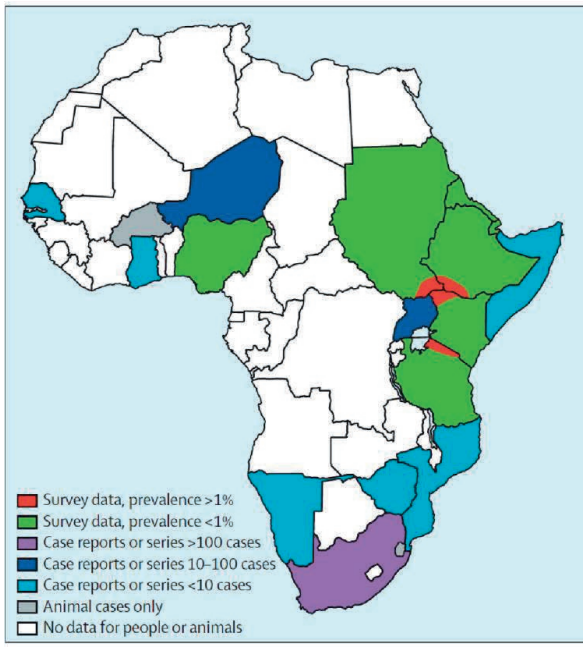



**Figure 4: Overview of the availability and nature of reported epidemiological Data**

Prevalences and numbers refer to human cases unless otherwise specified.

**Panel: Present knowledge and unanswered questions**

**Cystic echinococcosis is common in east Africa, especially in the Turkana region, but rare in west Africa**

What is different in west Africa to make the disease rare? How important is the strain of Echinococcosis granulosus in this context?

**Hepatic disease is the most common clinical presentation**

Why is pulmonary disease the most common presentation in some areas, in people and livestock?

**A female predominance is noted in clinical cases, but not in serological surveys**

What effect does immunosuppression have on the clinical course of cystic echinococcosis?

**Different strains of E granulosus are prevalent in Africa**

What effect does the strain of E granulosus have on the clinical presentation and course of disease? Why are most people in the Turkana affected by the G1 strain, when most animals in the region are infected with the G6 strain? How is the lifecycle of the G1 strain sustained?

**High prevalences in livestock do not correspond to high prevalences in people and vice versa** What factors influence the transmission of Echinococcus spp? What role do genetic factors of host and parasite play?

**Cases of severe disseminated disease have been reported with co-infection with HIV or tuberculosis**

What role do HIV and tuberculosis have in the context of cystic echinococcosis? How does co-infection affect the epidemiology and clinical course of disease?

The suspicion that immune-suppression affects the clinical course of cystic echinococcosis might have implications for the future, particularly in view of the ongoing HIV and tuberculosis epidemics in Africa. In view of the long incubation period of this disorder and its unknown

epidemiology, increasing prevalence could easily be overlooked in countries overwhelmed by HIV and tuberculosis. So far only very few case reports of coinfection exist, but some of them are concerning,90 reporting severe disseminated disease. Further research is necessary to identify the different genotypes of E granulosus prevalent in Africa and the clinical signs and symptoms associated with them, in addition to further work investigating the hostparasite interaction.

| **Search strategy and selection criteria** |
| --- |
| We searched PubMed with the terms ―cystic echinococcosis/Africa hydatid disease/ echinococcosis disease/echinococcosis/Africa‖ and ―hydatid disease/echinococcosis‖ for each country of<br>Sub-Saharan. Africa (as defined by the UN), for all available articles without time period restrictions up to May, 2012. We selected case reports, case series, epidemiological studies of human disease and disease in livestock, and studies of prevalence in animal hosts, published in English. |

**Contributors**

MPG, KW, and CNM conceived the paper. KW wrote the first draft. All authors contributed to and approved the final version.

**Conflicts of interest**

We declare that we have no conflicts of interest.

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EGYPT
Northern
Sudan
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# Chapter 3

## First Report on Circulation of Echinococcus ortleppi in the one Humped Camel (Camelus dromedaries), Sudan

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**Abstract**

**Background**: Echinococcus granulosus (EG) complex, the cause of cystic echinococcosis (CE), infects humans and several other animal species worldwide and hence the disease is of public health importance. Ten genetic variants, or genotypes designated as (G1-G10), are distributed worldwide based on genetic diversity. The objective of this study was to provide some sequence data and phylogeny of EG isolates recovered from the Sudanese one-humped camel (Camelus dromedaries). Fifty samples of hydatid cysts were collected from the one-humped camels (Camelus dromedaries) at Tamboul slaughter house, central Sudan. DNAs were extracted from protoscolices and/or associated germinal layers of hydatid cysts using a commercial kit. The mitochondrial NADH dehydrogenase subunit 1 (NADH1) gene and the cytochrome C oxidase subunit 1 (cox1) gene were used as targets for polymerase chain reaction (PCR) amplification. The PCR products were purified and partial sequences were generated. Sequences were further examined by sequence analysis and subsequent phylogeny to compare these sequences to those from known strains of EG circulating globally.

**Results**: The identity of the PCR products were confirmed as NADH1 and cox1 nucleotide sequences using the Basic Local Alignment Search Tool (BLAST) of NCBI (National Center for Biotechnology Information, Bethesda, MD). The phylogenetic analysis showed that 98% (n = 49) of the isolates clustered with Echinococcus canadensis genotype 6 (G6), whereas only one isolate (2%) clustered with Echinococcus ortleppi (G5).

**Conclusions**: This investigation expands on the existing sequence data generated from EG isolates recovered from camel in the Sudan. The circulation of the cattle genotype (G5) in the one-humped camel is reported here for the first time.

**Keywords**: Echinococcus granulosus, NADH 1 gene, cox1 gene, Genotypes, Phylogenetic analysis, Sudan

**Background**

The larval stage of Echinococcus granulosus complex (EG) causes cystic echinococcosis (CE) in domestic live- stock and humans. Because of the involvement of the vital organs, CE in humans is considered a critical public health problem. In addition to human health concerns, infections in cattle breeding areas may result in economic losses [1]. Moreover, CE represents one of the neglected tropical diseases, especially in the Sub- Saharan Africa [2]. In the Sudan, many reports of cystic echinococcosis have been described in humans and animals [3-12]. In addition to its importance as a major public health problem in the country, CE is also considered as one of the major causes of condemnation of sheep carcasses during meat inspection [13]. Currently, 10 distinct genotypes of EG designated as G1-G10 have been described worldwide on the basis of genetic diversity related to nucleotide sequences of the mitochondrial cytochrome C oxidase subunit 1 (cox1) and NADH de- hydrogenase subunit 1 (NADH 1) genes. These different genotypes are associated with distinct intermediate hosts including sheep, pigs, cattle, horses, camels, goats and cervids [14-25]. Of the ten genotypes of EG, the sheep (G1), the cattle (G5) and the camel (G6) strains were reported in humans and livestock in the Sudan [8-10,26-28]. Recent epidemiological studies indicated that the camel strain (G6) was the most prevalent strain in Sudan [10,27,28]. EG complex comprises a number of intra- specific variants, strains or genotypes at the genetic level [29-32]. It was suggested that the exten-sive intra-specific genetic variation of E. granulosus could be better understood within the context of variations in the life cycle pattern [29]. In addition, different genotypes would probably exhibit different antigenicity, transmission profiles, and sensitivity to chemotherapeutic agents as well as different pathological consequences [29,33,34]. These biological variations should be considered in developing vaccines, diagnostic kits and pharmacological therapies for control of CE. Therefore, due to epidemiological implementation and control strategies, it is essential that circulating EG genotypes in a given area of endemicity should be clearly defined [30,35]. In this study, hydatid cysts recovered from the one- humped camel (Camelus dromedaries) in an endemic area of Tamboul, Central Sudan, were defined by genetic studies and subsequent phylogenetic analysis. The molecular characterization was made possible by targeting fragments of the mitochondrial NADH 1 and cox1 subunit 1 genes to define the circulating genetic variants in the hyper endemic area of Tmboul, Central Sudan.

## Methods

### Collection of samples

Hydatid cysts (n = 50) were collected over a period of one month from camel at the slaughterhouse of Tamboul, a village located at the camel producing region of Central Sudan. This slaughterhouse represents one of the major abattoirs of camel in the Sudan. The hydatid cysts were obtained from camels soon after slaughtering and transferred in thermos flasks to the Molecular Biology Laboratory at the Faculty of Veterinary Medicine, University of Khartoum, for processing and molecular characterization studies. The cysts were mainly located in the lung (n = 45) and the rest were found in the liver (n = 5). All cysts were fertile and measured 2–10 cm in diameter. Hydatid cysts containing protoscolices and associated germinal layers were aspirated with sterile needles. The aspirates were transferred to clean sterile 50 ml tubes to which 70% alcohol was added as preservative.

### DNA Extraction from intact cysts

The suspensions containing protoscolices and/or associated germinal layers were washed in nucleic acid free water to remove excess alcohol. Extraction of DNA from hydatid cysts was made possible using a commercially available QIAamp tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer‘s instructions. Briefly, 200 µl of the suspended aspirate, 20 µl of proteinase K stock solution, and 200 µl of lysing buffer were pipetted into 1.5 ml eppendorf tube. The mixture was incubated at 37°C for 1 h and then at 70°C for 30 min before the addition of 200 µl of absolute alcohol and mixing by vortexing. The mixture was then transferred to the QIAamp spin column placed in a clean 2 ml collection tube and centrifuged at 8000 RPM in MiniSpin centrifuge (Eppendorf, Wesseling-Berzdorf, Germany) for 1 min at room temperature. The QIAamp spin column was washed twice with 500 µl of the washing buffers by spinning for 1 min. The QIAamp spin column was placed in a clean 1.5 ml eppendorf tube and the DNA was eluted with 200 µl of double distilled water preheated at 70°C. Maximum DNA yield was obtained by spinning at 12,000 RPM for 1 min at room temperature. From the suspended nucleic acid 5 µl was used in the PCR amplification.

**Selection of primers**

The primers were selected from mitochondrial NADH dehydrogenase subunit 1 (NADH 1) gene and cytochrome C oxidase subunit 1 gene (cox1) gene. The NADH 1 primers used in this study were basically described previously [17]. For the first amplification step, a pair of outer primers JBl1: 5' AGATTCGTAAGGGGCCTAATA 3' and JB12: 5' ACCACTAACTAATTCACTTTC 3' were used to amplify a 530 bp PCR product from EG isolates. A pair of internal sequencing primers JB11.5: 5' TTATGGTAGA TATTATAG 3' and JB12.5: 5' CACACACATAAAACA AGC 3' designed to conserved segments of the EG NADH 1 sequences, were used to generate a 471 bp PCR product. The cox1 primers used in this study include a forward primer CO1: 5' GAG GTT TAT TTT TTT GGG CAT CCT 3' and a reverse primer CO1: 5' TAA AGA AAG ATA ATG AAA ATG 3' as described before [17]. CO1 and CO2 primers would produce a 415 bp specific PCR product.

**Polymerase chain reaction**

A stock buffered solution containing 150 µl 10x PCR buffer, 100 µl of 25 mM MgCl2, 12.5 µl of each dATP, dTTP, dGTP and dCTP at a concentration of 10 mM was prepared in 1.5 ml eppendorf tube. The primers were used at a concentration of 20 pg/µl, and double distilled water was added to bring the volume of the stock buffer solution to 1.5 ml. Each 0.5 ml PCR reaction tube contained 2 µl of the primers, 1 µl (5.0 U) of Taq DNA polymerase (QIAGEN), 5.0 µl of the target DNA and 42 µl of the stock buffered solution. For nested PCR, 2 µl of the primary PCR product was used as DNA template. The thermal cycling profiles were as follows: a 2 min initial incubation at 95°C, followed by 40 cycles of 95°C for 1 min, 55°C for 30 sec and 72°C for 45 sec, and a final incubation at 72°C for 10 min. Thermal profiles were performed on a Techne TC-412 thermal cycler (Techne, Staffordshire, UK). Following amplification, 15 µl from each PCR containing amplified products were loaded onto gels of 1.0% agarose and electrophoresed for 1 h. The gels were stained with ethidium bromide and the PCR products were easily identified following visualization under UV light. aligned with the corresponding regions of NADH 1 and cox1 subunit genes of known genotypes from other countries. The phylogenetic trees were constructed using Unweighted Pair Group Method with Arithmetic

mean (UPGMA) implemented in MEGA software version 5.0 [36]. The country of origin, the GenBank accession numbers and the genotype were given for each EG isolate when available. Bootstrap analysis of 1000 replicates was applied and values were given at relevant nodes of the constructed tree. Corresponding nucleotide sequences of NADH 1 and cox 1 of Taenia multiceps with GenBank accession umbers HM143887.1 and JX535576.1, respectively; were used as out groups in the constructed phylogenetic trees.

**Results**

Polymerase chain reaction (PCR): The PCR-based assay with primers specific for NADH1and cox1 genes yielded amplification products from of all of the fifty hydatid cysts obtained from naturally infected camels. For NADH1, the outer pair of primers produced a primary 530 bp PCR product and the nested primers produced a 471 bp PCR product. Cox 1 primers produced 415 bp PCR products. Sequence analysis and phylogenetic relationship. The sequences obtained from the PCR products were ound to align with corresponding regions for NADH1 and cox1 genes in the GenBank confirming the cysts to contain the EG complex. Aligned with BioEdit, partial sequences for NADH 1 and cox1 showed 100% homology among 49 out of 50 EG isolates recovered in this study. To investigate for the relationship between these EG isolates and the other EG genotypes identified glo- bally, phylogenetic trees were constructed (Figures 1 and 2). Forty nine (98%) of EG isolates (represented by one sequence in the tree) clustered with E. canadensis genotype 6 (G6), the camel genotype, of EG complex obtained from other parts of the world with a strong bootstrap (1000 replicates). However, only one EG iso- late (2%) clustered with E. ortleppi, the cattle genotype (G5). The partial sequences of NADH 1 gene representing the camel genotype (G6) and of the cattle genotype (G5) were submitted to the GenBank under accession numbers JN637176, JN637177, respectively. The partial sequences generated from mitochondrial cytochrome C oxidase subunit 1 (cox1) for G6 and G5 strains were submitted to the GenBank under accession numbers KC417036, JX912709, respectively.

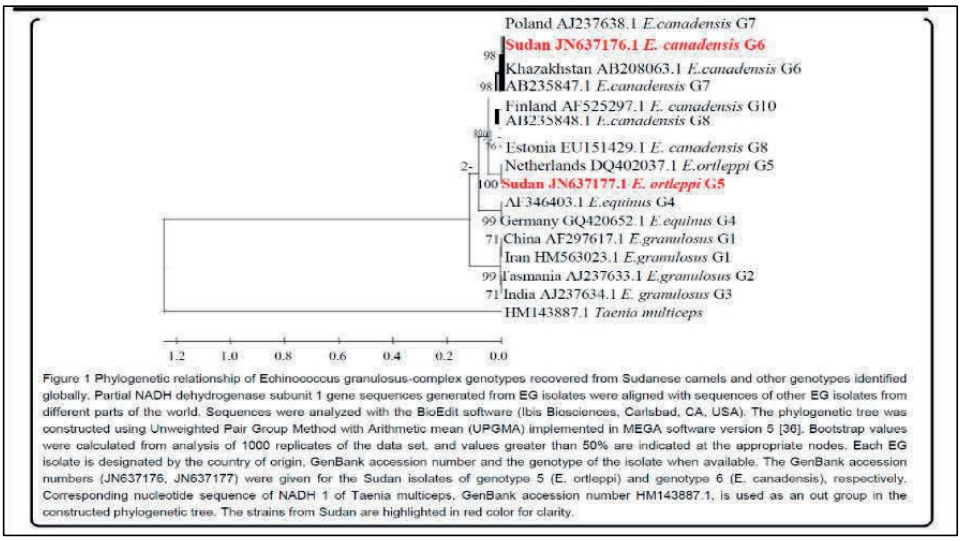


Figure 1 Phylogenetic relationship of Echinococcus granulosus-complex genotypes recovered from Sudanese camels and other genotypes identified globally. Partial NADH dehydrogenase subunit 1 gene sequences generated from EG isolates were aligned with sequences of other EG isolates from different parts of the world. Sequences were analyzed with the BioEdit software (Ibis Biosciences, Carlsbad, CA, USA). The phylogenetic tree was constructed using Unweighted Pair Group Method with Arithmetic mean (UPGMA) implemented in MEGA software version 5 [36]. Bootstrap values were calculated from analysis of 1000 replicates of the data set, and values greater than 50% are indicated at the appropriate nodes. Each EG isolate is designated by the country of origin, GenBank accession number and the genotype of the isolate when available. The GenBank accession numbers (JN637176, JN637177) were given for the Sudan isolates of genotype 5 (E. ortleppi) and genotype 6 (E. canadensis), respectively. Corresponding nucleotide sequence of NADH 1 of Taenia multiceps, GenBank accession number HM143887.1, is used as an out group in the constructed phylogenetic tree. The strains from Sudan are highlighted in red color for clarity.

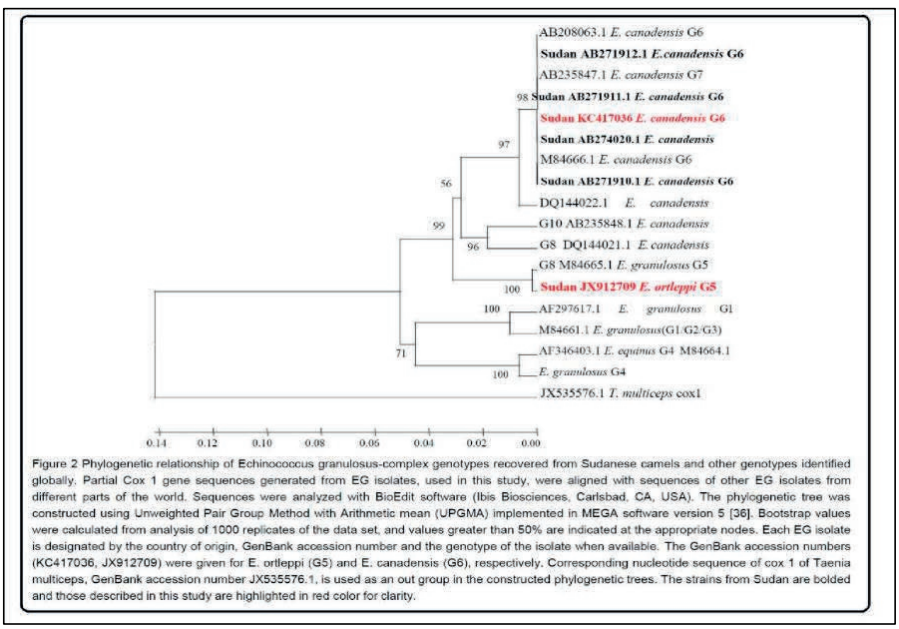


Figure 2 Phylogenetic relationship of Echinococcus granulosus-complex genotypes recovered from Sudanese camels and other genotypes identified globally. Partial Cox 1 gene sequences generated from EG isolates, used in this study, were aligned with sequences of other EG isolates from different parts of the world. Sequences were analyzed with BioEdit software (Ibis Biosciences, Carlsbad, CA, USA). The phylogenetic tree was constructed using Unweighted Pair Group Method with Arithmetic mean (UPGMA) implemented in MEGA software version 5 [36]. Bootstrap values were calculated from analysis of 1000 replicates of the data set, and values greater than 50% are indicated at the appropriate nodes. Each EG isolate is designated by the country of origin, GenBank accession number and the genotype of the isolate when available. The GenBank accession numbers (KC417036, JX912709) were given for E. ortleppi (G5) and E. canadensis (G6), respectively. Corresponding nucleotide sequence of cox 1 of Taenia multiceps, GenBank accession number JX535576.1, is used as an out group in the constructed phylogenetic trees. The strains from Sudan are bolded and those described in this study are highlighted in red color for clarity.

## Discussion

In Sudan, camels are owned by migratory pastoralists as a source of milk, meat, riding animals, as well as sign of wealth. Extensive research has been conducted to evaluate the role played by

camels in transmission of parasitic infections with special emphasis on cystic echinococosis [9,10,37]. The camel strain (G6) was reported to be the most prevalent genotype of EG in the Sudan, and that camels seem to play an important role in the transmission cycle of the parasite and the epidemiology of the disease. In contrast, the majority of the Sudanese eco-types of desert sheep and Nubian goats seem to harbor calcified or infertile cysts of EG-complex [9,10,38]. It was, therefore, suggested that sheep and goats have natural resistance to infection with EG-complex. However, this assumption requires further investigation. To advance beyond the current knowledge of the epidemiology of the disease in camel, attempts were made to better understand the life cycle and molecular epidemiology of EG isolates circulating in different parts of the Sudan. An initial step in controlling the life cycle of EG and minimizing infections is to determine the genotype(s) involved in the transmission cycle. In a previous study, different genotypes including G5 and G6 were confirmed in the Sudan by partial sequence analysis of the mitochondrial cytochrome oxidase 1 (cox1) and NADH dehydrogenase subunit 1 gene [8]. However, no phylogenetic study was conducted to determine the phylogenetic relationship of the identified genotypes of EG in the Sudan. In this study, the phylogenetic analysis illustrated that Echinococcus canadensis genotype 6 (G6) is the most infectious and widespread genotype in the Sudan, confirming the results of our previous studies [8,27]. Nevertheless, the present study indicated that Echinococcus ortleppi, the cattle genotype 5 (G5) should equally be considered as an infectious form of EG-complex in the one humped camels in Sudan. Sequence analysis and subsequent phylogenetic studies of fragments of the mitochondrial NADH subunit 1 gene indicated the presence of a single isolate of the cattle genotype (G5). However, the remaining 49 (98%) isolates belonged to E. canadensis (G6). Similar results were obtained when constructing phylogenetic trees using partial sequences derived from cox1 subunit gene, thus confirming the identity of the presence of cattle genotype (G5). This finding provides alarming evidence for the circulation of the cattle genotype (G5) in the one-humped camels. It is, however, uncertain whether the G5-infected camel was maintained in Tamboul area, Central Sudan, or brought from a neighboring area for the purpose of slaughtering. The circulation of a major variant (G6) in Sudanese camels suggests that specific mechanisms are responsible for its persistence in the endemic area of Tamboul, Central Sudan. This is probably due to close relationships between dogs and camels in the study area [39]. In rural communities with resource-poor settings, such as this study area, the practice of animal slaughtering is usually

performed in the open space. Under these conditions, dogs would have free access to feed on livestock viscera, which may harbor hydatid cysts, the infective stage. Therefore, it is believed that this practice of livestock slaughtering could effectively contribute towards the persistence of the camel genotype (G6) in the study area. The addition of EG strains sequences from Sudan enhances our understanding of the expansion and, to some degree, maintenance of the parasites in the intermediate hosts. Ongoing surveillance and EG strains characterization should also aid in determining the distribution of this cestode parasite in the country. As more sequencing data and prediction tools become more accurate and avail- able, these data will provide the public health authorities an opportunity to anticipate and prepare for treatment and subsequent control programs for the disease.

**Conclusion**

In conclusion, the result of this study indicates the circulation of E. ortleppi, the cattle genotype (G5) in the one humped camel in the Sudan for the first time. Therefore, the G5 strain should be considered during epidemiological surveys for this important parasitic infection in Sudan. In addition, this investigation expands on the existing data on sequences generated from EG isolates recovered from the one humped camel in the endemic area of Tamboul, Central Sudan.

**Competing interests**

The authors declare that they have no competing interests.

**Acknowledgement**

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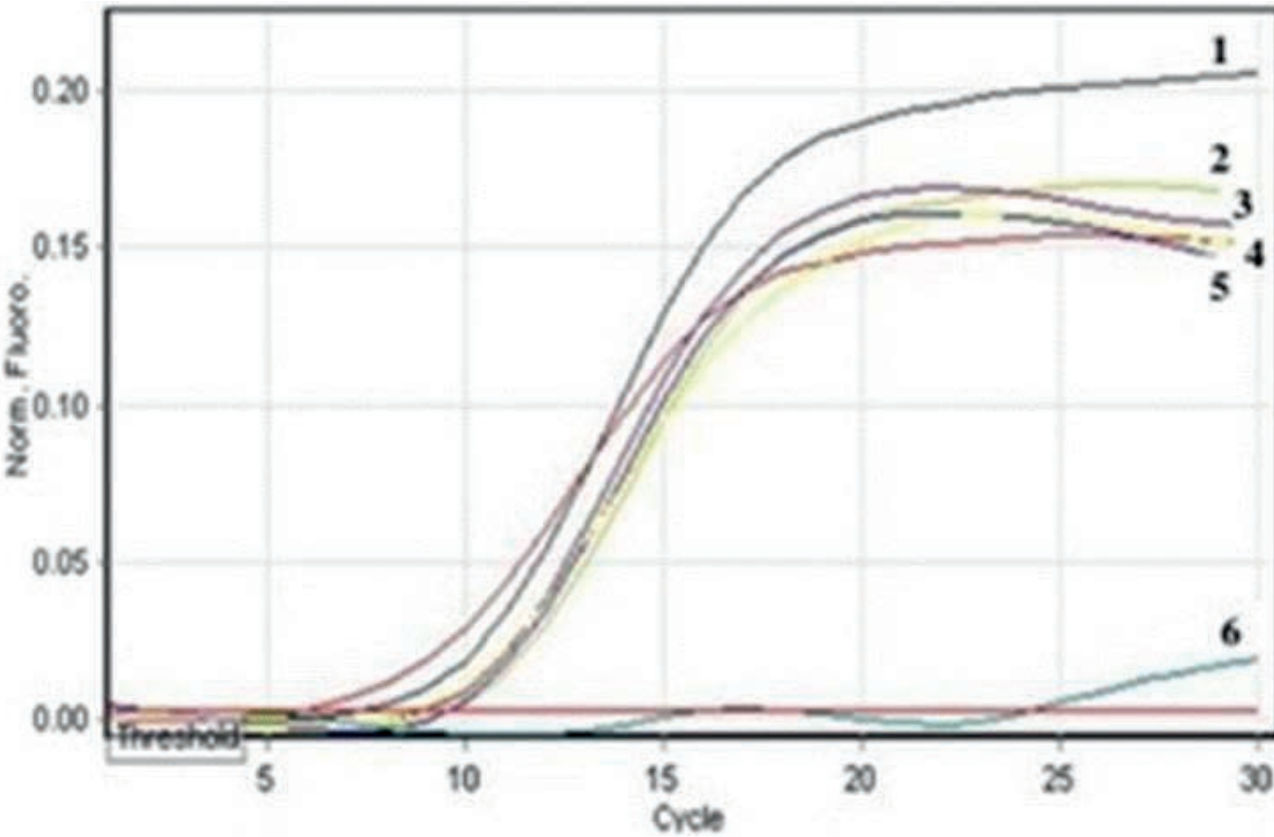
Section

II

# Molecular epidemiology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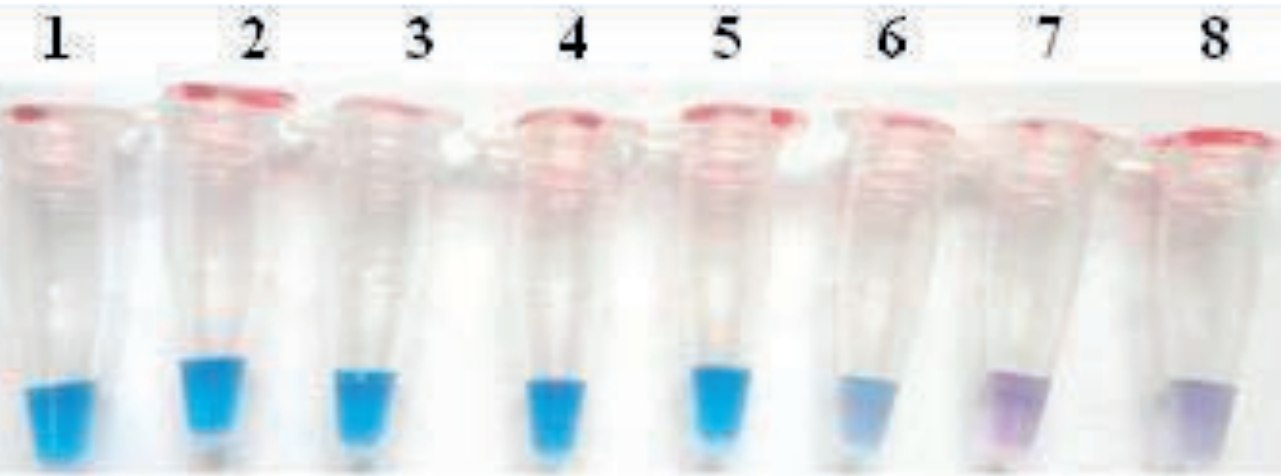
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# Chapter 4

## Development and evaluation of real-time loop-mediated isothermal amplification assay for rapid detection of cystic echinococcosis

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**Abstract**

**Background**: Cystic echinococcosis (CE) or hydatidosis, caused by the larval stage of Echinococcus granulosus (EG)-complex, is a neglected parasitic disease of public health importance. The disease is endemic in many African and Mediterranean countries including the Sudan. The objective of the present study was to develop and evaluate a real-time loop-mediated isothermal amplification (LAMP) assay for simple and rapid detection of CE in humans and domestic live stock in Sudan.

**Methods**: A set of six LAMP primers, designed from the mitochondrial NADH-1 gene of EG cattle strain of genotype 5 (G5), was used as a target for LAMP assay. The assay was performed at a constant temperature (63 °C), with a real-time follow-up using a Light Cycler and fluorochrome dye. Following amplification cycles in a simple water bath, LAMP products were observed for color change by naked eye and were visualized under UV light source using agarose gel electrophoresis.

**Results:** The real-time LAMP assay identified a variety of hydatid cysts strains recovered in the Sudan, including Echinococcus canadenses (G6) and Echinococcus ortleppi (G5). Real-time LAMP positive results were detected by the presence of an amplification curve, whereas negative results were indicated by absence of fluorescence detection. Positive LAMP results appeared as a bluish-colored reaction as observed by naked eye, whereas negative LAMP results were observed as purple-colored reaction. The sensitivity studies indicated that the LAMP assay detected as little as a 10 fg of parasite DNA. There was 100 % agreement between results of the LAMP assay and our previously described nested PCR when testing 10-fold serial dilution of DNA extracted from EG-complex hydatid cyst. However, there was no cross-reactivity with other parasites including cysticercus bovis, Fasciola gigantica, and Schistosoma bovis and nucleic acid free samples.

**Conclusion**: The developed LAMP assay would be expected to prove highly significant in epidemiological surveys of CE in developing countries or areas of resource-poor settings for both ease of use and cost.

**Keywords:** LAMP, Cystic echinococcosis, Echinococcus granulosus-complex, Hydatid cysts, Sudan

**Background**

Cystic echinococcosis (CE) in humans and susceptible animal populations is caused by the larval stage of Echinococcus granulosus (EG)-complex. In humans CE is considered a critical public health problem as vital organs may be severely involved. In addition, CE infection is of concern to camel producer especially in areas of endemicity, such as Tamboul region of Central Sudan [1]. Moreover, CE represents one of the neglected tropical diseases, especially in the Sub-Saharan Africa [2]. Several reports of CE have been described in humans and animals in different parts of the Sudan [3–13]. Ten dis- tinct genotypes/strains of EG-complex designated as G1– G10 are recognized worldwide on the basis of gen-etic diversity. These different genotypes are associated with distinct intermediate hosts including sheep, pigs, cattle, horses, camels, goats and cervids [14–25]. So far, three EG-complex genotypes including, the sheep (G1), the cattle (G5) and the camel (G6) strains were reported in humans and livestock in the Sudan [8–10]. Epidemiological studies indicated that the camel strain (G6) rep- resents the most prevalent genotype circulating in Sudan [26–28]. Recently, we reported, on occurrence of Echinococcus ortleppi (G5) in Sudanese ecotype of a dromedary camel [1]. In addition, circulating EGc omplex genotypes in humans and animals is especially important in the Sudan given the large number of livestock and their im- portance to the national economy and rural communities. The genotypes/strains of hydatid cysts strains in areas of endemicity should be clearly defined for the purpose of epidemiological implementation and subsequent effective control measures [29–35]. In the past few years CE has been repeatedly reported as an import-ant emerging infectious parasitic disease in Central Sudan [1, 27, 28]. It is, therefore, becoming increasingly obvious that the development of a simple and rapid molecular assay for detection of EG-complex is urgently needed particularly, in remote areas with resource-poor settings. Molecular-based techniques are useful for detection and genotyping of EG complex hydatid cysts. Conven-tional PCR assays were developed and evaluated for detection of CE [1, 36, 37]. However, most of the developed conventional PCR assays utilized a second round of nested amplification to increase the sensitivity of the assay and to confirm the identity of the primary amplified PCR product [36–38]. In addition, the PCR products may further require digestion by an endonuclease enzyme using PCR-RFLPs for genotyping of the associated EG strain. PCR-RFLPs technique is tedious, laborious and time consuming procedure [8, 16, 28, 38]. It is well

documented that nested PCR is prone to error and is complicated by cross contamination due to multiple manipulations of the primary PCR products [36, 37]. To address these problems, quantitative real-time PCR (qRT- PCR) were developed instead [39, 40]. However, the developed real-time PCR assays are sophisticated techniques, which require expensive automated thermal cycler and associated PCR kits. In addition, the application of real-time PCR requires an acceptable level of training and infrastructure, which does not exist in many African countries. Recently, loop-mediated isothermal amplification (LAMP) assay has been shown to be highly accurate for the detection of echinococcosis in canine definitive hosts [41–43]. However, the previously de- scribed LAMP assays for detection of EG-complex were not monitored by real-time accelerated devices. The previously reported LAMP assays utilized sets of four LAMP primers only. In the present study, the rapidity of the LAMP assay was improved by incorporating an additional pair of loop primers (LF and LB), designed from the mitochondrial NADH-1 gene of the recently identified Sudanese strain of E. orteleppi [1]. In addition, the assay was performed at a constant temperature (63°C), with a real-time follow-up using a Light Cycler and fluorochrome dye. Following amplification cycles in a simple water bath, LAMP products were observed for color change by naked eye and visualized under UV light using agarose gel electrophoresis. The outer pair of LAMP primers (F3 and B3) was employed in a conventional PCR to generate a 200 bp-specific PCR product. PCR products were purified and sequenced to determine the genotype of the EG-complex hydatid cysts strain as previously described [1].

## Methods

### Collection of samples

The study was conducted during April-October, 2014. A total of hundred hydatid cysts were used in this study. Fifty hydatid cysts (n = 50) were collected from camel at the slaughterhouse of Tamboul, a village located at the camel producing region of Central Sudan. This slaughter house represents one of the major abattoirs of camel in Central Sudan. Tamboul abattoir receives animals for slaughtering from different states in Sudan including AL Gezira State, River Nile State and Khartoum State, the national capital of Sudan. Forty hydatid cysts were

collected from cattle at ElKadaro slaughterhouse, Khartoum North. Ten hydatid cysts were collected from humans during surgical operations at the Khartoum Medical Teaching Hospital, Khartoum. The hydatid cysts were transferred in thermo-flasks to the Molecular Biology Laboratory at the Faculty of Veterinary Medicine, University of Khartoum, for processing and molecular detection by conventional PCR and LAMP assay. Hydatid cysts containing protoscolices and associated germinal layers were aspirated with sterile needles. The aspirates were transferred to clean sterile 50 ml tubes to which 70 % alcohol was added as preservative and stored at room temperature until used.

**DNA Extraction from hydatid cysts**

The suspensions containing protoscolices and/or associated germinal layers were washed in nucleic acid free water to remove excess alcohol. Extraction of DNA from hydatid cysts was made possible using a commercially available QIAamp tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer‘s instructions. Briefly, 200 µl of the suspended aspirate, 20 µl of proteinase K stock solution, and 200 µl of lysing buffer were pipetted into 1.5 ml eppendorf tube. The mixture was incubated at 37 °C for 1 h and then at 70 °C for 30 min before the addition of 200 µl of absolute alcohol and mixing by vortexing. The mixture was then transferred to the QIAamp spin column placed in a clean 2 ml collection tube and centrifuged at 8000 RPM in MiniSpin centrifuge (Eppendorf, Wesseling-Berzdorf, Germany) for 1 min at room temperature. The QIAamp spin column was washed twice with 500 µl of the washing buffers by spinning for 1 min. The QIAamp spin column was placed in a clean 1.5 ml eppendorf tube and the DNA was eluted with 200 µl of double distilled water preheated at 70 °C. Max-imum DNA yield was obtained by spinning at 12,000 RPM for 1 min at room temperature. From the suspended nucleic acid 5 µl was used in the PCR amplification. The extracted DNA was quantified using spectrophotometer at 260 nm wave length.

**Design of primers for LAMP assay**

The primers used for LAMP amplification were de-signed from the nucleotide sequence of the mitochondrial NADH dehydrogenase subunit 1 (NADH 1) gene of Echinococcus ortleppi. The

nucleotide sequence was retrieved from GenBank accession number JN637177 and aligned with the available sequences of cognate genes of other EG-complex genotypes circulating glo-bally to identify conserved regions by using CLUSTALW software version 1.83 (DNA Data Bank of Japan; http://clustalw.ddbj.nig.ac.jp/top-e.html.A potential target region was selected from the aligned sequences.

A set of six primers comprising two outer (F3 and B3), two inner (FIP and BIP), and two loop primers (LF and LB) were selected. FIP contained F1c (complementary to F1), and the F2 sequence. BIP contained the B1c sequence (complementary to B1), and the B2 sequence as shown in (Table 1). LAMP primers were designed using software PrimerExplorer V4 (http://primerexplorer.jp/elamp4.0.0/index.html;Eiken Chemical Co., Japan), as described previously by Nagamine et al. [44]. Insertion of ECO-R1 restriction sites in LAMP assay Restriction enzyme recognition sites were inserted into each primer set. For each LAMP assay the inner primers were modified by the insertion of an EcoR1 restriction site between the F1c and F2 segments of the FIP, and the B1c and B2 segment of the BIP primer (Table 2).

**Table 1** Design of LAMP primers for detection of EG-complex hydatid cysts based on the NADH 1 gene of E. ortleppi recovered from a dromedary camel in the Sudan (GenBank accession number JN637177)

**LAMP Primers**

| Primer | Position | Sequence |
|---|---|---|
| F3 | 164-186 | ATAGAGTTAGGTATAGTGGTCTT |
| B3 | 345-363 | CCTACAACAACACAAAGCA |
| FIP (F2&F1c) | | ACCTGCGCACAACAAAGAAT-TCTGTATTATGATTTTTAGCTGCTG |
| BIP (B2&B1c) | | TCTTTTTTAAGGTCGGTTCGATGT-CACACATAAAACAAGCCTCAA |
| F2 | 187-211 | TCTGTATTATGATTTTTAGCTGCTG |
| F1c | 227-246 | ACCTGCGCACAACAAAGAAT |
| B2 | 311-331 | CACACATAAAACAAGCCTCAA |
| B1c | 268-291 | TCTTTTTTAAGGTCGGTTCGATGT |
| LF | 209-223 | TGCTAGAAATTCTAG |
| LB | 300-317 | GCTTTTGGATCTGTTAGG |

**Table 2** Insertion of Eco R1 restriction sites between FIP and BIP LAMP primers based on the NADH 1 gene of E. ortleppi recovered from a dromedary camel in Sudan (GenBank accession number JN637177)

FIP: ACCTGCGCACAACAAAGAATGAATTCTCTGTATTATGATTTTTAGCTGCTG

BIP: TCTTTTTTAAGGTCGGTTCGATGTGAATTCCACACATAAAACAAGCCTCAA

| Primer | Color of sequence |
|---|---|
| F2 | Green |
| F1c | Blue |
| B2 | Brown |
| B1c | black |

Eco R1 restriction site is highlighted in red color

**LAMP reaction conditions**

The real-time LAMP assay was performed using a com- mercially available LAMP kit (LAMP kit, Mast Company, South Africa). The reaction condition for the LAMP assay was performed in a final volume of 25 µl per tube containing 12.5 µl 2× LAMP reaction mix. 1.0 µl of fluorochrome dye was used for realtime monitoring. 1.0 µl of detection dye was used instead for detection of color change as observed by the naked eye. 1.0 µl of Bst DNA polymerase at a concentration of 8 units per µl was used per reaction. A volume of 2.0 µl primer mixture containing (40 pmol each of the FIP and BIP primers, 20 pmol each of the LF and LB primers,

and 5 pmol each of the F3 and B3 primers) was added to the LAMP reaction mix. 5.0 µl of the target DNA were added. The final volume of the LAMP reaction mix was brought to 25 µl by adding nucleic acid-free water. Positive DNA controls (EG- ortleppi and EG-cana-densis) and negative DNAs controls including cysticercus bovis, Fasciola gigantica, and Schistosoma bovis and nucleic acid free samples were included in each LAMP reaction assay. The control and test DNA samples were incubated at 60–65 °C for 60 min in the LAMP assay.

**Purification and digestion of LAMP products**

LAMP products generated by the modified primer mix- tures containing restriction sites were purified by the QIAquick PCR Purification Kit (Qiagen, Germany) ac- cording to the manufacturer‘s protocol. The products were then digested using EcoR1 enzyme (New England Biolabs, Japan) at 37 °C for 2 h.

**Real-time monitoring of LAMP assay using light thermal cycler**

LAMP assay was also monitored by light thermal cycler (Rotergene Q, Australia) and a fluorochrome dye pro- vided in the commercial LAMP kit.

**Detection of color change by the naked eye in LAMP products**

Following LAMP assay in a simple water bath, Lamp products were observed by the naked eye for color change in the LAMP reaction mix using 1.0 µl of the detection dye provided in the commercial LAMP kit.

**Visualization of LAMP product by electrophoresis**

LAMP products were also visualized by electrophoresis onto 2 % ethidium bromide-stained agarose gel using gel documentation system (Uvitech, UK).

**Analysis of LAMP product with Eco R1**

The generated LAMP products were digested with EC0- R1 and analyzed with a 2 % ethidium bromidestained agarose gel electrophoresis.

**Analytical sensitivities and Specificity of the LAMP assay**

The analytical sensitivities of the LAMP assay for the detection of decreasing number of hydatid cysts copies, 10- folds dilution series of the DNA standard, ranging from $10^6$to $10^1$per reactions, were tested in the LAMP assay. For evaluation of the specificity of the LAMP assay, DNAs extracted from other parasites including cysticercus bovis, Fasciola gigantic, and Schistosoma bovis and nucleic acid free water were used to determine the specificity of the LAMP assay for specific detection EG-complex hydatid cysts using the specific primer sets.

**Conventional PCR using LAMP outer primers (F3 and B3)**

A stock buffered solution containing 150 µl 10× PCR buffer, 100 µl of 25 mM MgCl2, 12.5 µl of each dATP, dTTP, dGTP and dCTP at a concentration on 10 mM was prepared in 1.5 ml eppendorf tube. The primers were used at a concentration of 20 pg/µl, and double distilled water was added to bring the volume of the stock buffer solution to 1.5 ml. Each 0.5 ml PCR reaction tube contained 2 µl of the primers, 1 µl (5.0 U) of Taq DNA polymerase (QIAGEN), 5.0 µl of the target DNA and 42 µl of the stock buffered solution. The ther-mal cycling profiles were as follows: a 2 min initial incubation at 95 °C, followed by 40 cycles of 95 °C for 1 min, 54 °C for 30 s and 72 °C for 45 s, and a final incubation at 72 °C for 10 min. Thermal profiles were performed on a Techne TC-412 thermal cycler (Techne, Staffordshire, UK). Following amplification, 15 µl from each PCR containing amplified products were loaded onto gels of 2.0 % agarose and electrophoresed for 1 h. The gels were stained with ethidium bromide and the PCR products were easily identified using UV light source.

**Sequence analysis and genotyping**

The PCR products generated by (F3 and B3) were puri- fied using QIAquick PCR purification kit (QIAGEN) and sent to a commercial company (Macrogen, Seoul, Korea) for sequencing. Resulted sequences were edited and aligned using BioEdit software (Ibis Biosciences, Carlsbad, CA, USA). The Basic Local Alignment Search Tool (BLAST) of NCBI (National Center for Biotechnology Information, Bethesda, MD, USA) was used to confirm the identity of the generated sequences in relation to the GenBank nucleotide database. The sequences were then aligned with the corresponding regions of NADH 1 sub- unit genes of known genotypes from other countries to determine the genotype.

**Results**

**Optimization condition and visualization of LAMP product**

The optimization condition and visualization of LAMP products were determined using 10 pg of DNA extracted from Sudanese cattle strain (G5), which was incubated at a range of 60 to 65 °C. Optimum specific amplification for LAMP assay was achieved at 63 °C for 60 min.

**Detection of color change by naked eye in LAMP products**

Positive LAMP products were identified by detection of development of blue color in the LAMP reaction mix where as the negative samples appeared purple in color using the intercalating detection dye provided in the kit (Fig. 1).

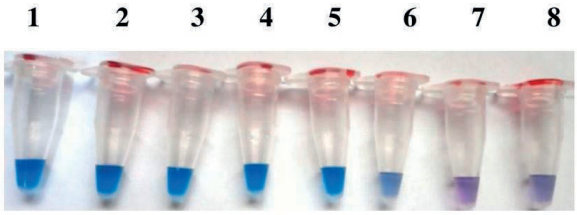


**Fig. 1** Detection by the naked eye of color change using serial dilutions of known concentration of *E. ortleppi* DNA recovered from a dromedary camel in Sudan. Blue color indicates positive LAMP result whereas purple color indicates negative LAMP result. Tube 1–8: 10-fold serial dilutions of 1.0 ng,100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1.0 fg, and DNA-free sample (negative control), respectively

**Real-time monitoring of LAMP assay**

LAMP assay was also monitored by light thermal cycler (Rotergene Q, Australia) and a fluorochrome dye for presence of amplification curve. Positive LAMP result was indicated by the presence of amplification curve where as negative result was indicated by absence of fluorescence detection. Real-time monitoring of LAMP reaction with light thermal cycler and the fluorochrome dye provided faster results compared with the naked eye observation, where positive results could be obtained as early as 10–15 min (Fig. 2).

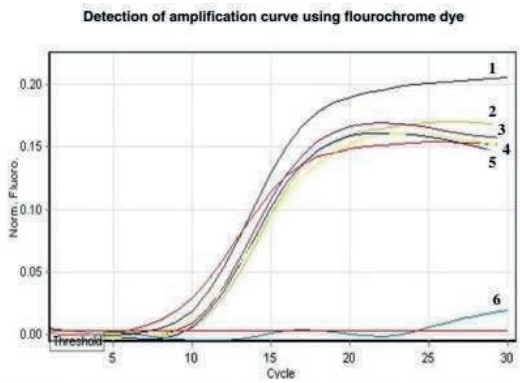


**Fig. 2** Real-time monitoring of LAMP assay using lightCycler and a fluorochrome dye. The detection of amplification curves using 1.0 pg DNA from hydatid cysts strains recovered from different animal species. Curve 1: hydatid cyst of cattle origin; curve 2–4: hydatid cyst of camel origin; curve 5: Hydatid cyst of human origin; curve 6: negative control

**Analytical sensitivity of the LAMP assay**

All hydatid cyst samples employed in this study were found positive in the described LAMP assay. The sensitivity of the LAMP assay was determined by testing 10-fold serial dilutions of DNA extracted from E. ortleppi recovered from a dromedary camel. The LAMP products were visualized by ethidium bromide-stained agarose gel electrophoresis, which produced the typical ladder-like pattern with UV irradiation. The LAMP assay has a detection limit, which span over 6 logs. High levels of analytical sensitivity were demonstrated by measuring decreasing numbers of DNA copies. The LAMP assays had 100 % sensitivity in detecting ≥ 1.0 pg of Parasite DNA (Fig. 3).

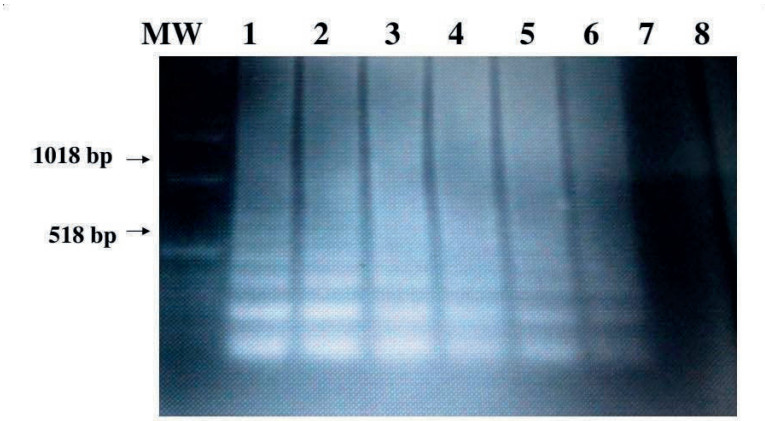


**Fig. 3** Sensitivities of the LAMP assay for detection of EG-complex hydatid cyst using ethidium bromide-stained agarose gel electrophoresis. The LAMP assay was performed with serial dilutions of known concentration of *E. ortleppi* DNA recovered from a dromedary camel in Sudan.. Lane MW: molecular weight marker; Lane 1–7: 10-fold serial dilutions of 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1.0 fg, of parasite DNA, respectively. Lane 8: nucleic acid-free sample (negative control)

Visualization of LAMP product from Sudanese EG-complex genotypes using a simple water bath set at 63 °C, and 1.0 pg of hydatid cyst DNA target, the LAMP product was de- tected from fresh and archive samples of EG-complex, including cattle strain (G5) and camel strain (G6) using ethidium bromide stained agarose gel electrophoresis (Fig. 4).

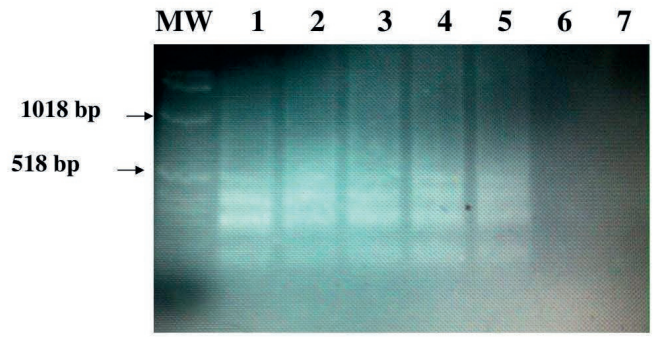


**Fig. 4** Visualization of Lamp products from fresh and archived hydatid cyst samples onto 2 % agarose gel using simple water bath. Lanes MW: Molecular marker; Lane 1: fresh sample of hydatid cyst of cattle origin; Lane 2: fresh sample of hydatid cyst camel origin; Lane 3–4: archived sample of hydatid cyst of camel origin; Lane 5–6: archived sample of hydatid cyst of human origin; Lane 7: nucleic acid-free water

**Specificity of LAMP assay**

The specificity studies for the LAMP assay indicated that there were no amplification products when using the specific LAMP primer set with DNA extracted from other parasites including cysticercus bovis, Fasciola gigantica, and Schistosoma bovis and nucleic acid free samples (Fig. 5).

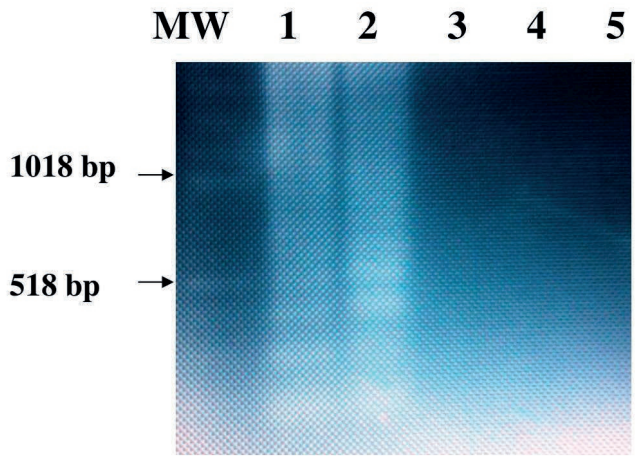


**Fig. 5** Specificity of the LAMP primers for the detection of EG-complex using *E. ortleppi* DNA recovered from hydatid cyst of a dromedary camel in the Sudan and analyzed in a 2 % agarose gel. Lanes MW: Molecular marker; Lane 1: 1.0 pg *E. ortleppi (G5)* DNA (positive control); Lane 2: 1.0 pg *E. canadensis (G6)* DNA (positive control); Lane 3: *cysticercus bovis*: Lane 4: *Fasciola gigantic*; Lane 5: *Schistosoma bovis*

**Digestion of LAMP product with Eco R1**

The specificity of the LAMP assay was further con- firmed by digestion of the LAMP product with EcoR1 restriction enzyme, which resulted in the predicted amplified products as shown in (Fig. 6).

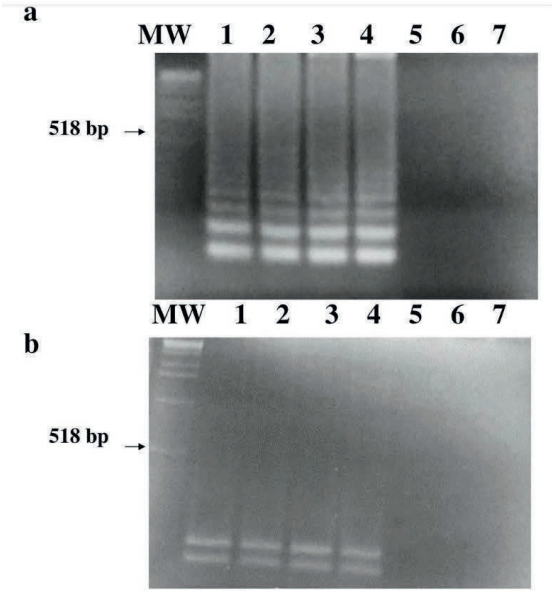


**Fig. 6** Restriction enzyme digestion of the LAMP products from hydatid cyst strains. **a** Visualization of the LAMP products from hydatid cyst strains. Lane MW: molecular weight marker; lanes 1and 2: 1.0 pg DNA from *E. ortleppi (G5)* DNA; Lane3 and 4: 1.0 pg DNA from *E. canadensis (G6)* DNA: Lane 5: *cysticercus bovis*: Lane 6: *Fasciola gigantica*; Lane 7: *Schistosoma bovis*. **b** Visualization of the restriction patterns of the digested LAMP products using Eco R1 restriction enzyme for the above gel

Conventional PCR using LAMP outer primers (F3 and B3) The conventional PCR, using LAMP outer pair of primers (F3 and B3), resulted in amplification of a specific 200- bp PCR products. The specific PCR products were detected from 1.0 pg DNA extracted from all Sudanese genotypes of hydatid cyst including G5 and G6 strains. However, no amplification products were obtained from cysticercus bovis, Fasciola gigantic, and Schistosoma bovis and nucleic acid free samples (Fig. 7).

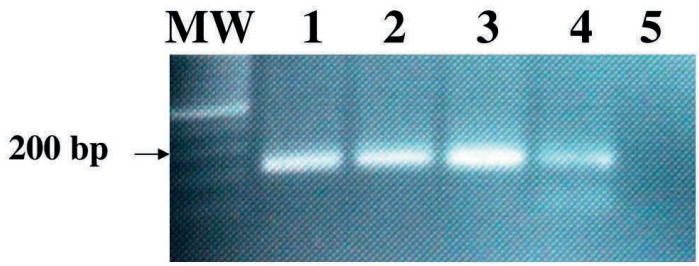


**Fig. 7** Specificity of the LAMP outer primers (F3 and B3) for amplification of the Sudanese strains of EG-complex using conventional PCR. Visualization of the 200-bp specific DNA PCR products on ethidium bromide-stained agarose gels. Lane MW: molecular weight marker; lanes 1–2: 1.0 pg *E.ortleppi* (G5) DNA (positive control); Lane 3–4: 1.0 pg *E.canadensis* (G6) DNA; Lane 5: nucleic acid-free water

**Sequence analysis and genotyping**

The PCR products generated by (F3 and B3) were purified using QIAquick PCR purification kit
(QIAGEN). Resulted sequences were edited and aligned using BioEdit software (Ibis Biosciences,
Carlsbad, CA, USA). The Basic Local Alignment Search Tool (BLAST) of NCBI (National Center for Biotechnology Information, Bethesda, MD, USA) confirmed the identity of the generated sequences and the genotypes of all hydatid cyst used in this study were confirmed as G5 or G6 strains.

**Discussion**

Cystic hydatidosis is a zoonotic parasitic disease affecting both humans and livestock and has a cosmopolitan distribution [14–24]. Accumulated reports indicated that various livestock are susceptible to hydatid infection in Sudan, with particularly high prevalence in the dromedarycamels [3–13, 26–28, 38, 45–47]. Early detection and genotyping of cystic echinococcosis (CE), commonly known as hydatidosis, would be advantageous in a variety of circumstances including control of the disease and subsequent prevention of spread of the

infection. Rapid detection of emerging zoonotic parasitic disease, such as CE, is especially important in the Sudan given the large numbers of livestock in the country, and their importance to the economy and rural communities [45–47]. In the present investigation, we developed and evaluated a real-time and conventional LAMP assay for simple and rapid detection of fresh and archive samples of hydatid cysts using a set of six LAMP primers. The development and evaluation of a one- step, single-tube, real-time accelerated loop-mediated isothermal amplification (LAMP) for the detection of CE in humans and domestic live stock in Sudan is a simple and rapid procedure. The assay was performed at a constant temperature (63 °C), with a real-time follow-up using a Light Cycler and a fluorochrome dye. The assay was highly sensitive and comparable to real-time PCR, with a detection limit of 10.0 fg of parasite DNA [40]. However, the real-time LAMP assay was much faster and generates results within 10–15 min for most employed samples. In addition to real- time detection, positive LAMP results were indicated by color change in the LAMP reaction mixed. Observation of LAMP amplified products for color change by naked eye or visualization of the products using agarose gel electrophoresis would be appropriate for most laboratory settings in developing countries [41–43]. The LAMP assay was performed under isothermal conditions and no special apparatus was needed, which makes the assay more economical and practical than real-time PCR assays. In fact, a number of PCR assays for detection of CE were described [36–39]. Together with the present study, the described LAMP assay should facilitate rapid detection and genotyping of hydatid cyst strains in a resource-poor setting in the tropics. In the present study, the potential of LAMP assay for rapid and accurate detection of CE was investigated, on a practical scale for the first time in Sudan. The LAMP assay provides high levels of diagnostic sensitivity and specificity when testing a variety of cysts sampled from human and domestic live stock. Using the detection dye, processing, extraction of parasite DNA and application of LAMP assay could be completed in approximately 90 min after arrival of the samples in the laboratory. However, the estimated time for real-time detection of a LAMP positive result was significantly reduced when using Light Cycler and fluorochrome dye. Positive LAMP results could be monitored as early as 5–10 min before completion of the cycles, which last for 60 min. In addition, an important practical advantage of the LAMP technique is that it utilizes simple and relatively inexpensive equipment, such as a simple water bath or heat block, which renders the assay promising for use in rural and remote areas with

resource- poor settings. Moreover, only basic molecular and technical skills are re-quired for performance of the LAMP assay procedure, and interpretation of the results may be as simple as a vis-ual evaluation of color change in the reaction mix. The sensitivity studies indicated that the LAMP detected 10.0 fg of parasite DNA as indicated by color change in the reaction mix, which is most likely the way it would be read in a resource-poor setting. Using agar-ose gel electrophoresis, the LAMP assay detected as little as 10 fg of parasite DNA. Our results illustrate that the sensitivities of the developed LAMP assay and our previously described nested RT-PCR assays are in 100 % agreement and both assays exhibit high levels of analyt-ical sensitivity [36]. However, nested PCR is prone to error and is complicated by cross reaction due to mul-tiple manipulations of PCR products. The specificity studies indicated that no cross reactiv-ity was detected with 1.0 pg DNA from cysticercus bovis, Fasciola gigantica, and Schistosoma bovis nucleic acid free samples under the same stringency condition de- scribed in this study. In the present study the LAMP assay was evaluated for detection of the Sudanese genotypes of EG-complex hydatid cysts. This study does not deal with sensitivity/specificity testing on a large prac-tical scale but rather constitutes a principle for application of LAMP assay for diagnosis of CE.Since the LAMP primers were designed based on mul- tiple sequence alignment of several published sequences of the NADH 1 gene, using BioEidit software (Carlsbad, CA, USA), and were selected from a highly conserved fragment of the gene, they would be expected to amplify DNA from all genotypes of EG-complex hydatid cyst strains circulating globally. However, DNAs from other genotypes of hydatid cyst strains were not available in the Sudan to be included in this LAMP assay. Therefore, additional research would be necessary to confirm this assumption. The described LAMP assay can have great potential in developing African countries, such as Sudan, where the disease is endemic and equipment and expert technical staff is scarce. The cost of the described LAMP assay should be around that of the conventional PCR assay, if not less expensive. In fact, the LAMP assay uti- lizes Bst enzyme for amplification of the target sequence. However, Taq DNA polymerase enzyme is required for conventional or real time PCR amplification, which is more expensive than Bst enzyme. The described real-time LAMP assay could very easily be adjusted for coprodiagnosis of EG-complex eggs in fecal samples from infected canines. The role of this LAMP assay in coprodiagnosis and its application in epidemiological studies and disease control programs should be promising and highly significant. It is worth mentioning that conventional

parasitological method could be useful for diagnosis of hydatid cyst under the microscope but has no significance in genotyping of the parasite. However, the LAMP assay, described in this study, could be employed for simultaneous detection and genotyping of cysts recovered from infected livestock. It is well documented that different genotypes exhibit different pathological consequences, transmission profiles, and sensitivity to chemotherapeutic agents. These biological variations should be considered in developing vaccines, diagnostic kits and pharmacological therapies for control of CE. In the present study, genotyping of the hydatid cyst strains was made possible by using the outer pair of
LAMP primers (F3 and B3) in a conventional PCR assay and subsequent sequencing of the specific PCR product. The genotypes of all strains of hydatid cyst used in this study were confirmed as Echinococcus canadensis (G6) or Echinococcus ortleppi (G5) using ClustalX (http://www.clustal.org/) as described previously [1].

**Conclusion**

In conclusion, the LAMP assay, described in this study, could be used for simple and rapid detection and genotyping of EG-complex hydatid cysts strains. There was 100 % agreement between results of the LAMP and our previously described nested RT-PCR when testing 10-fold serial dilution of parasite DNA. The LAMP assay provides very high levels of diagnostic sensitivity and specificity when testing a variety of archived hydatid cysts sampled from human or susceptible animal populations. Real-time monitoring of the LAMP assay using Light Cycler and fluorochrome dye enhanced the rapidity of the assay and a positive result could be obtained as early as 10–15 min post amplification reaction. The performance of the LAMP assay under isothermal conditions without the need of special apparatus, and visualization of results by the naked eye, makes the assay more economical and practical in remote areas or resource-poor settings. Partial sequences produced by LAMP outer primers (F3 and B3) could be targeted for sequencing and subsequent identification of the genotype of the hydatid cyst genotype/strain.

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**Availability of data and materials**

Any additional data and materials can be requested from the corresponding author.

**Authors' contributions**

MEA collected hydatid cyst samples, extracted the DNA and optimized the LAMP assay; MHE helped with Lamp assay optimization; FME and IAA helped with experimental design; MPG, helped with experimental design and preparation of the draft and the final version of the draft manuscript; IEA designed the experiment and prepared the final manuscript. All authors read and approved the final version of the manuscript.

**Competing interests**

The authors declare that they have no competing interests.

Consent for publication Not applicable.

**Ethical approval and consent to participate**

The study was approved by the Institutional Research Board (IRB) Committee, Alneelain University, Khartoum, Sudan. Hydatid cysts were collected from humans during surgical operations by qualified physicians; and from slaughtered animals during post-mortem inspection by veterinary officers at the slaughter houses. Informed consent from all human patients was provided through an ethical clearance form, which permits the use of clinical samples for diagnosis and subsequent research purposes. No experimental infection was conducted on live animals.

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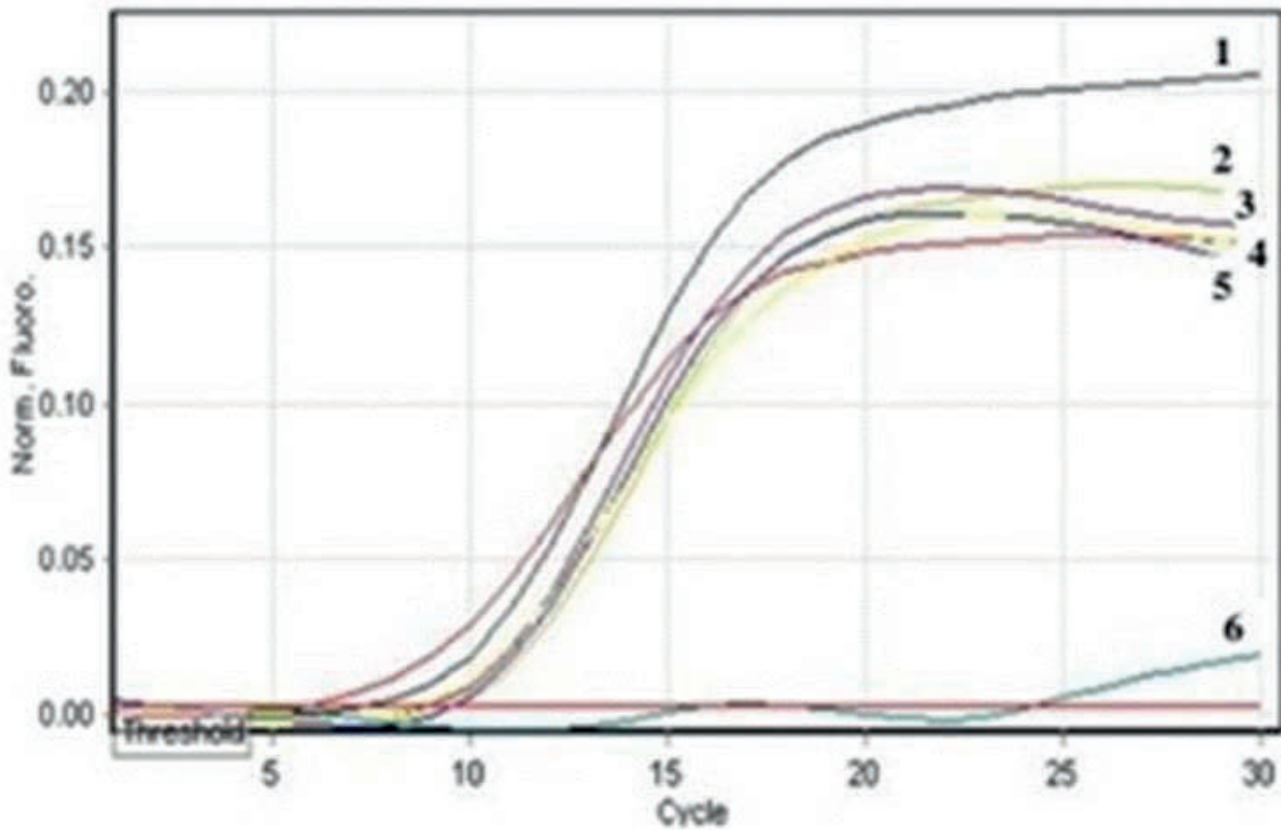


**Real-time PCR**

# Chapter 5

## Development of real-time PCR assay for simultaneous detection and genotyping of cystic echinococcosis in humans and livestock

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**Abstract**

**Objective:** To develop and evaluate a single-tube one-step real-time quantitative PCR (qPCR) assay for simultaneous diagnosis and genotyping of cystic echinococcosis (CE) in humans and livestock in the Sudan, and to compare it with conventional PCR assay.

**Methods:** Hydatid cysts were obtained from slaughtered animals and from humans after surgical interventions. DNA from the hydatid cysts and associated germ layers was extracted using a commercially available kit. The mitochondrial NADH dehydrogenase subunit 1 (NAD1) was used as a target for PCR amplifications. qPCR and conventional nested PCR assays were compared in this study.

**Results:** The qPCR assay amplified the NAD 1 gene of hydatid cysts on melting temperature generated at 80 $^0$C. Ten-folds serial dilutions of DNA with known dilution of $1 \times 10^6$ to $1 \times 10^1$ (1 ng–1 fg) resulted in detection of as little as 1 fg of DNA with an $R^2$ value equivalent to 0.997. Similar sensitivities were encountered from both qPCR and the conventional nested PCR. The two assays did not amplify DNAs from Fasciola gigantica, Taenia saginata, Schistosoma bovis and DNA-free samples (negative controls). The PCR amplified products were purified for subsequent sequencing. The sequence data were analysed to insure the specificity of the amplified PCR products and to identify the genotype(s) of hydatid cysts.

All cysts were identified as E. canadensis genotype 6 (G6).

**Conclusions**: The developed qPCR should be used as a rapid and reliable assay for diagnosis and genotyping of CE. The assay is highly recommended for the epidemiological surveillance in humans and livestock in endemic countries.

**Keywords:** Cystic echinococcosis, Diagnosis, Genotyping, Real time PCR, Sudan

## Introduction

In sub-Saharan Africa, including the Sudan [1-10]. Echinococcus granulosus s.l includes a number of genetically similar variants, Cystic echinococcosis (CE), due to the larval stages of Echinococcus granulosus senso lato (s.l), affects humans and a variety of domestic livestock and wild animals. The poor hygienic measures in developing countries, limited community awareness of the deleterious effect of CE on the health of affected individuals, presence of stray dogs, and home slaughtering of food animals are factors contributing towards the epidemiology of the disease strains or genotypes [11-13]. Ten distinct genotypes identified as G1-G10 have been described globally, based on nucleotide sequence analysis of the mitochondrial cytochrome C oxidase subunit 1 (CO1) and NADH dehydrogenase subunit 1 (NAH1) genes [14,15]. The genotypes have been related to the specific intermediate hosts including sheep, goats, horses, cattle, camels, pig, deer, elk, reindeer, moose and wapiti [16-26]. In humans, the disease is considered a deleterious health problem, while in animals infection may result in serious economic losses as the result of condemnations of affected organs in food animals [26,27]. Currently, infection with CE is diagnosed by radiology, microscopic demonstration of protoscolices, serology and molecular-based techniques. Serological reactions are useful for the identification of past infection during disease surveillance. However, cross-reactions are likely to occur with other cestode parasites. These limitations necessitate development of molecular techniques for rapid diagnosis of the parasite genome. The sensitivity of the assay is crucial in programs aiming at the prevention and control of the disease. Previous researchers have described conventional PCR assays for the diagnosis of CE [7,28]. In addition to the conventional PCR assays used for the detection of CE, most of the researchers have also utilized the nested amplification for the purpose of increasing the sensitivity and confirming the identity of the primary amplified PCR product [9,29,30]. Moreover, the amplified PCR requires digestion by restriction enzyme using PCR-RFLPs techniques for genotyping of EG strain, which is a rather expensive, laborious and time consuming technique [6,31]. As an alternative the real-time quantitative PCR (qPCR) was developed [30, 32]. Loop-mediated isothermal amplification (LAMP) assays have been developed and proved highly sensitive and specific for diagnosis of echinococcosis in the canine definitive hosts [33-35]. However, LAMP assays were described for limited applications and were not used on a wide scale. In the current investigation,

a SYBR green-based qPCR assay was developed and compared to the conventional nested gel-based PCR for direct diagnosis of hydatid cysts obtained from humans and domestic animals, and the generated PCR products were sequenced and used for subsequent genotyping.

## Materials and methods

### Ethics statement

The study was approved by the Ethical Approval Committee, Faculty of Veterinary Medicine, University of Khartoum, Sudan, and the Institutional Review Board of Al-Neelain University, Khartoum, Sudan. Human hydatid cysts were collected from patients subjected to surgical interventions at Khartoum Medical Teaching Hospital. The patients were informed of the objective of the sampling. Hydatid cysts from animals were collected during post-mortem examination by qualified veterinarians at the different abattoirs. Informed consent and permission for research to use the hydatid cysts were obtained from the patients and veterinarians in charge of the abattoirs. No experiment was conducted on humans or living animals.

### Collection of hydatid cysts

One hundred hydatid cysts were obtained from human patients and domestic animals. Ten of these hydatid cysts were recovered from humans during surgical interventions at Khartoum Medical Teaching Hospital. The remaining 90 cysts were obtained from animals during meat inspection at slaughter houses (70 cysts were collected from camels in Tamboul abattoir, 10 hydatid cysts from sheep, and 10 cysts from cattle in Omdurman slaughter house). Most of the hydatid cysts collected from sheep were rudimentary or calcified, whereas those collected from human patients and from cattle and camels were fertile.

Protoscolices and associated germinal layers of the hydatid cysts were aspirated with sterile needles. The aspirates were transferred to clean sterile 50 mL bottles, to which 70% alcohol was added as preservative.

**DNA extraction from intact cysts**

The suspensions containing protoscolices and associated germinal layers were washed in nucleic acid free water to remove alcohol. Extraction of DNA from alcohol free cyst suspension was made possible using a commercially available QIAamp tissue kit (Qiagen Hilden, Germany) according to the manufacturer‘s instructions. The detail for the extraction procedure was previously described [35]. A maximum yield of DNA was made possible by spinning the product at 12 000 r/min for 1 min at room temperature. The DNA concentration was determined by spectrophotometer at 260 nm wave length.

**Selection of primers for nested and real time PCR assays**

All primers were designed from the published sequences of NADH dehydrogenase 1 gene of E. granulosus genotype 6 (G6) [8]. In brief, a pair of outer primers (EGL1 and EGR2) were designed for the synthesis of the primary E. granulosus-specific PCR product. Primer EGL1 included bases 32-53 of the positive sense strand (5)-TGA AGT TAG TAA TTA AGT TTA A. EGR2 included bases 447-466 of the complementary strand (5)-AAT CAA ATG GAG TAC GAT TA. Using primers EGL1 and EGR2, the primary PCR amplification will produce a 435 bp PCR product. The second pair of internal primers (EGL3 and EGR4) were designed from the same DNA sequence cited above and used for nested PCR amplification. EGL3 included bases 162-181 of the positive sense strand (5)-TTA TAG TAT GCT TTCTGT GT. EGR4 included bases 420-437 of the complementary strand (5)-AAC ACA CAC ACC AAGAAT. The nested primers will result in amplification of a 276 bp PCR product, internal to the annealing cites of primers EGL1 and EGR 2. The nested pair of primers (EGL3 and EGR4) were also employed in SYBR green-based qPCR amplification assay. All primers were synthesized on a DNA synthesizer (Milliigen/ Biosearch, a division of Millipore Burlington, MA) and purified using oligo-pak oligonucleotide purification columns (Glen Research Corporation, Sterling, VA) as per manufacturer‘s instructions.

**Conventional single-round PCR assay**

A stock buffered solution containing 150 µL of 10× PCR buffer, 100 µL of 25 mmol/L $MgCl_2$, 12.5 µL of each dATP, dTTP, dGTP and dCTP at a concentration of 10 mmol/L was prepared in 1.5 mL Eppendorf tube and double distilled water was added to bring the volume of the stock buffer solution to
1.5 mL. The primers were used at a concentration of 20 pg/µL. The PCR reaction mixture contained 2 µL of the primers, 5 µL of the target DNA and 42 µL of the stock solution, and 1 µL of Taq DNA polymerase at a concentration of 5 IU/µL was used. The thermal cycling profiles were as follows: a 5 min initial incubation at 95 $^0$C, followed by 40 cycles of 95 $^0$C for 500 bp 1 min, 55 $^0$C for 30 s and 72 $^0$C for 45 s, and a final incubation at 72 $^0$C for 10 min. The primary and nested PCR amplification products were visualized onto ethidium bromide-stained agarose gels.

**SYBR green-based real time qPCR assay**

A single-tube amplification reaction was carried out using One- Step QIAgen kit (QIAgen). In brief, a standard 25 µL reaction mixture contained in final concentration 0.4 mmol/L dNTP mix, 3.0 mmol/L $MgSO_4$, DNA polymerase, 250 nmol/L of each nested primers, (Macrogen, Seol, Korea), 0.5 µL SYBR green 1 dye (Molecular Probe, USA) diluted 1:1 000 in RNase free water. Target genes were amplified in low-profile 0.2 mL tubes. The amplification was carried out in a Light Cycler Rotor Gene machine (QIAgen, Australia). No template control (NTC) was used as negative control. The cycling program consisted of initial pre-denaturation at 94 C for 5 min followed by 40 cycles of denaturation at 94 $^0$C for 15 s, and annealing at 55 $^0$C for 45 s. Finally, a melting curve analysis was done from 55–95 °C. The fluorescence threshold limit of the Rotor Gene system was set at 0.02.

**Results**

**Sensitivity of the conventional primary PCR**

The conventional PCR detected DNA extracted from all hydatid cyst used in this study. The outer pair of primers EGL1 and EGR2 produced a primary 435 bp PCR product from ≥100 fg

DNA. The primary PCR amplification products were visualized on ethidium bromide-stained agarose gels (Figure 1A).

**Sensitivity of the nested PCR**

The nested primers (EGL3 and EGR4) produced a 276 bp PCR product internal to the annealing sites of the outer primers. The nested amplification increased the sensitivity of the PCR assay and as little as 1 fg of DNA was detected by this assay as visualized on ethidium bromide-stained agarose gels (Figure 1B).

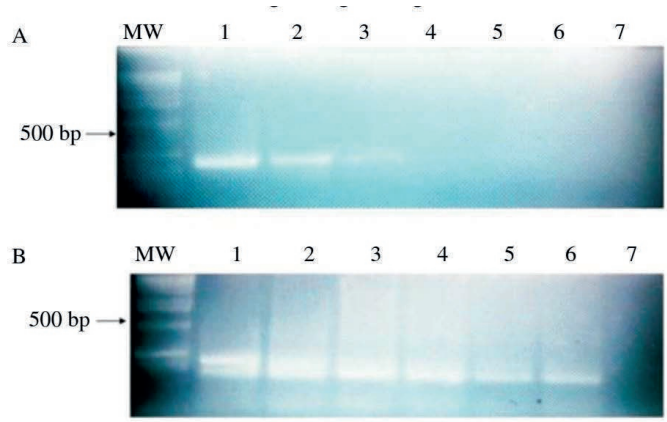


**Figure 1.** Sensitivity of the conventional and nested PCR assay for detection of *E. granulosus* NADH 1 gene using an ethidium bromide-stained agarose gel electrophoresis.
A: Amplification of the primary 435 bp-specific PCR product of *E. granulosus* visualized on an ethidium bromide-stained agarose gel. Lane MW: DNA ladder; Lane 1–6: DNA extracted from different serially diluted *E. granulosus* ($1 \times 10^6$ to $1 \times 10^1$) equivalent to 1.0 ng–1 fg of DNA, yielding a detection limit of as little as 1. fg of DNA. Lane 7: Negative control; B: Nested PCR amplification using the internal primers EGL3 and EGR4 on the above gel.

**Specificity of the nested PCR and qPCR**

The specificity studies of both conventional and qPCR assays indicated that the PCR product was specific and did not cross-amplify DNA of Taenia saginata, Fasciola

gigantica, and Schistosoma bovis and nucleic acid free samples. The result of the specificity of the conventional nested PCR is presented in (Figure 2).

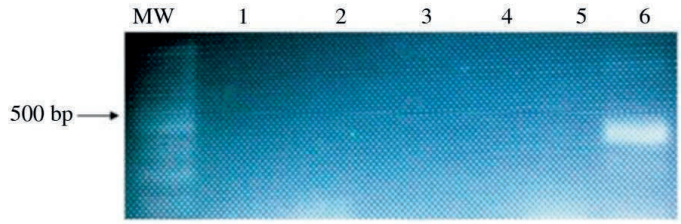


**Figure 2.** Specificity of the conventional PCR assay for detection of *E. granulosus* NADH 1 gene using an ethidium bromide-stained agarose gel electrophoresis.
The PCR product was specific and did not cross amplify DNA of other parasites. Lane MW: Molecular weight marker; Lane 1: Nucleic acid free samples; Lane 2: Cysticercus bovis; Lane 3: *Fasciola gigantica*; Lane 4: *Schistosoma bovis*; Lane 5: Nucleic acid free sample; Lane 6: 1 pg DNA extracted from hydatid cyst (positive control).

**Sensitivity of the SYBR green-based real time qPCR**

The one-step qPCR based on SYBR green 1 chemistry enabled rapid detection of hydatid cysts. Serially diluted E. granulosus DNA ($1 \times 10^6$ to $1 \times 10^1$, i.e. 1 ng to 1 fg) yielded a detection limit of as little as 1 fg of DNA (Figure 3). The standard curve generated from the amplification profile of the one-step SYBR green-based qPCR of the serially diluted E. granulosus DNA showed a linear range of 6 logs of dilution with a $R^2$ value equivalent to 0.997 59 (Figure 4). The melting curve analysis of the amplified DNA products from hydatid cysts obtained a distinct melting peak (Tm value) at 80 $^0$C (Figure 5).

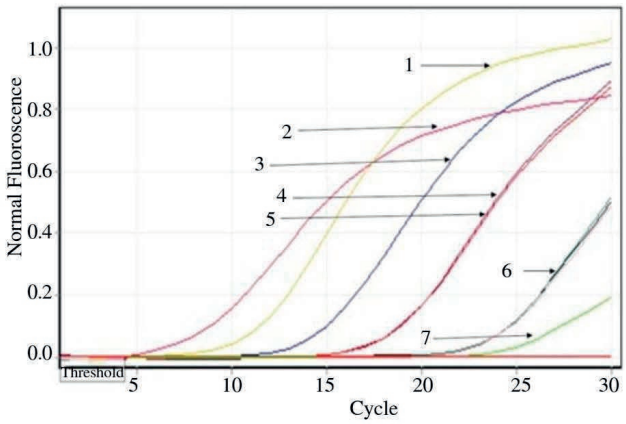


**Figure 3.** Sensitivity of the amplification profile of the one-step SYBR green-based qPCR of serially diluted *E. granulosus* DNA ($1 \times 10^6$ to $1 \times 10^1$) as represented by numbers 1–7, respectively.

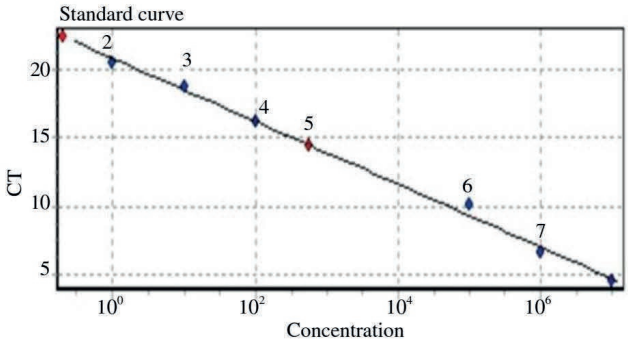


**Figure 4.** Standard curve generated from the amplification profile of the one-step SYBR green-based qPCR of serially diluted *E. granulosus* DNA ($1 \times 10^7$ to $1 \times 10^1$) as represented by numbers 1–7, respectively. The figure shows a linear range of 7 logs of dilution with a $R^2$ value equivalent to 0.99759.

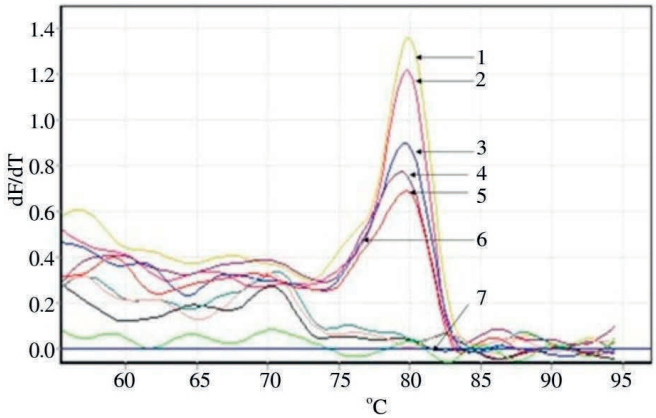


**Figure 5.** Melting curve analysis of the amplified DNA products from hydatid cysts, with a distinct melting peak (Tm value) at 80 °C.

**Sequencing and genotyping of hydatid cysts**

Partial sequences produced by PCR amplifications were found to align with the corresponding regions for NADH 1 gene in the GenBank confirming that the cysts contained the E. granulosus complex. Aligned with BioEdit, partial sequences for NADH 1 showed high similarity among all E. granulosus isolates recovered in this study. All cysts were identified as camel genotype (G6).

**Discussion**

The performance of SYBR green-based real-time qPCR assay gel-based nested PCR assay. Both conventional and qPCR assays showed similar sensitivity and specificity for the rapid diagnosis and differentiation of hydatid cysts from humans and animals. The developed qPCR assay showed a dynamic detection limit, which spans over a 6 $\log_{10}$ concentration range. However, the nested PCR was found to be time consuming, prone to errors and complication by cross contamination resulting from multiple manipulations of the primary PCR products. Whereas, SYBR green-based qPCR required approximately 45 min from sample submission until the

assay is accomplished giving final results, while the time spent for nested PCR amplification and subsequent visualization of results required at least five consecutive hours. Accordingly, the qPCR has been optimized in this study to develop an efficient qPCR assay for diagnosis and quantification of E. granulosus hydatid cysts because of its simplicity, high sensitivity, and specificity and cost efficiency. The additional advantage of utilizing SYBR green 1 based qPCR is that the test primer pairs are relatively easy to design and are suitable for conventional PCR analysis. To generate a standard curve and to insure the possible detection limits of the SYBR green-based qPCR assay, 10-fold serial dilutions ($1 \times 10^6$ to $1 \times 10^1$) of a known concentration of the parasite DNA were tested in the current study. The assay showed linear results for the 6 logs of the serially diluted DNA. The detection limit of the SYBR green $1_5$-based assay was calculated to be 1 fg equivalent to DNA extracted from 10 protoscolices. Melting curve analysis was conducted to insure the existence of the specific amplicon in the reaction tube. The melting peak temperature (Tm value) was calculated to be 80 C from the PCR products. The conventional gel-based nested PCR assay was proved to be highly specific in detecting the E. granulosus DNA. The first round of the conventional PCR was far less sensitive for the detection of E. granulosus DNA when compared to the nested PCR or SYBR green-based qPCR assay. The sensitivity was, however, significantly increased (1 000 times) using a second round of nested amplification with the nested primers. Similar result was obtained with SYBR green-based qPCR and as little as 1 fg of parasite DNA was detected in both assays. The SYBR green-based qPCR is a single-tube one-step assay that does not require post amplification steps. The specificity studies of both conventional and qPCR assays indicated that the PCR products were specific and did not cross-amplify DNA of other parasites including cysticercus bovis, Fasciola gigantica, and Schistosoma bovis and nucleic acid free samples. It is worth mentioning that surgical removal of hydatid cysts in hospitalized human patients requires intercostal intubation, which may result in accidental rupture of pulmonary cysts. The ruptured cyst is likely to be invaded by secondary bacteria [36]. In addition, calcification of hydatid cysts is not an uncommon finding in infected patients. Infertile or calcified hydatid cysts are common in animals, as reported in the majority of the Sudanese desert sheep and Nubian goats [6,7,28,37]. In these circumstances, the diagnosis of cystic hydatidosis by conventional techniques would be extremely difficult, if not impossible. However, in the present investigation, the detection of ruptured and calcified cysts was made possible by both the conventional and qPCR assays. The

qPCR assay was preferred for its convenience and minimum sample handling, thus preventing the occurrence of cross-contamination which may decrease the quantitative reliability of the assay. It is well documented that SYBR green-based qPCR assays are less specific than the TaqMan qPCR assays. In addition, the qPCR assay can be implemented in a research laboratory setting for the purpose of rapid diagnosis and epidemiological surveillance of CE in humans and animals in developing countries, such as Sudan. CE is widespread worldwide including the Sudan and identification of the genotype of the cyst would be advantageous for the prevention and control of the disease [37-40]. In Sudan, genotypes G5 and G6 were described in cattle and dromedary camel (Camelus dromedarius). The sequence analysis showed that Echinococcus canadensis genotype 6 (G6) is the most infectious and widespread genotype in the Sudan, which is in agreement with previous studies [28,30,41]. In conclusion, this study demonstrates that SYBR green based qPCR can serve as a useful tool during survey of the disease among humans and susceptible animal populations. The qPCR offers advantages over the conventional gel-based nested PCR, being less time consuming and preventing cross contaminations.

**Conflict of interest statement**

We declare that we have no conflict of interest.

**Acknowledgment**

The study was made possible by the invaluable assistance provided by the veterinary medical staff at Omdurman slaughter house and the staff of the Khartoum Medical Teaching Hospital, Khartoum, Sudan. The authors are very grateful to Mr. Abdall M. Fadl Elmoula for technical assistance.

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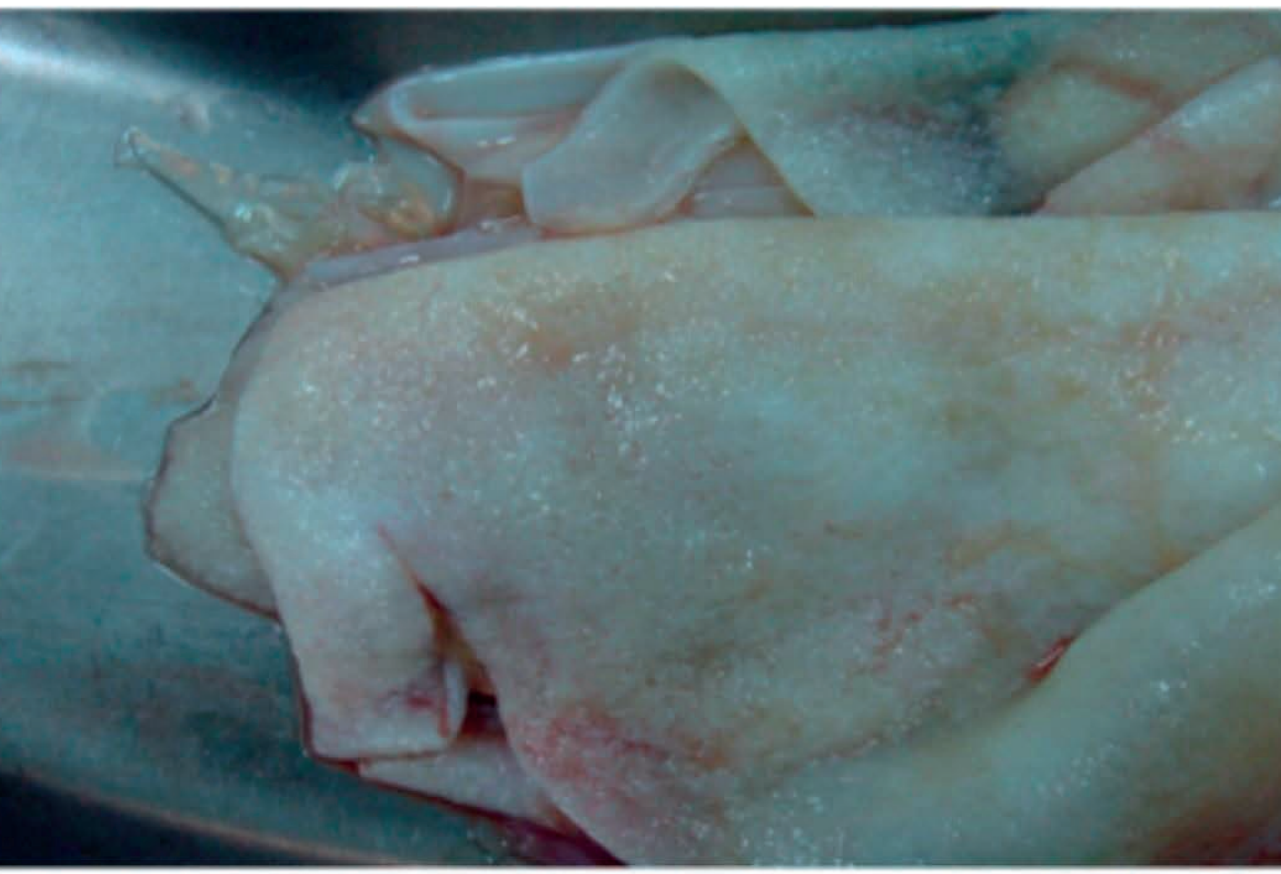

# Chapter 6

## First molecular characterization of Echinococcus granulosus (sensu stricto) genotype 1 among cattle in Sudan

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**Abstract**

**Background:** Echinococcus granulosus sensu lato (s.l.) is the causative agent of cystic echinococcosis (CE), which is a cosmopolitan zoonotic parasitic disease infecting humans and a wide range of mammalian species including cattle. Currently, little information is available on the genetic diversity of Echinococcus species among livestock in Sudan. In the present study, fifty (n = 50) hydatid cysts were collected from cattle carcasses (one cyst sample per animal) at Alkadarou slaughterhouse, Khartoum North, Sudan. DNA was extracted from protoscolices and the germinal layer of each cyst and subsequently amplified by PCR targeting the mitochondrial NADH dehydrogenase subunit 1 (NADH1) gene. The amplified PCR products were purified and subjected to direct sequencing for subsequent construction of phylogenetic tree and net work analysis.

**Results:** The phylogenetic tree revealed the presence of Echinococcus canadenesis genotype 6 (G6) in 44 cysts (88. 0%), Echinococcus ortleppi genotype 5 (G5) in 4 cysts (8.0%) and Echinococcus granulosus sensu stricto (s.s) genotype 1 (G1) in 2 cysts (4.0%). The phylogenetic network analysis revealed genetic variation among the different haplotypes/genotypes. This report has provided, for the first time, an insight of the role of cattle in the transmission of the zoonotic G1 echinococosis.

**Conclusions:** The results of the study illustrate that Sudanese breeds of cattle may play an important role in the transmission dynamics and the epidemiology of cystic echinococcosis in Sudan. This study reports the first molecular identification of E. granulosus s.s. in cattle in Central Sudan.

## Background

Cystic echinococcosis (CE) is a significant public health problem with high endemicity in east and central Africa including Sudan [1–4]. The larval stage of Echinococcus granulosus sensu lato (s.l.) causes CE in humans and a wide range of mammalian species. The life cycle involves the ingestion of parasite eggs by an intermediate host belonging to wildlife and domestic livestock species, including cattle. The dog is considered as the definitive host for this parasitic infection [5]. Humans are accidental dead end hosts. It is estimated that CE results in economic losses in the livestock sector due to morbidity. In addition, partial or total condemnations of infected organs of slaughtered animals are frequently encountered in endemic areas [5–9]. Echinococcosis has recently been included by the World Health Organization (WHO) as a neglected tropical disease [10]. CE may significantly affect the overall development and work productivity in endemic areas. In pastoral Sudanese communities, CE remains highly endemic with higher prevalence compared to agricultural communities. CE is endemic in most parts of the world, including regions of South America, the Mediterranean, Eastern Europe, East Africa, the Near and Middle East, Central Asia, China and Russia [7, 10–13]. Currently, ten distinct genotypes of E. granulosus s.l., designated as G1-G10, have been described worldwide on the basis of genetic diversity related to nucleotide sequences of the mitochondrial NADH dehydrogenase subunit 1 (NADH 1) and cytochrome C oxidase subunit 1 (COX1) genes. These different genotypes are associated with distinct intermediate hosts including sheep, goats, horses, cattles, pigs, camels and and members of the cervid family [14–19]. Of the ten genotypes of E. granulosus s.l., the cattle (G5) and the camel (G6) strains have already been reported among humans and livestock in Sudan [2, 20, 21]. Recent epidemiological studies indicated that the camel genotype (G6) was the most prevalent strain in Sudan [4, 22]. The extensive intra-specific genetic variation of E. granulosus s.l. could be better understood within the context of variations in the life cycle pattern [23, 24]. It is suggested that, different genotypes would probably exhibit different antigenicity, transmission profiles, pathological consequences, and different sensitivity to chemotherapeutic agents [25]. A lot of research efforts have been directed towards the epidemiology of CE in Sudan [26–29]. However, only few reports of the genetic diversity of the parasite among the cattle in Sudan employed sequence analysis of mitochondrial markers [2, 4,

21]. It is, therefore, becoming increasingly obvious that expanding the existing sequence data on the genetic diversity of E. granulosus s.l. is necessary to better understand the biology, ecology and molecular epidemiology of this parasite. In this investigation, a molecular characterization was conducted to identify hydatid cysts recovered from local cattle breed in Central Sudan.

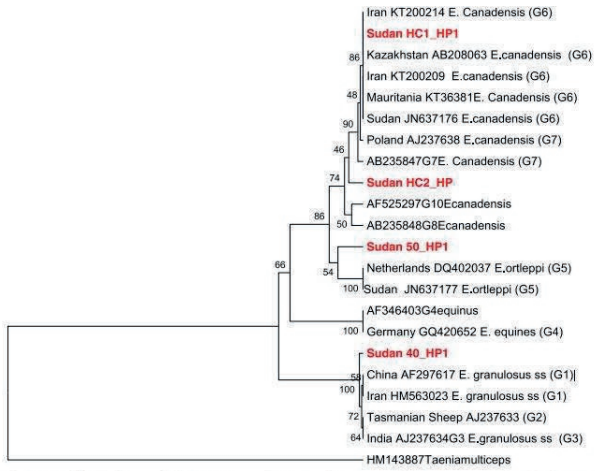


**Fig. 1** Phylogenetic relationship of hydatid cysts of *Echinococcus* granulosus sensu lato recovered from Sudanese cattle and other genotypes identified globally. NADH dehydrogenase subunit 1 (NADH-1) partial sequences generated from this study were aligned with sequences of other strains from different parts of the world. Sequences were analyzed with the BioEdit software (Ibis Biosciences, Carlsbad, CA, USA). The phylogenetic tree was constructed using unweighted pair group method with arithmetic mean (UPGMA) implemented in MEGA software version 6.0 [31]. Bootstrap values were calculated from analysis of 500 replicates of the data set, and values greater than 50% are indicated at the appropriate nodes. Each genotype was designated by its GenBank accession number and the country of origin when available. The GenBank accession numbers (LC167080, LC167081) were given for *Echinococcus granulossus* sensu stricto (G1) and *Echinococcus orteleppi* (G5), respectively. *Echinococcus canadensis* genotypes (G6) were given accession numbers LC167082 and LC167083). Corresponding nucleotide sequence of NADH 1 of *Taenia multiceps*, GenBank accession number HM143887, was used as an out group. The partial NADH-1gene sequences identified in this study were highlighted in red color for clarity of the constructed phylogenetic tree

## Methods

### Collection of samples and processing

Fifty hydatid cysts (n = 50) were collected over a period of 6 months from cattle during April–October, 2016, at Al kadarou slaughterhouse, Khartoum North, Central Sudan. This slaughter house is the major cattle battoir in Khartoum North, Sudan. Hydatid cysts were obtained from cattle instantly after slaughtering and transferred in thermo-flasks to the Molecular Biology Laboratory at the Faculty of Veterinary Medicine, University of Khartoum, for processing and molecular characterization.

**DNA extraction from hydatid cysts**

Parasite genomic DNA was extracted from hydatid cysts as described by Ahmed and his coworkers [4]. Maximum DNA yield was obtained by spinning at 12,000 rpm for 1 min at room temperature. From the suspended nucleic acid, 5 µl was used in the PCR amplification.

**Primers design and PCR assays**

The primers were designed based on the published sequences of NADH dehydrogenase subunit 1 (NADH-1) gene of E. granulosus genotype 6 (G6) reported by Bowles and McManus [15]. Briefly, primer EGL1: 5′TGA AGT TAG TAA TTA AGT TTA A′3 and primer EGR2:

5′AAT CAA ATG GAG TAC GAT TA′3 were designed to amp-lify a fragment of 435 bp of E. granulosus s.l. by PCR. The details of PCR amplification, visualization and of results were described previously [4].

**Sequence processing and phylogenetic analysis**

The PCR products were purified using QIAquick PCR puri- fication kit (Felden, Germany) and submitted for sequencing to a commercial company (Macrogen, Seoul, Korea). Bidir- ectional sequence fragments of the forward and reverse primers were generated for each sample. These were edited manually to correct possible base calling errors using BIOE- DIT 7.0 and were subsequently joined to reconstruct a fragment of 344 bp of the parasite (NADH-1) gene. The consensus sequences were aligned with the corresponding region of NADH-1 gene of known genotypes circulating globally using CLUSTAL-X 2.1 [30]. The phylogenetic tree was constructed using the unweighted pair group method with arithmetic mean (UPGAM) implemented in MEGA software version 6.0 with 1000 bootstrap replicates [31]. Corresponding nucleotide sequences of NADH-1 of Taenia multiceps with GenBank accession number HM143887 were used as out groups in the constructed phylogenetic trees.

**Phylogenetic network analysis**

To measure the genetic variability, the number of haplo- types was determined using DNASP v5 [31] with insertions and deletions considered as variable sites. We used the median-joining (MJ) network algorithm [32] implemented in NETWORK 4.6 (www.fluxus-engineering.com).

**Results and discussion**

Microscopic examination revealed that all hydatid cysts were fertile and measured 2–10 cm in diameter. The predilection sites of the cysts were found to be the lung and the liver. All fifty DNA samples were amplified by PCR and generated a fragment of 435 bp of the NADH-1 gene. The partial sequences of the NADH-1 gene representing genotypes G1 (accession number LC167080), G5 (accession number

LC167081) and G6 (accession numbers LC167082 and LC167083) were submitted to GenBank, DNA

Data Base of Japan (DDBJ). The sequence analysis indicated a prevalence of (88.0%, n = 44), (8.0%, n = 4), (4.0%, n = 2) for Echinococcus canadenesis (G6), Echino- coccus ortleppi (G5), and E. granulosus sensu stricto s.s (G1), respectively. The phylogenetic network analysis revealed clear genetic variation between the different genotypes and haplotypes. The present investigation indicated that at least three different genotypes of E. gran- ulosus s.l. are actively circulating in cattle in Sudan as illustrated by the phylogenetic tree (Fig. 1) and phylogenetic network analyses (Fig. 2). The sample Sudan HC1_HP was well grouped with haplotype 6 and samples

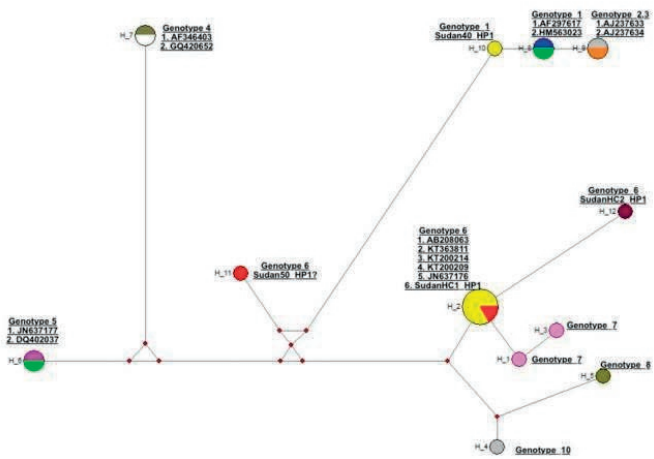


**Fig. 2** Phylogenetic network analysis of haplotypes. The number of haplotypes was determined with insertions and deletions considered as variable sites. Median-joining (MJ) network algorithm [32] implemented in NETWORK 4.6 was used to construct the phylogenetic network. The GenBank accession numbers were the same as indicated for the phylogenetic tree

Sudan HC2-HP1 that was clustered with genotype 6 in the phylogenetic tree was six SNPs different from the haplo- type 6. Sudan 40_HP was grouped with genotype 1 and differed with only 2 SNPs from the previously known genotype 1. the three Echinococcus genotypes (G1, G5 and G6) reported in this study are all known human pathogens of significant public health concern [33]. The exclusive occurrence and a predominant circulation of the camel genotype (G6) in the bovine species suggested that cattle can play an important role in the transmission dynamic and the epidemiology of the disease [4]. The present study indicated that E. granulosus s.s., the sheep strain (G1), should equally be considered as an important infectious form of CE among cattle in Central Sudan.

**Conclusions**

The present study represents the first molecular record o v E. granulosus s.s G1, thus reinforcing its role as a source of infection among Sudanese cattle breeds. In addition, this investigation provides additional information on the existing data indicating that Echinococcus granulosus s.s. G1, which was previously restricted to other region in the African continent, is now becoming broadly distributed in the country. Active surveillance is required to determine the distribution and prevalence of CE and to identify the genotypes/strains circulating in different regions of Sudan.

**Abbreviations**

CE: Cystic echinococcosis; cox1: Cytochrome C oxidase subunit 1; DDBJ: DNA Data Base of Japan; E. granulosus s.l.: Echinococcus granulosus sensu lato; E. granulosus s.s.: Echinococcus granulosus sensu strict: s.s; G1: Genotype 1; G5: Genotype 5; G6: Genotype 6; N: Number; NADH-1: NADH dehydrogenase subunit 1

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**Availability of data and materials**

The data and materials of this study were linked to the Dryad repository via TreeBASE. The link to TreeBASE search ID is provided below: https://doi.org/105061/dryad.5qr6t.

**Authors' contributions**

MEA help with collection of hydatid cyst samples, extracted the DNA, optimized the polymerase chain reaction-based detection assay, editing of sequences and helped with the manuscript writting; BS collected hydatid cyst samples, edited and analyzed the sequence data; MPG designed the experiment and helped with preparation of the final manuscript; IEA designed the experiment, helped with collection of hydatid cyst samples and prepared the final manuscript. All authors read and approved the final version of the manuscript.

**Ethics approval and consent to participate**

The study protocol was approved by the Institutional Research Board (IRB), Hydatid cysts were collected from slaughtered cattle during post-mortem inspection by qualified veterinary officers at Alkadarou slaughter house, Khartoum North, Sudan. Formal consent and permission for research use of hydatid cysts were obtained from both the university and abattoir veterinarians. In this study, no experiment was conducted on live animals.

**Consent for publication:** Not applicable.

**Competing interests:** The authors declare that they have no competing interests. All authors have read and approved the final version of this manuscript.

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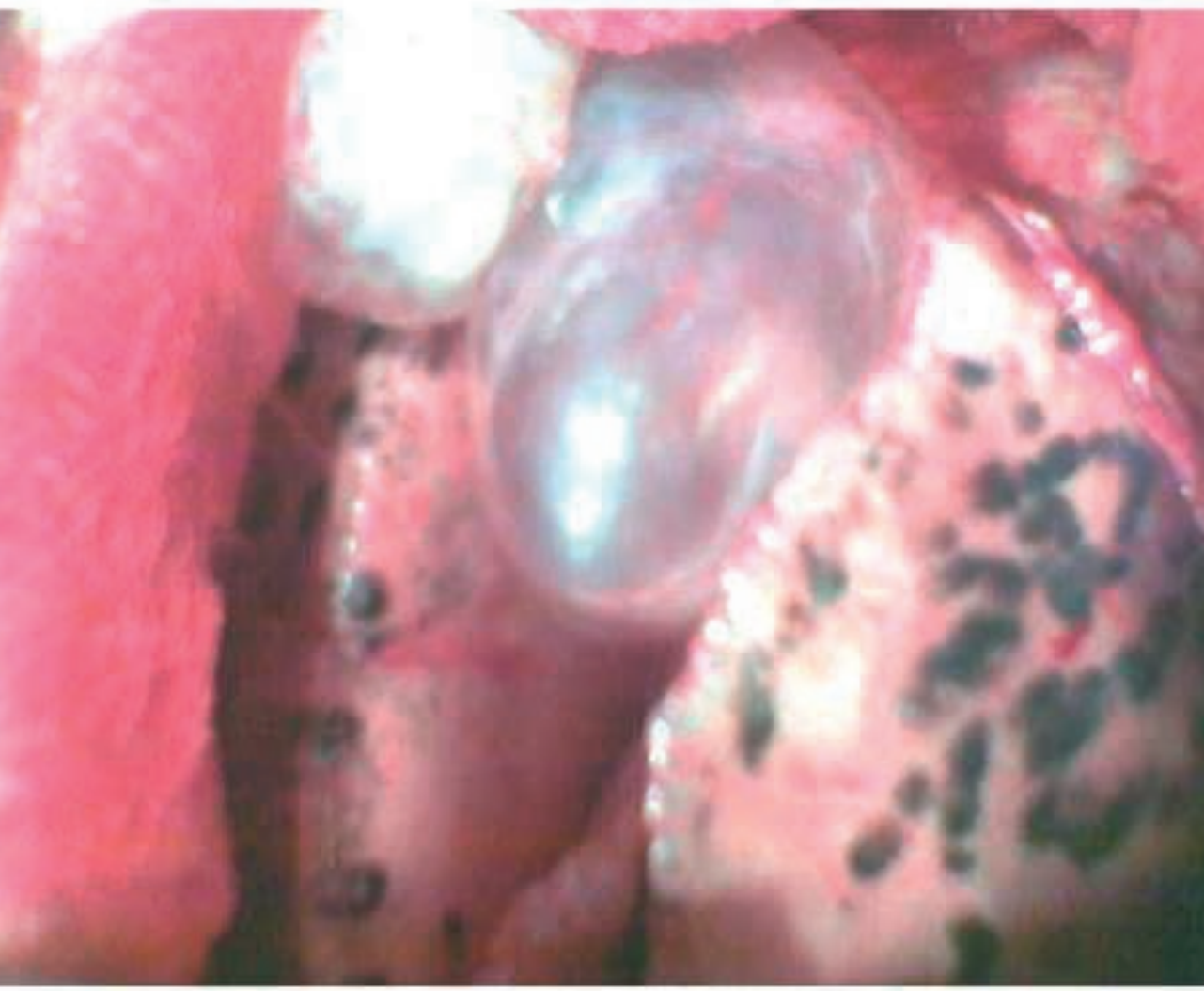

# Chapter 7

## Genetic characterization of cystic pulmonary echinococcosis in Sudan as determined by mitochondrial DNA sequences

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**Abstract**

**Background:** Cystic echinococcosis (CE) is a zoonotic parasitic disease caused by the larval stages of the cestode Echinococcus granulosus. It is a significant public health problem with high endemicity in east and central Africa including Sudan. Worldwide, pulmonary echinococcosis cyst is a significant problem medically, socially, and economically. Until now ten genetic variants, or genotypes designated as (G1-G10), are distributed worldwide based on genetic diversity. In this study we aimed to investigate molecular diversity of Echinococcus granulosus isolates collected from human clinical samples removed surgically from lung using mitochondrial gene nad1 in Sudan.

**Methods:** Seven human echinococcosis cysts originated from lung were collected through surgery from ElShaab Teaching Hospital in Khartoum state during 2016-2018. The fertility of cysts was detected microscopically by examination of the cysts fluid in which the protoscoleces were found. Protoscoleces were removed from each cyst and their total genomic DNAs were extracted. PCR was performed to amplify fragments of 530 base pair (bp) forNADH dehydrogenase subunit 1(NADH-1) gene. The amplified PCR products were purified and subjected to direct sequencing for subsequent construction of phylogenetic tree and network analysis.

**Results:** The identity of the PCR products were confirmed as NADH1 nucleotide sequences using the Basic Local Alignment Search Tool (BLAST) of NCBI (National Center for Biotechnology Information, Bethesda, MD). The phylogenetic tree revealed the presence of Echinococcus canadenesis genotype 6 (G6) in all cysts (100%).

**Conclusions:** According to the results of the previous and present observations which has been performed by PCR only and PCR followed by sequencing respectively, it can be concluded that the pul echinococcosis in Sudan are caused by genotype 6 (G6) camel strain of E. granulosus, which indicates the camel-dog cycle in the studied area.

**Keywords:** Echinococcosis, Pulmonary, PCR, DNA sequencing, Phylogony, Sudan

## Introduction

Cystic echinococcosis, caused by the metacestode of Echinococcus granulosus, is one of the most common zoonotic diseases associated with huge economic losses and great public health significance worldwide [1]. Dog and other carnivores that harbor the adult cestode in their small intestine are the definitive hosts for the parasite, while a wide range of mammalian species including domestic animals and man act as intermediate hosts [2]. The metacestode stage of E. granulosus inhabits the liver, lungs, and other internal organs of livestock and humans after oral uptake of the eggs, which are produced by adult worms in the canine small intestine [3,4]. Humans can be accidently infected by ingesting its eggs, which results in human cystic echinococcosis (CE) or hydatidosis [5]. CE may develop silently over years and even decades until it surfaces with clinical signs or symptoms. Clinical symptoms are mainly related to the localization, size and number of cysts. However, a recent study described that there seemed to be a relationship between the genotypes and the size of hydatid cysts, in which all the patients infected with G7 genotype showed smaller liver cysts than those infected with G1 genotype. CE is an important and sometimes life-threatening disease, which mostly affects lungs and liver, but other organ scan be also affected [6]. Fatal cases of human CE have been reported with inoperable CE cysts of the brain and anaphylactic shock caused by rupture of liver hydatid cysts [7]. In the Sudan, many reports of cystic echinococcosis have been described in humans and animals [8]. In addition to its importance as a major public health problem in the country, CE is also considered as one of the major causes of condemnation of sheep carcasses during meat inspection. Different genotypes of E. granulosus have been identified from a variety of hosts worldwide. Until now, 10 genotypes(G1-G10) has been described [9]. The genotypes are different in some criteria such as pathogenicity, host specificity, pattern of life cycle, transmission dynamics and developmental rates, human infectivity and response to chemotherapeutic drugs [10]. Of the ten genotypes of EG, the sheep(G1), the cattle (G5) and the camel (G6) strains were reported in humans and livestock in the Sudan [11,12]. The epidemiological situation in Sinnar area, Blue Nile state is characterized by intense transmission of Echinococcus canadensis(G6), thereby closely resembling the situation in most other regions of Sudan [13]. This study aimed to investigate genotypes of the echinococcosis cysts isolated from echinococcosis patients from different parts of Sudan. Echinococcosis cysts were defined by genetic studies and subsequent

phylogenetic analysis. The molecular characterization was made possible by targeting fragments of the mitochondrial NADH 1 to define the circulating genetic variants in the different part of Sudan.

## Materials and Methods

### Collection of samples

A total of 7 Echinococcus cysts were collected from 7 patients suffering from pulmonary CE. All patients came from different parts of Sudan and were operated surgically at Al-Shaab hospital. The cysts were preserved separately in 70% ethanol and formalin.

### Microscopic examination

Cysts or cyst material were examined microscopically toconfirm whether or not they are hydatid cysts. Fertility of the collected Echinococcus cysts was assured by detection of protoscolices in aspirated fluid samples.

### Extraction of genome DNA

Genomic DNA was extracted from sediment positive for protoscoleces using GF-1 Blood DNA Extraction Kit (Vivantis) according to manufacturer's instructions. Briefly, 200 µl of the suspended aspirate, 20 µl of proteinase K stock solution, and 200 µl of lysing buffer were pipetted into 1.5 ml eppendorf tube. The mixture was incubated at 65°C for 10 min before the addition of 200 µl of absolute alcohol and mixing by vortexing. The mixture was then transferred to the spin column placed in a clean 2 ml collection tube and centrifuged at 5000 RPM in MiniSpin centrifuge (Eppendorf, Wesseling-Berzdorf, Germany) for 1 min at room temperature. The spin column was washed twice with 500 µl of the washing buffersand centrifuge at 5000 RPM for 1 min and the DNA was eluted in 100µl of buffer and was stored at 4 ° c until further use in PCR analysis.

***Selection of primers:*** The primers were selected from mitochondrial NADH dehydrogenase subunit 1 (NADH 1) gene. The NADH 1 primers used in this study were basically described previously (Bowles J and McManus DP, 1993). For the first amplification step, a pair of outerprimers JBl1: 5' AGATTCGTAAGGGGCCTAATA 3' andJB12: 5' ACCACTAACTAATTCACTTTC 3' were used toamplify a 530 bp PCR product from EG isolates. A pair of internal sequencing primers JB11.5: 5'

TTATGGTAGATATTATAG 3' and JB12.5: 5' CACACACATAAAACAAGC 3' designed to conserved segments of the EG NADH1 sequences, were used to generate a 471 bp PCR product.

**Polymerase chain reaction (PCR)**

A stock buffered solution containing 150 µl 10x PCR buffer, 100 µl of 25 mM MgCl2, 12.5 µl of each dATP, dTTP, dGTP and dCTP at a concentration of 10 mM was prepared in 1.5 ml eppendorf tube. The primers were used at a concentration of 20 pg/µl, and double distilled water was added to bring the volume of the stock buffer solution to 1.5 ml. Each 0.5 ml PCR reaction tube contained 2 µl of the primers, 1 µl (5.0 U) of Taq DNA polymerase (Vivants), 5.0 µl of the target DNA and 42 µl of the stock buffered solution. For nested PCR, 2 µl of the primary PCR product was used as DNA template. The thermal cycling profiles were as follows: a 2 min initial incubation at 95°C, followed by40 cycles of 95°C for 1 min, 55°C for 30 sec and 72°C for 45 sec, and a final incubation at 72°C for 10 min. Thermal profiles were performed on a Techne TC-412 thermal cycler (Techne, Staffordshire, UK). Following amplification, 15 µl from each PCR containing amplified products wereloaded onto gels of 1.0% agarose and electrophoresed for 1 h. The gels were stained with ethidium bromide and the PCR products were easily identified following visualization under UV light.

**Sequence analysis and construction of phylogenetic tree**

The sequences obtained from the PCR products were sent for sequencing to commercial company (Macrogen, Seoul, Korea). Resulted sequences were edited and aligned using BioEdit software (Ibis Biosciences, Carlsbad, CA, USA). The Basic Local Alignment Search Tool (BLAST) of NCBI (National Centre for Biotechnology Information, Bethesda, MD, USA) was used to confirm the identity of the generated sequences in relation to GenBank nucleotide database. The sequences then aligned with the corresponding region of NADH1 subunit gene of known genotypes from other countries. All sequences were submitted to the GenBank (accession numbers MW660881 to MW660886). The phylogenetic tree was constructed using Neighbor joining tree implemented in MEGA software version 7.

## Results

### Polymerase chain reaction (PCR)

The PCR-based assay with primers specific for NADH-1 yielded amplification products from of all of the seven hydatid cysts obtained from patients. For NADH1, the outer pair of primers produced a primary 530 bp PCR product and the nested primers produced a 471 bp PCR product (figure 1). The PCR products submitted for sequencing to a commercial company (Macrogen, Seoul, Korea).

### Sequence analysis and phylogenetic relationship

The sequences obtained from the PCR products were found to aligned and compared with corresponding regions for NADH1 gene in the GenBank confirming the cysts to contain the EG complex, using BioEdit and also the BLAST program of GenBank. All sequences were submitted to the GenBank (accession nos. MW660881 to MW660886). Genotype identifications were in complete agreement for all isolates. Blast search of the obtained sequences derived from human isolates indicated the occurrence of G6 genotype (E. canadenesis) in all isolates. Presence of nucleotide substitution at position 16282(A to C) was detected among 2 strains with G6 genotype when compare to reference strain NC_044548.1 (complete genome), also found other substitution at position 7579 (G to C) in that same 2 strains when compared to reference strain NC_038227.1(G6 strain) (Figure 2). Among all the strains with G6 genotype, substitution at position 16132 (C to G) was observed in compare to reference strain NC_044548.1 (complete genome) and NC_038227.1(G6 strain) (Figure 3). The phylogenetic analysis of concatenated sequences of nad1gene showed that all strains related to G6 genotype (Figure 4). Moreover, reference sequences for G6 genotype available in the GenBank, were included in the comparative analysis.

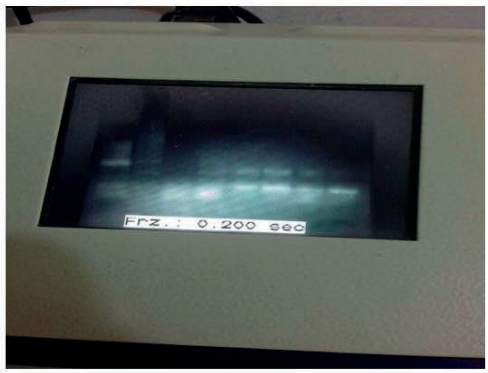


Figure 1: Nested PCR product, Lane 1,2,3,4,5: DNA extracted from echinococcosis cyst sample from patient`s lung (+ve), Lane Nc: negative control, Lane PC: E. granulosus (G6) strain DNA (positive control), MW: molecular weight marker (500bp).


Continued figure (1): nested PCR product for sample 6 and 7 were positive

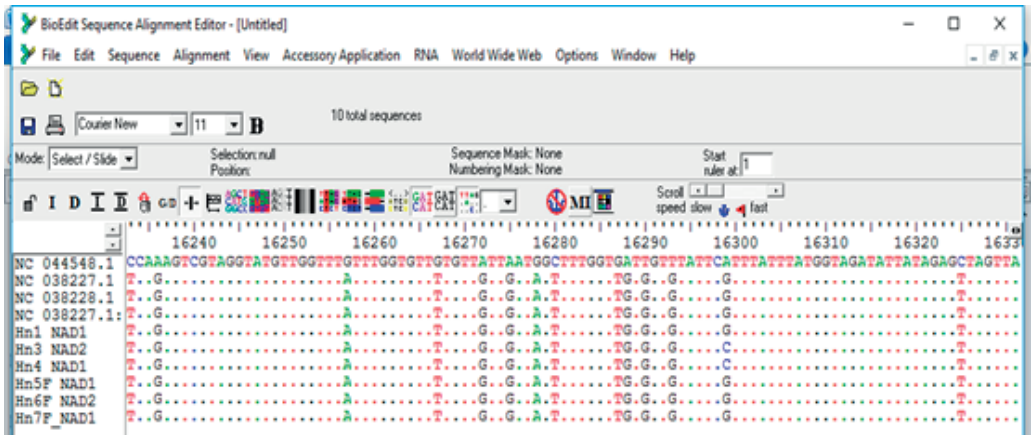


**Figure 2:** Multiple sequence alignment of nad1 gene of samples and Echinococcus granulosus mitochondrion, complete genome (NC_044548.1), Echinococcus granulosus sensu lato genotype G6 isolate 1 mitochondrion, complete genome (NC_038227.1) and Echinococcus granulosus sensu lato genotype G7 isolate 27 mitochondrion, complete genome (NC_038228.1) as reference samples.

*All samples except 3 and 4 showed nucleotide substitution A>G when compared with complete genome reference sequence, samples 3 and 4 showed nucleotide substitution G>C when compared with G6/7 reference sequence.

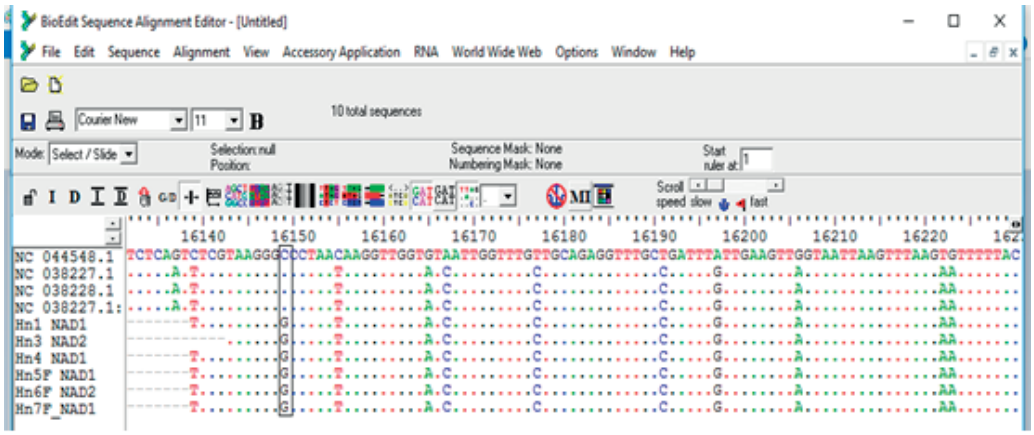


**Figure 3:** Multiple sequence alignment of nad1 gene of samples and Echinococcus granulosus mitochondrion, complete genome (NC_044548.1), Echinococcus granulosus sensulato genotype G6 isolate 1 mitochondrion, complete genome (NC_038227.1) and Echinococcus granulosus sensulato genotype G7 isolate 27 mitochondrion, complete genome (NC_038228.1) as reference samples.

*All samples showed nucleotide substitution C>G.

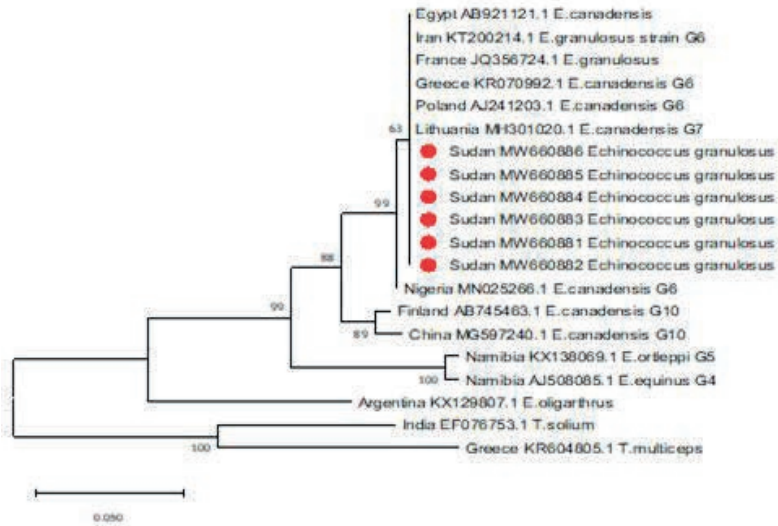


**Figure 4:** Phylogenetic relationship of Echinococcus granulosus-complex genotypes recovered from Sudanese human and other genotypes identified globally.

Partial NADH dehydrogenase subunit 1 gene sequences generated from EG isolates were aligned with sequences of other EG isolates from different parts of the world. Sequences were analyzed with the BioEdit software (Ibis Biosciences, Carlsbad, CA, USA). The phylogenetic tree was constructed using neighbor joining tree implemented in MEGA software version 7. Bootstrap values were calculated from analysis of 1000 replicates of the data set, and values greater than 50% are indicated at the appropriate nodes. Each EG isolate is designated by country of origin, GenBank accession number and the genotype of the isolate when available. The GenBank accession numbers (MW660881, MW660882, MW660883, MW660884, MW660885 and) were given for the Sudan isolates of genotype 6 (E. canadensis). The strains from Sudan those described in this study are highlighted in red color for clarity. T. solium and T. multiceps were used as the out group sequence data.

**Discussion**

Cystic pulmonary echinococcosis (CE), affects many people throughout the world, although, advances in diagnosis and treatment of CE had been achieved in the recent years. There is still a

limit to the disease control. As an endemic region with high incidence of CE, the disease is considered as a public health and socio-economic problem in Sudan. Investigations on the epidemiology and different genotypes of parasites in the intermediate and final hosts should be considered in any endemic area to achieve the evidence based control and management programs [14]. In the African countries, the G6 has been reported as the dominant genotype [15]. The genotype of all isolates from camel in Mauritania, Algeria and Sudan has been reported to be G6 [16,17]. However, other studies in Kenya and Libya have shown a noticeable prevalence of G1 strains in camel isolates [17,18]. Different genotypes of E. granulosus including G1, G 5 and G6 /G7 have been reported from different hosts in Sudan [13,19,20]. The study results showed that all the hydatid cysts analyzed for species/genotypes identification were G6 (camel strain). The exclusive occurrence and a predominant circulation of the camel genotype (G6) suggested that cattle can play an important role in the transmission dynamic and the epidemiology of the disease in Sudan. This study was in agreement with many studies in Sudan conducted to identify the genotypes of E. granulosus in human. Omer et al., Ahmed, Aradaib and Omer et al. documented the predominance of E. canadensis 'camel strain' (G6) [12,21,22]. However, genotyping of 22 patients isolates from Elshaab Teaching Hospital, Khartoum showed the G6 (Camel strain) in all except one patient showed G1 strain (sheep strain) [23]. As a lot of studies conducted in Sudan for molecular genotyping of E. granulosus that showed the predominant strain among livestock was E. canadensis 'camel strain' (G6) [13,24,25]. While other studies demonstrated that E. canadensis 'camel strain' (G6) coexist with E. ortleppi 'cattle strain' (G5) and E. granulosus senso stricto 'sheep strain' (G1) are circulating in production animals, where E. ortleppi was found in cattle only in Central, Eastern and western Sudan [12,17,20,26,27]. The exclusive occurrence and a predominant circulation of the camel genotype (G6) suggested that cattle can play an important role in the transmission dynamic and the epidemiology of the disease in Sudan. Unlike the study done in Iran which found all the human cases studied in that study were infected by G1 strain of E. granulosus (sheep strain). This finding is emphasis on the importance of sheep as a source of canine and subsequently human infections which support the idea that G1 strain is predominant in the East Azerbaijan Province [28]. The phylogenetic reconstruction method highly supported the existence of main strains, G6 genotype in humans from Sudan. However, few phylogenetic studies were conducted to determine the phylogenetic relationship of the identified the genotypes of EG in the Sudan. In this study the phylogenetic analysis illustrated that Echinococcus canadensis

genotype 6 (G6) is the most infectious and widespread genotypes in Sudan, confirming the results of previous studies [13,20]. Results of our study showed presence of various nucleotide substitution in nad1 genes that are region specific in compare to reference strains in the world. In conclusion, data on frequency of E. granulosus genotypes in human revealed no changing pattern of genotypes distribution between isolates in Sudan. G6 was identified as the most common species in human in Sudan.

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Section

# III

# Community based cross sectional study on KAP on EC

# Chapter 8

## Echinococcosis in Tambool, Central Sudan: a knowledge, attitude and practice (KAP) study

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**Abstract**

**Introduction**: In Sudan, echinococcosis (EC) is a chronic neglected zoonotic parasitic disease caused by Echinococcus granulosus. Studies have shown high prevalence rates in dogs (50–70%), camels (35%) and sheep, goats and cattle (10–11%). In total, 0.3–1.0% of humans in Central and South Sudan are infected with the G6 camel strain. This strain is almost exclusively the cause of human infections. The objective of this study was to explore knowledge, attitudes and practices (KAP) regarding the disease among people living around Tambool city, Central Sudan.

**Methods:** A cross-sectional survey was conducted in three villages around the city of Tambool in Central Sudan. Three-hundred-and-twelve households were selected from the administrative unit of the area for participation in the study, of which 300 agreed to partake. A standardized questionnaire was designed to collect data on EC in animals, humans and the environment. The questionnaire domains were socio-demographic characteristics, KAP regarding echinococcosis.

**Results:** The population surveyed showed that 68.7% (206/300) had never heard of the disease, while 31.3% (94/300) had heard about it. The level of knowledge among the 31.3% of those that had heard about the dis- ease was excellent (69/94; 73.4%); so were their attitudes (76/94; 80.9%). However, the majority of the participants (64/94; 68%) showed poor practice regarding this disease, enhancing the odds for further propagation of parasite circulation in the animal and human populations at risk. Knowledge was found to be significantly associated with marital status. Practice was found to be significantly associated with occupation.

**Conclusions:** There is a need for the implementation of a multidisciplinary program using the One Health approach to effectively control and prevent EC.

**Keywords:** Attitudes and practices, echinococcosis, knowledge, The One Health, Sudan

## Introduction

In Sudan, echinococcosis (EC) is a chronic neglected zoonotic disease caused by the parasite Echinococcus granulosus,1–3 affecting mainly carnivorous animals as definitive hosts (dogs, foxes) and other animals as intermediate hosts (camels, cattle, sheep). Humans may be accidentally infected through intimate direct contact with dogs or ingestion of contaminated water or food. Different types of animals are bred in Sudan, such as sheep, goats, cows and donkeys. Most of these animals are intermediate hosts of Echinococcus, while (stray) dogs are considered to be the main definitive host for Echinococcus in many parts of the country. Previous surveys conducted in Sudan have shown that EC is circulating in many parts of the country. For central (Tambool) and south (Kurdufan) Sudan, studies have established high prevalence rates of E. granulosus in stray dogs (50–70%), camels (35%) and sheep, goats and cattle (10–11%), with 0.3–1.04% of all humans being infected.1,4–9. Infection in humans often causes hydatid cysts, which may lead to serious complications if left untreated.4,6,10–15 Treatment is expensive and sometimes cumbersome, requiring surgery in some of the advanced cases.12,16. The region of this study is rich with host animals, particularly camels, having one of the biggest camel markets in the country and the region. Many animal cases of EC were detected at the city‘s abattoir, while human cases were detected at the rural hospital. These animal and human cases were the driving force behind developing this study to explore the awareness, attitudes and practices of the community towards EC in this area. The study area, three villages around Tambool city, is known for its large livestock population, predominantly camels. Most livestock are slaughtered in the only public abattoir, where stray dogs live in the streets and have access to the abattoir offal. Screening for EC in humans has led to successful medical management, by the use of portable ultrasound and chemotherapy in asymp- tomatic cases.6 Genotyping has revealed that in Sudan, G6 (the camel strain) is the cause for almost all infected human samples collected.1,4,7,17. EC is one of the diseases for which the One Health approach is used, an approach that acknowledges that the health of people is connected to the health of animals and the environ- ment.18,19 One Health was proposed as a concept to foster such interdisciplinary collaboration. It was therefore considered necessary to discover the knowledge level of people living around Tambool, and their attitudes and practices regarding EC, which might influence the disease and its spread.

## Materials and Methods

### Study design and area

This was a cross-sectional community-based study in three villages around Tambool city. The households included in the sur- vey numbered 300 out of 312 that were approached initially. Ethical approval was obtained from Al Neelain University IRB and Gezira Ministry of Health. Written informed consent was taken from all participants prior to starting the study

.

### Data collection and analysis

A standardized questionnaire was designed by the research group, which included a veterinarian, an epidemiologist and a physician to collect the data. The data covered different aspects of EC in animals, humans and their environment. The domains in the questionnaire were: socio-demographic characteristics of the participants, such as gender, age, income, education level, marital status and occupation; knowledge about EC, such as cause, transmission, symptoms and prevention; attitudes and practices towards EC, such as health seeking behavior, food habits, animal slaughter habits and meat inspection practices. The KAP scale was divided into three categories—poor, good and excellent. The knowledge scale consisted of 15 questions; the attitude domain consisted of seven questions; the practice domain was composed of five questions. The questionnaire was designed in Arabic, then translated into English (Supplementary file). Prior to the survey, a prefield visit was undertaken in order to visit the rural hospital and the abattoir in the study area. This was found to be helpful in understanding the context of the EC from both health and veterinarian perspectives. The questionnaire was piloted with 20 community members. The authors approached community leaders who explained to community members the purpose of the study, its objectives and the importance of their participation. Community members were mobilized as facilitators upporting data collection. A 1-day workshop was conducted to train a multidisciplinary team of young physicians and veterinary students of Butana University located near the study area. They became part of the data collection team and built basic knowledge capacity about the study objectives and how to conduct face-to-face interviews administering the questions from the KAP questionnaires, and could thus effectively participate in the study. The questionnaire was

administered to 300 out of 312 household heads living in the study area. The remaining 12 household heads were not interested in participating in the study and, therefore, were not included. House-to-house visits to interview household heads were conducted by the data collection team with significant support from the community members. Data cleaning was done in the field. Data was analyzed using SPSS version 19.0 (IBM, Armonk, NY, USA). Univariate analyses were performed using the $\chi 2$ test for categorical variables, and Student 's t-test and ANOVA for continuous variables. Results were considered statistically significant at a two sided p-value of less than 0.05.

**Results**

The total number of participants was 300, of which 162 were male. Regarding their educational level, 43% (130/300) were illiterate and 42.7% (128/300) had received primary or inter-mediate education; only 42/300 (14.0%) had received second- ary or higher education. In total, 24.3% (73/300) pursued animal-related occupations, 79.8% (227/300) did not have animal-related jobs. Of the 300 participants, 68.6% (206/300) had never heard about the disease, while only 31.3% (94/300) had heard about it (Table 1).

**Table 1** Socio -demographic characteristics of participants in the KAP study about echinococcosis living around thr ee villages in Tambool, central Sudan (n=300)

| Factor | n | % |
|---|---|---|
| Sex | | |
| Male | 162 | 54 % |
| Female | 138 | 46 % |
| Marital status | | |
| Married | 245 | 81.7 % |
| Unmarried | 55 | 18.3 % |
| Education level | | |
| Illiterate | 130 | 43.3 % |
| Intermediate or le ss | 128 | 42.7 % |
| Secondary and above | 42 | 14.0 % |
| Occupation | | |
| Animal -related job | 89 | 29.7 % |
| Non -animal related job | 211 | 70.3 % |

The authors asked the latter participants to partake in the KAP study, namely those who had heard of the disease. Of the participants of the KAP study, 36.2% (34/94) were male and 63.8% (60/94) were female. The mean age was 37.5 y. Most of the participants, constituting 45.7%

(43/94) of the sample, had a monthly income of less than 500 Sudanese Pounds (SDGs) (70 US$) per month, and 32 (34%) had no income at all. A total of 78.7% (74/94) were married. Regarding the participants 'educational level: 36.2% (34/94) were illiterate; 11.7% of the participants (11/94) had followed Khalwa (a reli- gious center education); and 41.5% (39/94) had a primary level of education (Table 2).

**Table 2.** Socio-demographic characteristics of participants who responded to the KAP survey (n=94)

| Factor | n | % |
|---|---|---|
| Gender | | |
| Male | 34 | 36.2 |
| Female | 60 | 63.8 |
| Age group (y) | | |
| 1–19 | 5 | 5.3 |
| 20–39 | 47 | 50 |
| 40–59 | 42 | 44.7 |
| Income per month (SDG) | | |
| No income | 32 | 34 |
| <500 | 43 | 45.7 |
| 500–1000 | 16 | 17 |
| 2001–3000 | 1 | 1.1 |
| 3001–4000 | 1 | 1.1 |
| Missing | 1 | 1.1 |
| Marital status | | |
| Never married | 14 | 14.9 |
| Married | 74 | 78.7 |
| Divorced | 3 | 3.2 |
| Widower | 3 | 3.2 |
| Education level | | |
| Illiterate | 34 | 36.2 |
| Khalwa | 11 | 11.7 |
| Primary | 39 | 41.5 |
| Secondary | 9 | 9.6 |
| University | 1 | 1.1 |

The most frequent occupation amongst women was housewife (44.7%, 42/94). The second most com- mon occupation was business owner (28.7%; 27/94; Table 3).

**Table 3.** Occupation of those who completed the KAP survey (n=94)

| Occupation | n | % |
|---|---|---|
| Student | 3 | 3.2 |
| Farmer | 5 | 5.3 |
| Housewife | 42 | 44.7 |
| Clerk | 1 | 1.1 |
| Business owner | 27 | 28.7 |
| Businessman | 2 | 2.1 |
| Craftsman | 1 | 1.1 |
| Unemployed | 6 | 6.4 |
| Other | 7 | 7.4 |
| Total | 94 | 100 |

Of those participants who had heard about the disease, 73.4% (69/94) had excellent knowledge regarding the disease, while 2.1% (2/94) had good knowledge and 24.5% (23/94) had poor knowledge (Table 4). Regarding attitudes, 19.1% (18/94) had a poor score regarding attitude towards dealing with the disease; no participant scored good for attitude and 80.9% (76/94) scored excellent for attitude (Table 4). Practice was a bit different, with 68% (64/94) exerting poor practices in dealing with the disease; 1.1% (1/94) exhibited good practices and 29.9% (28/94) exhibited excellent practices in dealing with the disease. There were five questions in the practice domain (Table 4). The knowledge scale was associated with gender, age group educational level, occupation and income; $\chi^2$ and p values were not significant.

Table 4. Scale of knowledge, attitude and practice of participants in the KAP study about echinococcosis living around three villages in Tambool, central Sudan (n=94; figures given as percentages)

| Scale | Range | | Poor | Good | Excellent |
|---|---|---|---|---|---|
| Knowledge | Poor | 0–21 | 23 (24.5%) | 2 (2.1%) | 69 (73.4%) |
| | Good | 22–43 | | | |
| | Excellent | 44–64 | | | |
| Attitude | Poor | 0–9 | 18 (19.1%) | 0 (0%) | 76 (80.9%) |
| | Good | 10–19 | | | |
| | Excellent | 20–28 | | | |
| Practice | Poor | 0–7 | 64 (68%) | 1(1.1%) | 28(29.9%) |
| | Good | 8–15 | | | |
| | Excellent | 16–22 | | | |

Only for the marital status was the p value significant at 0.000 (Table 5). Attitude of participants was associated with gender, age group, marital status, educational level, occupation and income per month; however, no association was found using $\chi 2$ and p values testing that were not significant for any of the domains (Table 5). In terms of attitudes, 95% of the participants who heard about the disease considered it to be contagious and about half of them believed that EC patients should be isolated. Practice domain was also associated with gender, age group, marital status, educational level and income per month, but $\chi 2$ and p values were not significant. p Value was found to be significant for occupation at 0.04 (Table 5).

Table 5. Knowledge, attitude and practice among participants

| Factor | Knowledg | | Attitude | | Practice | |
|---|---|---|---|---|---|---|
| | $\chi^2$ | p-value | $\chi^2$ | p-value | $\chi^2$ | p-value |
| Gender | 0.185 (2) | 0.912 | 0.071 (1) | 0.789 | 0.170 (2) | 0.919 |
| Age group | 3.067 (4) | 0.547 | 3.079 (2) | 0.214 | 1.526 (4) | 0.822 |
| Marital status | 27.99 (8) | 0.0001* | 3.423 (4) | 0.490 | 7.833 (8) | 0.450 |
| Education level | 9.238 (8) | 0.323 | 7.110 (4) | 0.130 | 9.933 (8) | 0.270 |
| Occupation | 9.140 (16) | 0.908 | 5.049 (8) | 0.752 | 25.415 (16) | 0.063 |
| Income per month | 7.754 (12) | 0.804 | 2.126 (6) | 0.908 | 6.041 (12) | 0.914 |

The numbers in brackets are the degrees of freedom for the relevant $\chi^2$.
*Indicates significance at $<0.05$.

## Discussion

The main aim of the study was to find out the level of KAP regarding EC of people around Tambool city, which might influence the prevalence of this disease. The results of this study showed that EC is truly neglected, and the majority of the participants had insufficient information about the disease. Out of the 300 participants, 68.7% (206/300) were completely ignorant about the disease, despite the fact they lived in an area rich with host animals for Echinococcus, particularly camels.

Comparing the result of only 31.3% of respondents (94/300) being aware of EC with a study in Gansu Province in China,20 quite the reverse picture was seen, with 65.9% (641/972) knowledgeable about EC. Similarly, a study in Morocco21 showed 50% of participants had heard of

the disease. These differences could be explained by the fact that in Sudan, both at the local and national health system levels, the disease is neglected, with a lack of preventive programs for the disease. The occupation of the 300 participants was as follows-29.7% (89/300) had animal-related jobs and the rest, 70.3% (211/300) held jobs unrelated to animals. Interestingly, the majority of those who had heard about EC were housewives and business owners. This could be explained by the fact that women take care of small ruminants at home and are responsible for preparing food for the family in that context. Therefore, such types of activities may increase their concern and knowledge about EC as a food-borne disease. In addition, most of the business owners in the study context are small scale, related to agriculture or the animal trade, and they can also win animals, factors that may increase their awareness about EC. Regarding the occupations of study participants, most interviewees were housewives, which reflect the majority of females who were interviewed in the study. The second most common occupation was business owner. Despite this, the income level was very low, at only 500 SDG per month, which is around 70 US$ a month. A proportion of the participants had no income at all, which corresponds with the low level of education.

The participants in the KAP study consisted of 31.3% subjects (94/300) who had heard of EC. The educational level of the participants was low; 36.2% were illiterate and 41.5% had only a primary level education. This result was not very much different from the study performed in China, 20 where the level of education of participants who could not read or write was 38.3%. This poor educational level could be explained by the fact that Tambool does not have many schools. Moreover, the curriculum in the available schools is not health-oriented. Primary and secondary curricula should be oriented more towards health- seeking behavior. Knowledge of those who participated in the KAP was found to be excellent at a level of 73.4% (69/94).

However, this study had a large majority of participants who had never heard of the disease (68.6%) and, therefore, could not be included in the KAP study. These results, when compared with other studies, such as that done in Morocco, which showed that 50% of the interviewees were graded as having good knowledge. 21 The poor knowledge results in Tambool could be related to the fact that no educational programs are available in the area regarding the disease. Knowledge was positively associated with marital status, indicating that marriage has increased the knowledge level; the information for females was probably obtained from their husbands, as well as from female gatherings, which are quite common practice among housewives. In the

attitude domain, 80.9% (96/94) were graded as having an excellent attitude. Attitude was not found to be positively associated with any characteristics, such as age, gender, occupation or income. Most attitudes people have are usually acquired from community culture and tribal beliefs; a positive association was therefore expected, although the insignificant result may be due to the small sample size of the study.

Practice domain was found to be different, where 68% (64/94) had poor practices in dealing with the disease, 1.1% (1/94) had good practices and only 29.8% (28/94) had excellent practices in dealing with the diseases. Occupation was found to be positively associated with the practice domain. This could be explained by the fact that those who work in animal-related jobs may have been educated in better practices than those who do not have animal-related jobs. Practice domain was not significantly associated with age, gender, marital status or income. As mentioned earlier, in a closed community like Tambool, which represents a homogeneous population, people tend to have the same practices towards health and the same health-seeking behavior, which may differ between tribes.

## Conclusions

This study is the first KAP study on EC in Tambool, where EC is prevalent in both animals and humans. It was found that the large majority of the participants had never heard of the dis- ease. A small proportion of those participants who had heard of the disease had excellent knowledge and attitude, but poor practice towards dealing with it. The One Health approach was useful in the collection of data for EC at the interplay between animals, humans and the environment. A national program should be implemented to take control of the training and education of community members, as well as health professionals, in order to increase KAPs of people in the area, which will reduce the prevalence of the disease in Tambool.

**Supplementary data:** Supplementary data are available at International Health online (https://academic.oup.com/inthealth).

**Authors' contributions:** MEA, OAH, AKA, EE, AAO conceived the study. OAH, MEA designed the questionnaire. MEA, OAH, AKA, EE, AAO con- ducted the field study and

collected the data. MMAE, AKA, AAO, SLB, OAH, MEA analyzed the data. All the authors interpreted the data. All authors drafted the manuscript and revised it.

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Section

# IV

# Determinants of cystic echinococcosis among Khartoum population

# Chapter 9

## Prevalence of Cystic Echinococcosis and Associated Risk Factors among Humans in Khartoum State, Central Sudan

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**Abstract**

**Background:** Hydatid disease or cystic echinococcosis (CE) is caused by the larval stages of the cestode parasite *Echinococcus granulosus*. The objectives of this study were to estimate the prevalence of seropositivity and to identify the risk factors associated with the disease among humans in Khartoum State, Central Sudan.

**Methods:** A cross-sectional study was conducted between November 2017 and April 2018. A total of 305 randomly selected consenting participants from three localities were included in the current investigation using a multistage probability sampling method. An in-house enzyme-linked immunosorbent assay was used to detect immunoglobulin G antibodies to *E. granulosus*. The $\chi^2$ test and logistic regression analysis were used to determine the risk factors associated with CE seropositivity.

**Results:** A seroprevalence of 6.5% (20/305) was recorded among humans in Khartoum State, Central Sudan. Age (odds ratio [OR] 16.61 [confidence interval {CI} 2.21 to 117.92], p=0.006), locality (OR 3.08 [CI 1.42 to 22.54], p=0.011) and contact with dogs (OR 2.34 [CI 0.026 to 0.646], p=0.013) were recorded as potential risk factors for seropositivity to CE in the study area.

**Conclusions:** The seroprevalence of CE (6.5%) is high among humans in Khartoum State, Central Sudan.

**Keywords:** *Echinococcus granulosus*, ELISA, epidemiology, hydatid disease, Sudan, survey.

## Introduction

Cystic echinococcosis (CE), caused by the dog tapeworm *Echinococcus granulosus* (*EG*), accounts for 2–3 million cases of human echinococcal infections worldwide and constitutes a major public health problem in many parts of the world,[1–5] including regions of South America, the Mediterranean countries, Eastern Europe, East Africa, the Near and Middle East, Central Asia and China.11–15

The life cycle of this cestode parasite requires a definitive carnivore host, usually dogs, and an intermediate host such as domestic or wild livestock. Equine and camelid animal species can also be infected with CE. Humans acquire the infection after accidental ingestion of eggs excreted with carnivore faeces and serve as a dead-end host of the parasite cycle.[9]

The disease is recognized as a neglected zoonotic disease by the World Health Organization.[6,7] The economic impact of the disease may significantly affect the overall development and work productivity of humans in the endemic areas. The disease mainly affects pastoral communities in resource-poor settings. Unlike agricultural communities, pastoral communities usually have a higher prevalence of the disease due to the presence of a large population of dogs in the environment.[7–10]

Across Africa, CE is an endemic zoonotic disease in many parts of the continent. This includes Sudan; although the disease is of public health importance, it has been largely neglected in the past. However, a recent increase in interest by the Sudanese health authorities has led to several studies investigating the epidemiology of CE and the risk factors associated with the disease in humans. In southern Sudan, the overall prevalence was shown to be 3.5%, with 60% of the cysts located in the liver.[8] In a survey conducted in humans in central Sudan, a cyst prevalence of 0.8% was reported using ultrasound.[9] The prevalence of human hydatidosis was reported to be 1.22% in South Darfur State, with the predominant site of infection being the liver.[10] Currently, 10 distinct genotypes of *EG*, designated as G1–G10, have been described worldwide. Of the 10 genotypes of *EG*, sheep (G1), cattle (G5) and camel (G6) strains have been reported among humans and livestock in Sudan.[2,16] It is worth mentioning that the epidemiological studies on CE in Sudan were almost exclusively conducted in animals based on abattoir records.[9,17–19] However, no information is available with regard to the prevalence and associated risk factors from a well-structured community-based study. Such epidemiological studies on CE in humans are essential to inform and appropriately implement control programs. Therefore, the present

investigation was conducted among residents of Khartoum State in Central Sudan to determine the prevalence of CE as determined by detection of anti-*EG*-specific immunoglobulin G (IgG) antibodies and to examine variables considered as risk factors for acquiring the disease by applying a standardized questionnaire.

## Materials and methods

### Study area

Khartoum State is one of the largest states in Sudan, which includes three major cities: Khartoum, Bahry (Khartoum North) and Omdurman. Khartoum is the capital of Sudan and is located in the centre of the country. The population of Khartoum State is comprised of people from different parts of Sudan and is estimated to be nearly 6 million. The state covers an area of approximately 23 000 $km^2$ and is situated at longitudes 31.5–34°E and latitudes 15–16°N, at the junction of the White Nile and the Blue Nile, forming the Nile River, which runs to the north throughout Sudan and Egypt. The climate is very hot and dry in the summer season, but cold and dry in the winter season. Average rainfall reaches 150 mm in the northeastern areas and 250 mm in the northwestern areas. The temperature in summer may reach up to 48°C from April to June. In winter, the temperature eventually declines to 15°C between November and January.

### Study design

A cross-sectional study was conducted from November 2017 to April 2018 within the three major cities of Khartoum State. Stratified sampling depended on the number of rural villages in each of the five municipalities. During field visits, epidemiological data were collected by a questionnaire that was written and administrated so all participants are asked precisely the same questions in an identical format. Responses were recorded in a uniform manner so as to increase its reliability. Blood samples were obtained for separation of sera and subsequently used in indirect enzyme-linked immunosorbent assays (ELISAs) for detection of seropositivity to anti-*EG*-specific IgG antibodies. A multistage probability sampling method was applied in this study. In the present study, a total of 305 randomly selected participants were included in the multistage probability sampling. The three localities (Khartoum, Omdurman and Khartoum North) were selected randomly from the five localities of Khartoum State. Ten villages were randomly selected in each locality and 10 houses were selected in each village. An additional five samples were taken, for a total of 305 participants.[20]

**Sample size**

To estimate sample sizes, we used an echinococcosis prevalence of 3.5% in humans.[8] A design effect of 2 and a non-response rate of 10% were used to adjust for the sampling technique. The formula for the calculation of the sample size was estimated using Epi Info 6.0 (www.cdc.gov/epiinfo/).[21] The required sample size at 95% confidence, 3.5% prevalence and 0.025 absolute precision was calculated to be 209. This number was approximated to 305 samples to increase the accuracy of data obtained, of which 27, 157 and 121 were assigned to Bahry, Omdurman and Khartoum localities, respectively.

**Ethical approval and consent to participate**

The study was approved by the Institutional Review Board of Al Neelain University, Khartoum, Sudan. Information regarding the study was initially communicated to potential human participants prior to their signing an informed consent. The structured questionnaire was employed to collect risk factor–associated information.

**Questionnaire**

The study included a questionnaire survey to determine the potential risk factors for transmission of CE in humans.[22] The questionnaire included basic demographic data of the participants, data on education and occupation, living standards including waste management and water supply as well as slaughtering practices and knowledge of the disease (using visual material). Consented persons were interviewed and their responses transcribed to the questionnaire. Furthermore, the questionnaire was presented as open-format questions to reduce bias. Interviews were conducted for all participants in all above localities. Interviewers were trained before conducting the survey to ensure that the questionnaires were well understood by the participants so as to avoid differences in the definitions and interpretations of concepts used. All participants included in this study responded to the questionnaire, which covers sociodemographic characteristics including age (young age, <18 y; old age ≥18 y), gender (male and female), disease awareness (yes, no), occupation (employed, unemployed), education (illiterate, primary, secondary, university), locality, dog contact, occupation, presence of a dog in the house, dog treatment and home slaughtering.

**Antigen preparation**

Scolices obtained from hydatids and hydatid cysts (the cyst originating from the lung after surgical removal) were washed five times in normal saline (0.9% sodium chloride). Soluble scolex antigen (SSA) was prepared after the disruption of whole scolices by frequent freezing and thawing. Scolices were further ultrasonically disrupted in a microcentrifuge ultrasonic disintegrator (Soniprep 150, MSE, Heathfield, UK).

The suspension was centrifuged at 12 000 rpm and the supernatant was collected. The deposit was suspended in phosphate buffered saline (PBS, pH 7.2) and ultrasonically disintegrated. The supernatant was collected and added to the earlier supernatant. The combined supernatant was used as SSA antigen.[23] The protein concentration of the antigen was estimated by the Biuret method using a spectrophotometer (UV-Vis Spectrophotometer UV/mini 1240, Shimadzu, Kyoto, Japan).[38]

**ELISA**

Indirect ELISA was performed to screen the sera for *EG*-specific IgG antibodies as described previously, with some modifications.[23] The ELISA was performed in 96-well immunoassay microplates (Nunc, Roskilde, Denmark) and optimal working dilutions of reagents were determined by chessboard titration.

Unless stated otherwise, 100 µl test volumes were used and incubations were performed for 1 h at 37°C. The plates were washed three times with PBS containing 1% Tween 20 (PBST; Merck, Darmstadt, Germany), wells were post-coated with 200 µl of PBS containing 2% bovine serum albumin (Calbiochem, La Jolla, CA, USA) and the diluents for reagents was PBS containing 10% skimmed milk (Khartoum, Vetrinary Molecular laboratory, Amba, Denmark). Briefly, the wells of polystyrene microtitration plates were coated with 100 µl of SSA, which was diluted in 0.05 M carbonate buffer of pH 8 to give a final concentration of 30 µg of protein/ml. The plates were then incubated overnight at 4°C. The plates were washed, and aliquots of test sera–positive and negative controls were added in separate wells at a dilution of 1:100. After another 1 h of incubation, the plates were washed and rabbit anti-human IgG conjugated horseradish peroxidase (HRP) was added to the plate at a dilution of 1:1000 and incubated again for 1 h. The plates were then washed and the substrate, 2,2-azino-bis(3-ethylbenthiazoline-6-sulfonic acid (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) was added. Crimean– Congo haemorrhagic fever virus–

infected camel serum was incorporated in each ELISA plate as a positive control to estimate the upper limit of the sensitivity. The results were read using an ELISA reader set at 405 nm. A presumptive diagnosis was made when the IgG antibody titre in the test sample had an optical density of <0.20. Positive control sera were obtained from human cases who were subjected to surgery for hydatid cyst removal. Sera from healthy uninfected individuals and patients infected with related cestodes *Taenia saginata* and *Taenia hydatigina* were used as negative controls.

**Statistical analyses**

SPSS for Windows (version 21.0; IBM, Armonk, NY, USA) was employed to enter and analyse data. Univariable analysis using a $\chi^2$ test was used to determine the associations between the outcome variable (status of CE seropositivity) and its potential risk factors. A significant association between CE and a risk factor was initially considered as $p<0.25$ (two-tailed; $\alpha=0.25$). The results of the univariable analysis were further subjected to multivariable analysis using logistic regression. The results were expressed as the odds ratio (OR) with 95% confidence interval (CI) for each risk factor. A p-value <0.05 was interpreted as representing a significant association between CE seropositivity and the associated risk factor.

**Results**

The initial ELISA screening for anti-*EG*-specific IgG antibody, revealed positive results in 20 of 305 serum samples, which accounts for a seroprevalence of 6.5%. The highest rate of CE seropositivity was recorded in Khartoum (14.8%), whereas the lowest rate of seropositivity was recorded in Omdurman (0.6%).

The univariate analysis using a $\chi^2$ test was conducted for the association between the potential risk factors and CE seropositivity. The initial results using univariate analysis showed that the independent variables were statistically significant; including age, locality, dog contact, occupation, presence of a dog in the house, dog treatment and home slaughtering ($p<0.25$; Table 1). The significant results in the univariable model were further subjected to a final multivariate model using logistic regression analysis to illuminate confounding factors. Age (OR 16.61 [CI 2.21 to 117.92], p=0.006), localities (OR 3.08 [CI 1.42 to 22.54], p=0.011) and contact with dogs (OR 2.34 [CI 0.026 to 0.646], p=0.013) were recorded as potential risk factors for seropositivity

to CE. The results are summarized in Table 2. Other potential factors did not show any significant association with CE seropositivity.

**Table 1.**
Summary of analysis for risk factors of echinococcosis (IgM) among humans in Khartoum State, Sudan (n=305) using a $\chi^2$ test

| Risk factors | Animals tested, n | Animals affected, n (%) | fd | $\chi^2$ | p-Value |
|---|---|---|---|---|---|
| Locality | | | 2 | 20.09 | 0.001 |
| Bahry | 27 | 2 (7.4) | | | |
| Omdurman | 157 | 1 (0.6) | | | |
| Khartoum | 121 | 17 (14.8) | | | |
| Age | | | 1 | 12.48 | 0. 001 |
| Young | 162 | 3 (1.9) | | | |
| Old | 143 | 17 (11.9) | | | |
| Gender | | | 1 | 0.027 | 0. 86 |
| Female | 193 | 13 (6.7) | | | |
| Male | 112 | 7 (6.2) | | | |
| Education | | | 3 | 0.74 | 0.87 |
| Illiterate | 55 | 5 (9.1) | | | |
| Primary | 72 | 4 (5.6) | | | |
| Secondary | 62 | 4 (6.5) | | | |
| University | 116 | 7(6.0%) | | | |

| Risk factors | Animals tested, n | Animals affected, n (%) | fd | $\chi^2$ | p-Value |
|---|---|---|---|---|---|
| Dog contact | | | 1 | 5.18 | 0.136 |
| No | 186 | 17 (9.1) | | | |
| Yes | 119 | 3 (2.5) | | | |
| Occupation | | | 4 | 6.90 | 0.141 |
| Teacher | 21 | 2 (9.5) | | | |
| Housewife | 109 | 6 (5.5) | | | |
| Retiree | 20 | 4 (20) | | | |
| Employer | 94 | 5 (5.3) | | | |
| Student | 61 | 3 (4.9) | | | |
| Presence of dog | | | 1 | 1.86 | 0.145 |
| No | 257 | 19 (7.4) | | | |
| Yes | 48 | 1 (2.1) | | | |
| Dog treatment | | | 1 | 10.2 | 0.001 |
| No | 206 | 20 (9.7) | | | |
| Yes | 99 | 0 (0.0) | | | |
| Home slaughterhouse | | | 1 | 1.50 | 0.220 |
| No | 24 | 3 (12.5) | | | |
| Yes | 281 | 17 (6.0) | | | |

**Table 2.**
Multivariate analysis using a logistic regression model for significant association ($p<0.05$) of 426 risk factors and seropositivity (IgG) among humans in Khartoum State

| Risk factors | OR | 95% CI | p-Value |
|---|---|---|---|
| Age | | | |
| Young | Ref. | 2.21 to 117.92 | 0.006 |
| Old | 16.61 | | |
| Locality | | | |
| Omdurman | Ref. | 1.42 to 22.54 | 0.011 |
| Khartoum | 3.08 | | |
| Dog contact | | | |
| No | Ref. | 0.026 to 0.646 | 0.013 |
| Yes | 2.34 | | |

## Discussion

CE in humans and animals is characterized by the development of metacestode larval stages in the liver and other organs. CE is a relevant public health and economic problem worldwide.[1,2,24–26] The disease is endemic in many areas of the African continent, including Sudan.[17–19,27–33] However, due to the lack of epidemiological data, problems associated with disease diagnosis and the chronic nature of infection and long-term treatment, it often has a low priority and is therefore part of the group of neglected tropical diseases.[7]

Several immunodiagnostic assays have been employed in serological studies to assess the prevalence and associated risk factors of CE in humans worldwide.[13,21,34] Most of the described serological assays did not detect all cases of CE in infected humans. This is mainly attributed to

the low sensitivity and specificity of the serological assays compared with field studies, which were conducted using imaging techniques such as portable ultrasound.[35,36]

The present study showed that the prevalence of African horse sickness virus (AHSV) IgG-specific antibodies was high (5.6%). IgG-specific antibodies against *EG* recorded in this study showed evidence of prior exposure of humans to CE in Khartoum State. The high prevalence rate (6.5%) indicates significant circulation of the disease among horses in Central Sudan. Seven parameters considered as risk factors with $p<0.25$ (two-tailed; $\alpha=0.25$) were initially considered to be associated with seropositivity to CE among humans in Khartoum State in the univariate analysis using the $\chi^2$ test. These included dog contact, age, dog treatment, presence of a dog in the house, localities, occupation and home slaughtering. However, the multivariate analysis using a logistic regression model illustrated that only three potential risk factors were significantly associated with CE seropositivity in the study area, including dog contact (OR 2.34 [CI 0.026 to 0.646], p=0.013). The study showed that humans in contact with dogs are at least twice as likely to be at risk of acquiring an echinococcal infection. This is attributed to the fact that the patients are likely to become infected through the contaminated environment with infected dog faeces that contains *EG* eggs. The highest rate of CE seropositivity was recorded among humans in Khartoum (14.8%). This is most probably due to the presence of a high density dog population in this locality, which provides parasite eggs for human infection with CE. There was also an association between CE seropositivity and Khartoum (OR 3.08 [CI 1.42 to 22.54], p=0.011), again due to high population density of dogs in this locality. Residents of Khartoum are at 3-fold risk of becoming infected with CE compared with other localities, suggesting an increased endemicity of this locality with echinococcosis infection. Age was also reported as a potential risk factor in this study (OR 16.61 [CI 2.21 to 117.92], p=0.006). Of interest, participants >18 y of age are at a very high risk, as they are 16 times more likely to become infected with CE. The present study illustrated that the prevalence of CE seropositivity is alarmingly high among the population of Khartoum State and is probably similarly high throughout the country.

It appears that the survey should be extended in the future to include prevalence and associated risk factors of echinococcal infection among dog populations. Molecular epidemiological studies

should also be considered to identify the genotypes of the parasites circulating in the country. No significant difference was reported between CE seropositivity and other potential risk factors included in the study. Males and females were equally affected by CE and hence gender had no significant association with CE seropositivity. In addition, no significant difference was observed between AHSV seropositivity and age or education status. Early diagnosis of CE is necessary for prevention and control of the disease.

It should be noted that long follow-up treatment with albendazole is recommended for treatment of CE in infected patients. Dog owners should also be educated and the risks of the disease and prevention of the infection through proper hygienic measures such the management of waste, waste handling, washing hands and the use of plastic gloves when cleaning a dog facility. Dewormer for dogs should be used at regular intervals to ensure the elimination of the adult *EG*. In this regard, effective animal husbandry and management systems should be applied to control dog breeding. In an endemic area such as Khartoum State, where the main factors for seropositivity are those linked to contact with dogs, it is extremely important to focus on interruption of the life cycle of the parasite to prevent the spread of infection from dog to human. However, it should be noted that in stray dogs, this method of control is extremely difficult, if not impossible. Therefore, an important intervention would be to increase the frequency of application of anthelmintic drugs. Control measures should be directed towards education and animal management to disrupt the life cycle of the parasite.[37]

Currently the Sudan Ministry of Health has taken these recommendations into account for a proper control program to combat this important zoonotic infection. The epidemiology of human CE is complex and depends on the presence of the parasite in the zoonotic cycle, which involves a larval stage host such as livestock, equines and wildlife. This study suggested that hydatid disease is maintained in the study area mainly by the existence of large numbers of dogs, which shed infective eggs into the environment.[38] Improper hygiene measures and the presence of stray dogs in the country provide ideal conditions for maintenance of the life cycle of this parasite, as they have an increased risk of acquiring *EG* infection by having free access to infected carcasses.[39–41] In this study, we estimated the prevalence of human echinococcosis and the associated risk factors in Khartoum State. We anticipate that this study will help to facilitate control programs aimed at controlling the infection in dogs and preventing new infections in humans.

**Limitations of the study**

SSA was employed in our ELISA. A more defined recombinant antigen would be recommended to improve the specificity of the ELISA by eliminating the possibility of cross-reaction with other related cestodes. Further studies will be required to verify this finding, such as prospective studies with a larger sample size. In addition, the questionnaire did not include information related to infection with *EG* in dogs. It is therefore recommended that further studies on canine echinococcosis should be conducted in parallel with human CE to better predict and respond to the disease in the study area of Central Sudan.

**Conclusions**

The results obtained from the present study confirmed the circulation of CE in Khartoum State as determined by indirect ELISA. The seroprevalence of CE is high (6.5%) among residents of this state. Age, locality and contact with dogs were identified as potential risk factors for contracting the disease. The genotypes of the hydatid cysts in infected patients in the area remain to be identified. Surveillance for CE among residents of the state and the distribution among the dog population should continue for a better understanding of the epidemiology of the disease. This study provides suggestions to the public health authorities regarding control of the disease and prevention of spread of the infection to the human population in Khartoum State.

**Authors' contributions:** MEA collected blood samples and hydatid cyst samples and helped with the manuscript writing. SS conducted the ELISAs and helped with manuscript writing. IAA conducted the statistical analysis. MPG designed the experiment and helped with preparation of the final manuscript. IEA designed the experiment and prepared the final manuscript. All authors read and approved the final version of the manuscript.

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**Ethical approval:** The study protocol was approved by the Ethics Committee of Al-Neelain University, Khartoum, Sudan. Participation was on a voluntary basis and the residents of Khartoum State were selected randomly. Written informed consent was obtained from all participants after explanation of the study purpose before the procedure of blood collection. The risk factors information was obtained from the residents through the structured questionnaire form, permitting the use of their blood samples for diagnostic and research purposes.

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# Chapter 10

## Summary and conclusion

The present investigation was conducted to improve the diagnostic potential of the currently used techniques for detection of *Echinococcus granulosus* senso lato (s.s.) and to identify the different genotypes of the parasite circulating in the Sudan using sequencing and phylogenetic analysis. Molecular characterization of parasitic zoonotic disease, such as CE, is especially important in the Sudan given the large numbers of livestock in the country, and their importance to the national economy and pastoral communities. Accumulated reports indicated that various livestock are susceptible to hydatidosis in Sudan, with particularly high prevalence among cattle and camels. In **section I** we provided data to elucidate the epidemiology of cystic echinococossis in some parts of the continent and in Sudan. We gave an overview on the prevalent species or strains of *Echinococcus granulosus* in sub-Saharan Africa, treatment (chemotherapy, percutaneous radiological techniques) and preventive strategies. Existing data are suggestive of unusual clinical presentations of cystic echinococcosis in some parts of the continent, for which the causes are speculative **(Chapter 2)**. Part of our research was conducted in Tamboul, Central Sudan to determine the prevalence of of CE genotypes and the result indicates the circulation of *E. ortleppi*, the cattle genotype (G5) in the one humped camel in the Sudan and this was shown for the first time **(Chapter 3)**. Therefore, the G5 strain should be considered during epidemiological surveys for this important parasitic infection in Sudan. In addition, this investigation expands on the existing data on sequences generated from EG isolates recovered from the one humped camel in the endemic area of Tamboul, Central Sudan. Thereafter we this investigation expanded the investigation on the existing sequence data generated from EG isolates recovered from camel in the Sudan. The circulation of the cattle genotype (G5) in the one-humped camel is reported here for the first time. conducted studies. In **section II**, to elucidate the molecular characteristic of cystic echinococcosis, we develope and evaluated a real-time loop-mediated isothermal amplification (LAMP) assay for simple and rapid detection of CE in humans and domestic live stock in Sudan **(Chapter 4)**. LAMP assay could be used for simple and rapid detection and genotyping of EG-complex hydatid cysts strains. There was 100 % agreement between results of the LAMP and our previously described nested RT-PCR when testing 10- fold serial dilution of parasite DNA. The LAMP assay provides very high levels of diagnostic sensitivity and specificity when testing a variety of archived hydatid cysts sampled from human or susceptible animal populations. Realtime monitoring of the LAMP assay using Light Cycler and fluorochrome dye enhanced the rapidity of the assay and a positive result could be obtained as early as 10–15 min post amplification reaction. The performance of the

LAMP assay under isothermal conditions without the need of special apparatus, and visualization of results by the naked eye, makes the assay more economical and practical in remote areas or resource-poor settings. Partial sequences produced by LAMP outer primers (F3 and B3) could be targeted for sequencing and subsequent identification of the genotype of the hydatid cyst genotype/strain. The developed LAMP assay would be expected to prove highly significant in epidemiological surveys of CE in developing countries or areas of resource-poor settings for both ease of use and cost.

For simultaneous diagnosis and genotyping of cystic echinococcosis (CE) in humans and livestock in the Sudan, develop and evaluate a single-tube one-step real-time quantitative PCR (qPCR). The assay has been described for the first time in Africa **(Chapter 5)**. The performance of SYBR green-based real-time qPCR assay for diagnosis of hydatid cyst was compared with the conventional gel-based nested PCR assay. Both conventional and qPCR assays showed similar sensitivity and specificity for the rapid diagnosis and differentiation of hydatid cysts from humans and animals. The developed qPCR assay showed a dynamic detection limit, which spans over a 7 log10 concentration range. However, the nested PCR was found to be time consuming, prone to errors and complication by cross contamination resulting from multiple manipulations of the primary PCR products. Whereas, SYBR green-based qPCR required approximately 45 min from sample submission until the assay is accomplished giving final results, while the time spent for nested PCR amplification and subsequent visualization of results required at least five consecutive hours. Accordingly, the qPCR has been optimized in this study to develop an efficient qPCR assay for diagnosis and quantification of E. granulosus hydatid cysts because of its simplicity, high sensitivity, and specificity and cost efficiency. The additional advantage of utilizing SYBR green 1 based qPCR is that the test primer pairs are relatively easy to design and are suitable for conventional PCR analysis. To generate a standard curve and to ensure the possible detection limits of the SYBR green-based qPCR assay, 10-fold serialdilutions (1 × 106 to 1 × 101) of a known concentration of the parasite DNA were tested in the current study. The assay showed linear results for the 6 logs of the serially diluted DNA. The detection limit of the SYBR green 1-based assay was calculated to be 1 fg equivalent to DNA extracted from 10 protoscolices. Melting curve analysis was conducted to insure the existence of the specific amplicon in the reaction tube. The melting peak temperature (Tm value) was calculated to be 80 °C from the PCR products. The conventional gel-based nested PCR assay was proved to be highly specific in detecting the *E. granulosus* DNA. The first round of the conventional PCR was far less sensitive for the detection

of *E. granulosus* DNA when compared to the nested PCR or SYBR green-based qPCR assay. The sensitivity was, however, significantly increased (1000 times) using a second round of nested amplification with the nested primers. Similar result was obtained with SYBR green-based qPCR and as little as 1 fg of parasite DNA was detected in both assays. The SYBR green-based qPCR is a single-tube one-step assay that does not require post amplification steps. The specificity studies of both conventional and qPCR assays indicated that the PCR products were specific and did not cross-amplify DNA of other parasites including *Cysticercus bovis*, *Fasciola gigantica*, *Schistosoma bovis* and nucleic acid free samples. It is worth mentioning that surgical removal of hydatid cysts in hospitalized human patients requires intercostal intubation, which may result in accidental rupture of pulmonary cysts. The ruptured cyst is likely to be invaded by secondary bacteria. In addition, calcification of hydatid cysts is not an uncommon finding in infected patients. Infertile or calcified hydatid cysts are common in animals, as reported in the majority of the Sudanese desert sheep and Nubian goats. In these circumstances, the diagnosis of cystic hydatidosis by conventional techniques would be extremely difficult, if not impossible. However, in the present investigation, the detection of ruptured and calcified cysts was made possible by both the conventional and qPCR assays. The qPCR assay was preferred for its convenience and minimum sample handling, thus preventing the occurrence of cross-contamination which may decrease the quantitative reliability of the assay. It is well documented that SYBR green-based qPCR assays are less specific than the TaqMan qPCR assays. In addition, the qPCR assay can be implemented in a research laboratory setting for the purpose of rapid diagnosis and epidemiological surveillance of CE in humans and animals in developing countries, such as Sudan. In Sudan, genotypes G5 and G6 were described in cattle and dromedary camel (*Camelus dromedarius*). The sequence analysis showed that Echinococcus canadensis genotype 6 (G6) is the most infectious and widespread genotype in the Sudan, which is in agreement with previous studies.

*Echinococcus granulosus* sensu lato (s.l.) is the causative agent of cystic echinococcosis (CE), which is a cosmopolitan zoonotic parasitic disease, infecting humans and a wide range of mammalian species including cattle. Currently, little information is available on the genetic diversity of Echinococcus species among livestock in Sudan. Since little information is available on the genetic diversity of *Echinococcus* species among livestock in Sudan (**Chapter 6**). To illuminate this, DNA was extracted from hydatid cysts collected from cattle carcasses (one cyst sample per animal) at Al-kadarou slaughterhouse, Khartoum North, Sudan. The phylogenetic tree revealed the presence of *Echinococcus canadenesis* genotype 6 (G6), *Echinococcus ortleppi*

genotype 5 (G5) and *Echinococcus granulosus* sensu stricto (s.s) genotype 1 (G1). The phylogenetic network analysis revealed genetic variation among the different haplotypes/genotypes. This report has provided, for the first time, an insight of the role of cattle in the transmission of the zoonotic G1 echinococosis.

Cystic echinococcosis (CE) is a significant public health problem with high endemicity in east and central Africa including Sudan. Worldwide, pulmonary echinococcosis cysts constitute a significant problem medically, socially, and economically. Until now ten genetic variants, or genotypes designated as (G1-G10), are distributed worldwide based on genetic diversity. In **Chapter 7**, we investigated molecular diversity of *Echinococcus granulosus* isolates collected from human clinical samples removed surgically from lung using mitochondrial gene nad1 in Sudan. It can be concluded that the pul echinococcosis in Sudan are caused by genotype 6 (G6) camel strain of *E. granulosus*, which indicates the camel-dog cycle in the studied area.

The other important leg needed to inform management improvement is to understand the base line knowledge, attitudes and practices in human population in quest, **section III (Chapter 8)**. Since the level of knowledge in the majority of participants and the practice regarding the disease was poor, there is a need for the implementation of a multidisciplinary program using the One Health approach to effectively control and prevent EC.

The epidemiological studies on CE in Sudan were almost exclusively conducted in animals based on abattoir records. However, no information is available with regard to the prevalence and associated risk factors from a well-structured community-based study. Such epidemiological studies on CE in humans are essential to inform and appropriately implement control programs. In **Section IV**, a cross sectional study was conducted among residents of Khartoum State in Central Sudan to determine the prevalence of CE (**Chapter 9**). The results obtained from the present study confirmed the circulation of CE in Khartoum State as determined by indirect ELISA. The seroprevalence of CE is high (6.5%) among residents of this state. Age, locality and contact with dogs were identified as potential risk factors for contracting the disease.

In conclusion, the investigations demonstrated that three different genotypes of E. granulossus s.l. are actively circulating in indigenous cattle in Central Sudan. The genotypes identified include E. granulosus s.s. (G1), *E. ortleppi* (G5), and *E. canadensis* (G6). The results of the study represent the first molecular record of *Echinococcus granulosus* s.s G1, thus reinforcing its role as a source of infection among Sudanese breed of cattle. In addition, this investigation provides additional

information, which will expand on the existing data indicating that Echinococcus granulosus s.s G1, which was previously restricted to other region in the African continent, is now becoming broadly distributed in the country. The availability of additional sequence data would be expected to enhance our understanding of the biology, ecology and molecular epidemiology of CE in humans and livestock. Active Surveillance would be necessary to determine the distribution and prevalence of CE and to identify the genotypes / strains circulating in different regions of Sudan. In addition to that, SYBR green-based qPCR should serve as a useful tool during survey of the disease among humans and susceptible animal populations. The qPCR offers advantages over the conventional gel-based nested PCR, being less time consuming and preventing cross contaminations. The developed qPCR should be used as a rapid and reliable assay for diagnosis and genotyping of CE. The assay is highly recommended for the epidemiological surveillance in humans and livestock in endemic countries. This thesis also concluded that LAMP assay could be used for simple and rapid detection and genotyping of EG-complex hydatid cysts strains. The role of this LAMP assay in coprodiagnosis and its application in epidemiological studies and disease control programs should be promising and highly significant. It is worth mentioning that conventional parasitological method could be useful for diagnosis of hydatid cyst under the microscope but has no significance in genotyping of the parasite. However, the LAMP assay, described in this study, could be employed for simultaneous detection and genotyping of cysts recovered from infected livestock. The performance of the LAMP assay under isothermal conditions without the need of special apparatus, and visualization of results by the naked eye, makes the assay more economical and practical in remote areas or resource-poor settings. The developed LAMP assay would be expected to prove highly significant in epidemiological surveys of CE in developing countries or areas of resource-poor settings for both ease of use and cost.

**Recommendations**

The role of stray dogs in maintenance of the life cycle of the parasite and increasing the risk of human infection should be given special attention. In Sudan, stray dogs are usually found roaming on streets and hanging around slaughterhouses, feeding on offals of slaughtered animals or carcasses of dead animals in rural areas (has been mentioned by Musa NO and others 2012, chapter 3 reference no. 13). This situation is most frequently observed during scarifying religious festival, where animal slaughtering is practiced in the back yards and the open space. Under these circumstances, stray dogs could have free access to yards and animal houses and

thereby contaminating the environment with *Echinococcus* spp. eggs. It is, therefore, recommended that after animal slaughtering, proper disposal of carcasses should follow the appropriate hygienic measures. Active Surveillance would be necessary to determine the distribution and prevalence of CE and to identify the genotypes/ strains circulating in different regions of Sudan. In rural communities with resource-poor settings, the practice of animal slaughtering is usually performed in the open space. Under these conditions, dogs would have free access to feed on livestock viscera, which may harbor hydatid cysts, the infective stage. Therefore, it is believed that this practice of livestock slaughtering could effectively contribute towards the persistence of the specific genotype in the study area. The addition of EG strains sequences from Sudan enhances our understanding of the expansion and, to some degree, maintenance of the parasites in the intermediate hosts. Ongoing surveillance and EG strains characterization should also aid in determining the distribution of *Echinococcus granulosus* senso lato s.s. in the Sudan. The One Health approach was useful to collect the data of EC at the interplay between animals, humans and the environment. A national program should be implemented to take control of the training and education of community members as well as health professionals so as to increase knowledge, attitudes and practices of people in the area, which will be aiming to reduce the prevalence of the disease in central where the disease is present.

# Chapter 11

## Nederlandse samenvatting

De conclusie van het onderzoek is dat drie verschillende genotypes van E. granulossus s.l. actief circuleren bij inheems vee in Centraal-Soedan. De geïdentificeerde genotypes zijn E. granulosus s.s. (G1), E. ortleppi (G5) en E. canadensis (G6). De resultaten van het onderzoek vertegenwoordigen de eerste moleculaire registratie van Echinococcus granulosus s.s G1, waardoor de rol van deze ziekte als infectiebron bij Soedanees runderras wordt versterkt. Bovendien levert dit onderzoek aanvullende informatie op, die de bestaande gegevens die erop wijzen dat Echinococcus granulosus s.s G1, die voorheen beperkt was tot andere regio's van het Afrikaanse continent, nu breed verspreid raakt in het land. De beschikbaarheid van aanvullende sequentiegegevens zal naar verwachting ons inzicht in de biologie, de ecologie en de moleculaire epidemiologie van CE bij mens en vee vergroten. Actieve bewaking zou nodig zijn om de verspreiding en prevalentie van CE te bepalen en de genotypes/stammen die in verschillende regio's van Soedan circuleren te identificeren. Daarnaast zou qPCR op basis van SYBR green een nuttig instrument moeten zijn bij het onderzoek naar de ziekte bij mensen en gevoelige dierpopulaties. De qPCR biedt voordelen boven de conventionele op gel gebaseerde nested PCR, is minder tijdrovend en voorkomt kruisbesmettingen. De ontwikkelde qPCR moet worden gebruikt als een snelle en betrouwbare test voor de diagnose en genotypering van CE. De test wordt ten zeerste aanbevolen voor epidemiologische surveillance bij mensen en vee in endemische landen. In dit proefschrift werd ook geconcludeerd dat de LAMP-test kan worden gebruikt voor eenvoudige en snelle detectie en genotypering van EG-complexe hydatidecystenstammen. De rol van deze LAMP-assay in coprodiagnose en de toepassing ervan in epidemiologische studies en ziektebestrijdingsprogramma's zou veelbelovend en zeer significant moeten zijn. Vermeldenswaard is dat de conventionele parasitologische methode nuttig kan zijn voor de diagnose van hydatidecysten onder de microscoop, maar geen betekenis heeft voor de genotypering van de parasiet. De in deze studie beschreven LAMP-test kan echter worden gebruikt voor de gelijktijdige detectie en genotypering van cysten van besmet vee. De uitvoering van de LAMP-test onder isotherme omstandigheden zonder de noodzaak van speciale apparatuur, en de visualisatie van de resultaten met het blote oog, maakt de test economischer en praktischer in afgelegen gebieden of gebieden met weinig middelen. De ontwikkelde LAMP-test zal naar verwachting van groot belang blijken bij epidemiologisch onderzoek naar CE in ontwikkelingslanden of gebieden met weinig middelen, zowel vanwege het gebruiksgemak als vanwege de kosten.

Aanbevelingen

De rol van zwerfhonden bij het in stand houden van de levenscyclus van de parasiet en het verhogen van het risico op infectie bij de mens moet speciale aandacht krijgen. In Soedan worden zwerfhonden meestal op straat aangetroffen en rond slachthuizen, waar ze zich voeden met slachtafval of karkassen van dode dieren in plattelandsgebieden. Deze situatie wordt het vaakst waargenomen tijdens religieuze feesten waarbij dieren worden geslacht in achtertuinen en op open plekken. Onder deze omstandigheden kunnen zwerfhonden vrije toegang hebben tot erven en dierenverblijven en zo de omgeving besmetten met eieren van Echinococcus spp. Daarom wordt aanbevolen om na het slachten van dieren de kadavers op passende hygiënische wijze te verwijderen. Actieve bewaking is nodig om de verspreiding en prevalentie van CE te bepalen en de genotypes/stammen die in verschillende regio's van Soedan circuleren te identificeren. In plattelandsgemeenschappen met weinig middelen wordt het slachten van dieren gewoonlijk in de open ruimte uitgevoerd. In deze omstandigheden hebben honden vrije toegang tot de ingewanden van het vee, waarin zich hydatide cysten, het besmettelijke stadium, kunnen bevinden. Daarom wordt aangenomen dat deze praktijk van het slachten van vee effectief kan bijdragen tot de persistentie van het specifieke genotype in het studiegebied. De toevoeging van EG-stammen uit Soedan vergroot ons inzicht in de uitbreiding en, tot op zekere hoogte, de instandhouding van de parasieten in de tussengastheren. Lopende surveillance en karakterisering van EG-stammen moet ook helpen bij het bepalen van de verspreiding van Echinococcus granulosus senso lato s.s. in Soedan. De One Health-aanpak was nuttig om de gegevens van EG te verzamelen in de wisselwerking tussen dier, mens en milieu. Er moet een nationaal programma worden uitgevoerd om de opleiding en vorming van zowel leden van de gemeenschap als gezondheidswerkers onder controle te krijgen teneinde de kennis, attitudes en praktijken van de mensen in het gebied te verbeteren, hetgeen tot doel heeft de prevalentie van de ziekte te verminderen in het centrum waar de ziekte aanwezig is.

# Appendix

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# List of Publications

**Publications included in the thesis:**

1. **Ahmed, M.E.;** Ahmed, M.E.; Siddig, S.A.; Adam, I.A.; Grobush M.P.; **Aradaib, I.E.** 2021. Prevalence of Cystic Echinococcosis and Associated Risk Factors among Humans in Khartoum State, Central Sudan. International Health 2021;13:327–333.
2. **Ahmed, M.E.,** Salim, B., Grobusch, M.P., Aradaib IE. 2018. First molecular characterization of *Echinococcus granulosus* (sensu stricto) genotype 1 among cattle in Sudan. **BMC Vet Res; 14 (1): 36**. doi: 10.1186/s12917-018-1348-9.
3. **Ahmed ME,** Hassan OA, Khalifa AKA, Elobied E, Osman AAA, Brair SL, Ahmed OIE, Elfadul MMA, Cremers AL, Grobusch MP. Echinococcosis in Tambool, Central Sudan: a knowledge, attitude and practice (KAP) study**. Int Health 2018;10:490–494.**
4. **Ahmed, M.E.,** , MH Eldigail, MP Grobusch, IE Aradaib. Development of real-time PCR assay for simultaneous detection and genotyping of cystic echinococcosis in humans and livestock. Asian Pac J Trop Dis 2017;7:738–743.
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6. **Ahmed M.E.,** Abdelrahim MI, Ahmed FM. Hydatid disease, a morbid drop needs awareness. **Sudan Med J 2011:5.**
7. **Ahmed, M.E.;** Eltom, K.A.; Musa, N.O., Ali, I.A.; Elamin, F.E.; Grobusch, M.P. Aradaib, I.E. 2013. First Report on Circulation of *Echinococcus ortleppi* in the one Humped Camel (Camelus dromedaries), Sudan. **BMC Veterinary Research** 9: 127.
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9. **Ahmed, M. E.;** Ali, I. A; Elsanousi, I. M., Aradaib, I. E**. 2010**. A clinical case of aortic root abscess caused by brusellosis. Molecular diagnosis surgical treatment and successful management. **The Cardiology 5: 1-43.**

10. **Ahmed, M, E;** Abu Aisha, H, Aradaib, I. E. **2006.** A clinical case of recurrent epleural effusions culminating in empyema-thorace in a Sudanese woman with yellow nail syndrome. **International Journal of Tropical Medicine 3: 137-139.**

11. **Ahmed, M. E.;** Aradaib, I. E. 2006. Association of *Pseudomonas stutzeri* with reptured infected hydatid cysts**. Surgery Journal 1: 32-34.**

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14. **Ahmed, M. E.;** Aradaib, I. E**.** 2006. Rapid Detection of Rift Valley Fever Virus in cell Culture. **International Journal of Tropical Medicine 1: 44-47.**

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**33**. Abdel Hamid MM, Abdallah WME, Hussien M, Mohammed NM, Malik EM, **Ahmed ME,** Mohamed AO. Absence of K13 gene mutations among artesunate/sulfadoxine–pyrimethamine treatment failures of Sudanese Plasmodium falciparum isolates from Damazin, southeast Sudan**. Trans R Soc Trop Med Hyg2019;113:428–430.**

**34.** Sara S Abdalla, **Mohamed E Ahmed**, Alsmawal awad Elimam, Mawahib H Eldigal, Imadeldin E. Aradaib, Martin P Grobusch, Genetic charachterization of cystic Pulmonary echinococcosis in Sudan: **J Surgery Research 2021;4 (3): 353-362**

# PhD portfolio

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**COURSES**

- Vascular International Leicester Oct.1992.
- Tracheal Surgery Symposium Liverpool July.1994.
- Practical Heart Surgery Workshop [SWISS Foundation] October2002and 2003. OPCAB &Mini invasive valve Surgery and endovascular prosthesis
- Beating Coronary Bypass course July 2004 Cairo Egypt
- 2nd General Thoracic Surgical Course 14-17 June 2006 Bursa, Turkey
- 3rd International Red Crescent (IRC) Course Nov.2006 Innisbrooke Austria
- Cardio genesis and Stem Cell Therapy course Feb and May 2007, Cairo Egypt
- Practical Heart Surgery Workshop (SWISS FOUNDATION) Oct. 2007 Zurich, Switzerland
- Cardiac Valve Repair July2007 Brussels Belgium
- Brussels Valve Course May 2008 Brussels Belgium
- Aortic valve Course May 2009 Brussels Belgium
- Austruma (Trauma Course), Sydney Australia Feb 2009
- Epidemiology and Clinical Research Design North Carolina Summer Course, July2010, Ulm, Germany
- Tropical Ultrasound (US) Course, May 2011 Pavia, Italy
- Advance Epidemiology and Genetic Epidemiology North Carolina summer Course, July2011 Ulm, German
- Biological Ethics Course, Khartoum, Sudan, March 2012.
- 3rd Stem cells conference Lugano, Switzerland 25-26 June 2012
- Basic molecular course, Veterinary molecular Laboratory, Khartoum University course 2013
- Advanced molecular, Ohio University, Ultrasound Course, Brazil 2015.

- 3rd Oxford Coronary Revascularization Course March 2014
- 4th Oxford Thoracic surgery course March 2015
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- World Cardiothoracic Congress
- World Echinococcosis Association
- American College of Chest Physicians
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- Jordanian International Cardiac Conferences, 3rd April.1996
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- Jordanian Thoracic Conference Nov.1999.
- European Cardiothoracic Conference Sept.1999. (Glasgow).
- Mediterranean & Syrian Cardiac Conference Oct.1999.
- Italian & Jordanian Cardiac Society Meeting 1st June.2000.
- European Cardiothoracic Conference Oct.2000 (Frankfurt).
- 17th World Congress for Heart Research July.2001(Winnipeg CANANDA]
- European Cardiology Conference August 2003 Vienna Austria
- European Cardiothoracic Conference Sep. 2003 Vienna Austria
- 3rd International Valve Conference June 2003 Paris France
- International Cardiothoracic Conference April 2004 Cairo Egypt
- Beating Coronary Bypass course July 2004 Cairo Egypt
- XX1st. International Congress of Hydatidology August 2004
- European Cardiothoracic Conference Sep. 2004 Leipzig Germany
- Biennial International Conference of the Pakistan Society of the Cardiovascular and Thoracic surgeons March 2005 Lahore
- Malaysian Cardiothoracic Conference August 2005 Kula lumpure Malaysia
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- Aortic valve Repair Symposium May2009Brussel Belgium
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- 4th International FIMA Conference on Health Problems in Relation to Displaced and Disaster Aug 2009 (Chairman)
- Symposium on Hydatid Disease as Neglected Disease Nov2009Friendship Hall Khartoum
- London Darfour Medical International Conference March 2010, UK
- DFG Research Presentations and meeting, April 2010, Berlin
- Brussels Aortic Valve Course May 2010
- 2nd European cardiovascular Stem cells conference May2010, Lugano, Switzerland
- Unusual cases of Hydatid disease, June 2010 Salam Rotana, Khartoum, Sudan
- Epidemiology and Clinical Design North Carolina Summer Course, July2010, Ulm, Germany
- Australian Ultrasound in Medicine (ASUM), Sept 2010 Gold Coast, Queensland, Australia
- First Regional Hydatid (CESSARi) Conference and meeting Nov 2010, Khartoum, Sudan
- 50 years Anniversary of Zurich Cardiac surgery, Zurich, Switzerland April 2011
- Tropical Ultrasound Course, May 2011 Pavia, Italy
- Advance Epidemiology and Genetic Epidemiology North Carolina summer Course, July2011 Ulm, Germany

- Australian Ultrasound in Medicine (ASUM) Sept 2011Melbourne, Australia
- XXIV World Congress of Hydatidology, Sept2011 Urumqi, China
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- 3rd Stem cells conference Lugano, Switzeland 25-26 June 2012
- Al Neelain Post graduate 3rd Conference Dec 2012
- Thirty- Ninth International Scientific Conference 8-10 March 2013
- 5th Sudan Chest Physicians conference April 2013
- XXV Echinococcosis World Congress Nov 2013
- First International Hypertension Conference Khartoum Dec 2013
- XXVI Echinococcosis World Congress Oct 2015, Bucharest, Romania
- 5th International Integrated Medicine disease conference, Feb 2016, Aswan, Egypt
- Heart Valve Society Conference, Feb 2017, Monte Carlo
- C3 invasive Cardiac Conference, June 2017, Orlando, Florida, USA
- XXVII Echinococcosis World Congress Oct 2017, Algeria
- 4th ICOPHAI, Nov 2017, Doha, Quarter
- C3 invasive Cardiac Conference, June 2018, Orlando, Florida, USA
- 7th International Integrated Medicine disease conference, Nov 2018, Khartoum Sudan
- XXVIII World Congress of Echinococcosis Nov 2019, Peru Lima
- 8th International Al-Neelain Postgraduate Conference, Dec 2019 Khartoum Sudan

**Awards**

1) Merits award in Echinococossis by IAH Sept 2011 Urumqi, China
2) Appreciation award in the management of Hydatid by IAH Nov 2013
3) Golden award in the field of echinococosis by IAH Nov 2013
4) Presidential Golden award in the field of Science and Arts of Sudan 2013
5) Al-Neelain University, Khartoum, Golden Scientific Research Award Dec 2015

## Presentations In Conferences, Symposia and Workshops

1. Osman,A. Ma.; **Ahmed, M. E.;** Omer, R. A.; Gameel, A. A.; Abu Aisha, H. H.; Aradaib, I. E. **2007**. Rapid Detection of *Echinoc occus granulossus*-Complex and Specific Identification of the Camel Genotype (G6) Using A Nested PCR. The 22nd **Internatioal Conference of Hydatidology, Athens, Greece.**
2. Mohamed E. Ahmed; Ibtisam A. Ali; Hassan H. AbuAisha; Imadeldin E**. Aradaib (2007).**

   A Complicated Case of Bilateral Pulmonary Hydatidosis, Thoracic Empyema and

   Bullus Emphysema: Clinical Investigations and Surgical Management . **The 17th**

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