Epidemiological and Biomolecular Studies on
*Echinococcus granulosus* in Sudan

By

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A thesis submitted in partial fulfillment of the requirements
of the degree of PhD
University of Khartoum

Supervisor
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April, 2006
Dedication

To the soul of my father who has dreamed a lot to see the day I get this title
To my mother who has supported me and gave me her blessing to overcome all the difficulties I faced during my work
To my sisters and brother
To my husband Ayman, the one who was always behind me providing all the support encouragement, power and strength
To my son Monzir, the beautiful smile which shone and enlightened my life during the last period of my work
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Acknowledgement

First, many thanks to Allah, my God who insert the desire of work in my sides, and give me the support to work and power to continue.

Many thanks to Prof. Imad Aradaib, my supervisor who helped in designing the research protocol and looked after me with their kind supervision and advices.

Thanks to the members of the Department of Parasitology, University of Hohenehim, Germany for helping me during the course of the study and for making my stay in Germany easy and enjoyable. Special thanks are due to Prof Ute Mackenstedt for her valuable supervision as well as official and personal helps. Many thank Dr. Thomas Romig for helping me in planning my work, and for his valuable advice and suggestions and for revising the manuscript. Special thanks to Dr. Anke Dinkel for her supervision and for helping me in performing the molecular biology techniques. May thanks are also due to Dr. Michael Merli for his useful guidance during my work in Hohenheim.

May thanks are also extended to Dr. Mohamed Elamin and International Red Cross Commission (ICRC), Khartoum for providing me with the human samples

My thanks are also due the family of the Central Veterinary Research laboratories, Soba, specially Prof. Mohamed Salih Algablabi, and Prof. Salah Mukhtar. Special thanks to the staff of Parasitology, Soba, specially Mr. Nasir, Khalil, Ahmed Elshik for technical help.
Thanks to my colleges, Dr. Osman Mukhtar, Dr. Yahia Hassan Beik, Dr. Wegdan Hassan Ali and Dr. Selma Osman. and all other friends in the Central Veterinary Research Laboratories (CVRL), Soba.

Thanks are also extended to the German Academic Exchange Service (DAAD) for financial support of this work and for being a real family during my stay in Germany.

I also acknowledge the important support offered from my mother, sisters and brother.

My thanks are also due to my husband Dr. Ayman Elnahas for being very helpful during the work and also for support and encouragement. Thanks Monzir for sharing this time with me.
Summary

This study represents an epidemiological and biomolecular study about *Echinococcus spp.* and hydatid disease in definitive and intermediate hosts including humans in Sudan. In this study, a survey of cystic echinococcosis was conducted during the period from May 2001 to July 2003 in different parts of the Sudan. The prevalence rates in camels, cattle, sheep and goats examined in different states of the Sudan was found to be 59.8% (466/779), 6.1% (299/4893), 11.3% (1180/10422) and 1.9% (106/5565) respectively. The encountered number of cysts was 2387 in camels, 333 in cattle, 1514 in sheep and 108 in goats. Fertility rates were found to be 73.7%, 77%, 19% and 31.5% in camel, cattle, sheep and goat respectively. The favorite site for cysts in camels was the lung (1627/2387). The liver was found to be the preferred site in cattle (206/333) whereas the peritoneum was the predilection site in sheep (1242/1514) and goats (53/108).

Strain characterization of the *E. granulosus* complex in human and livestock population was described for the first time by using polymerase chain reaction amplification and sequencing technology. Even though we were able to detect *E. ortleppi* and sheep strain (G1) in some samples, camel strain (G6) appears to be the predominant strain causing cystic echinococcosis in humans and animals in Sudan. 533 of a total of 542 of all isolates were characterized as belonging to this strain. In this study, the sheep strain of *E.granulosus* was reported for the first time in Sudan in two samples of human origin and five samples of sheep origin.

Secondly, 42 dogs shot as a part of the rabies control program in Tamboul and Rofa, central Sudan, were autopsied and their intestinal
contents were examined for the presence of *Echinococcus* worms. Faecal samples were taken for coprodiagnosis. Worm burden in positive dogs was determined using dilution method and the harvested worms were characterized using G5/6/7 and G1 PCRs. From the 42 euthanized dogs, 12 (28.5) were harboring *E.granulosus* worms. The worm burden was 22-80*10^3* in the positive dogs. All the DNA samples extracted from the worm suspension were characterized as camel (G6) strain of *E.granulosus*. 83.3% (10/12) of the DNA extracted from the faecal samples collected from the 12 dogs which were found to be positive at necropsy were also found positive with copro PCR and the strain was characterized as camel (G6) strain of *E.granulosus*. Two samples were considered inconclusive as there was no signal in the inhibition test. 93.3% (28/30) copro DNA samples from the 30 samples collected from the dogs which were reported negative at necropsy were also negative using copro-diagnostic PCR. The other two samples were positive and characterized as sheep (G1) strain of *E.granulosus*. This copro PCR method was used for the first time in such a survey. Disregarding the inhibited samples, the overall sensitivity of the test was found to be 100%.

For the purpose of this study, hydatid cysts were obtained from the lungs of naturally infected camels (*Camelus dromedarius*) in Tamboul slaughterhouse in central Sudan. Viable protoscolices were collected from these cysts and used for experimental infection of dogs at different doses. Ten dogs were divided into two groups (A and B) of five dogs each. Dogs in group A received a dose of $4 \times 10^3$ protoscolices each whereas dogs in group B received a dose of $8 \times 10^3$ protoscolices each. Fecal samples were examined for patent
infection during the study period. Dogs were necropsied at 45 dpi (group A) and 54 dpi (group B). No eggs were detected in fecal samples from group A throughout the experimental period (45 days). However, eggs were first demonstrated in faeces 52 dpi in group B. The experimental animals in both groups did not show any adverse clinical signs during the experimental study. *Echinococcus granulosus* worms were recovered from both groups at the time of necropsy.

Molecular characterization of the adult worms was made possible using the polymerase chain reaction (PCR)-based detection assay. The worms were identified as G6 (camel) strain of *E. granulosus*. It was found that the prepatency period in dogs after experimental infection with protoscolices of camel origin is longer than the reported for other strains of *E. granulosus*. These are the first data on prepatency periods of the camel strain G6 in dogs confirmed by molecular characterization.
ملخص الطرحة

Σῃ. όργη 2 διπλάτ Α νιπό λεγ. ιψιπ ίψιπ αινιγμάτων υπολογίζοντας άμεσα άνευ ψεφυγμένων ιστοματικών. (Echinococcus) Τούτα οι ιστοσκελεστικές ιστοπλοίωση και δημιουργία της συνάντησης οι ιστοματικές και επιστημονικές περιπτώσεις O1 και O2. Η διάρκεια της εξετάσεως οδηγεί σε ιστοσκελεστικές ιστοπλοίωση και δημιουργία της συνάντησης οι ιστοματικές και επιστημονικές περιπτώσεις O1 και O2. Παράλληλα, τα κατεξοχήν προσωπικά δεδομένα των ανθρώπων συμμετέχουν στην διάρκεια της εξετάσεως οδηγεί σε ιστοσκελεστικές ιστοπλοίωση και δημιουργία της συνάντησης οι ιστοματικές και επιστημονικές περιπτώσεις O1 και O2.

(6.1 %) Το πείραμα τόσο εξετάζεται ιστοπλοίωση και δημιουργία της συνάντησης οι ιστοματικές και επιστημονικές περιπτώσεις O1 και O2. Παράλληλα, τα κατεξοχήν προσωπικά δεδομένα των ανθρώπων συμμετέχουν στην διάρκεια της εξετάσεως οδηγεί σε ιστοσκελεστικές ιστοπλοίωση και δημιουργία της συνάντησης οι ιστοματικές και επιστημονικές περιπτώσεις O1 και O2.

1514 %

(299/4893) %

106/5565)

1.9, (1180/10422) %

1180/10422) %

1514

%1180/10422) %

(299/4893) %

1.9, (1180/10422) %

1180/10422) %

1514

%1180/10422) %

1514

%1180/10422) %

1514

%1180/10422) %

1514

%1180/10422) %

1514

%1180/10422) %

1514
لا يمكنني قراءة النص العربي بشكل طبيعي.

يرجى تحويل النص إلى نص طبيعي للوصول إلى النص الصحيح.

لا يمكنني قراءة النص العربي بشكل طبيعي.

يرجى تحويل النص إلى نص طبيعي للوصول إلى النص الصحيح.

لا يمكنني قراءة النص العربي بشكل طبيعي.

يرجى تحويل النص إلى نص طبيعي للوصول إلى النص الصحيح.

لا يمكنني قراءة النص العربي بشكل طبيعي.

يرجى تحويل النص إلى نص طبيعي للوصول إلى النص الصحيح.

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يرجى تحويل النص إلى نص طبيعي للوصول إلى النص الصحيح.
Chapter One

General Introduction and Literature Review

1.1 Introduction

Echinococcosis and hydatidosis are terms used to describe infection of animals and humans with the adult tapeworm or larval metacestode stage of cestode species belonging to the genus *Echinococcus*. Members of this genus within the family of *Taeniidae* are small tapeworms at 1.2-7mm in length, possessing only a maximum of 7 segments (proglottids). The parasite is of pathogenic and economic significance in intermediate and aberrant intermediate hosts, where the larval parasite develops into a hydatid cyst, a fluid-filled cystic or vesicular structure composed of two main layers or membranes. The laminated layer is a carbohydrate–rich, acellular structure which is unique for the genus *Echinococcus*. It is both supportive and also physically encloses the asexually produced protoscolecies which bud off from the living germinal layer and are the infective stage for the definitive host. Production of protoscolecies by *Echinococcus* spp. is prolific ensuring high worm burdens in the carnivore definitive host. Infection with *E.granulosus* metacestodes results in the development of one or several unilocular hydatid cysts that may grow for the life of the host. *Echinococcus* species are of medical and veterinary importance because infection with metacestodes may cause severe illness and death in the intermediate host (Jenkins *et al*, 2005). Tremendous research was conducted in
echinococcosis. In Sudan, in spite the sizeable work appeared on the subject, molecular biology of the parasite is not fully investigated which enhance the establishment of this work.

Objectives of the Study

- **Epidemiological and biomolecular study of cystic echinococcosis in humans and animals in the Sudan.**
- **Strain characterization and Copro diagnosis of *E.granulosus* in central Sudan.**
- **Determination of the prepatent period of the camel strain, G6 of *E.granulosus*.**
1.2 Classification

*Echinococcus* was classified as follows:

Kingdom       Protista
Sub/Kingdom   Metazoa
Phylum        Platyhelminthes
Class         Eucestoda
Orde          Cyclophyllidea
Family        Taeniidae
Genus         *Echinococcus*

Currently there are six species recognized within the genus  
*Echinococcus* (Jenkins *et al.*, 2005), these are *E.granulosus*,  
*E.multilocularis*, *E.vogeli*, *E.oligarthus*, *E.ortleppi* and *E.equinus*.  
Seventh species, *Echinococcus shiquicus*, was recently described  
(Xiao *et al.*, 2005). Based on morphology, host specificity and  
molecular characteristics (Pearson *et al.*, 2002, McManus and  
Thompson, 2003), the *E.granulosus* complex is divided into three  
species and eight defined strains (Table 1). The present recognition of  
*Echinococcus* species reflects a series of largely host adapted species  
that are maintained in distinct cycles of transmission (Thompson,  
2001., Thompson and McManus, 2002). These are characterized by  
the principal intermediate hosts which are; sheep, horses, cattle,  
camels and different species of rodents (Table 1). Although these  
cycles of transmission may overlap in some geographical areas, the  
parasites involved have been shown to maintain their genetic identity
(Thompson et al., 1995, Thompson and McManus 2001., 2002.,
McManus and Thompson, 2003, Haag et al., 2004).

*Echinococcus granulosus* is the most widely distributed species
which exists as a series of genetically distinct strains/genotypes, some
of these genotypes are likely to warrant species status in the future,
particularly those in pigs, camels and cervids (Harandi et al., 2002,
Thompson and McManus, 2002, Lavikainen et al., 2003). However,
more research is required to determine their host and geographic
ranges and whether their genetic characteristics are conserved between
different endemic regions (Jenkins et al., 2005).

Although the most frequent strain associated with human cystic
echinococcosis (CE) appears to be the common sheep strain (G1),
other strains such as the Tasmanian sheep strain (G2), camel strain
(G6), pig strain (G7/G9) and cervid strain (G8) occur in a significant
number of cases in some locations. *E. ortlepi* and *E. equinus* were
previously characterized as cattle (G5) and horse (G4) strains of
*E. granulosus* respectively (Le et al., 2002., McManus, 2002.,
Thompson and McManus, 2002) *E. multilocularis* species recognition
occurred as late as 1953 when the parasite and its life cycle in foxes
and rodents were described by Rausch (1954) and Vogel (1957).
Although a number of *E. multilocularis* isolates have been described
from different geographical areas, their genetic variability remains
undetermined (Jenkins et al., 2005).

The description of *E. vogeli* and *E. oligarthus* was relatively
To date, there is no evidence of significant intraspecific variation in *E.
equinus, E. ortleppi, E. oligarthus* and *E. vogeli* (Jenkins et al., 2005).
1.3 Life Cycle (Fig 1)

Like other cestodes, species of *Echinococcus* require two different host species to complete their life cycles. Definitive hosts harbouring the adult tapeworm in the small intestine are exclusively carnivores and intermediate hosts harbouring the larval stage (metacestodes) are herbivorous or omnivorous. The adult worms are less than 7mm in length. They feed on the intestinal contents of the host without causing any symptoms as they do not invade tissues. When mature, *Echinococcus* worms shed the terminal proglottids containing eggs which pass with the faeces to the environment. The intermediate hosts acquire the infection by accidental ingestion of the eggs with contaminated food or water. Larvae contained in the eggs (oncospheres) emerge from the eggs in the small intestine, invade blood vessels and may migrate into almost every part of the body. There, the metacestodes grow for months or years forming fluid-filled cysts or vesicles. Protoscolices are produced within the metacestode in a phase of non-sexual reproduction. Once the metacestode is eaten by a suitable definitive host, these protoscolices will grow into adult tapeworms. The occurrence of a parasite in a particular host assemblage like dog/sheep or dog/horse reflects a variable degree of host parasite adaptation (Torgerson and Budke, 2003).
Figure 1
Life cycle of *Echinococcus granulosus*
1.4 Distribution of cystic echinococcosis (Fig 2)

Cystic echinococcosis is the most widespread disease caused by Echinococcus species. The adaptation of Echinococcus to a wide variety of host species and the repeated introduction and movement of domestic animals throughout the world had made possible its broad geographical distribution (Schantz et al., 1995).

In Europe, zoonotic members of the E.granulosus complex have been reported in every country with the exception of Ireland, Iceland and Denmark. They are most intensely endemic in the Mediterranean areas and parts of Eastern Europe such as Bulgaria (Torgerson and Budke, 2003). In the UK, the parasite has a restricted distribution, being found mainly in mid and southern Wales. In Asia, the parasite is endemic in large parts of China and is an important reemerging zoonosis in the former Soviet Republics in Central Asia (Torgerson et al., 2002a, b). Parasites are also found throughout the Indian Subcontinent and the Middle East. In North America they are found in Canada and Alaska, mainly in a sylvatic cycle. In USA, the parasite is very sporadic with just a few foci such as certain communities in Utah and California. In South America the parasite is extensive, particularly in Argentina, Uruguay and Peruvian Andes. In Australia, the parasite is common due to a sylvatic cycle between dingoes and wallabies with over 25% of dingoes and up to 65% of macropod marsupials infected (Jenkins and Morris, 1995., Jenkins, 2002).
Figure 2
World Distribution of cystic echinococcosis

Approximate geographic distribution of *Echinococcus granulosus* (1999)
Institute of Parasitology, University of Zurich (J. Eckert, F. Grimm & H. Bucklar)
Note: exact identification of endemic and highly endemic areas in all regions is not possible because of incomplete or lacking data.
1.5 Cystic echinococcosis in Africa


The cystic hydatid disease focus in North Africa include Morocco (Pandey *et al.*, 1988., Quhelli and Dakkak, 1992), Algeria (Cherid and Nosny, 1972., Abada *et al.*, 1977., Larbaoui and Allyulya, 1979), Tunisia (Ben Rachid *et al.*, 1984., Bchir *et al.*, 1985., Gharbi *et al.*, 1985), Libya (Gebreel *et al.*, 1983., Aboudaya, 1985., Shambesh *et al.*, 1992) and to a lesser extent Egypt (Hegazi *et al.*, 1986). The human populations of the first four of these countries total approximately 60 million. The annual incidence of hydatidosis was between 3.4 –4.6, 6.5–7.8 per 100,000 inhabitants in Algeria and Morocco respectively (Larbaoui and Allyulya, 1979., Quhelli and Dakkak,1992). In Tunisia, between 800 and 1,200 new case of hydatidosis are diagnosed every year for an annual average incidence of 16.5 per 100,000 (Gharbi *et al.*, 1985., Achour *et al.*, 1989).

In East Africa, camels are important intermediate hosts. Sheep and goats are frequently infected in Kenya (Macpherson, 1985) and in Ethiopia (Bekele *et al.*, 1988., Wosene, 1991). Many pastoral peoples do not bury their dead, allowing dogs and wild carnivores access to human hydatid cysts. *Echinococcus* infection has been found in 39–70% of dogs in Turkana, Kenya (Nelson and Rausch, 1963.,
**Table 1: Species and Strains of *Echinococcus* complex**

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain/genotype</th>
<th>Known intermediate hosts</th>
<th>Infective to humans</th>
<th>Disease in humans</th>
<th>Known definitive hosts</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Echinococcus</em></td>
<td></td>
<td>Republic of</td>
<td>Sheep (cattle, pigs, camels, goats, macropods)</td>
<td>Yes</td>
<td>Cystic (Unilocular)</td>
</tr>
<tr>
<td><em>granulosus</em></td>
<td>Sheep/G1</td>
<td>sheep/G2</td>
<td>Sheep (cattle?)</td>
<td>Yes</td>
<td>Cystic (Unilocular)</td>
</tr>
<tr>
<td></td>
<td>Tasmanian</td>
<td>Buffalo/G3</td>
<td>Buffalo (cattle?)</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>Camel/G6</td>
<td>Camels (sheep)</td>
<td>Yes</td>
<td>Cystic (Unilocular)</td>
<td>Dog</td>
</tr>
<tr>
<td></td>
<td>Pig/G7</td>
<td>Pigs</td>
<td>Yes</td>
<td>Cystic (Unilocular)</td>
<td>Dog</td>
</tr>
<tr>
<td></td>
<td>Cervid/G8 and</td>
<td>Cervids</td>
<td>Yes</td>
<td>Cystic (Unilocular)</td>
<td>Dog</td>
</tr>
<tr>
<td></td>
<td>G10 ?/G9</td>
<td>Sheep (cattle?)</td>
<td>Yes</td>
<td>Cystic (Unilocular)</td>
<td>Dog</td>
</tr>
<tr>
<td></td>
<td>Lion?</td>
<td>Zebra, wildbeest, warthog, bushpig, buffalo, various antelope, giraffe?</td>
<td>Yes</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>E. equines</td>
<td>Horse/G4</td>
<td>Horses and other equines</td>
<td>No</td>
<td></td>
<td>Dog</td>
</tr>
<tr>
<td></td>
<td>E. ortleppi</td>
<td>Cattle/G5</td>
<td>Cattle</td>
<td>Yes</td>
<td>Cystic (Unilocular)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain/genotype</th>
<th>Known intermediate host</th>
<th>Infective to human</th>
<th>Disease in human</th>
<th>Known definitive hosts</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Echinococcus</em></td>
<td></td>
<td>Rodents, domestic and wild pigs, dog, monkey, (horse)</td>
<td>Yes</td>
<td>Alveolar (multivesicular’)</td>
<td>Fox, dog, cat, wolf, raccoon-dog, coyote</td>
</tr>
<tr>
<td><em>multilocularis</em></td>
<td>Some isolate</td>
<td>variation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. shiquicus</td>
<td>?</td>
<td>Lagomorphs (pika)</td>
<td>?</td>
<td>?</td>
<td>Tibetan fox</td>
</tr>
<tr>
<td>E. vogeli</td>
<td>None reported</td>
<td>Rodents</td>
<td>Yes</td>
<td>Polycystic</td>
<td>Bush dog</td>
</tr>
<tr>
<td>E. oligarthus</td>
<td>None reported</td>
<td>Rodents</td>
<td>Yes</td>
<td>Polycystic</td>
<td>Wild felids</td>
</tr>
</tbody>
</table>
Macpherson, 1985) and 23% in stray dogs in Somalia (Macchioni et al., 1985). The initial surgical incidence rate for Turkana, was estimated to be 40 per 100 000 per year (Schwabe, 1964).

1.6 Cystic echinococcosis in Sudan

In Sudan, the disease was first mentioned at the beginning of the 20th century (Christopherson, 1909). Abel Malek (1959), made a list of the parasites of the domesticated animals in Sudan, including a single case of liver hydatidosis in cattle, 3 cases in camels as well as 4 cases of *Echinococcus* in dogs. The work done by Eisa and Mustafa (1962) drew attention to this serious zoonosis in southern Sudan, specially in Kapoeta district in Equatoria where they reported 86.4% prevalence rate of *Echinococcus* in dogs and 21% in cattle in the Upper Nile Province. About 33% and 9.4% infection rates amongst sheep and goats were reported respectively (Eisa et al., 1962). Another survey carried out by Eisa (1982) in Malakal and Rank district revealed over all infection of 2.7% among cattle. Elkhawad et al (1977) encountered an infection rate of 25%, 12% and 10% in cattle, sheep and goats respectively in Western Province. In another survey in the central region of Sudan, Elkhawad et al., (1979) reported an infection rate of 4.3%, 8.1%, 3.2% and 53.3% in cattle, sheep, goats and camel respectively. In Elobied (Kordofan region), the prevalence of hydatid cysts among camels was 67% (Saad et al., 1989). 3.84% cattle and 48.69% camels were found to harbour the parasite in central Sudan (Saad and Magzoub, 1989). Recently, Elmahdi et al (2004) recorded a prevalence rate of 44.6%, 6.9% and 3.0% in camels, sheep and cattle respectively. Given the high rate of non-fertile or calcified cysts in sheep, these animals appear to play an unimportant role in the
transmission cycle of echinococcosis in the studied areas (Saad and Magzoub 1989a; Saad and Magzoub 1989b; Elmahdi, 1998; Omer et al., 2002; Elmahdi et al, 2004 and Omer et al, 2004).

The prevalence rate of the disease in animals is high, thus providing high risk of the disease for human populations in different parts of the Sudan. However, no hospital records are currently available. The disease seems to be endemic in rural communities where people live in close contact with dogs (definitive host) and large numbers of livestock (intermediate hosts). The disease was prevalent in 0.33% (1 from 300) villagers in a village in central Sudan (Elmahdi et al, 2004). A special situation may exist among the Taposá and neighbouring tribes in Equatoria where the hyper endemic focus of the disease among the Turkana extends into Sudan (Eisa et al., 1962). Cahill et al (1965) conducted a serological study on 152 individuals in Southern Sudan. The prevalence rate among these individuals was estimated by 13.3%. Cystic echinococcosis was found in 2% (132/6728) of the human population in selected areas of southern Sudan (Njoroge, 2001). Mann (1974) stated that 50% of the human cases of hydatidosis in Uganda were immigrants from Southern Sudan.

Concerning the presence of Echinococcus species and strains, so far there is only one study available (Dinkel et al, 2004), that 44 of 46 samples characterized from different intermediate host in central Sudan were identified as G6 (camel strain), the two exceptions being cattle samples identified as E. ortleppi (previously the cattle strain, G5).
1.7 Molecular epidemiology of cystic echinococcosis

The value of DNA analysis in molecular epidemiological studies of cystic echinococcosis, as well as in clarifying the complex issue of strain variation of *E. granulosus*, is well recognized. Some molecular epidemiological surveys are summarized below.

In China, examination by a combination of DNA techniques has indicated that the common sheep strain is the most predominant in the northwest region of China (McManus *et al.*, 1994).

In Argentina, several distinct genotypes including the common sheep strain (G1) and Tasmanian sheep strain (G2) in sheep and humans, the pig strain (G7) in pigs and the camel strain (G6) in humans where identified (Rozenzvit *et al.*, 1999). Furthermore, that was the first report about the presence of the Tasmanian sheep strain (G2) and the camel strain (G6) in humans. In Nepal, three strains of *E. granulosus*, namely the sheep strain (G1), cattle strain (*E. ortleppi*) and camel strain (G6) were identified in buffalo, sheep, goat and human hosts, of which two human isolates were identified as G6 (Zhang *et al.*, 2000). In Iran, the sheep strain was found to be the most common genotype of *E. granulosus* affecting sheep, cattle, goat and occasionally camels. The majority of camels were infected with the camel strain (G6), as were 3 of 33 human cases (Harandi *et al.*, 2002). Biochemical and genetic studies indicated that there may be at least three strains of *Echinococcus* in Africa, the sheep strain involving sheep, goats and humans, the camel strain in camels and goats and the cattle strain (McManus and Macpherson, 1984, McManus *et al*, 1987). The existence of predator–prey parasite transmission between lion and its main food animal suggests that a distinct wildlife form of the parasite may exist in some parts of sub–Saharan Africa. Evidence
for this is that the parasite found in the lion is morphologically distinct from *E.granulosus* (Ortlepp, 1937). In North Africa *E.granulosus* is propagated primarily by a domestic cycle involving domestic dogs as the definitive host and many species of livestock including camels, cattle, sheep, goats, pigs, horses, donkeys and buffaloes as intermediate hosts (El–Kordy, 1946., Dar and Taguri, 1978., Larbaoui *et al.*, 1980., Jaiem, 1984., Gusbi *et al.*, 1987., Pandey *et al.*, 1988., Ahmed, 1991). The local importance of each intermediate host species in maintaining the cycle varies in different regions. In Kenya, molecular analysis showed the presence of sheep (G1), camel (G6) and cattle (*E.ortleppi*) strains. The range of intermediate hosts for these strains appeared to be similar sheep, cattle, camel and humans except that the camel strain was detected in one case of human and *E.ortleppi* was detected in a single case from pig (Dinkel *et al.*, 2004). Additionally, the camel strain (G6) was characterized in two samples of human origin in Mauritania (Bardonnet *et al.*, 2001).

### 18 Techniques of strain characterization

Several techniques that investigated the morphology, chemical composition, metabolism, and developmental stages of *Echinococcus* had been implemented to characterize different strains and species. Epidemiological studies on hydatid disease reported the occurrence of different strains in various countries (Kumaratilake and Thompson., 1982., McManus and Macpherson, 1984., McManus and Thompson, 1985). These studies emphasized the importance of strain characterization for the reason that strains may differ in their infectivity to various intermediate and final hosts (Thompson, 1977).
The biochemical composition of various developmental stages of different strains demonstrated considerable discrepancies (McManus, 1981., Kumaratilake and Thompson., 1984). This may cause a variation in the susceptibility of the parasite to different chemotherapeutic agents (Schantz et al., 1982., McManus et al., 1985). Strain variation may have practical implication in vaccinations attempts and in control programs.

1.8.1 General morphology
The study of rostellar hook morphology was established by InDialy and Sweatman (1965). Protoscolex rostellum hooks as well as strobilar hooks of adult worms were used successfully in morphological characterization. Total lengths, width, blade length of hooks were all used to identify *Echinococcus* strains in various parts of the world (William and Sweatman., 1963., Kumaratilake and Thompson, 1984). *Echinococcus* of camel origin is distinct from the sheep strain with regard to the shape of the hooks and hook length whereas differences in hook measurement characteristics between camel, cattle and horse material were less marked (Eckert et al. 1989). Characteristics of the strobilar morphology such as total worm length, length of the terminal segment, maximal number of segments, position of the sexually mature segment, position of the genital pore, number and distribution of testes, shape and size of the cirrus sac, shape of the ovary and the female reproductive system are all considered as important tools for strain characterization. The sexually mature segment is always terminal in worms of camel origin and always penultimate in worms of cattle origin. Testes are found throughout the
segment in case of worms of camel, sheep and horse origin and confined to the centre of the segment in worms of cattle origin (Thompson et al, 1984., Kumaratilake et al, 1986, Eckert et al, 1989). Morphological characterization enabled the identification of two morphologically different strains that specifically affected sheep and horse. A virulent goat/dog strain was morphologically identified in India by Pandey (1972).

1.8.2 Biochemical Methods

Biochemical techniques using Sodium Dodycyl Sulphate Poly-acrylamide gel electrophoresis (SDS-PAGE), isoelectric focusing and isoenzyme analysis were reported to be highly useful in strain characterization of helminthes parasites in general and *E.granulosus* in particular. Hydatid cyst fluids collected from pigs and sheep showed different SDS-PAGE protein patterns as reported by Zvolinskene et al., 1977. Similar DNA contents collected from hydatid cysts in sheep and horse showed different composition of poly-saccharides and lipids (McManus and Smyth, 1982). In addition, protoscolices of the horse strain were found to produce more lactate than that of the sheep strain. Isoelectric focusing was also used to differentiate between strains in UK and Australia (Kumaratilake and Thompson, 1984).

1.8.3 Developmental method in vivo

Different strains of *Echinococcus* require different periods to reach maturity in dogs. In worms of cattle origin, thin-shelled eggs appeared in the uterus as early as 30 days post infection (PI), with the
majority of worms being in this condition at 35 days and certain to be infective by about 35-37 days (Thompson et al., 1984). In Australia, different prepatent periods have been recorded in isolates from different regions (Kumaratilake et al., 1893). A prepatent period of 35 days has also been reported in a dog infected with hydatid material from giraffe of South West African origin (Tscherner, 1978).

Maturation of a particular strain can vary with the host in which it develops (Thompson and Kumaratilake, 1985). Thus, although in Australia the domestic sheep strain of *E.granulosus* matures in both dogs and dingoes, the growth of the sylvatic form in dogs was found to be substantially retarded compared with that in dingoes.

1.8.4 Developmental method in vitro

Culturing of protoscolices using a special media of Parker 858 and 20% fetal serum was successfully implemented in differentiation between horse and sheep strains (Smyth and Davis, 1974). Horse protoscolices failed to segment in the culture system while those from sheep segmented and matured. This variation is presumably based on the nutritional requirements of different strains.

1.8.5 Genetic characterization

Methods used for strain typing include sequencing of partial mitochondrial cytochrome c oxidase subunit 1 (*cox1*) and of NADH dehydrogenase 1 (*nad1*) genes (Bowles et al., 1992, Bowles and McManus, 1993), analysis of ribosomal DNA regions (ITS1, ITS2) by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP., Bowles and McManus, 1993, Gasser and Chilton,
1995) and random amplification of polymorphic DNA (RAPD)-PCR (Scott and McManus, 1994., Siles-Lucas et al., 1994).

1.8.5. a Sequencing of partial mitochondrial cytochrome c oxidase subunit 1 (cox1). (Bowles et al., 1992).

Different isolates of *Echinococcus* were analyzed for sequence variation within a region of the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene. For each *Echinococcus* isolate examined, a single double-stranded PCR product that has a size of 446bp was visualized on an ethidium-bromide stained agarose gel. Eleven distinct partial CO1 sequences were detected amongst the examined *Echinococcus* isolates. This method enabled the division of 7 discrete groups (G1-G7) of *E.granulosus*. The CO1 sequences of some of these groups were very similar. The sequence obtained with the Tasmanian sheep sample (G2) differed from the standard sheep sequence at 3 of the 366 nucleotide sites examined. A common sequence (G4) was found in isolates of horse and donkey origin. A unique CO1 sequence (G5) was found with a cattle isolate from Holland, whereas camel isolates from Somalia and Sudan and a goat isolate from Turkana region shared the same sequence G6. This was found to be different from the sequence found in pig isolates from Poland (G7) at only one nucleotide position.

1.8.5 /b Analysis of ribosomal DNA regions ITS1 by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Bowles and McManus, 1993)

Due to the fact that most of the polymorphism detected in *Echinococcus* using pSM889 as a probe (McManus and Rishi, 1989)
results from sequence dissimilarity in the ITS1 and ITS2 spacer regions and it is also likely that the non-transcribed spacers are too variable for the purpose of identification. This method focused on the ITS region of the rDNA repeat. The PCR-RFLP allows *Echinococcus* isolates to be easily and rapidly distinguished using size and sequence of the nuclear genomic rDNA ITS1 region as a genetic marker. This method supports the existence and pattern of intraspecific genetic variation in the genus. The sharp delineation between the *E.granulosus* sheep, horse, cattle and camel/pig isolate groups indicated that various strains are not interbreeding and are following separate evolutionary paths.

1.8.5.c Analysis of ribosomal DNA region ITS2 by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Gasser and Chilton, 1995)

The PCR-RFLP of ITS2 rDNA provides a method of identifying some taeniid cestodes including different isolates of *E.granulosus*. This method enabled the differentiation between isolates from camel and horse and a representative of the sheep strain. In spite of this variation, it appears that digestion of ITS2 with the digestion enzyme RsaI provides a reliable specific marker for *E.granulosus*, since the restriction of isolates representing different strains shared a common restriction fragment of 310 bp.

1.8.5.d A PCR system for detection of species and genotypes of *E* granulosus complex (Dinkel et al, 2004)

With this method those *Echinococcus* species and genotypes, which are presently assumed to have the greatest public health impact,
can be easily and rapidly distinguished. It is a PCR system which uses a part of the mitochondrial 12S rRNA gene as a target sequence. This PCR system identifies g1 (sheep strain) and G5/6/7 (cattle, camel and pig strain P) each with a different pair of primers. To discriminate between *E. ortleppi* and *E. granulosus* G6/7, seminested PCRs are used in a second step.

1.9 Diagnosis of *Echinococcus* in definitive and intermediate hosts

1.9.1 Diagnosis of *Echinococcus* in definitive host

Diagnosis of *Echinococcus* in definitive hosts is difficult, because the eggs of all *Echinococcus* and *Taenia* species are morphologically indistinguishable from one another and the characteristic small segments of *Echinococcus* species may be absent from the faeces or can be easily overlooked. The two major parasitological methods are purgation with arecoline salts and examination of the small intestine at necropsy (Deplazes and Eckert, 1996). Both these techniques are time consuming, laborious and require special safety precautions.

Therefore, indirect methods such as detection of serum antibodies, coproantigens and parasite DNA in faecal sample are described to simplify and improve epidemiological investigations.

1.9.1.a Detection of serum antibodies

Taeniid cestode antigens derived from adult worms, juvenile intestinal stages or onchospheres interact with the immune system of the host and may lead to the production of specific antibodies (Jenkins
and Rickard, 1985). Enzyme Linked immunoassays (ELISA) based on detection of these antibodies have been developed for diagnosis of *Echinococcus* spp infection in final hosts (Jenkins and Rickard, 1986., Gasser *et al*, 1988). A problem encountered with this method is the persistence of antibodies after elimination of the cestodes (Gottstein *et al*, 1991., Gasser *et al*. 1993). Usage of an ELISA based on the detection of antibodies directed against Em2 antigen revealed a sensitivity of 60% (Gottstein *et al*, 1991). Even though no cross reactions were shown with sera from dogs with various helminthes when using this ELISA, animals without a detectable intestinal infection were also positive. Serological diagnosis of canine echinococcosis due to *E.granulosus* has been based on the detection of antibodies (IgG, IgA and IgE) directed against a protoscolex antigen preparation (Gasser *et al*. 1993). Overall specificity ranged from 97-100% but sensitivity varied (53-84%) according to geographic region, though it could be increased by combining the evaluation of various immunoglobulins classes (Gasser *et al*. 1993., Craig *et al*. 1995).

1.9.1.b Detection of coproantigens
Coproantigen ELISAs were developed for both *E.multilocularis* and *E.granulosus*. The most important advantage of these methods over the conventional serum antibody assays is that they indicate current infection (Allan *et al*. 1992., Deplazes *et al*. 1992, 1999). The sensitivity of coproantigen ELISAs developed for *E.multilocularis* was reported to be 83.6% whereas the overall sensitivity of the ELISAs against *E.granulosus* has shown different levels of sensitivity ranging between 50 and 87.5% (Allan *et al*. 1992., Craig *et al*. 1995.,
Moro et al. 1999). The sensitivity of the ELISAs developed for both species of *Echinococcus* depends on the worm burden (Craig et al. 2003).

**1.9.1.c Detection of copro-DNA**

The advantage of detecting *Echinococcus* DNA by polymerase chain reaction (PCR) in faecal samples is that it has the potential to provide absolute species specificity assays with high sensitivity. Additionally, as the test is aimed to mainly detect eggs in faecal samples, it provides a more accurate estimation of the potential risk for human infection than ELISA-based methods. Problems encountered when extracting DNA from faecal samples are that these samples often contain PCR inhibiting substances that create false negatives. Different kits have been utilized to purify DNA, eliminating inhibitory substances, but this method is time consuming and it may enhance the possibility of losing the DNA present in the faecal samples. Sieving of the faecal samples in several steps concentrates eggs from the sample to improve sensitivity (Monnier et al., 1996), but may make the protocol complicated and time consuming and may not be suitable for large epidemiological studies (Craig, 2003). Usage of a commercial kit, the Qiaamp Ministool kit (Qiagen, UK) combined with a single sieving step provided good results (Abbasi et al. 2003).

A PCR was described by Bretagne et al (1993) for the detection of *E. multilocularis* DNA in faecal samples of foxes by using the U1 snRNA. However, more specific results for the same parasite were
obtained by *E.multilocularis* specific PCR (Dinkel *et al*, 2004). This method can also be applied to *E.granulosus*

1.9.2 Diagnosis of cystic hydatidosis in intermediate host

The most frequent approach for the detection of cystic echinococcosis in domestic livestock is by examination at necropsy. Radiological (X-ray) diagnosis of ovine hydatidosis has been applied successfully on a very small scale (Wyn-Jones and Clarkson, 1984), but it is not a suitable system for epidemiological surveys. Use of sonography for hydatidosis screening in some animals such as sheep could be useful in the establishment of an epidemiological control system as well as in the analysis and assessment of risks that the disease poses for humans and animals (Eduardo *et al*. 2001). Prevalence of hydatid cysts in goats in northwestern Turkana, Kenya, and Taposa land, southern Sudan was determined using ultrasonography (Njoroge *et al*.2000). Unlike with abattoir surveys, the author considered the results of this survey as unbiased because it covers the entire flock.

1.9.2 Serodiagnosis of hydatid disease in animals

Even though serological diagnosis of *Echinococcus* infection in domestic livestock had not been successful, few studies had been undertaken to characterize the problems with sensitivity and specificity of immunodiagnosis in animals (Craig, 1993). The principal problem is that animals with fertile cysts have undetectable levels of specific antibodies and cross-reactivity occurs against other parasites (Verastegui *et al*, 1992). However, identification of exposure
to *E.granulosus* at the flock or herd level by use of mean values for serum antibody activity is possible using hydatid cyst fluid antigens in ELISA (Lightowlers and Gottstein, 1995). Three ELISA tests using a purified Antigen B subunit, a recombinant oncospheral antigen (EG95) and a crude protoscolex antigen were evaluated in naturally or experimentally infected sheep. The highest diagnostic sensitivity was 63% with the protoscolex antigen (Kittelberger *et al*. 2002).

1.9.3 Diagnosis of cystic hydatidosis in humans

1.9.3.a Imaging Techniques

Imaging methods for the detection of space occupying lesions are used for the diagnosis of cystic and alveolar echinococcosis. Computerized tomography (CT), magnetic resonance imaging (MRI), ultrasound (US) and radiography (X-ray) are currently the most useful imaging techniques. (Eckert *et al*., 2001). CT scans provide the best overall resolution for both diseases but are limited to well equipped hospitals and not applicable for field studies (Craig. *et al*, 2003). Introduction of ultrasound imaging (US) had improved both the diagnosis of the disease and the understanding of the natural history of the disease (Ammann and Eckert, 1995., Caremani *et al*, 1997., Von Sinner, 1997), but is not applicable when cysts are located in lungs, bone or brain. The US examination is a suitable technique for population studies aimed at detecting cases and determining the prevalence of the disease.

All imaging techniques rely on the expertise of the operator in differentiating *Echinococcus* infection from other space occupying lesions. The image can be confused with other cystic lesions such as tumors, and hepatic abscesses, but the identification is easier when
some pathognomonic lesions such as a visible laminated layer or daughter cysts are present (Craig. et al, 2003).

1.9.3.b Immunodiagnosis of human hydatidosis

Immunodiagnosis has been practiced for the detection of both cystic and alveolar echinococcosis. The source of the antigen used for the detection of cystic echinococcosis has been hydatid fluid removed from cysts in livestock. The crude cyst fluid can be used for procedures such as immunoelectrophoresis (detection of arc 5) and the indirect haemagglutination assay with good results, but now components such as Antigen B, a heat stable lipoprotein, are frequently purified from the cyst fluid (Wen and Craig, 1994). Recombinant Antigen B proteins have also been used but they are less sensitive than the native Antigen B (Rott et al. 2000).

For alveolar echinococcosis, the antigens used are derived either from cyst masses or protoscolices obtained from experimentally infected rodents. The carbohydrate rich Em2, which is affinity purified, or the commercially available preparation Em2 plus belong to the most useful antigens. (Siles-Lucas and Gottstein, 2002). Whole protoscolex homogenate has been used in ELISA with some success but it is better when used in immunoblotting as a band with relative mass of 18 kDa has been reported to be highly specific for alveolar echinococcosis infections (Ito, Schantz and Wilson, 1995).

Antigens available for the diagnosis of alveolar echinococcosis are usually more specific than those used for cystic echinococcosis (Craig et al.2003). The major problems regarding immunodiagnostic tests are due to the existence of significant numbers of both false
negative and false positive cases. The average sensitivity of ELISA-based diagnostics is around 80%. Specificities with reference to other, non cestode infections is usually high (95-100%) but significant levels of cross reactivity exist within the main larval taeniid infections. Serology is more problematic in geographical areas where *E. granulosus*, *E. multilocularis* and *Taenia solium* occur together. Individuals with previous exposure to the parasite without the development of further infection or significant pathology (Cohen *et al*. 1998, Bartholomot *et al*. 2002) are often antibody positive when serologically examined.

Benefits such as confirmation of the imaging or clinical evidence, identification of asymptomatic individuals and providing information on the state of the infection and the immune response against the parasite can be gained when serology is combined with other diagnostic procedures such as imaging techniques (Craig *et al*. 2003).
Chapter Two

Material and Methods

2.1 Epidemiological survey
During the period of May 2001 to July 2003 frequent visits were made to slaughterhouses in different representative areas of the Sudan (central, eastern, western and southern Sudan). A total number of 21,659 animals (779 camels, 4,893 cattle, 10,422 sheep and 5,565 goats) was examined (Table 2 and Fig.2). Different organs including lungs, liver, heart, spleen and kidneys of the slaughtered animals were thoroughly inspected. Hydatidosis positive animals and the number of cysts encountered were recorded.

2.2 Hydatid cysts
DNA was extracted from 527 ethanol preserved hydatid cyst samples (317 none calcified and 210 calcified) collected from camel (157, 50), cattle (62, 45), sheep (81, 60), goats (10, 55) and humans (7, 0) from different parts of central, western and southern Sudan during the epidemiological survey. Representative samples from cattle (4, 3) and camel (3, 5) were obtained from northern and eastern Sudan respectively for the purpose of DNA characterization. Additional 26 samples of sheep origin were also obtained from central Sudan for
DNA characterization. DNA was also extracted from adult *E.granulosus* worms obtained from 15 dogs in central Sudan.

Table 2: Numbers of the examined camels, cattle, sheep and goats in different study areas

<table>
<thead>
<tr>
<th>Location (Sudan)</th>
<th>Camels</th>
<th>Cattle</th>
<th>Sheep</th>
<th>Goats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central (Khartoum, Medani, Tamboul, Rofa)</td>
<td>214</td>
<td>250</td>
<td>400</td>
<td>0</td>
</tr>
<tr>
<td>Western (Darfur and Kordofan Staes)</td>
<td>565</td>
<td>4318</td>
<td>9727</td>
<td>5523</td>
</tr>
<tr>
<td>Southern (Juba and Malakal)</td>
<td>0</td>
<td>325</td>
<td>295</td>
<td>42</td>
</tr>
<tr>
<td>Total</td>
<td>779</td>
<td>4893</td>
<td>10422</td>
<td>5565</td>
</tr>
</tbody>
</table>

Figure 2

Study area
2.2.2 Collection of hydatid cysts or cyst material from human patients

Visits were made to hospitals in the same areas to estimate the numbers of human cystic echinococcosis cases. Hydatid cysts or cyst-
derived materials were obtained from 7 patients, admitted to Khartoum and Juba medical teaching hospitals, for diagnosis of the disease. Human samples were used for further molecular characterization.

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

2.2.4 Preservation of hydatid cysts and adult *E. granulosus* samples
Representative intact cysts or cyst material as well as *E.granulosus* worms were preserved in 70% alcohol for further DNA characterization.

2.2.5 Preservation of the faecal samples
Collected faecal samples were preserved in -20°C and later upon arrival in Germany at -80 °C for at least three days for the inactivation of eggs.

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

### 2.2.7 DNA extraction

DNA was isolated from ethanol preserved or frozen cysts as described by Dinkel et al, 1998, that up to 0.5 g protoscolices or cyst wall (germinal and laminar layer) or adult worm was cut into small pieces using sterilized scalpel or scissor and transferred to 1.5 ml Eppendorf reaction tube. 500 µl digestion buffer, 10 µl 1 M dithiothreitol and 60 µl proteinase K were added to the sample and incubated for 3-12 hours at 56°C with frequent agitation to allow the process of digestion until suspension was clear.

DNA was then extracted with phenol chloroform isoamyl (25:24:1), that 570 µl of the phenol chloroform isoamyl preparation was added to the sample which was then centrifuged at 13,200 rpm for 10 min. The upper watery phase (~500 µl) containing the DNA was transferred in a new 1, 5 ml reaction tube and subsequently 50 µl 3 M Na-acetate (0.1 volume of the aqueous volume) and 1000 µl (double the volume of the DNA containing phase) cold absolute ethanol (-20°C) were added and then incubated at -20°C for 2-12 hours. The DNA was precipitated by centrifugation at 14,000 g for 30 minutes at 4°C. The pellet was then washed with 70% cold (-20°C) ethanol and
Table 1: Prevalence of cystic echinococcosis in livestock in Central Sudan

<table>
<thead>
<tr>
<th>Examined animals</th>
<th>Total number examined</th>
<th>Prevalence %</th>
<th>Total number of cysts</th>
<th>Fertility %</th>
<th>Predilection site</th>
<th>Cysts examined for genotype n (n Non calcified-n calcified)</th>
<th>Genotype (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Camel</td>
<td>214</td>
<td>55.6%</td>
<td>360</td>
<td>75%</td>
<td>Lung (270/360)</td>
<td>87 (57-30)</td>
<td>G6 (87)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cattle</td>
<td>250</td>
<td>20%</td>
<td>63</td>
<td>100%</td>
<td>Liver (63/63)</td>
<td>27 (27-0)</td>
<td>G6 (27)</td>
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</tr>
<tr>
<td>Sheep</td>
<td>400</td>
<td>2.5%</td>
<td>12</td>
<td>0%</td>
<td>Liver (0/10)</td>
<td>12 (0-12)</td>
<td>G6 (10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Goat</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
subsequent centrifugation at 14,000 g for 10 minutes. Evaporation of the ethanol was allowed at 35-40 °C in an oven. The DNA was resuspended by the addition of 100-200 µl nuclease free water depending on the amount of the precipitated DNA and then left overnight at 4 °C.

2.2.8 DNA extraction from the faecal samples

0.5 gr of each faecal sample was diluted 1:2 (vol/vol) with distilled water and then 1500 µl of this suspension was transferred in a reaction tube (12 ml). Under the hood, 108 µl of 1 M KOH and 30 µl 1M Dithiothreitol were added, thoroughly mixed and left for 30 min in a water bath at 65 °C. For neutralization, 270 µl 2 M Tris-HCl (pH 8.3) and 40.5 µl HCl were added to each sample. 1950 µl phenol-chloroform-isoamylalcohol was then mixed with each sample and centrifuged at 10,000 g for 10 minutes. From the upper watery phase, which contains the DNA, 1800 µl was transferred into a new 12 ml reaction tube. After the addition of 5400 µl binding buffer and 30 µl of a silica matrix the samples were incubated at 37 °C for 60 minutes with frequent agitation in a hybridization oven. After that samples were centrifuged for 30 seconds at 10,000 g, and 1000 µl binding buffer was added to the pellet after discarding the supernatant. This was then transferred into a new 12 ml reaction tube and centrifuged at 13,000 g for 30 seconds. The pellet was then washed 3 times with washing buffer. After drying at 35-40 °C in an oven to eliminate ethanol, 100 µl nucleic acid free water was added to the pellet and then incubated in a water bath at 50°C for 15 minutes to resuspend
DNA. After centrifugation at 13.000 g for 60 seconds, 85 µl of this solution were transferred in a new 1.5 reaction tube.

2.2.9 Determination of the DNA concentration in samples

4 µl of the sample was diluted with 200 µl nuclease free water. The concentration of the DNA in the samples was measured photometrically at 260 nm (nucleic acids) and also at 280 nm (proteins) to calculate the purity of the DNA.

2.2.10 Polymerase Chain Reactions

2.2.10.1 Cestode specific PCR (cs PCR)

A PCR with the primer pairs P60for and P375rev. was performed as described by Dinkel et al. (1998, 2004). The 50 or 100 µl reaction mixture consisted of 5 or 10 µl of the PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl), 5 or 10µl of MgCl₂ (2.5 mM), 1 or 2µl of deoxynucleoside triphosphate mixture each at a concentration of 200 µM, 2 or 4µl of each primer (20 or 40 pmol) and 0.25 µl of Ampli-Taq Polymerase (1.25 units). The amount of the added DNA was calculated at 200ng. The reaction volume was then completed to 50 or 100µl using nucleic acid free water. PCR was running for 40 cycles (denaturation for 30 s at 94 °C, annealing for 1 min at 55 °C and elongation for 30 s at 72 °C). Positive and negative controls were used to assess the quality of the PCR.

2.2.10.2 PCR assay specific for E. granulosus G1 (g1 PCR)

As described by Dinkel et al, (2004), PCR was performed in a 50 µl volume containing 5µl PCR buffer consisted of 5 µl of the PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl), 4µl of MgCl₂
(2 mM), 1 µl of deoxynucleoside triphosphate mixture each at a concentration of 200 µM, 2.5 µl of each of the primer pair ss1for and ss1rev. (each 25 pmol) and 0.25 µl of Ampli-Taq Polymerase (1.25 units.) The amount of the DNA was calculated at 200 ng. The reaction volume was then completed to 50 µl using nucleic acid free water. PCR was performed with 40 cycles (denaturation for 30 s at 94 °C, annealing for 1 min at 57 °C and elongation for 40 s at 72 °C). Positive and negative controls are used to assess the quality of the PCR.

2.2.10.3 PCR assay specific for *E. granulosus* G6/7 and *E. ortleppi* (g5/6/7 PCR, g6/7 PCR, g5 PCR)

For the first PCR (g5/6/7), the primer pair E.gcs1for and E.gcs1rev. was used (Dinkel *et al*, 2004). 50 µl volume containing 5 µl PCR buffer consisted of 5 µl of the PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl), 4 µl of MgCl2 (2 mM), 1 µl of deoxynucleoside triphosphate mixture each at a concentration of 200 µM, 2.5 µl of each of the primer pair ss1 and ss1 rev. (each 25 pmol) and 0.25 µl of Ampli-Taq Polymerase (1.25 units) The amount of the DNA was calculated at 200 ng. The reaction volume was then completed to 50 µl using nucleic acid free water. PCR was running for 40 cycles (denaturation for 30 s at 94 °C, annealing for 1 min at 57 °C and elongation for 40 s at 72 °C). Positive and negative controls are used to assess the quality of the PCR.

2.2.10.4 Semi nested PCRs specific for *E. ortleppi* and *E. granulosus* (G6/7)

Seminested PCR was performed to discriminate between *E. ortleppi* and *E. granulosus* G6/7. The reaction mixture of 50 µl
contained 1.5 µl of the positive amplification product from the g5/6/7 PCR, 5µl PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl), 4µl of MgCl₂ (2.mM), 1µl of deoxynucloside triphosopate mixture each at a concentration of 200 µM, 2.5µl of each of the primer pair Eg camel or cattle for and cs1 rev. (each 25 pmol) and 0.25 µl of Ampli-Taq Polymerase (1.25 units.). PCR was performed for 3o cycles (denaturation for 30 s at 94 °C, annealing for 1 min at 60 °C and elongation for 30 s at 72 °C).

2.2.11 Detection of the PCR products (Agarose gel electrophoresis)

2.2.11.1 Preparation of the gel
Agarose gel was prepared by melting 0.75 g of the Agarose powder in 50 ml 1x TBE buffer until solution is completely clear. After cooling the Agarose solution to about 50 °C it was poured into electrophoresis plate and mixed with 1µl ethidium bromide, comb was placed and left to solidify. The chamber was then filled with 1 x TBE buffer.

2.2.11.2 Preparation of the sample and running of the gel
10 µl of each of the PCR products was mixed with 2 µl loading buffer and loaded onto the Agarose gel. 100bp ladder as a marker was also loaded with the samples to determine the different band sizes. Samples were then allowed to be separated using a power of 5v/cm.
2.2.12 Visualization of the PCR products

The gel was photographed under ultraviolet light using a computerized documentation system (Bio-Profil®, Image Analysis Software).

2.2.13 Mitochondrial gene sequencing

As a reference method, the sequence of a part of the mitochondrial cox1 with primer pair 2575 (‘5 TTT TTT GGG CAT CCT GAG GTT TAT 3’) and 3021 (‘5 TAA AGA AAG AAC ATA ATG AAA ATG 3’; Bowles et al., 1992) and nad1 using primer pair JB11 (5 AGA TTC GTA AGG GGC CTA ATA 3) and JB12 (5 ACC ACT AAC TAA TTC ACT TTC 3; Bowles and McManus, 1993b) were obtained. Both PCRs were performed as described previously (Bowles et al., 1992; Bowles and McManus, 1993). After purification of the PCR products over QIAquick™ columns, cycle sequencing was performed with AB1 Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit using the corresponding PCR primers (forward and reverse) as sequencing primers. Cycling conditions (25 cycles) were: denaturation for 10 s at 94°C and annealing for 4 min at 60°C. Electrophoresis was carried out on the AB1 Prism 310 Genetic Analyzer. Nucleotide sequence analysis was made using the National Centre for Biotechnology Information BLAST programs and databases.

2.3 Used Chemicals

Agarose, Roth, Seakem, AGS
Dithiothreitol (DTT), Roth
DNA-Molecular weight marker (100bp ladder), Fermentas
EDTA (Ethylendiaminetetra Acetic Acid), Merck
Ethidium bromide (10mg/ml), Roth
Ficol 400, Pharmacia
Phenol-Chloroform-Isoamylalcohol (25:24:1), Roth
Proteinase K, Fermentas
Potassiumhydroxide, Merck
Tris pure (Tris-(hydroxylmethyl)amino methane), Biomol
Tris-HCl (Tris-( hydroxylmethyl)amino methane. HCl), Biomol

2.4 Puffers and solutions

Digestion Buffer

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<tr>
<td>EDTA</td>
<td>pH 8.0</td>
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<tr>
<td>NaCl</td>
<td></td>
</tr>
<tr>
<td>SDS</td>
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DNA-Loading Buffer (6 x)

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<tr>
<td>Xyleneanol FF</td>
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</tr>
<tr>
<td>Ficoll 400</td>
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</tr>
<tr>
<td>in 1x TBE</td>
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TBE Buffer (5 x)

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<td>108 g</td>
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<tr>
<td>Boric acid</td>
<td>55 g</td>
</tr>
<tr>
<td>0.5 M EDTA</td>
<td>40 ml</td>
</tr>
<tr>
<td>H2O</td>
<td>2L</td>
</tr>
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</table>
2.5 Enzymes
Taq-Polymerase, Ampli-Taq, Applied Biosystem.

2/6 Oligonucleotides
E.g ss1 for. 5' GTA TTT TGT AAA GTT GTT CTA '3
E.g ss1 rev. 5' CTA AAT CAC ATC ATC TTA CAA T '3
E.g cs1 for 5' ATT TTT AAA ATG TTC GTC CTG '3
E.g cs1 rev 5' CTA AAT AAT ATC ATA TTA CAA C '3
E.g camelfor 5' ATG GTC CAC CTA TTA TTT CA '3
E.g cattlefor 5' ATG GTC CAC CTA TTA TTT TG '3
P60 for5' TT AAG ATA TAT GTG GTA CAG GAT TAG
ATA CCC '3
P375 rev.5' GGT ACA CAC CGC CCG TCA CCC TCG GTT
'T3
All primers were synthesized by Roth.

2.7 Disposables
Pipette tips; Sarstedt and Eurolab companies
Petri dishes; Greiner and Sarstedt
Reaction tubes; 0.2 ml; Applied Biosystem
0.5 ml; and 1.5 ml Sarstedt
2.0 ml Eppendorf
12 ml Greiner
15 and 50 ml Greiner
2.8 Instruments

Centrifuge: Eppendorf centrifuge 5415D
Cold Centrifuge: Eppendorf Centrifuge 5417 R
Gel electrophoresis: Bio Rad Mini-Sub® cell
Geldocumentation system: Ltf- Labortechnik GmbH and Co.Kg
(Camera: CCD video camera module, Computer: Belina,
Prinetr: Mitsubishi ).
Hybridization oven: Selutec hyp 10-S, Steuer Electrolabor and
Umweltechnik.
Incubator: Memmert, Germany
Laminar flow: Heraeus Instrument, Germany
PCR thermocycler: Gene Amp PCR system 2400 Applied
Biosystem.
Pipettes: Eppendorf, autoclavables
Power Supply: Bio Rad Power PAC 300
Printer: DPU-414 thermal printer
Refrigerators: Bosch electronics
Spectrophotometer: Eppendorf Biophotometer
Votrex: Genie 2, Bender and Hober AZ, Zuerich, Switzerland
Waterbath: Julabo P
Chapter Three

Epidemiological and Biomolecular Study of Cystic Echinococcosis in Humans and Animals in Sudan

Abstract

A survey of cystic echinococcosis was conducted during the period from May 2001 to July 2003 in different parts of the Sudan. The prevalence rates in camel, cattle, sheep and goats examined in different states of the Sudan was found to be 59.8% (466/779), 6.1% (299/4893), 11.3% (1180/10422) and 1.9% (106/5565) respectively. The encountered number of cysts was 2387 in camel, 333 in cattle, 1514 in sheep and 108 in goats. Fertility rates were found to be 73.7%, 77%, 19% and 31.5% in camel, cattle, sheep and goat respectively. The favorite site for cysts in camels was the lung (1627/2387). The liver was found to be preferred site in cattle (206/333) whereas the peritoneum was the predilection site in sheep (1242/1514) and goats (53/108).

Strain characterization of E. granulosus in human and livestock population was described for the first time by using polymerase chain reaction amplification and sequencing technology. Even though we were able to detect E. ortleppi and sheep strain (G1) of E. granulosus in some samples, the camel strain (G6) of E. granulosus appears to be
the predominant strain causing cystic echinococcosis in humans and animals in Sudan as 533 of the 542 examined DNA samples were characterized as belonging to this strain. In this study, the sheep strain of E. granulosus was reported for the first time in Sudan in two samples of human origin and five samples of sheep origin.

Introduction

Echinococcosis is a cyclozoontic infection of a world wide distribution and a variable geographic incidence. The disease is caused by the larval cystic stage of the E. granulosus complex and it constitutes a serious health hazard with increasing incidence rates in many endemic areas of the world. Parasite transmission may occur in domestic, sylvatic or semi-domestic animal host cycles depending on the Echinococcus species (and strains) (Craig et al. 2003). The E. granulosus complex is known to exist as biological distinct taxa, which may vary in their infectivity to domestic animals and possibly to man (Thompson, 1986., McManus and Smyth, 1986). These species or strains vary in features such as morphology, biochemistry, physiology, pathogenicity, developmental patterns and infectivity to human and domestic animals (Eckert et al. 1989) with important implications for the epidemiology of hydatid disease.

The disease has been extensively studied in a number of different geographical areas and is now present in Asia, Africa, South and Central America and the Mediterranean region (McManus and Smyth, 1986). It is found to be common in parts of the United Kingdom, Europe and Australia (Cook, 1989; Schantz, 1990).
In the Sudan, studies on animal echinococcosis were conducted by several workers (Saad and Magzoub 1989a; Saad and Magzoub 1989b; Elmahdi, 1998; Omer et al., 2002; Elmahdi et al, 2004 and Omer et al, 2004). While the disease is most abundant in camel and cattle, sheep appear to play an unimportant role in the transmission cycle of echinococcosis. The prevalence rate of the disease in animals is high, thus providing high risk of the disease for human populations in different parts of the Sudan. However, no hospital records are currently available. The disease seems to be endemic in rural communities where people live in close contact with dogs (definitive host) and large numbers of livestock (intermediate host). Cystic echinococcosis was found in 2% (132/6728) of the human population in selected areas of southern Sudan (Njoroge, 2001) and in 0.33% (1 from 300) of villagers in central Sudan (Elmahdi et al, 2004). Forty cases of human hydatidosis were recorded in the National Health Institute, Khartoum, Sudan in 2001 (Personal communication).

Currently, there is no information available about the dominant strain of the parasite and its infectivity to human. The objective of the present investigation is to provide information about the epidemiology and the molecular characterization of the prevalent taxa of *E. granulosus* complex in human and animals in the Sudan.

**Materials and Methods**

1. **Epidemiological survey**

   Frequent visits were made to slaughterhouses in different representative areas of the Sudan (Central: Khartoum, Tamboul and Medani, Eastern: Kassala and Gedarif, Western: Darfur state and Southern: Juba, Malakal) during the period of May 2001 to July 2003.
A total number of 21659 animals (779 camels, 4893 cattle, 10422 sheep and 5565 goats) was examined. Different organs including lungs, liver, heart, spleen and kidneys of the slaughtered animals were thoroughly inspected and hydatidosis positive animals and the number of cysts encountered were recorded. Visits were also made to hospitals in the same vicinity to estimate the number of human cystic echinococcosis cases. Hydatid cysts or cyst-derived materials were obtained from 7 patients, admitted to Khartoum (5) and Juba medical teaching hospitals (2), for diagnosis of the disease. Theses samples were used for the molecular characterization.

Epidemiological surveys about the disease were not conducted in Eastern and Northern Sudan but some hydatid cyst samples from camels (8) and cattle (7) were obtained for DNA characterization.

2. Parasitological studies
Cysts or cyst materials were examined macro and microscopically. Fertility of the collected hydatid cysts was determined by detection of protoscolices in aspirated fluid samples. Sterile or calcified cysts were considered infertile.

Exemplary samples of intact cysts or cyst materials were preserved in 70% alcohol for DNA characterization.

3. DNA extraction
DNA was extracted from 527 ethanol preserved hydatid cyst samples (317 none calcified and 210 calcified) collected from camel (157, 50), cattle (62, 45), sheep (81, 60), goats (10, 55) and humans (7, 0) from different parts of central, western and southern Sudan during the epidemiological survey. Some samples from cattle (4, 3)
and camel (3, 5) were obtained from northern and eastern Sudan respectively for the purpose of DNA characterization.

DNA extraction was done as by Dinkel et al. (1998). Briefly, 500ul of hydatid fluid containing protoscolices were digested by lysing buffer containing 500 µl digestion buffer, 10 µl 1 M dithiothreitol and 60 µl proteinase K. The total nucleic acid was then extracted using the standard phenol-chloroform-isooamylalcohol (25:24:1) and precipitated with absolute alcohol. The DNA concentration was quantified using spectrophotometer at 260 nm wave length.

4. Polymerase chain reaction (PCR) and molecular characterization

Strain characterization was done using a previously described G5/6/7 polymerase chain reaction (G5/6/7 PCR) and G1 (g1 PCR) for the samples which were negative with the G5/6/7 PCR (Dinkel et al., 2004), that the PCR was performed in a 50 µl volume containing 5 µl PCR buffer consisted of 5 µl of the PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl), 4 µl of MgCl₂ (2 mM), 1 µl of deoxynucleoside triphosphate mixture each at a concentration of 200 µM, 2.5 µl of each of the primer pairs cs1forward and cs1reverse (each 25 pmol) for the first PCR and ss1for and ss1rev for the G1 PCR and 0.25 µl of Ampli-Taq Polymerase (1.25 units) The amount of the DNA was calculated at 200 ng. Positive and negative controls were used to assess the quality of the PCR. Samples which were positive with the G5/6/7 PCR when visualized on ethidium bromide gel were then subjected to a second semi-nested PCR amplification using the primer pair E.g.camel or E.g.cattle for and cs1rev to determine if the sample
is camel/pig or cattle strain of *E.granulosus*. Some calcified cysts were negative when tested with G5/6/7 PCR or G1 PCR, these samples were subjected to cestode specific PCR using the primer pair P60.for and P375.rev as described by Dinkel,, *et al.* (1998). When positive, 0.5-1 µl of this PCR product was used with the G5/6/7 or G1 PCRs.

5. Visualization of the PCR products
   The etidium bromide stained agarose gel was photographed under ultraviolet light using a computerized documentation system (Bio-Profil®, Image Analysis Software).

6. Mitochondrial gene sequencing
   For confirmation of the PCR results, sequencing of a part of the mitochondrial cox1 with primer pair 2575 and 3021 (Bowles *et al.* 1992) and nad1 using primer pair JB11 and JB12 (Bowles and McManus, 1993b) was performed
Results

1. Epidemiological survey

In Central Sudan, the prevalence rate of cystic echinococcosis in camels was found to be 55.6% (119/214). The total number of cysts was 360. These were primarily located in the lungs 84% (304/360) followed by the liver and spleen. No cysts were found in the heart and kidneys. In cattle and sheep, the prevalence rates were 20% (50/250) and 2.5% (10/400), respectively. The predilection site in cattle and sheep was the liver with a percentage of 77% and 60% respectively. Fertility was found to be 100% in cattle and 75% in camels whereas none of the cysts was found to be fertile in sheep (Table 1, Figures 1 and 2).

In Western Sudan, the highest prevalence rate was recorded in camels as 61.4% (347/565). In contrast, the prevalence rate in cattle, sheep and goats was found to be 5.2% (226/4318), 11.9% (1162/9727) and 1.9% (103/5523), respectively.

Predilection site was the lung in camel (1323/2018) and the liver in cattle (138/247), whereas mesentery was the predilection site in sheep (1242/1494) and goats (51/76). The fertility rate was 73.8% (1490/2018) in camel, 82.3% (186/226) in cattle, 19% (289/1494) in sheep and 32.4% (34/105) in goats (Table 2, Figures 3 and 4).

In Southern Sudan, the prevalence rate was 7% (23/325) in cattle, 3% (8/295) in sheep and 7% (3/42) in goat. Camels were not examined. Predilection site was the liver in cattle (82.6%), sheep (100%) and goats (100%). The fertility rate was 3% (8/23) in cattle,
whereas in sheep and goats the fertility was found to be 0% (Table 3, Figures 5 and 6).

2. Strain characterization
   The camel strain of *E. granulosus* was detected by polymerase chain reaction and sequencing of DNA samples extracted from hydatid cysts of camel, cattle and goat origin. The cattle strain was found in two samples of cattle from Eastern and Southern Sudan. The sheep strain (G1) was found in five DNA samples extracted from hydatid cysts of sheep origin in central Sudan.

   The camel (G6) strain was found in five DNA samples extracted from hydatid cysts of humans whereas the common sheep strain (G1) was found in two samples.

   Two calcified cysts of sheep origin were negative (Table 4, Figures 8, 9, 10 and 11).
Figures 1 and 2: Prevalence and fertility of hydatid cysts from camel, cattle, sheep and goats in central Sudan

Prevalence of hydatid disease in camel, cattle, sheep and goats in central Sudan

Fertility Percentage of hydatid cysts collected from camel, cattle and sheeps in central Sudan
Table 2: Prevalence of cystic echinococcosis in livestock in Western Sudan

<table>
<thead>
<tr>
<th>Examine\nd animals</th>
<th>Total number examined</th>
<th>Prevalence %</th>
<th>Total number of cysts</th>
<th>Fertility %</th>
<th>Predilection site</th>
<th>Cysts examined for genotype n (n fertile - n infertile)</th>
<th>Genotype (n)</th>
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<tbody>
<tr>
<td>Camel</td>
<td>565</td>
<td>61.4% (347/565)</td>
<td>2018 (73.8%)</td>
<td>1490/2018</td>
<td>Lung 1323/2018</td>
<td>120 (100-20)</td>
<td>G6 (120)</td>
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<tr>
<td>Cattle</td>
<td>4318</td>
<td>5.2% (226/4318)</td>
<td>247</td>
<td>186/247</td>
<td>Liver 138/247</td>
<td>60 (30-30)</td>
<td>G6(60)</td>
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<tr>
<td>Sheep</td>
<td>9727</td>
<td>12% (1162/9727)</td>
<td>1494</td>
<td>289/1494</td>
<td>Peritoneum 1242/1494</td>
<td>95 (55-40)</td>
<td>G6 (93)</td>
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<tr>
<td>Goats</td>
<td>5523</td>
<td>1.9% (103/5523)</td>
<td>105</td>
<td>34/105</td>
<td>Peritoneum 51/76</td>
<td>62 (10-52)</td>
<td>G6 (62)</td>
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Figures 3 and 4: Prevalence and fertility of hydatid cysts from camel, cattle, sheep and goats in western Sudan
Table 3: Prevalence of cystic echinococcosis in livestock in Southern Sudan

<table>
<thead>
<tr>
<th>Examined animals</th>
<th>Total number examined</th>
<th>Prevalence %</th>
<th>Total number of cysts</th>
<th>Fertility %</th>
<th>Predilection site</th>
<th>Cysts examined for genotype n (n fertile-n infertile)</th>
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<td>0</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cattle</td>
<td>325</td>
<td>7% (23/325)</td>
<td>23</td>
<td>34.7% (8/23)</td>
<td>Liver 19/23</td>
<td>20 (5-15) E. ortleppi (1) G6 (19)</td>
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<td>Sheep</td>
<td>295</td>
<td>2.7% (8/295)</td>
<td>8</td>
<td>0%</td>
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<td>8 (0-8) G6 (8)</td>
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<tr>
<td>Goat</td>
<td>42</td>
<td>7% (3/42)</td>
<td>3</td>
<td>0%</td>
<td>Liver 3/3</td>
<td>3 (0-3) G6 (3)</td>
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Figures 5 and 6: Prevalence and fertility of hydatid cysts from camel, cattle, sheep and goats in southern Sudan
Figure 7: Predilection sites of hydatid cysts in camel, cattle, sheep, and goats in different parts of Sudan.
Table 4: Strain characterization of hydatid cyst samples from camel, cattle, sheep, and goats from different parts of Sudan

<table>
<thead>
<tr>
<th>ANIMALS LOCATION</th>
<th>CAMEL</th>
<th>CATTLE</th>
<th>SHEEP</th>
<th>GOATS</th>
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<td>120 (G6)</td>
<td>60 (G6)</td>
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<td>62 (G6)</td>
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<td>4 (G6)</td>
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<td>Southern Sudan</td>
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<td>20, 19 (G6)</td>
<td>8 (G6)</td>
<td>3 (G6)</td>
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</table>
Figure 8: Visualization of the first amplified 254 bp PCR products from different cyst samples.

Lane MW: molecular weight marker, Lane 1: G5/6/7 DNA (positive control), Lane 2: negative control, Lane 3-11: DNA extracted from hydatid cyst samples from different representative animals.
Figure 9: Semi-nested PCR amplification of the 171 bp specific PCR product of the camel (G6) strain from the first 254 bp PCR product for the above gel.

Lane MW: molecular weight marker, Lane 1: *E. granulosus* (G6) strain DNA (positive control), Lane 2: negative control, Lane 3-11: DNA extracted from hydatid cyst samples from different representative animals.
Figure 10: Semi-nested PCR amplification of the 171 bp specific PCR product of the cattle (*E. ortleppi*) strain from the first 254 bp PCR product for the G5/6/7 PCR.

Lane MW: molecular weight marker, Lane PC: *E. ortleppi* DNA (positive control), Lane NC: negative control, Lane 1-2: DNA extracted from hydatid cyst samples from cattle from eastern and southern Sudan.
Figure 11: Visualization of the 254 bp G1 (sheep strain) PCR products from different cyst samples.

Lane MW: molecular weight marker, Lane PC: G1 DNA (positive control), Lane NC: negative control, Lane 1-6: DNA extracted from hydatid cyst samples from sheep and humans.
Discussion

Hydatidosis (cystic echinococcosis) is a highly endemic zoonotic disease in most of the Mediterranean countries, North Africa and Middle East (Matossian et al., 1977). The disease is of public health importance and requires both surgical and chemotherapeutic interventions (Ibrahim, 1990; El-Mufti et al., 1993).

In Sudan, several studies were conducted to investigate the epidemiology of this disease. Tola (1987) reported prevalence rates of 56.4%, 2.1% and 2.0% in camels, cattle and sheep respectively, whereas Saad and Magzoub (1989), reported prevalence rates of 48.6% and 38.4% in camel and cattle, respectively.

In the present study, hydatidosis was investigated in a total number of 21659 animals (779 camels, 4893 cattle, 10422 sheep and 5565 goats) in the Sudan. The highest rate of infection in the total examined number was encountered in camel (60%, 466/779) followed by sheep (11.3%, 1180/10422). The prevalence rate of cystic echinococcosis in camel was the highest compared with other examined animals in central and western Sudan (55.6% and 61.4% respectively). The number of cysts encountered in camels were high comparing with the other animals (2387). The high prevalence of infection in camels may be due to the fact that, in nomadic environments, camels are usually found in close contact with dogs (definitive host), thus providing infection to more susceptible livestock or human populations. The prevalence rate of the disease in sheep in Western Sudan was the highest comparing to its prevalence in the same animal in other investigated areas Sudan (19%).
Fertility rates of the cysts in camel and cattle in central Sudan was 75% and 100% respectively (Table 1) whereas in western Sudan it was found to be 73.8% and 75% respectively. However, the fertility rate in sheep and goats in western Sudan was 19% and 32.4% respectively. This fertility was reduced to 0% in both animals in central and southern Sudan. It is well documented that sheep and goats are unimportant hosts in the transmission cycle of cystic echinococcosis in the Sudan (Saad and Magzoub, 1989, Omer et al., 2002, Elmahdi et al., 2004). This could be attributed to the fact that sheep and goats are usually slaughtered at younger age before development of mature cysts. However, in the present study the animals (3-5 years) developed calcified or infertile cysts. This may be due to immunoresponcne of sheep to hydatidosis infection (Saad and Magzoub, 1989a; Omer et al, 2002), or due to genetic characteristics of the parasite strain or species (Elmahdi et al, 2004).

Postmortem inspection of all examined animals indicated that the predilection sites of hydatid cysts appeared to be the lung for camels, liver for cattle and peritoneum for sheep and goats.

Elkhawad (1978) reported the liver to be the predilection site of hydatidosis in sheep whereas camels were mostly affected at the lungs. This result is in disagreement with Dada et al (1981) who reported the pulmonary cysts to be common in sheep and goats.

It is not known yet to what extent epidemiological significant differences (such as host range, infectivity, virulence and drug susceptibility) reflect genetic heterogeneity within Echinococcus populations (Bowles et al, 1992). However, in Sudan, with the exception of the report on strain characterization of E. granulosus described by Dinkel et al, 2004, no information is currently available in this regard. The author reported the presence of camel strain in central Sudan, and
cattle strain of *E. granulosus* in 25% (2/8) of the samples obtained from cattle origin. In our study we detected by PCR and DNA sequencing twice the cattle strain in cattle in Eastern and Southern Sudan. The hydatid cyst samples obtained from camels and cattle in eastern and central Sudan and sheep in central Sudan were identified as camel (G6) strain. Five of the DNA samples extracted from human cysts were characterized as camel (G6) strain. The other two samples as well as five of the samples obtained from sheep in central Sudan were found to be G1 (sheep strain). It is obvious that the high prevalence and fertility rates of hydatid cysts in camel indicate the important role of camel for maintenance of hydatidosis in Sudan. This is in contrast to many other countries where the sheep strain of *E. granulosus* is the responsible strain for most of cystic echinococcosis. In the human population, the camel strain of *E. granulosus* was reported in Argentina, Iran, Kenya and Nepal (Dinkel *et al.*, 2004). In the present study, we report the first records of camel strain of *E. granulosus* in the Sudanese population as well as the presence of the sheep strain in human and sheep in Sudan. The link between genetically homogenous strains and features of epidemiological importance can be useful in implementation of control programs.

In conclusion, prevalence and strain characterization of *E. granulosus* for livestock and human population was described for the first time in Sudan using polymerase chain reaction and DNA sequencing technologies.
Chapter Four

Strain Characterization and Coprodiagnostic PCR of *Echinococcus. granulosus* in Central Sudan

Abstract

42 dogs shot as a part of the rabies control program in Tamboul and Rofa, central Sudan were autopsied and their intestinal contents were examined for the presence of *E. granulosus* worms. Faecal samples were taken as well for coprodiagnosis. The worm burden in positive dogs was determined using a dilution method and the harvested worms were characterized using G5/6/7 and G1 PCRs. From the 42 euthanized dogs, 12 (28.5%) were harboring *E. granulosus* worms. The worm burden was 22-80*10^3 in the positive dogs. All the DNA samples extracted from the worms were characterized as camel (G6) strain of *E. granulosus*. 83.3% (10/12) of the DNA extracted from the faecal samples collected from the 12 dogs which were found to be positive at necropsy were found positive with copro-PCR and the strain was characterized as the camel (G6) strain of *E. granulosus*. Two samples were considered inconclusive as there was no signal in the inhibition test. 93.3% (28/30) copro- DNA samples from the 30 samples collected from the dogs which were reported negative at necropsy were negative using copro-diagnostic PCR. The other two samples were positive and characterized as sheep (G1) strain of *E.granulosus*. This copro-PCR method is used for the first time in such a survey. The overall sensitivity of the test was found to be 100% as all the necropsy positive dogs were found to be positive using copro-PCR.

Introduction
Hydatidosis is a disease caused by cestodes of the *E. granulosus* complex which possesses a two host life cycle involving dogs as definitive hosts and different animals and humans as intermediate hosts. Hydatidosis in intermediate hosts results from accidental ingestion of tapeworm eggs passed into the environment with faeces from definitive hosts. The disease is of an economic importance in intermediate and aberrant intermediate hosts (Torgerson and Budke, 2003). Diagnosis of the disease in the definitive host is an important tool in establishing a successful control program especially in areas where there are a lot of stray dogs and uncontrolled slaughtering. Diagnosis of *E. granulosus* in dogs is difficult, because the eggs of all *Echinococcus* and *Taenia* species are morphologically indistinguishable from one another and the characteristic small segments of *Echinococcus* species may be absent from the faeces or can be easily overlooked. Methods like purgation with arecoline salts and examination of the small intestine at necropsy (Deplazes and Eckert, 1996) are not suitable for large scale epidemiological studies as they are time consuming, laborious and require special safety precautions. Serum antibody detection and copro-ELISA have been successfully employed for the diagnosis of *Echinococcus* spp. in dogs but the sensitivity of the former varied according to geographic region and the sensitivity of the latter depends on the worm burden (Craig *et al.* 2003). CoproPCR have been used for the detection of *E. multilocularis* in foxes (Bretagne *et al.* 1993, Dinkel *et al.*, 1998) and also for the detection of *E.granulosus* (Abbasi *et al.* 2003)

In this study we used for the first time the copro-PCR assay which was developed for the detection of *E. multilocularis* in foxes (Dinkel *et al.*, 1998) in a survey for the prevalence and strain characterization of *E. granulosus* in stray dogs in central Sudan.
Materials and Methods

1. Stray dogs
   As a part of rabies control program, a campaign was done in Central state (Tamboul and Rofa), central Sudan, for the purpose of controlling the increasing population of stray dogs at that area. The whole intestines (small and large intestines) of all the shot dogs (42) were securely removed avoiding hazards of contamination to the environment and workers.

2. Scaping
   Within 2-4 hours, the whole intestine of each individual animal was opened after securing its content by making tight double adjacent ligatures at both ends of the intestine. The mucosa was scraped with a scalpel. The intestinal contents and the mucosal scrapings were washed through an 80-mesh-per-inch brass sieve. The material retained by the sieve was then washed into a glass container and examined for worms with a hand lens. The number of worms recovered from each individual material was estimated using a dilution method. Faecal samples were collected from the rectum of each intestine (42 samples) and labeled.

3. Preservation of the faecal samples and harvested worms
   Harvested *E. granulosus* adult worms were preserved in 70% alcohol for further molecular characterization. Collected fecal samples were frozen at -20°C and later upon arrival in Germany at -80 °C for 3 days for the inactivation of eggs.
4. DNA extraction from *E. granulosus* adult worms

DNA was extracted from worm suspensions obtained from 12 dogs. After washing the worms three times from the alcohol using nucleic acid free water, DNA was extracted as previously described by Dinkel *et al* (2004). Briefly, 500 ul of hydatid fluid containing protoscolices were digested by lysing buffer containing 500 µl digestion buffer, 10 µl 1 M dithiothreitol and 60 µl proteinase K. The total nucleic acid was then extracted using the standard phenol-chloroform-isoamylalcohol (25:24:1) and precipitated with absolute alcohol.

5. DNA extraction from the faecal samples

DNA was extracted from the faecal samples as previously described by Dinkel *et al* (1989). Briefly, 0.5 g of the sample were diluted 1:2 (vol/vol) with distilled water. A total of 1500 of the resulting solution was used for the DNA extraction starting with a stage of alkaline hydrolysis using 108 of 1M KOH and 30 of 1 M dithiothreitol. After vortexing and incubation at 65° C for 30 min, the samples were neutralized with 270 µl of 2 M Tris-HCl (pH 8.3) and 40.5 µl of 25% HCl. 1950 µl phenol-chloroform-isoamylalcohol was then mixed with each sample. The aqueous phase was transferred to a new tube and the DNA was precipitated by the addition of 5400µl binding buffer and 30 µl of a silica matrix. The samples were then incubated at 37 °C for 60 minutes with frequent agitation centrifuged for 30 seconds at 10,000 g, and 1000 µl binding buffer was added to the pellet after discarding the supernatant. This was then transferred into a new 12 ml reaction tube and centrifuged at 13,000 g for 30 seconds. The pellet was washed 3 times with washing buffer. After drying at 35-40 °C in an oven to eliminate ethanol, 100 µl nucleic acid free water was added to the pellet and incubated in a water bath at 50°C for 15 minutes to re-suspend DNA.
After centrifugation at 13.000g for 60 seconds, 85 µl of this solution were transferred in a new 1.5 µl reaction tube.

6. Polymerase Chain Reactions (PCRs)

For the DNA extracted from the *E. granulosus* adult worms, strain characterization was determined using our previously described G5/6/7 polymerase chain reaction (G5/6/7 PCR) (Dinkel *et al* 2004). The PCR was performed in a 50 µl volume containing 5µl PCR buffer consisting of 5 µl of the PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl), 4 µl of MgCl$_2$ (2mM), 1 µl of deoxynucleoside triphosphate mixture each at a concentration of 200 µM, 2.5 µl of each of the primer pair cs1forward and cs1reverse (each 25 pmol) and 0.25µl of Ampli-Taq Polymerase (1.25 units) The amount of the DNA was calculated at 200 ng. Positive and negative controls were used to assess the quality of the PCR.

After visualization on ethidium bromide gel, samples were subjected to a second semi-nested PCR amplification using the primer pair E.g. camel or E.g. cattle for and cs1rev to determine if the sample is camel/pig or cattle strain of *E. granulosus*. For the DNA obtained from the faecal samples, first a cestode specific PCR was performed as described by Dinkel *et al* (1998, 2004). The 100 µl reaction mixture consisted of 10 µl of the PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl), 10µl of MgCl$_2$ (2.5 mM), 2µl of deoxynucleoside triphosphate mixture each at a concentration of 200 µM, 4 µl of each primer (20 or 40 pmol) and 0.25 µl of Ampli-Taq Polymerase (1.25 units). 10 µl of the DNA was added and the volume was completed to 100 using nucleic acid free water. PCR was running for 40 cycles (denaturation for 30 s at 94 °C, annealing for 1 min at 55 °C and elongation for 30 s at 72 °C). Positive and negative controls are used to assess the quality of the PCR.
In a second step, a nested G5/6/7 PCR was performed using 1,5 of the PCR products of the first PCR and consequently a seminested PCR were performed as previously described.

7. Visualization of the PCR products
The ethidium bromide stained agarose gel was photographed under ultraviolet light using a computerized documentation system (Bio-Profil®, Image Analysis Software).

8. Control for inhibition
Due to unknown factors present in some faecal samples, PCRs may occasionally be inhibited and may therefore give false negative results (Wilde et al., 1990). To control such inhibitions 10 µl of *E. granulosus* DNA was added to each negative sample and the first PCR was repeated (Dinkel et al.1998). The test sample was recorded as negative only if a signal was obtained, if not, the result was considered inconclusive.

9. Mitochondrial gene sequencing
The sequence of a part of the mitochondrial cox1 gene with primer pair 2575 and 3021 (Bowles et al. 1992) and nad1 gene using primer pair JB11 and JB12 (Bowles and McManus, 1993b) was performed.
Results

1. Necropsy

From the 42 euthanized dogs, 12 (28.5%) were harboring *E. granulosus* worms (table 1). The worm burden was 22-80*10^3 in the positive dogs.

2. PCR of the *E. granulosus* worms

All the DNA samples extracted from the worm suspension were characterized as camel (G6) strain of *E. granulosus* Fig (1, 2).

3. Copro-PCR

83.3% (10/12) of the Copro DNA extracted from the faecal samples collected from the 12 dogs which were found to be positive at necropsy were found positive with copro PCR and the strain was characterized as camel (G6) strain of *E. granulosus* samples (Table 1 and Fig 3,4,5). The result of the other two samples was considered inconclusive as there was no signal in the inhibition test. 93.3% (28/30) copro-DNA samples from the 30 samples collected from the dogs which were reported negative at necropsy were negative using copro-diagnostic PCR. The other two samples were positive and characterized as the sheep (G1) strain of *E. granulosus* (Table 1 and Fig 6).
Table 1: Necropsy and Copro-PCR results of the 42 dogs shot in central Sudan

<table>
<thead>
<tr>
<th>Necropsy</th>
<th>CoproPCR and strain characterization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive: n=12</td>
<td>Positiv: n=10 (G6)</td>
</tr>
<tr>
<td></td>
<td>PCR inhibited n=2</td>
</tr>
<tr>
<td>Negatives: n=30</td>
<td>Negative: n=28</td>
</tr>
<tr>
<td></td>
<td>Positive: n=2 (G1)</td>
</tr>
</tbody>
</table>
Figure 1: Visualization of the first amplified 254 bp PCR products from *E. granulosus* worms.

Lane MW: Molecular weight marker, Lane PC: *E. granulosus* positive DNA, Lane NC: DNA negative control, Lanes 1-12, DNA from *E. granulosus* worms
Figure 2: Semi-nested PCR amplification of the 171 bp specific PCR products of the camel (G6) strain from the first 254bp

Lane MW: Molecular weight marker, Lane PC: *E. granulosus* positive DNA, Lane NC: DNA negative control, Lanes 1-12, DNA from *E. granulosus* worms
Figure 3: Visualization of the first amplified 373bp PCR product from copro DNA.

Lane MW: Molecular weight marker, Lane PC: *E. granulosus* positive DNA, Lane NC: DNA negative control, Lanes 1-12, Copro-DNA.

Figure 4: Visualization of the 254bp G5/6/7 nested PCR from the first cestode specific PCR
Lane M: Molecular weight marker, Lane PC: *E. granulosus* positive DNA, Lane NC: DNA negative control, Lanes 1-12: CoproDNA.

Figure 5: Semi-nested PCR amplification of the 171bp specific PCR products of the camel (G6) strain from the G5/6/7 PCR
Lane MW: Molecular weight marker, Lane PC: *E. granulosus* positive DNA, Lane NC: DNA negative control, Lanes 1-12: CoproDNA.

**Figure 6:** Visualization of the amplified 254 bp G1 (sheep strain) PCR products from copro DNA.
Discussion

Sudan is the largest country in Africa and of the Arab world and it has a large livestock population. Preliminary data about the prevalence of hydatidosis in different intermediate hosts in Sudan revealed that the

Lane MW: Molecular weight marker, Lane PC: *E. granulosus* positive DNA, Lane NC: DNA negative control, Lanes 1-2: CoproDNA
disease occurs in all domesticated intermediate host species in Sudan (camel, cattle, sheep and goats) with the highest infection rates in camels (Elmahdi et al 2004, Omer et al., 2002 and 2004). Infection rates in humans are considerable. The study area is located in Central Sudan and considered the largest market for camel meat in the country. This area is inhabited by villagers who own dogs for the purpose of guarding. In most of the cases these dogs are fed on the offal obtained from the slaughterhouse. These are mostly hydatidosis infected camel lungs. This in turn ensures a continuous focus of echinococcosis in that area. In our survey we examined a number of 42 dogs, 12 of these (28.5%) were found to be positive at necropsy. From the data encountered about the prevalence of hydatidosis in camels in that area (55.6%) and the high rate of cyst fertility (Saad and Magzoub, 1988., Omer et al., 2002., Elmahdie et al., 2004), it was suggested that the infection rate in dogs should be high, however the relatively low prevalence rate reported here may be due to particular risk behaviours in dogs like having access to offal which can markedly differ according to the area and season (Macpherson et al. 1985., Wachira et al. 1994). The worm burden in these dogs was very high (22-80*10^3) and the worms were mostly mature. This may explain the high prevalence of the disease in intermediate hosts, because most of the animals are reared under open grazing conditions and dogs are always accompanying the herd. CoproPCR is used for the first time in such studies in Sudan. 10 of the faecal samples obtained from the necropsy of positive dogs were found positive using PCR and the strain was characterized as the camel strain. Thus, the sensitivity of this test is 100%. The result of the other two positive necropsy samples was considered inconclusive as the DNA was inhibited. From the 30 samples obtained from the dogs which were negative at necropsy, 2 samples were found to be positive using copro-PCR and they were characterized as
sheep strain (G1) of *E.granulosus*. This strain of *E.granulosus* has always
thought to be absent from Sudan since it was never detected in the
previous surveys about strain identification in Sudan (Omer *et al.*, 2004.,
Dinkel *et al.*, 2004). Nevertheless, we found the sheep strain in 1.3%
(7/542) of the samples that we have characterized from different
intermediate hosts in Sudan (Omer *et al.* 2006). The reason for this
apparent rarity of the G1 strain is not clear from the available
epidemiological data, and there is an urgent need for additional studies.

In conclusion, 100% of the DNA samples extracted from the fecal
samples obtained from the positive dogs at necropsy were also found
positive at PCR. Sensitivity of this coproPCR is 100%. Thus, integration
of such method in future surveys of the disease is very useful comparing
with the traditional methods concerning specificity, sensitivity, cost and
the processing time needed (Dinkel *et al.*, 1998).

Chapter Five

Experimental Infection of Dogs with Protoscolices of Camel
Origin and Molecular Characterization of the Maturing
Adult Worms Using PCR

ABSTRACT

For the purpose of this study, hydatid cysts were obtained from the
lungs of naturally infected camels (*Camelus dromedarius*) in Tamboul
slaughterhouse in central Sudan. Viable protoscolices were collected from these cysts and used for experimental infection of dogs at different doses. Ten dogs were divided into two groups (A and B) of five dogs each. Dogs in group A received a dose of $4 \times 10^3$ protoscolices whereas dogs in group B received a dose of $8 \times 10^3$ protoscolices each. Fecal samples were examined for patent infection during the study period. Dogs were necropsied at 45 dpi (group A) and 54 dpi (group B). No eggs were detected in fecal samples from group A throughout the experimental period (45 days). However, eggs were first demonstrated in faeces 52 dpi in group B. The experimental animals in both groups did not show any adverse clinical signs during the experimental study. 

*Echinococcus granulosus* worms were recovered from both groups at the time of necropsy.

Molecular characterization of the adult worms was made possible using the Polymerase chain reaction (PCR)-based detection assay. The worms were identified as G6 (camel) strain of *E. granulosus*. It was found that the prepatency period in dogs after experimental infection with protoscolices of camel origin is longer than the reported for other strains of *E. granulosus*. These are the first data on prepatency periods of the camel strain G6 in dogs confirmed by molecular characterization.
INTRODUCTION

Cystic echinococcosis or hydatidosis is an important cyclo-zoonotic parasitic disease caused by a specific tapeworm, *E. granulosus* which cycles in a predator/prey relationship between a carnivore (definitive host) and a herbivore (intermediate host). Humans can accidentally become infected by ingestion of eggs of the adult worm. The disease is common in open range livestock raising areas. In addition, uncontrolled slaughtering of meat-animals and the presence of large stray dog population also contribute towards the endemicity of the disease (Anderson *et al.*., 1997). The parasite causes serious public health problems in certain parts of the world including the Sudan (Saad and Magzoub, 1988). The camel is an important host of *E. granulosus* and is commonly infected throughout Africa and the Middle East (Schwabe, 1986). The camel has attracted much interest as an intermediate host of *E. granulosus*, particularly in its role as a reservoir of infection in humans (Eckert *et al.* 1989). In addition, it has been proposed that the camel form of *Echinococcus* may be a different subspecies or strain (Pandey *et al.* 1986). However, very little comparative work has been carried out to characterize isolates from *E. granulosus* from camels (Thompson and Lymbery, 1988).

The prepatent period of *E. granulosus* in camels was reported to be as early as 5 weeks in the definitive host (Eckert *et al.*, 1989). However, some workers reported the prepatent period to be as long as 12 weeks post infection (Saad and Magzoub., 1988). Currently, no information is available about the prepatent period in regard to the strain of the parasite. In a previous report, it has been suggested that different strains of the parasite may have different prepatent period in the final host (Eckert *et al.*, 1989). The morphological and biological characteristics of the
intestinal stages of *E. granulosus* of camel origin were previously studied by Eckert *et al.*, 1989 without performing the molecular characterization.

The objectives of the present study were to experimentally transmit infection to dogs using different doses of protoscolices of camel origin, and to determine the parasitological data of the infection using conventional methods as well as the strain characterization using polymerase chain reaction (PCR).
Materials and Methods

1. Experimental Animals
   Ten stray dogs (four males and six females) about 1-2 weeks –old were selected. Young dogs are usually easy to handle and are more susceptible to infection. The dogs were divided randomly into two groups (five dogs each). Dogs in each group were housed together and received milk, cooked meat, bread and water. The animals were subjected to parasitological examination to eliminate the possibility of helminth infection. The experimental animals received a total dose of $4 \times 10^3$ and $8 \times 10^3$ protoscolices for dogs in group A and B, respectively.

2. Infective Materials
   Hydatid cysts were obtained from camels in Tamboul slaughterhouse (Central Sudan). The cysts were collected from the lungs of the infected camels. Hydatid fluid containing protoscolices was aspirated with sterile needles. The protoscolices were then kept in clean sterile jars. The viability of the protoscolices was determined by eosin exclusion and observation for flame cell movement basically as described by Smyth et al., 1980. Viable protoscolices were counted individually with dilution method, mixed with milk and fed orally to the dogs at the prescribed dose.

3. Parasitological Parameters
   Fecal samples were collected from both groups and were examined every two days from day 28-35 post infection and daily from day 36 to 54 post infection for the presence of eggs of the parasite in feces. Conventional parasitological methods including both direct faecal
examination and centrifugal flotation methods (Soulsby, 1968) were used to examine the faeces for presence of proglottids or eggs.

4. Necropsy

Dogs in groups A and B were necropsied after euthanasia at days 45 and 54 (pi), respectively. The whole small intestine was removed. Tight double adjacent ligatures were made at both ends of the intestine to secure the contents. The intestine was then opened and the mucosa was scraped with a scalpel. The intestinal contents and the mucosal scrapings were washed through 80-mesh-per-inch brass sieve. The material retained by the sieve was then washed into a glass container and examined for worms with a hand lens. Adult worms were examined microscopically to determine the extent of development. Approximate worm counts were obtained by a dilution technique. Worms from each dog were preserved in 70% alcohol for molecular characterization.

5. Molecular characterization of the adult worms

Adult worms recovered from each dog in each group were washed thoroughly with nucleic acid free water by centrifugation to remove the 70% alcohol. DNA was then extracted from the washed worms according to the method described by Dinkel et al (1998). Briefly, the worms were homogenized and the homogenate was lysed using lysing buffer (500 µl digestion buffer, 10 µl 1 M dithiothreitol and 60 µl proteinase K). The total nucleic acid was then extracted using the standard phenol-chloroform-isoamylalcohol (25:24:1) and precipitated with absolute alcohol. The DNA concentration was quantified using spectrophotometer at 260 nm wave length. Strain characterization was determined using G5/6/7 polymerase chain reaction (G5/6/7 PCR) (Dinkel et al., 2004), that the PCR was performed in a 50 µl volume containing 5 µl PCR
buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl), 4 µl of MgCl$_2$ (2 mM), 1µl of deoxynucleoside triphosphate mixture each at a concentration of 200 µM, 2.5 µl of each of the primer pair cs1forward and cs1reverse (each 25 pmol) and 0.25 µl of Ampli-Taq Polymerase (1.25 units) The amount of the DNA was calculated at 200 ng. Positive and negative controls were used to assess the quality of the PCR. After visualization on ethidium bromide gel, samples were then subjected to a second semi-nested PCR amplification using the primer pair Eg.camel or E.g.cattle forward and cs1reverse to determine if the sample is camel/pig or cattle strain of *E. granulosus.*
RESULTS

1. Parasitological findings

Microscopic examination of the faecal samples collected from dogs in group A revealed that there were no eggs detected throughout the course of the study. The examination of the faecal samples from dogs in group B revealed the first detection of the eggs at day 52 d.p.i.

2. Necropsy

Dogs in group A were necropsied at day 45 d.p.i. The recovered number of worms was estimated to be $15-35 \times 10^2$ / animal (37.5%-87.5%). The infection was considered prepatent as none of the recovered worms were gravid. However, in group B the number of the recovered worms was estimated to be $50-75 \times 10^2$ (62.5%-93.8%). The infection in this group was considered patent as all harvested worms had gravid segments. (Table1).


Application of PCR to DNA extracted from adult worm resulted in amplification of a 254 bp PCR product. The semi-nested amplification produced a 171 bp PCR which is specific for G6 strain (camel strain) of *E. granulosus*. 
Table 1: Results of the experimental infection of dogs in groups A and B with protoscolices of camel origin.

<table>
<thead>
<tr>
<th>Group Number</th>
<th>Infection dose (Protoscolices)</th>
<th>Duration of the study period</th>
<th>The first appearance of eggs</th>
<th>No of recovered worms</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (n=5)</td>
<td>$4 \times 10^3$</td>
<td>45</td>
<td>-</td>
<td>$15-35 \times 10^2$</td>
</tr>
<tr>
<td>2 (n=5)</td>
<td>$8 \times 10^3$</td>
<td>56</td>
<td>52</td>
<td>$50-75 \times 10^2$</td>
</tr>
</tbody>
</table>
Figure 1: Visualization of the first amplified 254 bp PCR products from different worm samples.

Lane MW: molecular weight marker, Lane 1: G5/6/7 DNA (positive control), Lane 2: negative control, Lane 3-11: DNA extracted from *E. granulosus* worm samples from different animals
Figure 2: Seminested PCR amplification of the 171 bp specific PCR product of the camel (G6) strain from the first 254 bp PCR product for the above gel.

Lane MW: molecular weight marker, Lane 1: *E. granulosus* (G6) strain DNA (positive control), Lane 2: negative control, Lane 3-11: DNA extracted from *E. granulosus* worms from dogs in group A and B.
DISCUSSION

The camel population in the Sudan exceeds three million and is raised mainly in a belt north of 12°N latitude (Majid et al., 2001). The most densely populated areas are Kordofan, Eastern and Darfur states, followed by other regions in the Central and Northern provinces. The camels are owned by migratory pastoralists as a source of milk and meat as well as pack and riding animals. Extensive research has been conducted to evaluate the role played by Sudanese camels for transmission of parasitic diseases with special emphasis on diseases of public health importance. A few epidemiological studies were carried out to determine the prevalence of cystic echinococcosis in camels in Sudan. The prevalence rate of the disease in camel was found to be 48.69% (Saad and Magzoub, 1989), 56.6% (Omer et al., 2004) and (Elmahdi et al., 2004). In addition, studies were also conducted to determine the prepatent period of the parasite in dogs using protoscolices of camel origin. Saad and Magzoub (1988) found all of four puppies tested to be readily susceptible to infection with E. granulosus from camel in Sudan. In Egypt, El-Hafez Ahmed (1977) infected five 1.5-month-old puppies, of which only three harboured intestinal stages 6 days after infection. In the same country, three dogs were successfully infected with protoscolices of camel origin (Aba-Yazed, 1988). The prepatent period was reported to be ranging from 35-97 days in the definitive host (Slepnev et al., 1977; Saad and Magzoub, 1988). Eckert et al. (1989) reported the prepatent period of E. granulosus in puppies infected with protoscolices of camel origin to be 40 days. However, in the present study the experimentally infected dogs have successfully developed adult worms and the prepatent period was found to be 52 days. This is in agreement to the study conducted in Nyala, Western Sudan by Adam (2004) who reported the prepatent period...
to be 54 d.p.i. This result indicated the important role played by camel for maintenance of the life cycle of the parasite in Sudan. Despite the fact that all experimental dogs developed adult worm, only dogs in group B developed patent infection. In addition, very little information is available in regard to influence of the strain of the parasite on the prepatent period. The number of the harvested worms was extremely high compared with the infection dose in both groups (37.5%-87.5% in group A and 62.5%-93.8% in group B). It was suggested that protoscolices of camel strain would probably take longer time to produce patent infection in dogs. The molecular characterization study showed that the recovered worms were belonging to the G6 strain (camel strain) of *E. granulosus*. This should be the reason for the prolongation of the prepatent period in the experimental dogs. To advance beyond the current knowledge of the epidemiology of the disease, we propose that attempts should be made to conduct comparative experimental infection with echinococcosis in dog using protoscolices of different strains. We recognize that we had a small sample size (n=5 for each group), but we believe that the experiment supports the important role of the camel in the transmission cycle of echinococcosis in Sudan.

In conclusion, the scientific data presented in this study indicated that camel can serve as intermediate host for maintenance of the adult worm of the parasite in dogs. In addition protoscolices of camel origin take longer time to develop in dogs compared to other strains.

**Chapter Six**
General Discussion

Sudan is the largest country in Africa, with an area of 2.44 million square kilometers, extending from 4°N to 22°N. It has a population of about 25 million, mostly living in rural areas. Climatic condition is diverse, with average rain fall varying from less than 25 mm in the north to 1500 mm in the south. Sudan has the second largest animal population in Africa. In 1995 the livestock was estimated by 103 million head, of which 30 million were cattle, 37 million were sheep, 33 million goats and about 3 million camels. Western Sudan has the most livestock (40%), followed by southern Sudan (27%) and central Sudan (23%). The majority of breeds are well adapted to harsh environment and often trek long distances in search of feed and water. This trip is mostly accompanied by dogs as guard animals.

In this study we tried to examine the general feature of echinococcosis in both definitive and intermediate hosts. To determine the epidemiological status of the disease in different intermediate host, a survey was conducted in the period from May 2001 to July 2003 in parts of central, western and southern Sudan. The prevalence rates in camel, cattle, sheep and goats examined in different states of the Sudan was found to be 59.8% (466/779), 6.1% (299/4893), 11.3% (1180/10422) and 1.9% (106/5565) respectively. The encountered number of cysts was 2387 in camel, 333 in cattle, 1514 in sheep and 108 in goats. Fertility rates were found to be 73.7%, 77%, 19% and 31.5% in camel, cattle, sheep and goat respectively. The favorite site for cysts in camels was the lung (1627/2387). The liver was found to be predilection site for cyst in cattle (206/333) whereas the peritoneum was the predilection site in sheep (1242/1514) and goats (53/108). Other sites of infection were the liver and spleen.
From this result it is obvious that the higher prevalence rate of the disease was encountered in camels followed by cattle and this was also true regarding the fertility of cysts. Elkhawad et al. (1979) reported a prevalence rate of 35.3% in camels in central Sudan. Among the 141 camels examined at Tamboul area, central Sudan, 64 (45.4%) were harbouring hydatid cysts (Saad et al. 1983). Tola (1987) mentioned the prevalence rates of hydatid disease in camel and cattle as 56.4% and 2.1% respectively which were higher than that in sheep and goats. Even hydatid cysts were reported in sheep and goats in this study, but the fertility of the encountered cysts was very low. The prevalence rates of cystic hydatidosis in camel and cattle in western Sudan was found to be 79.5% and 6.4% in Nyala slaughterhouse in western Sudan (Mohamed and Elmalik, 2000). Abattoir survey in areas in central Sudan revealed the highest infection rate and fertility rate of hydatid cysts in camel and cattle. The prevalence rates and fertility percentages in sheep and goats was mentioned to be of no significance by all authors in Sudan that cysts encountered in sheep and goats were calcified or semicalcified (Elkhawad et al., 1979., Idris et al., 1985., Tola., 1987, Saad and Magzoub., 1989a and 1989b., Omer et al., 2002 and 2004 and Elmahdi et al., 2004).

These results may lead to the suggestion that in contrast to many parts of the Africa including parts of southern Sudan, Kenya and the countries of Maghreb, where sheep play the most important role in the cycle of hydatid disease (Macpherson and Wachira, 1997), camels are the most important animals in the life cycle of *E. granulosus* in Sudan. Although sheep in Kenya may be no more likely to be infected than those in central Sudan, with prevalence of 8.1 % recorded in the sheep of Masailand (Macpherson, 1985) and 3.6% in the sheep of Turkana district (Njoroge et al, 2002), they are far more likely to harbour fertile cysts that -50%-88% of Kenyan sheep cysts are fertile. The high level of fertility
seen in the cysts of Kenyan sheep is comparable with that seen in regions with much higher prevalences of sheep infection, such as Tunisia (Lahmar et al., 1999). Peru (Dueger and Gilman, 2001) and Kazakhstan (Torgerson et al., 2003).

The camel (Camelus dromedarius) is an important livestock species uniquely adapted to hot and arid environments. It produces milk, meat, wool, hair and hides, serves for riding and as a draft animal for agriculture and short distance transport. The camel is considered a browsing animal, but they acquire diseases which are transmitted by contaminated food or water such as fascioliasis and schistosomosis beside echinococcosis. This may be due to the fact that camels are changing their habitat (Majid et al. 2002).

In this study it was assumed that the prevalence rate in the human population should be high in areas with high prevalence rates in livestock and dogs, the disease should be particularly prevalent in rural communities where there is close contact between dogs and livestock, and the unhygienic conditions in some slaughterhouses should lead to higher infection rates. Human hydatidosis in Sudan was first reported by Christopherson (1909). Eisa et al (1962) reported the occurrence of hydatidosis as an endemic disease amongst Taposia tribe in Kapoeta district of the Equatoria Province in southern Sudan. It was reported that about half of the hydatidosis patients in Uganda were immigrants from southern Sudan (Ower and Bitakarmine, 1975). Studies about hydatidosis conducted in southern Sudan reported a prevalence ranging from 0.5%-3.5% in humans (Magambo et al. 1996). Data obtained about the prevalence of cystic echinococcosis in humans considered insufficient. This is because estimation of such data depends primarily on the evaluation of hospital records. Unfortunately, no such records exist, in any reliable form, in Sudan.
Strain characterization was conducted in a number of 542 hydatid cyst samples obtained from different intermediate hosts (camel, cattle, sheep, goats and humans) in different parts of the Sudan. The camel strain of *E. granulosus* was detected by polymerase chain reaction and DNA sequencing of all DNA samples extracted from hydatid cysts of camel, cattle and goat origin. The cattle strain (*E. ortleppi*) was found in two samples of cattle origin from eastern and southern Sudan. Sheep strain (G1) was found in five DNA samples extracted from hydatid cysts of sheep origin in central Sudan.

Camel (G6) strain was found in five DNA samples extracted from hydatid cysts of humans whereas as the common sheep strain (G1) was found in two samples. There is only one previous study about strain characterization of *E. granulosus* in Sudan (Dinkel et al., 2004). From 46 samples obtained from camels, cattle, sheep and goats in central Sudan, two samples of cattle origin were characterized as *E. ortleppi* whereas the other samples were characterized as camel strain (G6) of *E. granulosus*. Hydatid cysts samples of human origin are characterized for the first time in Sudan in this study. Camel strain (G6) was found in 71.4% (5/7) of the surgical obtained cysts. The infectivity of this strain to human was previously reported in sporadic cases from one case in Argentina (Roenzwit et al., 1999), two cases from Nepal (Zhang et al. 2000), two samples from Mauritania (Bardonnet et al., 2001) 3 of 33 samples from Iran (Harandi et al., 2002) and in 1 of 178 in Kenya (Dinkel et al. 2004). It is obvious that the percentage of human infection due to the camel strain of *E. granulosus* in Sudan is higher than in other countries. This is may be due to the fact that this strain is more prevalent and more fertile in Sudanese livestock ensuring the source of infection to the definitive host.
The sheep strain (G1) of *E. granulosus* was reported for the first time in Sudan in this study in both animals and humans. However, the fact that 7 of 542 from Sudanese samples of human and sheep origin belonged to this strain indicates that this strain may be one of the genotypes which are not predominant in the country. The prevalence of echinococcosis in stray dogs in selected area in central Sudan was determined by examining the intestinal contents of shot dogs at necropsy as well as by means of coprodiagnostic PCR. From the 42 dogs examined 12 (28.5%) were harboring *E. granulosus* worms. The worm burden was (22-80*10^3) in the positive dogs. This rate was lower than that reported by Saad and Magzoub (1986) in the same study area (central Sudan) as they stated that 51% of the dogs were found to be infected The prevalence rate of *E. granulosus* in dogs was found to be 86.5% in Kapoeta district in southern Sudan and 6% of 33 dogs in Khartoum (Eisa *et al.* 1962 and 1977). In 1985, the prevalence rate of *E. granulosus* in dogs in Khartoum was reported to be 17.5% (Idris, 1985). These variable figures about the infection rate of *E. granulosus* are certainly due to particular risk behaviours in dogs like access to offal which can markedly differ according to the area and season (Macpherson *et al*., 1985, Wachira *et al*., 1994).

CoproPCR was involved for the first time in Sudan in this survey, from the 12 dogs found positive at necropsy, 10 were found positive by PCR and the strain was characterized as camel (G6) strain. The result of the other two samples was considered inconclusive as the DNA extracted from the faecal samples obtained from these animals was inhibited. Hence the sensitivity of the test is considered 100%.

From the 30 dogs which where found negative at necropsy, two were positive using copro-PCR and the strain was characterized as sheep (G1) strain. This result may be considered normal when compared with
identified strains in the intermediate host. That as mentioned before, the camel strain was found to be the common strain in intermediate host and hence the available strain for dog infection. Characterization of sheep strain (G1) supported the suggestion that the sheep strain (G1) is present but to a lesser extent in Sudan.

For the determination of the prepatent period of *E. granulosus* of camel origin in experimentaly dogs, ten dogs were divided into two groups, five dogs each. The first group (A) received a dose of protoscolices $4 \times 10^3$ whereas the second one (B) received a total dose of $8 \times 10^3$ protoscolices. Fecal samples were examined for patent infection during the study period. No eggs were detected in fecal samples from group A through out the experimental period (45 days). However, eggs were first demonstrated in faces at day 52 pi in group B which was necropsied at day 54 pi. None of the groups showed any adverse reactions throughout the course of the infection. Previous studies to determine the prepatent period required by protoscolices of camel origin in experimentally infected dogs indicated that protoscolices of camel origin are infective to dogs. Saad and Magzoub (1988) found all of four puppies tested to be readily susceptible to infection with *E. granulosus* from camel in Sudan. In Egypt, El-Hafez Ahmed (1977) infected five 1.5-month-old puppies, of which only three harboured intestinal stages 66 days after infection. In the same country, three dogs were successfully infected with protoscolices of camel origin (Aba-Yazed, 1988). The production of fully developed egg in *E. granulosus* of camel origin was mentioned to be 54 days (Adam, 2004) which agree with our findings. Some strains of *E.granulosus* mature earlier like *E.ortleppi* which requires 35 days. *E.granulosus* of camel origin is distinct in various morphological features and does not conform to any described subspecies of *E. granulosus* (Eckert *et al.*, 1989) and hence the different and
relatively long prepatent period mentioned in *E. granulosus* of camel origin may be attributed to this difference.

*E. granulosus* worms harvested during this study were characterized by means of PCR and were reported to be camel (G6). This match well with our previous mentioned results that camel strain is the most abundant strain of *E. granulosus* in definitive and intermediate hosts in Sudan.

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