

HUMAN CYSTIC ECHINOCOCCOSIS IN SOUTH AFRICA

Benjamin Kgaile Mogoye

**Dissertation submitted to the Faculty of Health Sciences, University of the
Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of
Master of Science in Medicine.**

Johannesburg, May 2013

DECLARATION

I, Benjamin Kgaile Mogoye, declare that this dissertation is a result of my own work. It is being submitted for the Degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



(Signature of candidate)

31 May 2013

(Date)

DEDICATION

I would like to dedicate this dissertation to my parents; my loving mother Maserame M.E Mogoye and my late dear father, Ntsinyana 'Bra Zoo' Mogoye. Thank you for nurturing and moulding me into the person I am today. I salute you!

PUBLICATIONS AND PRESENTATIONS

Mogoye KB, Menezes CN, Grobusch M, Wahlers K, Frean J. Human cystic echinococcosis in South Africa (oral presentation). Proceedings of the Conference of the Southern African Centre for Infectious Disease Surveillance 'One Health'; 2011 Jul 14-15; National Institute for Communicable Diseases, Johannesburg.

Mogoye KB, Menezes CN, Grobusch M, Wahlers K, Frean J. Human cystic echinococcosis in South Africa (Abstract). Onderstepoort J Vet Res 2012;79(2). Available from: <<http://dx.doi.org/10.4102/ojvr.v7i2.469>>

Mogoye KB. Human cystic echinococcosis in South Africa. Presentation at the National Institute for Communicable Diseases Research Forum; 2012 Oct 15, Johannesburg.

Mogoye KB, Menezes CN, Wong ML, Stacey S, von Delft D, Wahlers K, et al. First insights into species and genotypes of *Echinococcus* in South Africa (In Press). Vet Parasitol, Apr 2013.

ABSTRACT

Cystic echinococcosis is a neglected parasitic zoonosis that is regarded as an emerging disease worldwide. Effective control of the disease is based on understanding the variability of *Echinococcus granulosus* (sensu lato), as genotypic characteristics may influence lifecycle patterns, development rate, and transmission. No molecular epidemiological research has previously been conducted to shed light on genotypes responsible for the disease in South Africa. To identify strains circulating in the country, parasite material was collected from patients between August 2010 and September 2012 and analyzed by PCR/RFLP methods. A total of 32 samples was characterized as *E. granulosus* (G1) (81%), *E. canadensis* (G6/7) (16%) and *E. ortleppi* (G5) (3%). Furthermore, two co-amplifying G6/7 genotypes were confirmed as G7 by sequencing. This is the first report on genotyping of *Echinococcus* species in South Africa, and, to our knowledge, the first report of the G5 and G7 genotypes from humans in Africa.

ACKNOWLEDGEMENTS

I would like to gratefully thank my supervisors Prof. John Freaan and Dr. Colin Menezes for the assistance and guidance received throughout the course of this research. Your critical inputs and advice have greatly helped enrich this work and have helped in my personal development as a young medical scientist.

I am very grateful to Dr. Dirk von Delft, a surgeon from the Eastern Cape Province for donating most of the samples for this research, and also wish to thank all patients who participated in this study and cardiothoracic surgeons from Charlotte Maxeke Johannesburg Academic Hospital; the meat inspectors from a Krugersdorp abattoir (Chamdor) for sample donation and the Parasitology Reference Laboratory, NICD, for providing facilities to conduct this research.

A special thanks to Dr. Marion Wasserman at Hohenheim University, Germany, for the training provided in molecular genotyping methods.

I also wish to thank Ms. Rindidzani Magobo, Nomsa Tau and Mrs. Anastasia Trataris-Rebisz for their kind assistance with the analysis and interpretation of DNA sequences, Mrs. Desiree du Plessis for her assistance with Reference Manager Software and Mrs. Lisa Ming Sun for assisting with proofreading of the dissertation.

Lastly, thank you to the German Research Foundation (DFG), project KE 282/7-1 and RO 3753/1-1, within the framework of CESSARi (Cystic Echinococcosis in Sub-Saharan Africa Research Initiative), for funding this research.

TABLE OF CONTENTS

	Page
DECLARATION.....	ii
DEDICATION.....	iii
PUBLICATIONS AND PRESENTATIONS.....	iv
ABSTRACT.....	v
ACKNOWLEDGEMENTS.....	vi
TABLE OF CONTENTS.....	vii
LIST OF FIGURES.....	x
LIST OF TABLES.....	xi
NOMENCLATURE.....	xi
CHAPTER 1 - INTRODUCTION.....	1
1.1 <i>Echinococcus</i> species.....	1
1.1.1 The parasite.....	1
1.1.2 Lifecycle.....	3
1.1.3 Taxonomy.....	6
1.2 Cystic echinococcosis/hydatid disease.....	9
1.2.1 Clinical manifestations.....	9
1.2.2 Diagnosis.....	11
1.2.3 Treatment.....	13
1.2.4 Prevention/control.....	14
1.3 <i>Echinococcus granulosus</i> (sensu lato) epidemiology.....	20
1.3.1 Characteristics of various strains/ variants.....	20
1.3.1.1 <i>E. granulosus</i> sensu stricto (G1-G3).....	20
1.3.1.2 <i>E. ortleppi</i> (formerly G5).....	21

1.3.1.3 <i>E. equinus</i> (formerly G4).....	21
1.3.1.4 <i>E. canadensis</i> (G6-G10).....	22
i. Camel strain (G6).....	22
ii. Pig strains (G7 and G9).....	23
iii. Cervid strains (G8 and G10).....	23
1.3.1.5 <i>E. felidis</i> (former lion strain).....	23
1.3.2 Molecular genotyping methods.....	24
1.3.3 The epidemiological situation in South Africa.....	26
1.4 Study aims.....	28
CHAPTER 2 - MATERIALS AND METHODS.....	30
2.1 Patient recruitment and sample collection.....	30
2.1.1 Patient recruitment from the collaborative study.....	30
2.1.2 Sample collection from other sources.....	31
2.1.2.1 NHLS Laboratory Information System (LIS).....	31
2.1.2.2 Individual donors.....	31
2.1.2.3 Abattoirs.....	33
2.2 DNA extraction, PCR and product sequencing.....	33
2.2.1 DNA extraction	33
2.2.2 <i>nad1</i> PCR/RFLP.....	34
2.2.2.1 Primary PCR (1073-1078 bp).....	34
2.2.2.2 Nested PCR.....	35
2.2.2.3 Restriction Fragment Length Polymorphism (RFLP).....	36
2.2.3 12S rRNA PCR.....	36
2.2.3.1 Cestode-specific PCR (373 bp fragment).....	36
2.2.3.2 PCR assay specific for <i>E. granulosus</i> G1-G3 (254 bp).....	37
2.2.3.3 PCR assay specific for <i>E. canadensis</i> G6/7 and <i>E. ortleppi</i> (254 bp).....	37
2.2.3.4 Semi-nested PCR (171 bp fragment).....	38
2.2.4 Gel electrophoresis.....	39
2.2.5 Sequencing of PCR products.....	39
2.3 Microscopic diagnosis.....	40
2.4 Data analysis.....	40
2.4.1 Alignment and editing of sequences.....	40
2.4.2 NCBI-BLAST.....	40
2.4.3 Statistical analysis.....	40
2.5 Ethical clearance.....	41

CHAPTER 3 - RESULTS	42
3.1 Patient recruitment and sample collection	42
3.2 Organ involvement	44
3.3 Influence of genotypes on cyst localization	45
3.4 Genotype distribution by province	45
3.5 DNA extraction, PCR and product sequencing	46
3.5.1 DNA extraction	46
3.5.2 <i>nadI</i> PCR/RFLP	47
3.5.3 12S rRNA PCR	48
3.5.4 Sequencing of PCR products	50
3.6 Microscopic diagnosis	51
3.6.1 Fertility status of cysts	51
3.6.2 Viability investigation	52
3.6.3 Cattle isolate	52
CHAPTER 4 - DISCUSSION	54
4.1 Genotype characteristics	55
4.1.1 <i>E. granulosus</i> (G1)	55
4.1.2 <i>E. canadensis</i> (G6/7)	55
4.1.3 <i>E. ortleppi</i>	56
4.1.4 <i>E. equinus</i>	57
4.2 Genotype vs. organ of cyst localization	57
4.3 Genotype distribution by province	59
4.4 Diagnostic methods	59
4.4.1 Microscopy	59
4.4.2 Comparison of <i>nadI</i> PCR/RFLP to 12S rRNA PCR method	60
CHAPTER 5 - CONCLUSIONS	62
CHAPTER 6 - RECOMMENDED FUTURE WORK	65
APPENDICES	66
REFERENCES	88

LIST OF FIGURES

Figure	Page
Figure 1.1 <i>Echinococcus granulosus</i> (sensu lato) tapeworms of cattle and sheep origin showing differences in size and maturation.....	2
Figure 1.2 Diagrammatic representation of the <i>E. granulosus</i> metacestode.....	3
Figure 1.3 A typical domestic lifecycle of <i>Echinococcus granulosus</i> (sensu lato).....	5
Figure 1.4 Phylogenetic tree showing genetic relationships of <i>Echinococcus</i> species.....	9
Figure 1.5 Pictures showing lung cyst on chest radiograph; macroscopic appearance of the <i>E. granulosus</i> cyst in liver tissue and protoscolices under a light microscope... 12	
Figure 1.6 Cycles supporting the major species and/or strains of <i>Echinococcus granulosus</i> (sensu lato), showing major definitive and intermediate hosts and other hosts that might be involved.....	18
Figure 2 Viable and non-viable protoscolices after 5 min eosin staining.....	32
Figure 3.1 Provenance of hydatid cyst samples recruited from 3 provinces, South Africa, 2010-2012 (n = 36).....	44
Figure 3.2 Organ distribution of cyst localization	44
Figure 3.3 Comparison of <i>Echinococcus</i> genotypes by number and organ localization of the cyst.....	45
Figure 3.4 The frequency distribution of <i>Echinococcus</i> genotypes by province.....	46
Figure 3.5 RFLP-PCR showing examples of the <i>nad 1</i> gene digested with restriction enzyme.....	47
Figure 3.6 Flow diagram summarizing <i>nad1</i> PCR/RFLP results.....	48
Figure 3.7 Flow diagram summarizing 12S rRNA PCR results.....	49
Figure 3.8 Gel electrophoresis showing an example of <i>E. granulosus</i> (G1-G3) specific PCR.....	50
Figure 3.9 Microscopic image of viable <i>Echinococcus ortleppi</i> protoscolices from a cow sample.....	53

LIST OF TABLES

Table	Page
Table 1 A summary of <i>Echinococcus</i> species/strains showing current taxonomic status	8
Table 3.1 Organ involvement by hydatid cysts	43
Table 3.2 Comparison of PCR and sequencing results	51
Table 3.3 Fertility status of <i>Echinococcus</i> genotypes.....	52

NOMENCLATURE

<i>ACT II</i>	Actin 2 gene
<i>AgB-I</i>	Antigen B gene
BLAST	Basic local alignment search tool
bp	Base pair
CE	Cystic echinococcosis
CNS	Central nervous system
<i>COX I</i>	Cytochrome c oxidase I gene
dNTP	Deoxyribonucleotide phosphate
EC	Eastern Cape Province
ELISA	Enzyme-linked immunosorbent assay
FS	Free State Province
G1 – G10	Genotype 1 to genotype 10
GP	Gauteng Province
<i>Hbx 2</i>	Homeodomain protein 2 gene
IMQAS	International meat quality assurance
IHA	Indirect haemagglutination assay
KZN	KwaZulu-Natal Province
LIS	Laboratory information system
mtDNA	Mitochondrial DNA
<i>nad1</i>	NADH dehydrogenase subunit 1

NCBI	National Center for Biotechnology Information
NHLS	National Health Laboratory Service
NICD	National Institute for Communicable Diseases
NTD	Neglected tropical disease
OIA	World Organisation for Animal Health
PAIR	Puncture, aspiration, injection, re-aspiration
PCR	Polymerase chain reaction
PRL	Parasitology Reference Laboratory
RFLP	Restriction fragment length polymorphism
Sensu lato	In the broad sense
Sensu stricto	In the most strict sense
spp.	species
UV	Ultraviolet
vs.	Versus
WHO	World Health Organization

CHAPTER 1 - INTRODUCTION

1.1 *Echinococcus* species

1.1.1 The Parasite

Echinococcus species are small cestodes (tapeworms), measuring approximately 2-7 mm in length and typically consist of a scolex and three segments, and have a rostellum surrounded by large and small hooks (Figure 1.1) (1-3). The metacestode stage of *E. granulosus* (sensu lato) is responsible for the disease called cystic echinococcosis (CE) or hydatid disease, that is regarded as a serious parasitic zoonosis and an emerging disease worldwide (4). The metacestode consists of a fluid-filled sac (Figure 1.2), with an outer laminated layer and an inner nucleated germinal layer, which gives rise to brood capsules by asexual budding; protoscolices arise from the germinal layer and from the inner wall of the brood capsules (5).

The species are classified in the Phylum Platyhelminthes, Class Cestoda, Order Cyclophyllidea, and Family Taeniidae (2;3;5). Apart from *E. granulosus* (sensu lato), which is the primary subject of this review, other species in the *Echinococcus* genus are *E. multilocularis*, *E. oligarthrus*, *E. vogeli* and *E. shiquicus*, causing either alveolar or polycystic echinococcosis disease (6;7). Of all forms of echinococcosis occurring in humans, only *E. granulosus* and *E. multilocularis* are the most important species due to their wide geographic distribution and their medical and economic impact (6;8). The name *E. granulosus* was first derived from the early work that was done by A. Batsch in 1786, although the classical description was given by H. Vogel in 1957 (9).

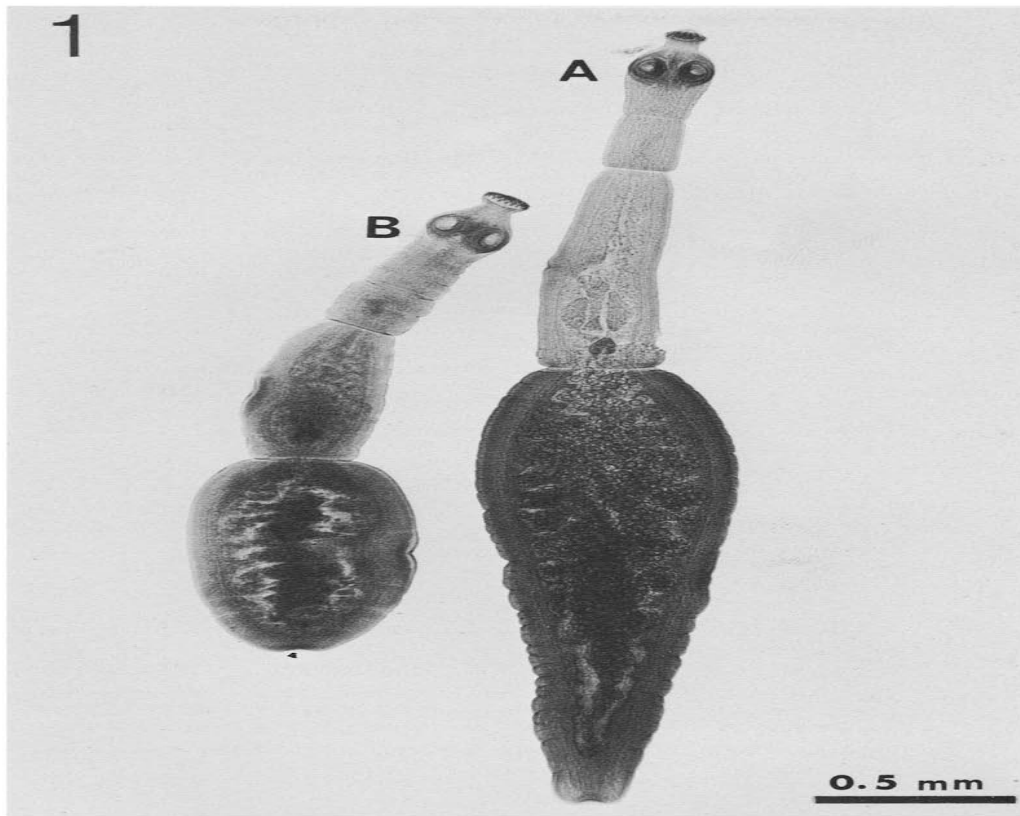


Figure 1.1 Comparison of adult 35-day-old *E. granulosus* of Swiss cattle origin (A) and Australian sheep origin (B). Specimens shown represent the most advanced stages of maturation attained in dogs. Note differences in size, particularly the length of the terminal segment in relation to the rest of the worm. In (B), the uterus in the terminal segment is only partially dilated and contains developing, unshelled eggs, whereas in (A), the uterus is fully dilated and contains fully developed thick-shelled eggs. Source: Thompson *et al*, 1984 (10).

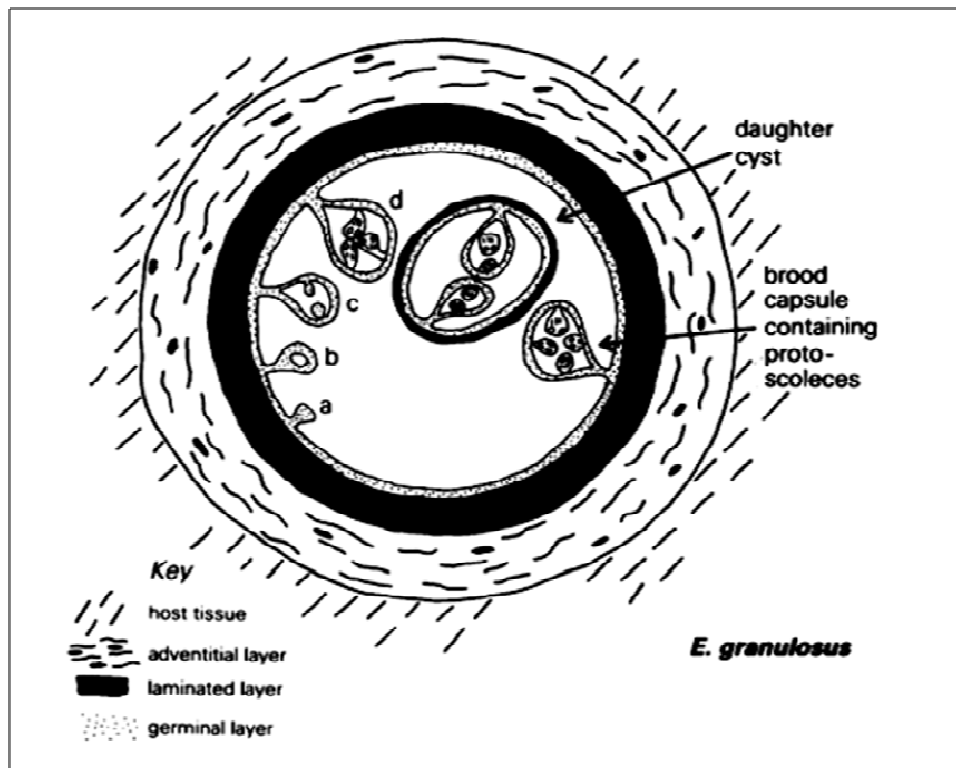


Figure 1.2 Diagrammatic representation of the *E. granulosus* metacestode. Source: Eckert *et al*, 2001 (5).

1.1.2 Lifecycle

The *Echinococcus* organisms have a biphasic propagation manner comprising a sexual reproductive phase in the hermaphroditic adult worms and an asexual proliferation of protoscolices in the larval stage (11). The adult tapeworms reside in the small intestines of definitive hosts (usually dogs) while the hydatid cyst (metacestode) develops in internal organs of the intermediate hosts (8). The adult tapeworm releases eggs that are passed with faeces and the intermediate hosts (sheep, cattle, goats, pigs, etc.) become infected when grazing in faecally-contaminated areas. Upon ingestion of tapeworm eggs, they hatch and release invasive oncospheres (hexacanth embryos) that penetrate the gut wall, gain access to

the circulation, and lodge in organs or tissues as metacestodes. Protoscolices develop within the metacestode and these must be consumed by a definitive host to complete the lifecycle (8). The dog (definitive host) usually starts shedding gravid proglottids or parasite eggs in faeces within 4-6 weeks of infection, and the detached proglottids may perform rhythmic contractions and relaxations which assists in egg expulsion in the environment (12;13). It was also shown that flies (and possibly other insects) may mechanically get contaminated from dog faeces and transport tapeworm eggs over considerable distances whilst feeding or laying eggs (14).

Humans serve as accidental intermediate hosts, acquiring infection through ingestion of faecally-contaminated material, and the metacestode development is similar to that of other intermediate animal hosts (Figure 1.3).

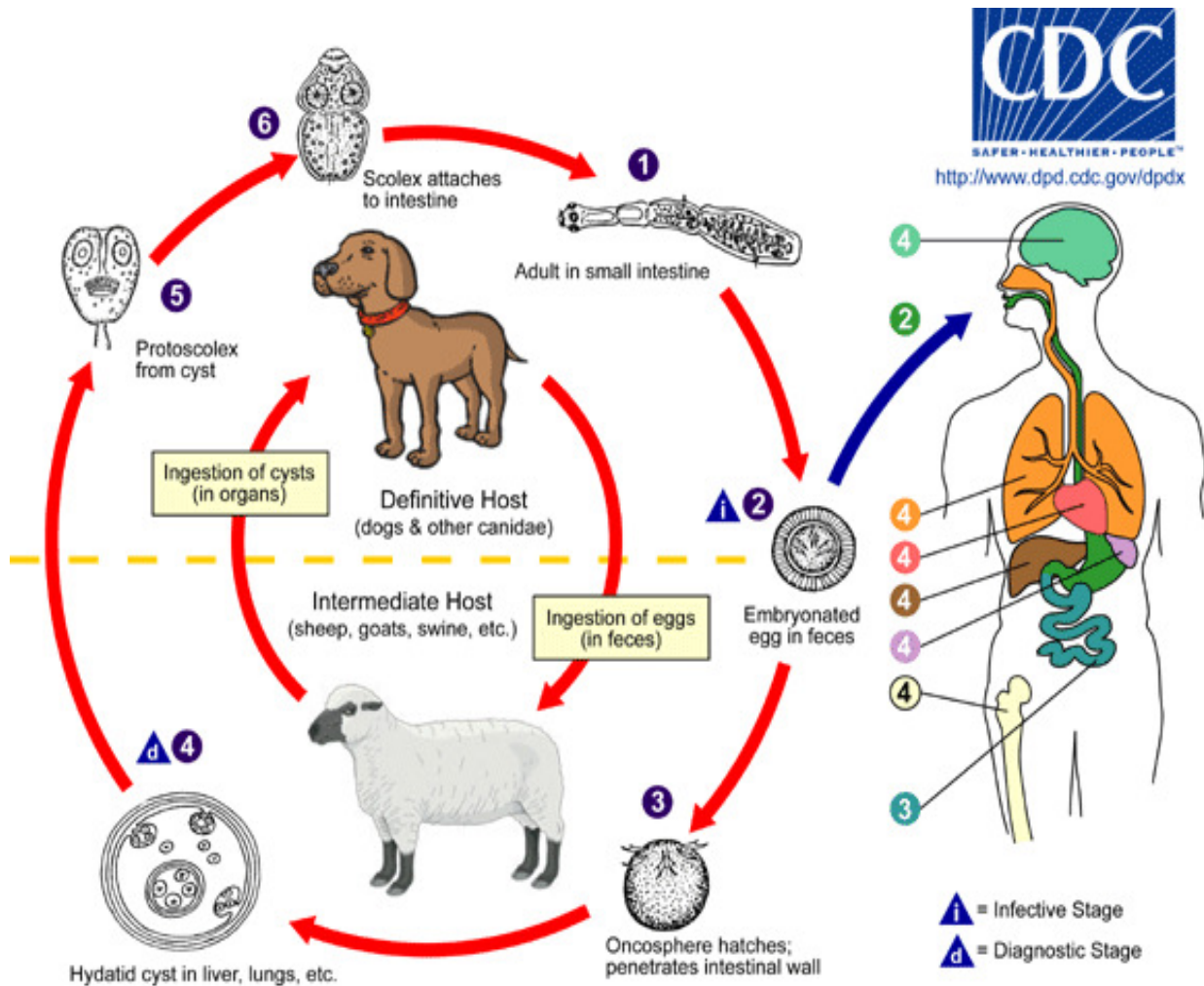


Figure 1.3 A typical domestic lifecycle of *Echinococcus granulosus* (sensu lato). Source: Centers for Disease Control and Prevention (15).

The lifecycle of the parasite can either be maintained in a synanthropic cycle involving dogs and domestic ungulates or in a sylvatic cycle involving wild caniid carnivores as definitive hosts and wild ungulates as intermediate hosts (16). Epidemiologically, it is important to be aware that synanthropic and sylvatic lifecycles can be independent or inter-linked, resulting in ‘spill-over’ situations as some strains are infective in both cycles (16). It is believed that the ancestral form of *E. granulosus* originated from the sylvatic cycle involving wolves as

definitive hosts and cervids (North American and Eurasian moose and reindeer) as intermediate hosts, with the domestic form of *E. granulosus* evolving from cervids and adapting to domestic ungulates with the development of animal husbandry (5).

Domestic transmission of the infection is usually through deliberate feeding of infected livestock or wildlife offal to dogs or through dogs scavenging carcasses of infected intermediate hosts. Transmission in the wild is usually through predator/prey relationships (8).

1.1.3 Taxonomy

The classification of causative agents of CE has been a controversial subject for many years. This taxonomic uncertainty was largely due to the limitations of morphological descriptions and lack of evidence for geographical or ecological segregation of the parasites (9;17;18). Although the classification of most variants of the parasite was mainly based on host-parasite specificity characteristics, they were nonetheless mainly grouped within the *E. granulosus* 'complex' and regarded as synonyms of *E. granulosus* (19-21).

This intraspecific variation within the *E. granulosus* 'complex' has epidemiological significance, as variants of this parasite have been shown to develop at different rates in the definitive host, thus affecting the timing of administering antihelmintic drugs in dog deworming programs (10;18;22). Given the international efforts aimed at controlling the spread of the disease, an informal nomenclature was needed to reflect the phenotypic

variability between host-derived populations of *E. granulosus* in different geographic locations (9).

Thus, the 'strain' concept was developed. This was defined as variants that differ statistically from other groups of the same species in gene frequencies, and in one or more characters of actual or potential significance to the epidemiology and control of echinococcosis (23). This variability may be reflected in characters which affect the lifecycle pattern, host specificity, development rate, pathogenicity, antigenicity and sensitivity to chemotherapeutic agents, transmission dynamics, epidemiology and control of echinococcosis (5).

Although historically, the characterization of *E. granulosus* strains/species relied mainly on morphologic techniques in combination with biological, epidemiological, biochemical and other features, the development and improvement of molecular methods has given more legitimacy to the strain concept. These molecular tools have shown evidence of genetic differences correlating to phenotypic variability within the isolates of *E. granulosus* 'complex' from different intermediate hosts (11;16;18;24).

The recognition of host-adapted strains and development of molecular methods eventually led to the development and confirmation of 11 genetically-distinct strains of *E. granulosus*, designated G1-G10, and the 'lion strain' (6;7;25-27). These strains were originally named after the intermediate host thought to be most important in perpetuating the lifecycle, and are still referred to as such by some researchers in the literature. G1 is generally referred to as the sheep strain, G2 as the Tasmanian sheep strain, G3 as the buffalo strain, G4 as the horse

strain, G5 as the cattle strain, G6 as the camel strain, G7 and G9 as the pig strains, and G8 and G10 as the cervid strains.

Table 1 A summary of *Echinococcus* species/strains, showing current taxonomic status.
Source: Hüttner and Romig, 2009 (7).

(Taxa recorded from sub-Saharan Africa are highlighted in bold)			
Conventional taxonomy Species	Strain	Genotype	Molecular taxonomy* Species
<i>E. granulosus</i>	Sheep strain	G1	<i>E. granulosus sensu stricto</i>
	Tasmanian sheep strain	G2	
	Buffalo strain	G3	
	Horse strain	G4	<i>E. equinus</i>
	Cattle strain	G5	<i>E. ortleppi</i>
	Camel strain	G6	<i>E. canadensis</i>**
	Pig strain	G7	
	Cervid strain	G8	
	?	G9	
	Fennoscandian cervid strain	G10	
	Lion strain	—	<i>E. felidis</i>
<i>E. multilocularis</i>			<i>E. multilocularis</i>
<i>E. shiquicus</i>			<i>E. shiquicus</i>
<i>E. oligarthra</i>			<i>E. oligarthra</i>
<i>E. vogeli</i>			<i>E. vogeli</i>

* Combined from Nakao *et al.* (2007) and Hüttner *et al.* (2008).
** The camel and pig strain might be split from *E. canadensis* as a separate species (Thompson, 2008; Saarma *et al.* 2009).

These ‘strains’ have since been assigned to a number of distinct species based on an increasing amount of genetic information (Figure 1.4) (11;17;18;24;28;29); and are now split into five species, namely *E. granulosus sensu stricto* (G1-G3); *E. equinus* (G4); *E. ortleppi* (G5); *E. canadensis* (G6-G10) and *E. felidis* (Table 1). There have also been suggestions to

remove the camel, pig and cervid strains from *E. canadensis*. However, molecular characterization of more isolates from different geographic areas will be needed (18).

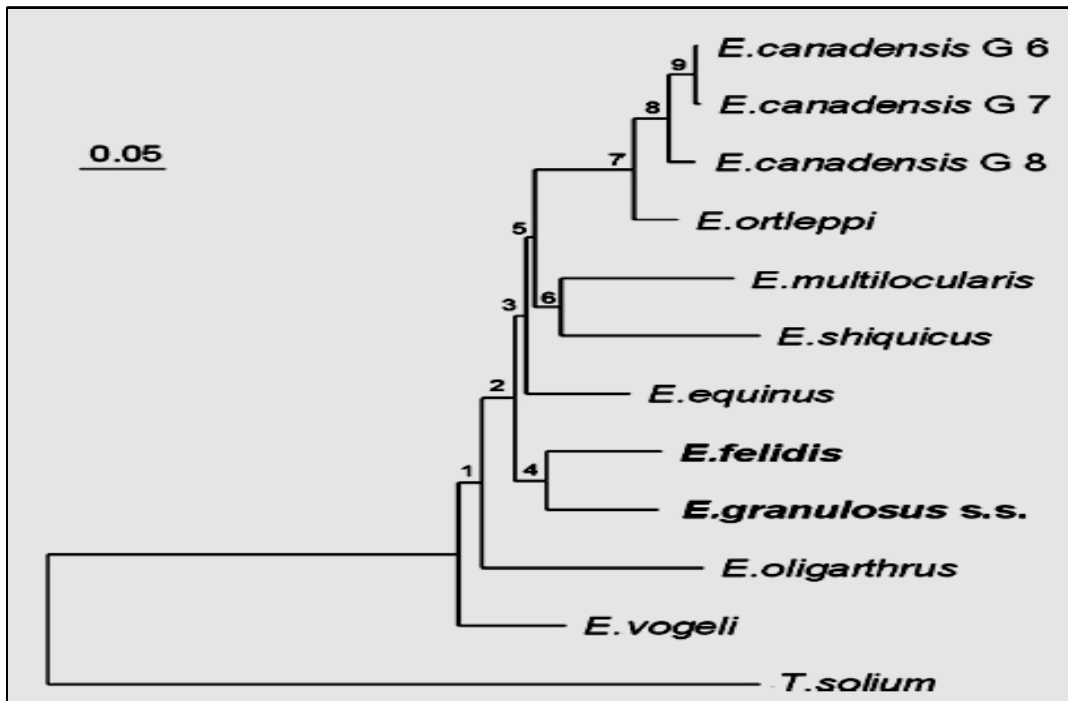


Figure 1.4 Phylogenetic tree showing genetic relationships of *Echinococcus* species. Source: Hüttner *et al*, 2008 (24). The scale bar represents the estimated number of nucleotide substitutions per nucleotide site. The sister species relationship between *E. felidis* and *E. granulosus* sensu stricto is shown in boldface.

1.2 Cystic echinococcosis/ hydatid disease

1.2.1 Clinical manifestations

The *Echinococcus* species are of medical and veterinary importance, since infection with the metacestode can cause severe illness (even death of the intermediate host) and livestock-associated production losses (4;8;30;31). Clinical manifestations of the disease in intermediate hosts primarily depend on the size, location and number of cysts (5;8;12;13).

Patients can remain asymptomatic for months, years, and even longer, with clinical symptoms showing from below one year to over 75 years of age in all sexes (5;12;32). The clinical complications are therefore a result of mechanical pressure exerted on surrounding vital organs by the growing cyst. Accidental rupture of cysts can also happen, releasing fluid filled with protoscolices and possibly leading to anaphylactic reactions and secondary multiple cyst infections (as protoscolices can develop into secondary cysts within the intermediate host) (12).

Lung infection may present as chronic cough, pneumothorax, pleuritis, lung abscess and parasitic embolism (5;12). Heart involvement can present as a tumour, complete heart block and sudden death (5). The effects in the brain are usually headache and mass effects with neurological symptoms, and patients with eye infection can present with pain, ptosis and visual disturbances (5).

Although estimations of anatomical distribution in the literature vary, the tapeworm cysts in humans can be found mostly in the liver (62 to 70%), lungs (20%), and other organs (brain, wall of the heart, kidneys, spleen, orbit of the eye, marrow cavity of bones, etc.) can be involved in 10% of the cases (12;33). Multiple cysts or multiple organ involvement can also be seen in 20% to 40% of patients (34). In other intermediate hosts such as sheep, cysts have been found localized in the liver in up to 90% of cases (35). The biological explanation for CE preponderance in the liver and lungs is thought to be due to the many capillary sites that the migrating *Echinococcus* oncospheres primarily encounter in the hepatic or pulmonary filtering system before they reach any other peripheral organ (36-38).

Very little information is currently available regarding the influence of genotypes on the pathogenicity of the disease in humans and animals (39). There is some evidence, however, that certain strains have low or no infectivity to humans, whereas some are thought to be more virulent (5;40;41). The *E. granulosus* G8 strain has been reported to result in less severe disease than other genotypes, which has led to recommendations to treat CE patients in areas where G8 is prevalent by careful medical management, rather than by aggressive surgery commonly used with other genotypes (27). However, subsequent studies have since shown that the G8 strain also has the potential to lead to severe clinical disease (42).

The clinical manifestations of cystic echinococcosis can also be complicated by other factors such as co-infection with human immunodeficiency virus (HIV) (43). It was found that the profound immunosuppression of the patient can result in extensive CE disease and can interfere with immunodiagnostic tests, leading to false-negative results (43).

The adult tapeworms are considered to be rather harmless to the definitive host, except when they occur in large numbers, when they might cause severe enteritis (13).

1.2.2 Diagnosis

Imaging procedures together with serology are usually the most effective way of yielding a diagnosis for the infection with *Echinococcus* spp. The use of ultrasonography is regarded as the safest and most convenient way of diagnosing echinococcosis, and is also helpful in following up treated patients (Figure 1.5 A and B) (5;12;44). Other imaging techniques used in more specialised centres include computed tomography (CT) scan, standard radiology

(x-ray) and magnetic resonance imaging (MRI) (5). The CT scan of the abdomen can give better information concerning the location, accessibility and possible complications in patients, and is also important where there is diagnostic uncertainty or where recurrent disease is diagnosed (45). MRI has been found to add little value to CT scanning and is also not cost-effective (45).

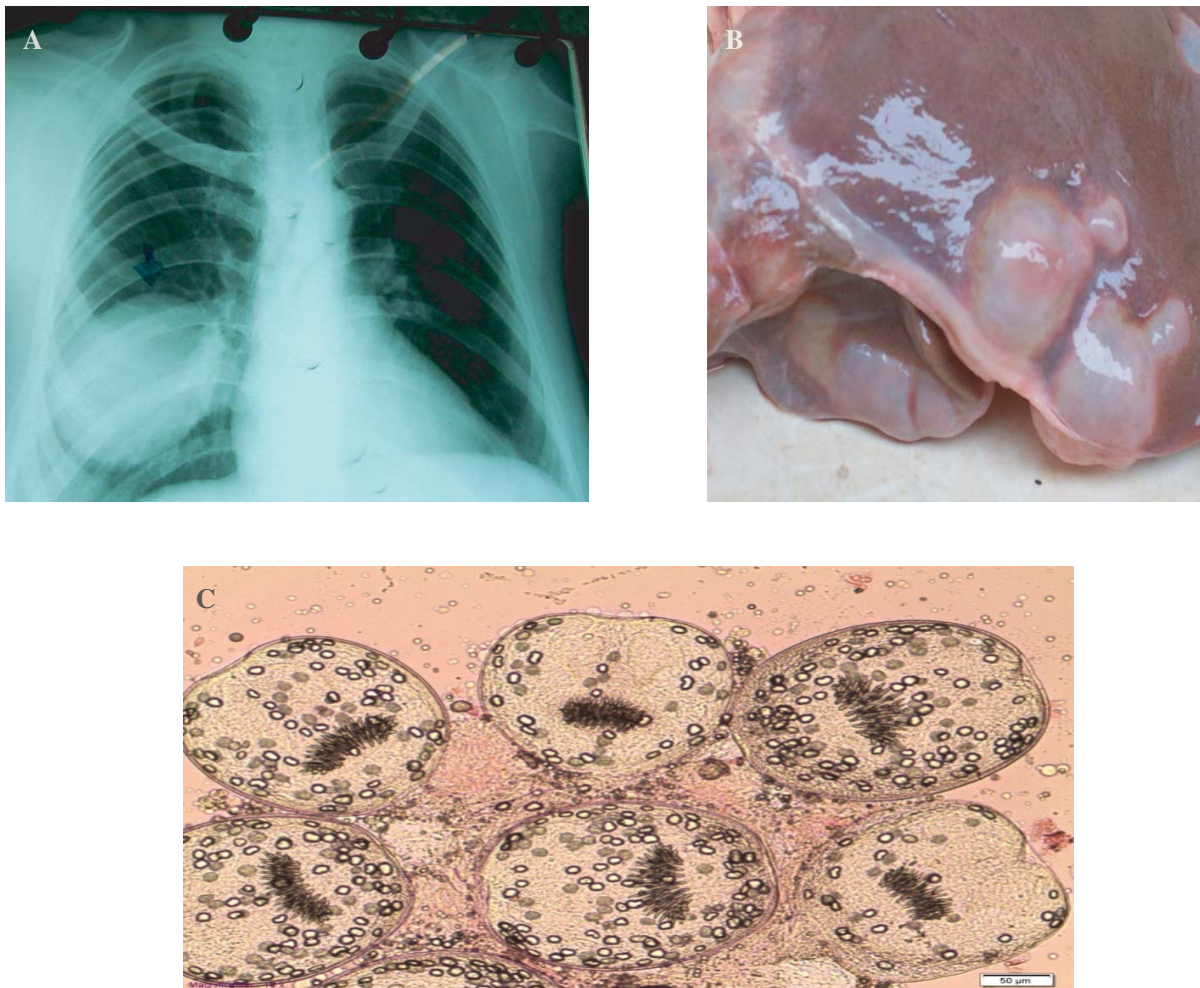


Figure 1.5 (A) Lung cyst on chest radiograph (46); (B) Macroscopic appearance of the *E. granulosus* cyst in liver tissue (46); (C) Protoscolices under a light microscope (100 x magnification).

The most accurate way of confirming the infection is through the simple use of a basic light microscope to find protoscolices or hydatid hooklets from aspirated cyst fluid (Figure 1.5 C); however, diagnosis through this method is not common as the sampling requires an invasive procedure such as removal of the cyst or therapeutic puncture, aspiration, injection and re-aspiration (PAIR) technique (5).

Immunodiagnostic tests can also be used to diagnose infections, or in sero-epidemiological surveys, to screen for the prevalence of CE and as a means of evaluating the efficacy of control measures (47-49). Some of the assays that have been used in screening studies are indirect haemagglutination assays (IHA), latex agglutination, complement fixation, as well as ELISA tests (47;48-51). The prevalence of infection has also been determined by collecting dog faecal samples and performing copro-antigen ELISA surveys (47). The shortcoming of these assays, however, is their low sensitivities as they have been found to produce false negative results of up to 50% (48;49).

1.2.3 Treatment

Surgery remains the mainstay treatment of echinococcosis (12;52). Where surgery is contra-indicated, i.e. by patients refusing surgery, very old patients, pregnant women or in cases of multiple cysts or cysts difficult to access, treatment of CE can be through PAIR, chemotherapy, or a 'wait and see' approach (12;52). The injection of protoscolicides when performing the PAIR procedure is important to inactivate viable protoscolices and reduce the chances of secondary infections in cases of accidental cyst rupture in patients. The agents (protoscolicides) with relatively low toxicity that are usually injected during the PAIR

procedure are 70%-95% alcohol, 15%-20% hypertonic saline solution or 0.5% cetrimide solution (52). It is also important to carefully choose less toxic scolical solutions as some can lead to adverse patient outcomes (53). Formalin was the original scolical solution that was used for CE treatment, but it has since been shown to be unsafe, as it has led to death from acidosis and cholangitis (54;55). There was also a clinical case report cautioning against the use of 20% hypertonic saline at a paediatric unit of the University of Cape Town, where it led to anuria, renal failure, bradycardia and eventually, patient death (53).

The preferred chemotherapeutic agents that are used in pre- and post-operative procedures and in non-invasive interventions are the benzimidazoles, albendazole and mebendazole (45;52;56). Both drugs interfere with glucose absorption through the wall of the parasite, leading to glycogen depletion (45;56). It has been proposed that praziquantel be administered as an additional anti-protoscolical drug alongside benzimidazoles, especially before and after surgery where there is risk of cyst rupture and release of protoscolices (12). It is important however, to take into consideration the possible side effects on patients as 10% to 20% of patients may develop transient elevation in transaminases, and both albendazole and mebendazole are contraindicated in pregnancy due to their teratogenic and embryotoxic effects in animals (45;57).

1.2.4 Prevention/control

The domestic transmission of *Echinococcus* species is mainly influenced by human activities and behaviour, politics and the presence of wildlife intermediate reservoirs (8). Because of the nature of transmission, the infection will usually be facilitated by uncontrolled

slaughtering practices, especially in pastoral communities where there is close proximity of humans, dogs and livestock and where offal is fed to dogs. In Australia, New Zealand and Iceland, successful or provisional interruption of *E. granulosus* transmission was achieved through concerted action and political will (58;59). The role of free-roaming dogs has also been demonstrated to have epidemiological significance, as soil samples in some areas have been found PCR positive for the G1 strain (60), thus showing that direct contact with dogs is not always the sole risk factor in areas that are not properly fenced. In the Turkana district of Kenya, which has an unusually high prevalence of CE (61;62), women were shown to have a higher incidence of the disease than men (63). This was especially the case when they were at child-bearing age as they used dogs to guard children against wild animals and also used dogs to remove excrement from babies, thus putting them at greater infection risk (63). Several control approaches were introduced to combat the high infection rate in that district, such as community education on the lifecycle of the parasite, including compilation of songs in local dialect as part of community-based health care programs (64).

Traditionally, prevention of the disease primarily focuses on veterinary interventions to control the intensity of infection in definitive hosts, by regular deworming of dogs and education on sanitary precautions when handling pets (33). However, collaboration between veterinarians and public health workers is essential for the successful control of echinococcosis (65). The epidemiological importance of CE is also underestimated due to underreporting and lack of compulsory notification (34).

The holistic control approach also needs to consider the 'spill-over' situations from sylvatic cycles; therefore careful epidemiological studies and strain identification are important as

basic requirements for effective control (16). The influence of the sylvatic cycle as reservoir for the domestic cycle is best illustrated through the Australian situation, where, although provisional eradication was achieved, CE started to resurface in eastern Australia, where the wildlife cycle was believed to be the main source of infection through farmers who fed offal from kangaroos and feral pigs to their dogs (8). The compatibility of the two transmission cycles was also demonstrated when a silver-backed jackal in South Africa was successfully infected with hydatid material from humans, cattle, pigs and sheep (20). Therefore, molecular epidemiological studies are necessary to understand genotypes responsible for transmission in a given area and to also allow rapid identification of 'spill-over' situations.

Perhaps the most important tool provided by molecular epidemiology in the control and prevention of CE, is the detailed knowledge on the species and genotypes present in a particular area that allows public health officials to properly time the administration of antihelminthic drugs in dogs before patency. It was found by Eckert *et al* (2001) that the pig (G7) and Tasmanian sheep (G2) strains have a shorter maturation time in dogs compared to the common sheep strain (G1) (5). The former cattle strain, *E. ortleppi*, has also been shown to reach patency a week earlier than the G1 strain (1). Therefore, dog treatment programs that are timed on the presumed presence of the common *E. granulosus* G1 strain will not efficiently reduce the intensity of transmission in areas where non-G1 strains are preponderant.

Although the G1 strain has a more dominant global presence than other genotypes, Elmahdi *et al* (2004) could not find any isolate of G1 strain in central Sudan, despite the large numbers of sheep in the area; only G6 and *E. ortleppi* were found (35). The sheep and goats in Sudan

seem to be considered unimportant in the transmission and maintenance of the infection, as most of them were found to harbour infertile or calcified cysts of *E. granulosus* (66;67). The knowledge of species/strains responsible for maintaining the lifecycle should therefore form an integral part of any control program (22).

Education and continued surveillance therefore underpin control efforts in order to break the cycle of transmission, and it is also essential to recognize which strains are present and which hosts support the perpetuation of the lifecycle (Figure 1.6) (18).

There are also efforts to control infection of intermediate host animals through the use of vaccines, such as the use of EgP-29 antigen cloned from *E. granulosus* and expressed in *Escherichia coli*, giving up to 96.6% protection against secondary echinococcosis (68-71).

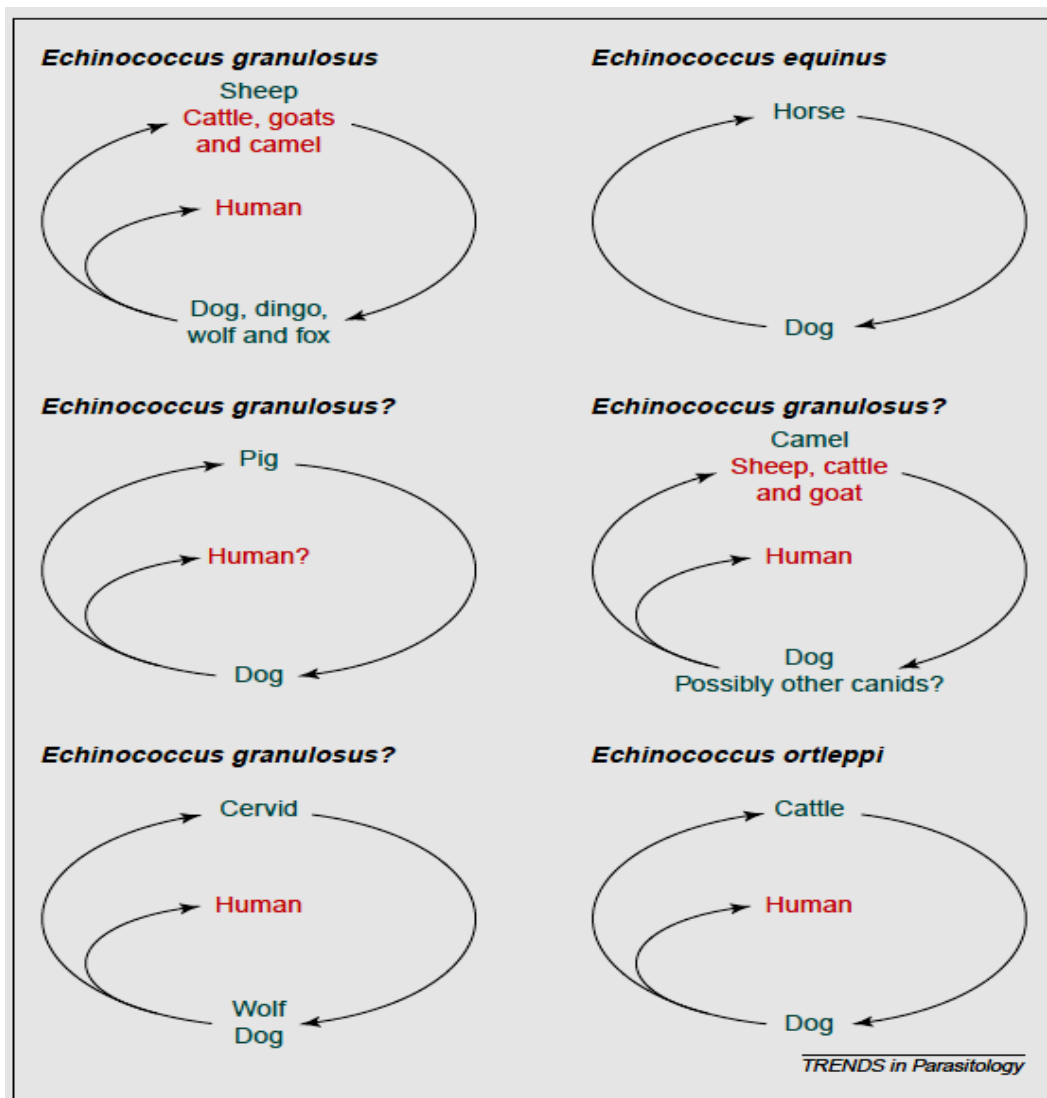


Figure 1.6 Cycles supporting the major species and/or strains of *Echinococcus granulosus* (sensu lato), showing major definitive and intermediate hosts (green) and other hosts (red) that might be involved. Question marks (?) indicate that the host's susceptibility to infection is uncertain. Source: Thompson and McManus, 2002 (18).

The WHO/OIE manual on echinococcosis in humans and animals identifies six preconditions for eradication of CE as follows: absence of adverse ecological factors; adequate administration, operational and financial resources; availability of effective tools; favourable epidemiological features; socio-economic importance and specific reasons for preferring

eradication over control (5). In addition, it identifies four phases of a control programme as summarized below (5).

1. Planning

- Introduction of special legislation on hydatid control
- Data on rural dog populations, incidence and age prevalence in human hydatid disease and ensuring that there are enough veterinarians available for the diagnosis and treatment of dogs
- Development of computer-based surveillance programmes
- Appropriate funding of the programme

2. Attack

- Mass dog deworming campaigns and the introduction of restrictive regulations on dog feeding practices
- Serological surveillance of the population
- Public education on the transmission lifecycle

3. Consolidation

- Ongoing surveillance of high-risk areas and introduction of appropriate control measures

- Quarantining of affected farms or farms deemed to be at risk
- Introduction of penalties for having infected dogs, where appropriate

4. Maintaining eradication

- Continued meat inspection services together with border controls to prevent reintroduction of the infection
- Cessation of some special activities in the above phases, and resources diverted to continuous meat inspection
- Continuous surveillance programs on humans, dogs and livestock

1.3 *Echinococcus granulosus* (sensu lato) epidemiology

1.3.1 Characteristics of various strains/variants

Epidemiologically, it is important to differentiate between various *Echinococcus* species and genotypes due to the intraspecific variability of these genotypes, differing in morphologic, phenotypic and other developmental characteristics, with implications for the control programs (18).

1.3.1.1 *E. granulosus* sensu stricto (G1-G3)

The common sheep (G1), Tasmanian sheep (G2) and buffalo (G3) strains are now regarded as a single species within the *E. granulosus* 'complex' and constitute the species *E. granulosus* sensu stricto (17). The validity of this characterization has further been

confirmed using phylogenetic studies (11). The high similarity of the three strains has also in the past been confirmed using the mtDNA sequences (72). The common sheep strain (G1) is the most epidemiologically important genotype with regard to its public health importance and geographic range in all continents (8;39;73-76). The Tasmanian sheep strain (G2) is thought to have evolved from G1 or exists as a rare genotype (77). Apart from Tasmania, it has also been described in human and other intermediate hosts in Argentina (78-80).

1.3.1.2 *E. ortleppi* (formerly G5)

E. ortleppi was given a unique species name and removed from the *E. granulosus* 'complex' because of its genetic distinctness, intermediate host preference and sympatric occurrence without interbreeding (10;18). As cited by Thompson, this species was initially described morphologically by Lopez-Neyra and Soler Planas in 1943, based on South African adult cattle worms that were originally described by Ortlepp in 1934 (9;81). The species status was later confirmed due to the morphologic and genetic distinctness of this tapeworm (11;72;82). The species has been found in Europe, Asia, South America and in some parts of Africa (40;82-85), and appears to have low pathogenicity for humans, as only two human CE cases have been described in the reviewed literature (8;83;86). Although cattle are poor intermediate hosts of other *Echinococcus* species, *E. ortleppi* has been found to be well adapted to this intermediate host animal in endemic regions (8-10).

1.3.1.3 *E. equinus* (formerly G4)

As with *E. ortleppi*, the former G4 strain has been given unique species status, namely *E. equinus*, due to its genetic distinctness, intermediate host preference and sympatric

occurrence without interbreeding (18). It has been found in Europe, the Middle East and South Africa and appears to exclusively infect equines (horses, donkeys and zebras) as intermediate hosts (8). No human CE cases have been reported, and increasing epidemiological evidence suggests that this species may either not be infective to humans, or its infectivity is very low (1;17).

1.3.1.4 *E. canadensis* (G6-G10)

This species comprises a number of *Echinococcus* genotypes derived from the camel (G6), pig (G7 and G9) and cervid strains (G8 and G10). They have been shown to cluster into a monophyletic group, and evidence suggests that they belong to a single species (11;87). As a result, the genotypes have tentatively been unified into a single species, *E. canadensis* (11). Furthermore, there are suggestions to remove the camel and pig strains (G6, G7 and G9) from *E. canadensis* and designate them a unique species name due to their close phylogenetic relationships, morphologic and other similarities, but further comprehensive population genetic, morphological and ecological studies are required for final decision about their taxonomic status (11). The individual strains are discussed below.

i. Camel strain (G6)

The camel strain, *E. canadensis* G6, is found in the Middle East, Africa, southern Asia and South America, and principally affects camels and goats, but sporadic human cases have also been reported in Nepal, Iran, Mauritania, Kenya and Argentina (18;22;80;85;88;89).

ii. Pig strains (G7 and G9)

This strain, *E. canadensis* G7, is transmitted by domestic pigs and is found in Europe, Asia, South America and Australia (8;25). Although previously, there was doubt surrounding infectivity of this strain to humans, molecular studies have since shown evidence of this strain from human CE cases in Turkey and Australia (16;25;28;90).

Another strain, similar to the pig strain (G7), was found to infect patients in Poland, but had some clear differences and has tentatively been designated as a new genotype, G9 (91).

iii. Cervid strains (G8 and G10)

The cervid strains can be found in arctic and subarctic regions of Europe, Asia and North America and are primarily maintained in the wild lifecycle (8). As reviewed by Jenkins *et al* (2005), human CE cases are known, but the disease appears to be more benign than CE cases caused by other *Echinococcus* species (8).

1.3.1.5 *E. felidis* (former lion strain)

This former lion strain appears to only circulate in the wildlife cycle in Africa as no records exist reporting the infection elsewhere (1;92). In the review by Hüttner and Romig (2009), tapeworms of this species have been found in South African and Ugandan lions, as well as in spotted hyenas in east Africa (7). In one of the national parks in Uganda, a 72% prevalence of *E. felidis* was found in lion faecal samples (92). The tapeworms were first described by Ortlepp from South African lions and their unique taxonomic status as *E. felidis* has since been demonstrated through phylogenetic studies (19;24). The chances of this wildlife cycle

spilling over to the domestic cycle appears to be limited, as experiments attempting to infect dogs with cysts presumed to be of *E. felidis* origin failed to establish infection (93).

1.3.2 Molecular genotyping methods

Molecular techniques allow a direct characterization of the parasite genome without the shortcomings associated with traditional methods, which rely on morphological and biological characteristics of the parasites. The latter methods have led to controversy regarding the correct taxonomical classification of *Echinococcus* species due to the variable nature of these organisms in different intermediate hosts from different geographical localities, where key phenotypic/biological features considered important for species characterization often overlap, leading to taxonomic confusion. However, molecular methods are not confounded by such host-induced variability (94). Both the mitochondrial and nuclear genomes have been used for characterizing variation in the *Echinococcus* species and genotypes, and the analysis of mitochondrial DNA (mtDNA) is especially useful in discriminating closely related organisms due to its relatively rapid rate of evolution (16).

Several methods have been used in genotyping studies, such as analysis of ribosomal or mitochondrial genes (*nad1*, ITS1, ITS2) by PCR and/or RFLP; PCR amplification of mitochondrial or nuclear genes (*COX1*, *nad1*, *Hbx 2*, *ACT II*, *AgB-I*) followed by sequencing (22;25;40;69;73;75;92;95-98). However, Dinkel *et al* (2004) found that some of these methods were either costly, time-consuming or required high quality DNA and were not always practical in the screening of large samples, especially in developing countries like Africa (89). As a result, a simpler, cost-effective PCR method especially suitable in

developing countries was developed. This method involves a PCR/semi-nested PCR system targeting the 12S rRNA mitochondrial genes and was found useful for the rapid diagnosis of *E. granulosus* G1, *E. canadensis* G6/7 and *E. ortleppi* (G5) (89). Another study conducted in East Africa by Hüttner *et al* (2009), revealed the presence of *E. felidis* (lion strain), and later showed that the PCR/semi-nested PCR method targeting the 12S rRNA failed to discriminate between *E. granulosus* sensu stricto (G1-G3) and the closely related sister taxon, *E. felidis* (92). For this reason, a new PCR-RFLP method was developed, which targeted a *nad1* gene and permitted the differentiation of all *Echinococcus* species known to be endemic in Africa, as well as some *Taenia* species (24;92). The problem with this latter method, however, is the size of the DNA fragment needed, as it targets an approximately 1078bp fragment, which is difficult to amplify on old hydatid cyst samples. This shows that no single method is optimal in all situations, but that a variety of molecular methods is available and researchers can adapt methods practical to their research settings.

The material needed for DNA extractions can either be tapeworm eggs or hydatid cyst material. For fertile cysts, protoscolices (hydatid sand) of the same cyst can be pooled and analyzed as one, since it was shown that there is no polymorphism in a microsatellite locus among protoscolices as they reproduce asexually within a cyst (99). Several DNA extraction techniques can be used, such as lysis of eggs in 0.02 M NaOH and directly using the lysed solution as PCR template (11). DNA can also be extracted by mincing the cyst material and digesting it with proteinase K, followed by phenol-chloroform/ethanol DNA precipitation or extractions using commercial tissue protocols as recommended by manufacturers (25;73;74;92;100).

1.3.3 The epidemiological situation in South Africa

Cystic echinococcosis has been reported in West African and East African countries and is considered rare or not documented in central and southern African countries (101). A study conducted by Verster *et al* (1966) in South Africa, estimated the average incidence of CE in slaughtered livestock to be 1%, with the incidence varying by region and livestock species (102). Cattle had a high CE prevalence in Eastern Cape and Limpopo provinces, whilst sheep had a higher prevalence in parts of the Free State and North West provinces and pigs in Western Cape Province and Mokopane (previously called Potgietersrus) in Limpopo Province (102).

To our knowledge, no molecular genotyping study has previously been conducted in South Africa to elucidate the prevalent genotypes. There is some published data that suggests that some work was done in the 1930s to study species of the *E. granulosus* 'complex' found in the country using morphological approaches. Orllepp morphologically described species of *Echinococcus* of horse and cattle origin, which were confirmed about 70 years later as *E. equinus* and *E. ortleppi* on the basis of their genetic distinctness, different intermediate host preferences and sympatric occurrence without interbreeding (11;18-20;72;81;83). As the name suggests, *E. ortleppi* was named in honour of the work that was done by Orllepp in 1930s.

Another species which was first described in South Africa is *E. felidis*, the former lion strain. This was based on the work by Orllepp in 1937, who isolated and described tapeworms from lions, and also found that cysts from warthogs could infect lions but failed to infect dogs (19). Although initially regarded as a strain, these tapeworms were later assigned the unique

species status of *E. felidis*, due to genetic distinctness from the other *E. granulosus* variants (24).

Another study was done to describe the helminth parasites of common wildlife species in the northern and western parts of the Limpopo Province in South Africa, where *E. granulosus* cysts were reported from the lungs of one of the six warthogs sampled (103). The species identification was, however, based only on morphological methods, thus not excluding the possibility that this was a different *Echinococcus* species, since *E. felidis* has already previously been implicated as occurring in warthogs (7).

A retrospective analysis done in 2011 to shed light on the situation of the disease in South Africa, found that CE is recognized as a clinical problem in the country, although its epidemiology and clinical spectrum was unknown (104). Some of the key epidemiological findings in the retrospective study are summarized below:

- Median age of patients from analyzed data was 28 years;
- There was no gender bias between males and females;
- Contrary to literature reports that 70% of cysts are localized in the liver, the study found 64% of cysts were localized in the lungs, with the liver accounting for only 9% of cases;
- There was an overall prevalence (serology and microscopy) of 8 to 28% in patients with suspected infections, according to laboratory test requests in 8 South African provinces (no data was available from the KZN Province);
- The Eastern and Northern Cape provinces had the highest prevalence rates;
- A conservative estimate of 137 CE cases should be expected per year in the country;

- About 25 articles (individual reports and small case series) related to South Africa are available in the literature, mainly covering unusual clinical presentations, such as cysts in CNS, spine, heart and the orbit.

From the above, it is clear that human CE is a neglected zoonosis in the country and more research is needed to properly understand the intensity and epidemiological aspects of the disease in the country. As shown in this literature review, efficiency of control measures is also intrinsically dependent on the understanding of *Echinococcus* species and genotypes circulating in a given area, and this is a shortfall in South Africa as to our knowledge, no molecular epidemiology has previously been done. Molecular techniques contribute towards rapid clarification of the epidemiological situation in a given area, but such studies have to be carried out by experienced reference laboratories (5). The geographically nearest country to South Africa where information on *Echinococcus* species and genotypes exists is Kenya, where the epidemiological situation is highly complex (involving at least four species of *Echinococcus*) and highly variable between regions of the country (105).

1.4 Study aims

Primary aim: To characterize species and genotypes of *Echinococcus* causing CE in South Africa as a first step towards understanding the cestode biology and host species responsible for transmission of the disease in the country.

Secondary aims:

1. To determine if there is an association between genotype and organs of cyst localization.
2. To determine if there is regional variability of strains in different South African provinces.
3. And lastly, if fertility of cysts can be readily determined. Fertility can play an important epidemiological role in terms of transmission potential, especially in livestock.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Patient recruitment and sample collection

2.1.1 Patient recruitment from the collaborative study

This research was embedded in a larger collaborative study involving clinical researchers at Chris Hani Baragwanath Hospital and Charlotte Maxeke Johannesburg Academic Hospital, who were evaluating new cases of cystic echinococcosis for demographic information, clinical presentation and course of disease, and associated co-infections. The risk factors, geographical distribution as well as the impact of co-infections like HIV, hepatitis B and TB on the clinical course, treatment and outcome of hydatid disease were also investigated.

Patients were required to complete informed consent or (for patients younger than 18 years) assent forms (Appendix A and B) and a standard patient questionnaire was used to collect the relevant clinical and epidemiological data (Appendix C). This larger study commenced in August 2010 and will run until July 2013. After surgery, the excised cysts and aspirated cyst contents were sent to PRL for molecular genotyping. A separate analysis for the larger collaborative study will be made of all data covering both the clinical and genotyping study; for this project, only relevant data (patient location, organ of cyst localization etc.) was analyzed.

2.1.2 Sample collection from other sources

2.1.2.1 NHLS Laboratory Information System (LIS)

As part of efforts to increase recruitment of human CE samples, the National Health Laboratory Service's diagnostic laboratory information system (LIS) national data repository was interrogated repeatedly for positive CE results registered on the LIS, so that we were able to recruit samples from the relevant laboratories.

2.1.2.2 Individual donors

A surgeon based at Frere Hospital in the Eastern Cape also donated samples to the study. The surgeon's interest was in finding out whether or not the CE drug treatment was successful in inactivating viable protoscolices of *Echinococcus* species to prevent further cyst growth or to reduce the risk of re-infection in case of spillage of hydatid fluid during surgical removal of the cysts. Generally, for each patient, two samples were sent: one 15 ml Falcon tube containing a mixture of hydatid cyst fluid and sterile preservative Krebs-Ringer solution (Appendix K) for viability checks (106), and the other sample being the whole cyst (or hydatid sand) for the genotyping study.

In summary, viability of protoscolices was confirmed either through demonstration of flame cell activity or through the procedure summarized below (106):

1. Protoscolices in the preservative solution were allowed to settle at the bottom of the Falcon tube for about 30 minutes.

2. 0.01 ml solution of pooled protoscolices was mixed with 0.01 ml of 0.1% aqueous eosin stain.

3. The mixture was left for 5 minutes at room temperature and observed microscopically at low magnification.

Viable protoscolices were confirmed when eosin stain failed to penetrate through the wall of protoscolices after 5 minutes; and in case of non-viable protoscolices, the pink eosin stain could be seen inside the protoscolices (Figure 2 A and B) (106). The data on cyst viability was included as part of the analysis for this research.

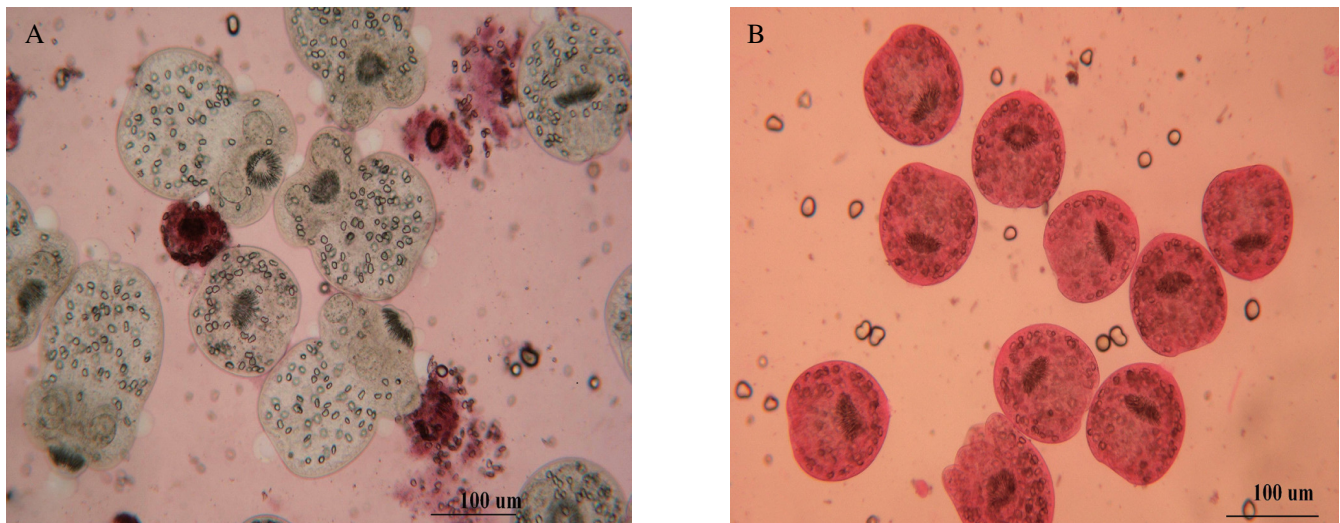


Figure 2 (A) Viable protoscolices after 5 min eosin staining. (B) Non-viable protoscolices after 5 min staining (106).

2.1.2.3 Abattoirs

Individual abattoirs and state veterinarians were contacted to donate samples for this study since livestock is affected by the same transmission lifecycle as humans and serves as a reservoir for human infection. The South African Department of Agriculture, Forestry and Fisheries was also contacted for assistance with sample collections, but meat inspection is subcontracted by the Department to a private company called International Meat Quality Assurance (IMQAS), which we contacted.

CE cases reported from abattoirs were also checked by running a query on the disease database available within the website of the department, from January 1993 to December 2011. The information was not useful as the database was last updated in 2001, ruling out the possibility of using the site to trace relevant abattoirs where cyst samples could currently be sourced (Appendix L).

2.2 DNA extraction, PCR and product sequencing

2.2.1 DNA extraction

All samples were preserved in 70% ethanol until DNA extraction. The DNA was initially extracted using either an automated DNA extraction instrument (Roche MagNA Pure Compact: Roche Diagnostics, Basel, Switzerland) or a manual commercial kit (QIAGEN DNA Mini Kit: QIAGEN, Valencia, California, USA). For the automated method, the cyst tissue was cut into small pieces and about 25 mg was incubated overnight in a solution containing 150 µl bacterial lysis buffer (Roche Diagnostics, Basel, Switzerland) and 20 µl of proteinase K (Roche Diagnostics, Basel, Switzerland) at 56°C. The digested material was

then loaded into the automated instrument and MagNA Pure Compact Nucleic Acid Isolation kit (Roche Diagnostics, Basel, Switzerland) was used following the manufacturer's instructions (Appendix G).

For the manual extraction method, samples were pre-digested and DNA extracted using a QIAGEN DNA Mini Kit (QIAGEN, Valencia, California, USA), and either a body fluid protocol (for hydatid cyst fluid) or a tissue protocol (for endocysts or protoscolices) was used as per the manufacturer's instructions (Appendix H and I).

For quality control, all samples were extracted together with a negative control (non-template nuclease-free water control) and all PCR amplifications were run with positive and negative controls. Preparation of master mix, loading of DNA samples and gel electrophoresis runs were all performed in separate rooms to reduce the risk of contamination.

2.2.2 *nad1* PCR/RFLP

These PCRs were performed as described by Hüttner *et al*, 2009 (92), with some modifications.

2.2.2.1 Primary PCR (1073-1078 bp)

This was run in a 25 µl reaction mixture containing 2.5 µl 10x Maxima Hot Start buffer (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 4 µl of 1.25mM dNTPs (Life Technologies, Carlsbad, California, USA), 2 µl of 2 mM MgCl₂ (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 0.6 µl of 6.25 pmol of each primer (*nadA* for. 5' TGT TTT TGA GAT CAG TTC GGT GTG 3' and *nadC* rev. 5' CAT AAT CAA ACG GAG TAC

GAT TAG 3'), 0.13 µl (0.625 units) Maxima Hot Start *Taq* DNA polymerase, 5 µl DNA template and topped to 25 µl with nuclease-free water.

The reactions were run in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the following cycle parameters: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at 55°C and 1 minute elongation at 72°C. The amplicons were stored at -20°C for further use.

2.2.2.2 Nested PCR

This was run in a 25 µl reaction mixture containing 2.5 µl 10x Maxima Hot Start buffer (Thermo Fisher Scientific, Massachusetts, USA), 4 µl of 1.25mM dNTPs (Life Technologies, Carlsbad, California, USA), 2 µl of 2 mM MgCl₂ (Thermo Fisher Scientific, Massachusetts, USA), 0.6 µl of 6.25 pmol of each primer (nadB for. 5' CAG TTC GGT GTG CTT TTG GGT CTG 3' and nadD rev. 5' GAG TAC GAT TAG TCT CAC ACA GCA 3'), 0.13 µl (0.625 units) Maxima Hot Start *Taq* DNA polymerase, 2 µl DNA template and topped to 25 µl with nuclease-free water.

The reactions were run in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the following cycle parameters: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at 55°C and 1 minute elongation at 72°C. The amplicons were stored at -20°C for further use.

2.2.2.3 Restriction fragment length polymorphism (RFLP)

The nested PCR product was digested with *Hph*I restriction enzyme in a 37 °C incubator for ~5 hrs. The reaction mixture contained 10 µl DNA, 18 µl nuclease-free water, 2 µl of 10x Buffer B (Fermentas), and 0.5 µl *Hph*I (Life Technologies, Carlsbad, California, USA). After digestion, 5-10 µl of the product was run in a 3% ethidium bromide-stained agarose gel. Restriction fragment patterns distinguished between *E. granulosus* G1, *E. equinus* G4, *E. canadensis* G6/7 complex, *E. ortleppi* G5 and *E. felidis* (92).

2.2.3 12S rRNA PCR

These PCRs were performed as described by Dinkel *et al*, 1998 and 2004 (89; 100), with minor modifications, as follows.

2.2.3.1 Cestode-specific PCR (373 bp fragment)

This was run in a 25 µl reaction mixture containing 2.5 µl 10x Maxima Hot Start buffer (Fermentas), 4 µl of 1.25mM dNTPs (Life Technologies, Carlsbad, California, USA), 2.5 µl of 2 mM MgCl₂ (Life Technologies, Carlsbad, California, USA), 1 µl of 10 pmol of each primer (P60.for. and P375.rev. (100)), 0.13 µl (0.625 units) Maxima Hot Start *Taq* DNA polymerase, 5 µl DNA template and topped to 25 µl with nuclease-free water.

The reactions were run in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the following cycle parameters: initial denaturation at 94°C for 5 minutes, followed by 40 cycles of 30 seconds denaturation at 94°C, 1 minute annealing at 55°C and 30 seconds elongation at 72°C. The amplicons were stored at -20°C for further use.

2.2.3.2 PCR assay specific for *E. granulosus* G1-G3 (254 bp)

This was run in a 25 µl reaction mixture containing 2.5 µl 10x Maxima Hot Start buffer (Life Technologies, Carlsbad, California, USA), 4 µl of 1.25mM dNTPs (Life Technologies, Carlsbad, California, USA), 2.0 µl of 2 mM MgCl₂ (Life Technologies, Carlsbad, California, USA), 1.25 µl of 12.5 pmol of each primer (E.g.ss1 for. 5' GTA TTT TGT AAA GTT GTT CTA 3' and E.g.ss1 rev. 5' CTA AAT CAC ATC ATC TTA CAA T 3'), 0.13 µl (0.625 units) Maxima Hot Start *Taq* DNA polymerase, 5 µl DNA template and topped to 25 µl with nuclease-free water.

The reactions were run in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the following cycle parameters: initial denaturation at 94°C for 5 minutes, followed by 40 cycles of 30 seconds denaturation at 94°C, 1 minute annealing at 57°C and 40 seconds elongation at 72°C. The amplicons were stored at -20°C for further use.

2.2.3.3 PCR assay specific for *E. canadensis* G6/7 and *E. ortleppi* (254 bp)

This was run in a 25 µl reaction mixture containing 2.5 µl 10x Maxima Hot Start buffer (Life Technologies, Carlsbad, California, USA), 4 µl of 1.25mM dNTPs (Life Technologies, Carlsbad, California, USA), 2.0 µl of 2 mM MgCl₂ (Life Technologies, Carlsbad, California, USA), 1.25 µl of 12.5 pmol of each primer (E.g.cs1 for. 5' ATT TTT AAA ATG TTC GTC CTG 3' and E.g.cs1 rev. 5' CTA AAT AAT ATC ATA TTA CAA C 3'), 0.13 µl (0.625 units) Maxima Hot Start *Taq* DNA polymerase, 5 µl DNA template and topped to 25 µl with nuclease-free water.

The reactions were run in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the following cycle parameters: Initial denaturation at 94°C for 5 minutes, followed by 40 cycles of 30 seconds denaturation at 94°C, 1 minute annealing at 53°C and 40 seconds elongation at 72°C. The amplicons were stored at -20°C for further use.

2.2.3.4 Semi-nested PCR (171 bp fragment)

To discriminate *E. orteppi* (G5) from *E. canadensis* G6/7, a semi-nested PCR specific for G6/7 or for *E. orteppi* (G5) was used in the second step, each amplifying a different fragment of 171 bp.

This was run in a 25 µl reaction mixture containing 2.5 µl 10x Maxima Hot Start buffer (Life Technologies, Carlsbad, California, USA), 4 µl of 1.25mM dNTPs (Life Technologies, Carlsbad, California, USA), 2.0 µl of 2 mM MgCl₂ (Life Technologies, Carlsbad, California, USA), 1.25 µl of 12.5 pmol of each primer, 0.13 µl (0.625 units) Maxima Hot Start *Taq* DNA polymerase, 2 µl DNA template and topped to 25 µl with nuclease-free water. For G6/7 PCR, the primers E.g.camel.for. 5' ATG GTC CAC CTA TTA TTT CA 3' and E.g.cs1 rev (as above) were used; and for *E. orteppi* (G5 PCR), the primers E.g.cattle.for. 5' ATG GTC CAC CTA TTA TTT TG 3' and E.g.cs1rev (as above) were used.

The reactions were run in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the following cycle parameters: Initial denaturation at 94°C for 5 minutes, followed by 30 cycles of 30 seconds denaturation at 94°C, 1 minute annealing at 60°C and 30 seconds elongation at 72°C. The amplicons were stored at -20°C for further use.

2.2.4 Gel electrophoresis

All PCR products (except digested RFLP products) were resolved on a 1.5% agarose gel stained with 10 mg/ml ethidium bromide. The RFLP products were resolved on a 3% agarose gel stained with ethidium bromide. The gels were made by adding either 0.45 g (1.5%) or 0.9 g (3%) of agarose to 30 ml 1x TAE buffer (Appendix J). This was dissolved in a microwave oven by heating for 1 minute. After cooling, 1.5 µl of ethidium bromide was added and the solution was poured into the gel-setting chamber to set for ~45 minutes.

Five microlitres (10 µl for RFLP) of product and 1 µl of 6x DNA loading dye (Life Technologies, Carlsbad, California, USA) were mixed and loaded into the gel wells. Four microlitres of a 100 bp Gene ruler (Life Technologies, Carlsbad, California, USA) were added to the first well of the gel. The samples were run at 100 volts for 15 to 30 minutes and the gel was viewed with an ultraviolet transilluminator (GelDoc, Vacutec, Johannesburg, South Africa) to confirm the presence of an amplicon. All samples were run with a known positive control (kindly donated by the Department of Parasitology, Hohenheim University, Germany).

2.2.5 Sequencing of PCR products

A representative number of samples and/or any samples that were not conclusively genotyped by the PCR/RFLP methods were sent for purification and sequencing of PCR products (Inqaba Biotechnical Industries, Pretoria, South Africa). In summary, the PCR products were purified using a sequencing clean-up kit (Zymo Research, Irvine, California, USA) following manufacturer's instructions (Appendix M). The sequencing reactions were performed using Big-Dye ver3.1 (Life Technologies, Carlsbad, California, USA) according to manufactures

instructions (Appendix N). The cost and time-saving benefits of sending samples away for sequencing outweighed that of purchasing the purification and sequencing kits and running them in PRL. Our laboratory also does not have a Big-Dye terminator machine.

2.3 Microscopic diagnosis

To microscopically check fertility status of cysts, the hydatid fluid was centrifuged at 3000 rpm (Hettich universal centrifuge, Germany) for 5 minutes; then the supernatant was poured out and a wet preparation was made from the sediment. The slides were viewed at 100x and 400 x magnifications using a light microscope. The cysts that contained hydatid hooks and/or protoscolices were regarded as fertile, and all others were considered infertile.

2.4 Data analysis

2.4.1 Alignment and editing of sequences

The sequences were analyzed with the BioEdit sequence alignment editor (107), before comparing them to other sequences available on GenBank.

2.4.2 NCBI-BLAST

All sequences were compared to published sequences in GenBank using the NCBI-BLAST software (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>).

2.4.3 Statistical analysis

Data analysis was performed using GraphPad (GraphPad Software Inc., San Diego, California, USA). Two-tailed Fisher's exact test was used, and results were considered statistically significant if $p < 0.05$.

2.5 Ethical clearance

- Ethical approval for the larger collaborative study was granted by the University of the Witwatersrand Human Research Ethics Committee, clearance # M10719 (Appendix D).

- Ethical approval for the collection of routine patient samples for this study was granted by the University of the Witwatersrand Human Research Ethics Committee, clearance # M110805 (Appendix E).

- The University of the Witwatersrand Animal Ethics Screening Committee ruled that no ethical clearance was needed for the use of animal cyst samples from abattoirs (Appendix F).

CHAPTER 3 – RESULTS

3.1 Patient recruitment and sample collection

A total of 36 samples from 33 CE patients was obtained between August 2010 and November 2012. Three patients had multiple cysts that were treated as separate cases, as the possibility of different genotypes co-habiting in the same patient could not be overlooked; thus, sample numbers were slightly higher than the total number of patients (Table 3.1). Seven (19%) samples were collected from the academic hospitals' collaborative study, nine through the NHLS LIS (25%) and 20 (56%) from the Eastern Cape Province hospitals (Figure 3.1).

The recruitment of animal samples from abattoirs was unsuccessful. Although the main company (IMQAS, Pretoria, South Africa) subcontracted to perform meat inspections on behalf of the Department of Agriculture, Forestry and Fisheries, was contacted and did their best to spread the message to its inspectors, only one lung cyst from a cow was eventually received. Most of the other smaller abattoirs that we contacted reported that CE samples were either very rare or even unheard of at their facilities. This one cow sample is not included in the analysis of human samples (unless specified) and is discussed separately.

Table 3.1 Organ involvement by hydatid cysts

Organ	Number of cases
Lung	14
Right middle lobe	1
Upper lobe	1
Lung, not defined	12
Liver	12
Pelvis	1
Deep lobe of parotid	1
Abdomen	1
Brain	1
Unknown	6
Total infected organs	36*

*The total number of samples exceed the number of patients (n = 33) due to three patients presenting with multiple cysts that were treated as separate cases. Two patients had multiple organ involvement (liver and pelvis, and, lung and liver, respectively). The third patient had two separate cysts from the liver.

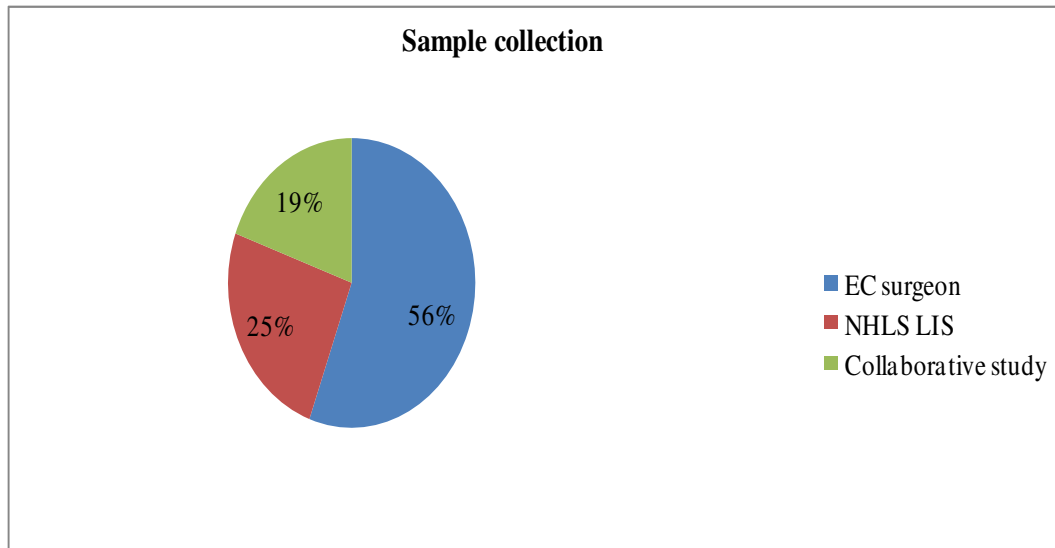


Figure 3.1 Provenance of hydatid cyst samples recruited from 3 provinces, South Africa, 2010-2012 (n = 36). NHLS LIS: National Health Laboratory Service laboratory information system; EC: Eastern Cape Province.

3.2 Organ involvement

The highest number of cysts was localized in the lungs (39%), followed by the liver (33%), with less commonly affected sites being the pelvis, abdomen, parotid gland and brain (collectively 11%) (Figure 3.2). No information was available for six (17%) cases.

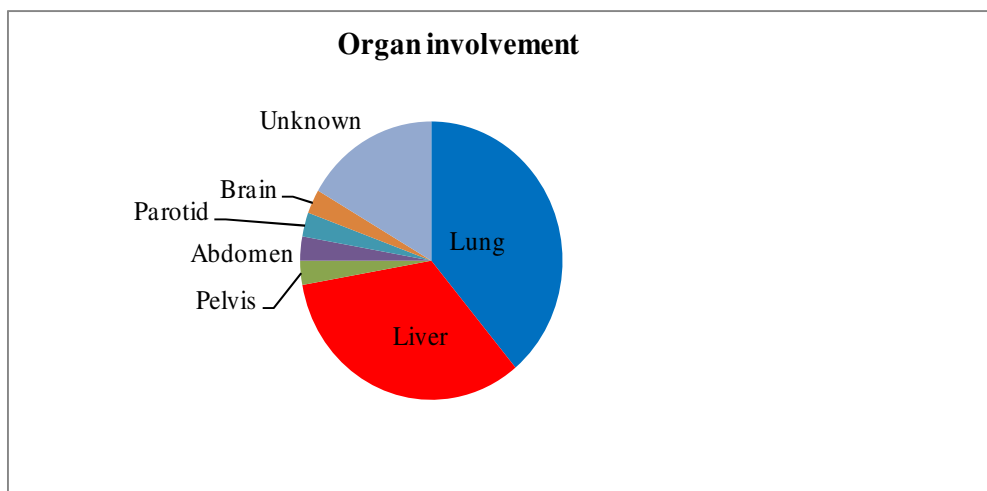


Figure 3.2 Organ distribution of cyst localization. Unknown: Information on affected organs not available.

3.3 Influence of genotype on cyst localization

Of the 36 samples, 32 (89%) were successfully genotyped by PCR and/or sequencing. For 27 of these cases (84%), information on both the genotype and organ localization was available. When looking at cases with pulmonary or hepatic involvement only (n = 23), there were slightly more cases of pulmonary disease (n = 14) than of hepatic disease (n = 9) (Figure 3.3). *E. granulosus* G1 made up a higher proportion of the liver than of the lung cysts. However, the association between the G1 and G6/7 genotypes and any organ of cyst localization was not significant ($p > 0.05$).

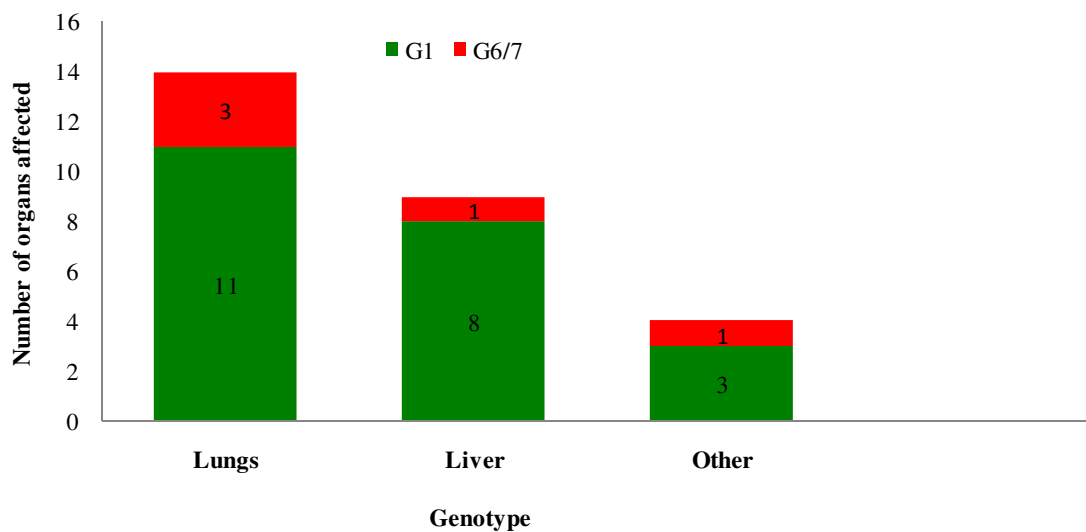


Figure 3.3 Comparison of *Echinococcus* genotypes by number and organ localization of the cyst (n = 27).

3.4 Genotype distribution by province

The overall distribution of genotypes in humans showed that G1 was the predominant genotype in the studied areas (26/32; 81%) followed by *E. canadensis* G6/7 (5/32; 16%) and

E. ortleppi (1/32; 3%). Samples were only received from three provinces, mostly Eastern Cape Province, followed by Gauteng and Free State provinces (Figure 3.4). The association between the prevalence of genotypes (G1 vs. G6/7) and province (EC vs. GP) was considered to be statistically significant ($p = 0.03$).

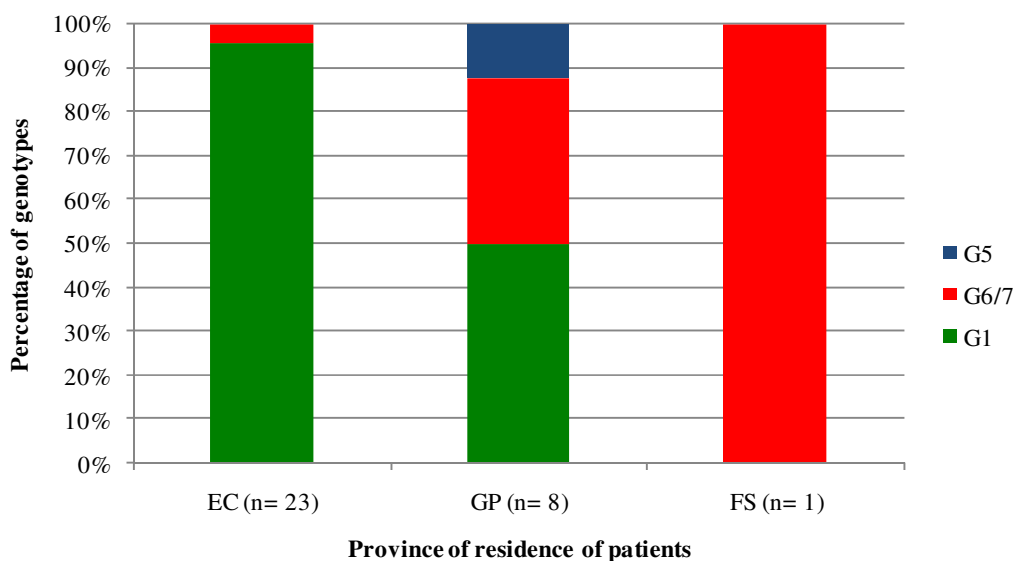


Figure 3.4 The frequency distribution of *Echinococcus* genotypes by province, South Africa (n = 32). EC: Eastern Cape Province; GP: Gauteng Province; FS: Free State Province.

3.5 DNA extraction, PCR and product sequencing

3.5.1 DNA extraction

Seven formalin-fixed samples were received. All these samples failed to amplify a 1073-1078 bp fragment on *nadI* PCR/RFLP method, but three of the samples eventually amplified successfully after removal of formalin by repeatedly washing the sample with PBS, centrifuging and discarding the supernatant, followed by rehydration in 70% ethanol. The

DNA was then re-extracted from the sample and successfully amplified by a 12S rRNA PCR that targets a 254 bp fragment.

The remaining four samples failed to amplify this way, and also did not amplify when prolonging incubation of the lysis/digestion step. A few published methods on extraction of DNA from formalin-fixed samples also failed PCR (108;109). The samples were also sent to Inqaba Biotechnical Industries for DNA extraction and amplification, with no success.

3.5.2 *nadI* PCR/ RFLP

Eleven samples were processed using the *nadI* PCR/FRLP, and nine (82%) samples were successfully genotyped (Figure 3.6). Of these, five samples were genotyped as *E. granulosus* (G1), three as *E. canadensis* (G6/7) and one as *E. ortleppi*. (Figure 3.5). The single sample from a cow was also genotyped as *E. ortleppi* by this PCR method.

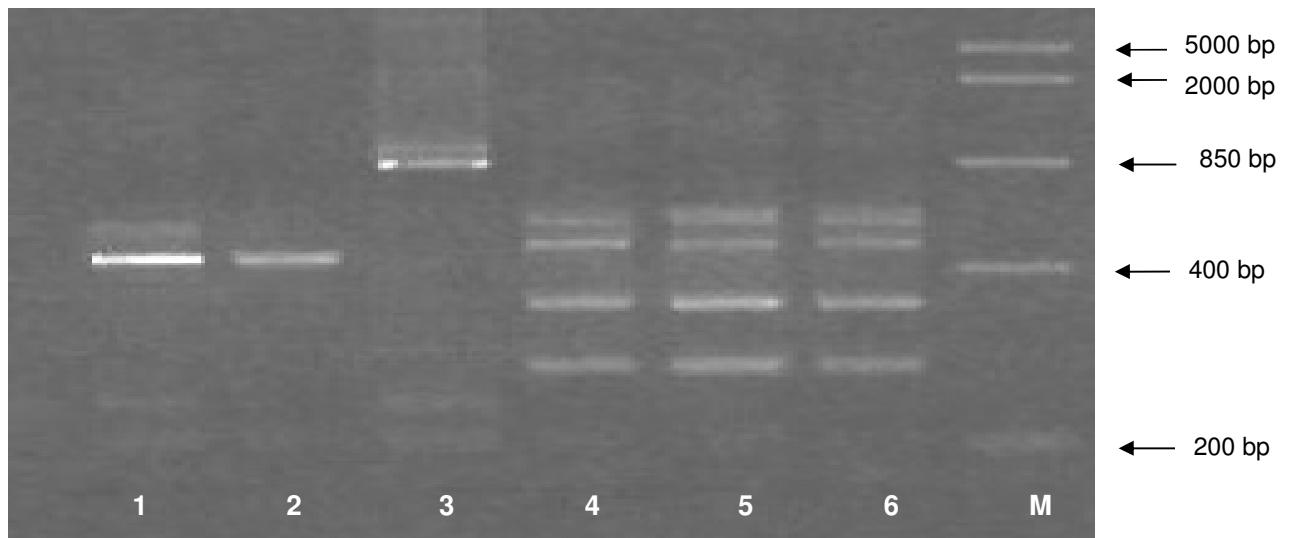


Figure 3.5 RFLP gel image showing examples of the *nadI* gene digested with restriction enzyme *HphI*. Patient samples: 1, *E. canadensis* G6/7*; 3, *E. ortleppi*; 5, *E. granulosus* sensu stricto**. Positive controls: 2, *E. canadensis* G6/7 positive control; 4 & 6, *E. granulosus* sensu stricto positive control; M, 5000 bp size marker.

*RFLP not able to differentiate G6 from G7 genotypes; **includes *E. granulosus* genotypes G1-G3, but G1 confirmed by sequencing.

The other two samples (MB and MT) that did not amplify by this method targeting a 1073-1078 bp fragment were later confirmed as G1 and G6/7 genotypes respectively, using a 12S rRNA PCR method.

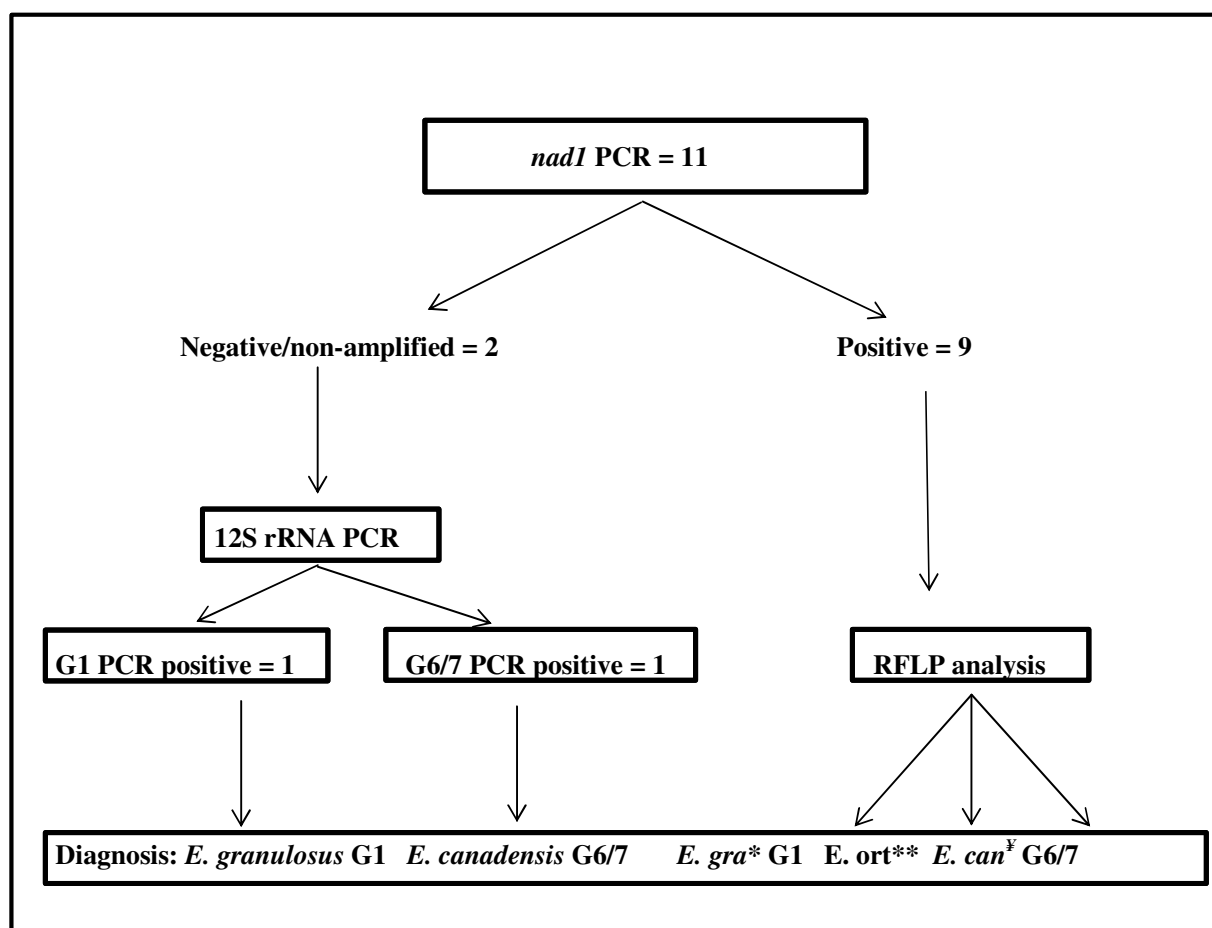


Figure 3.6 Flow diagram summarizing *nadI* PCR/RFLP results.

E. granulosus*; *E. ortleppi*; ^y*E. canadensis*.

3.5.3 12S rRNA PCR

A total of 27 samples (including samples that failed to amplify with *nadI* PCR) was run using the 12S rRNA method (Figure 3.7). All samples were first run with the G1 PCR, due to the high prevalence of this genotype (Figure 3.8). Twenty-one samples (78%) were confirmed as

G1, and the remaining six G1-negative samples were run on a PCR specific for G5/6/7 genotypes. Two samples were successfully genotyped as G6/7, with the other four samples failing to amplify. The latter samples were the same samples reported in section 3.5.1, which were degraded by formalin.

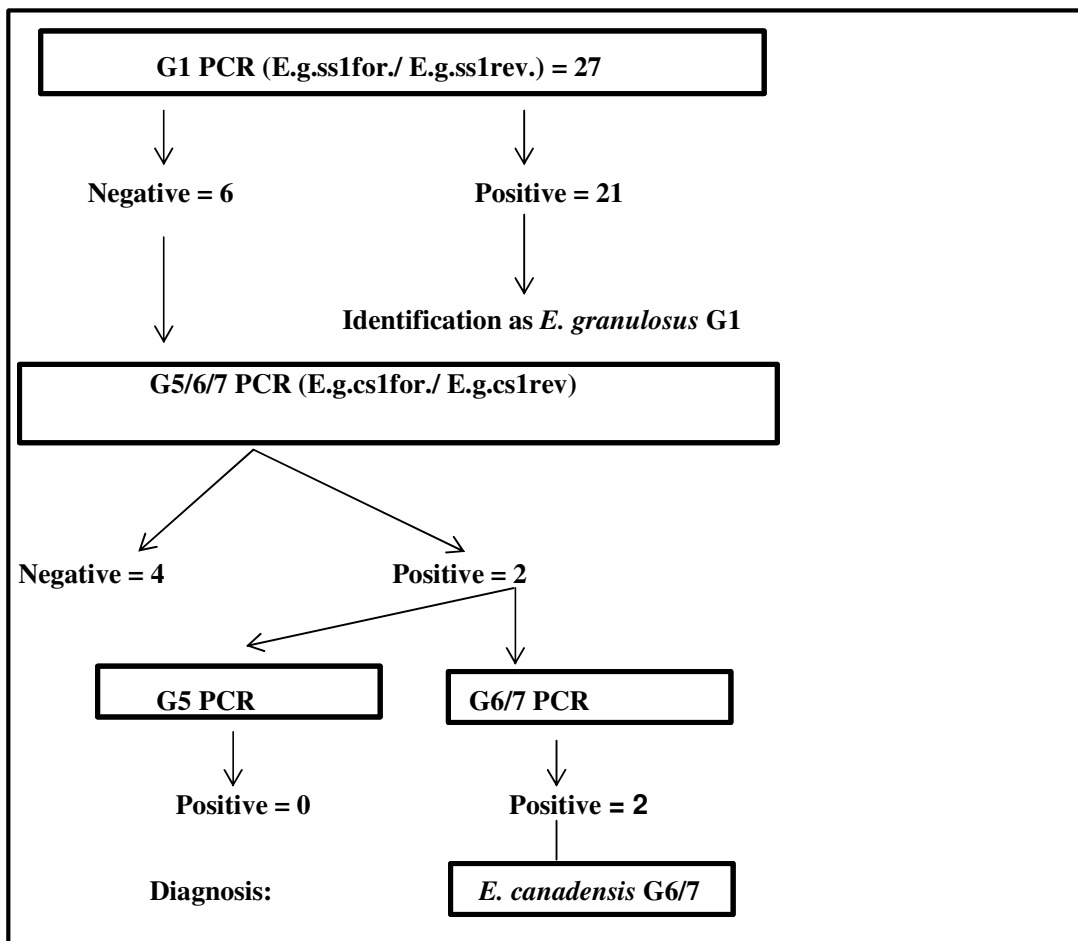


Figure 3.7 Flow diagram summarizing 12S rRNA PCR results.

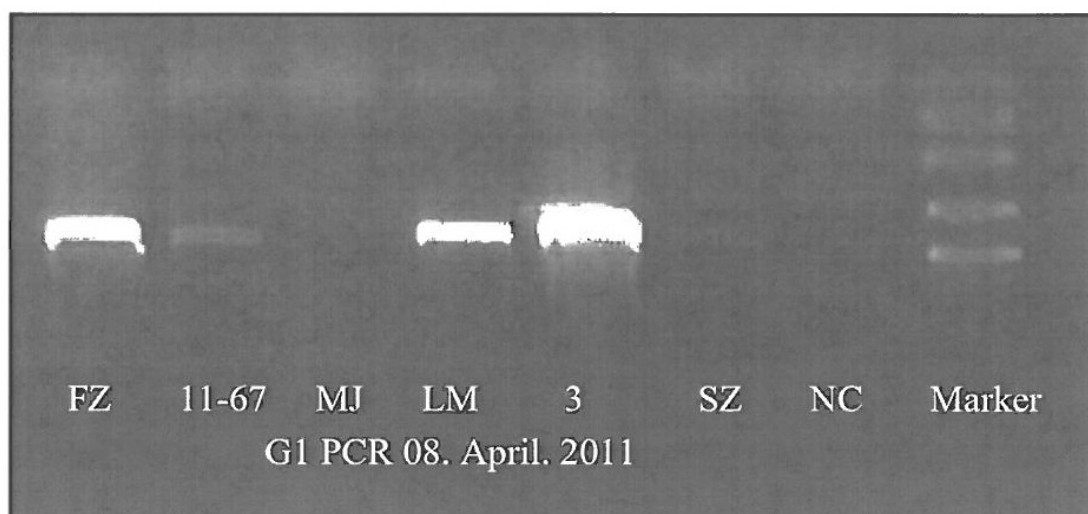


Figure 3.8 Gel electrophoresis showing an example of *E. granulosus* (G1) specific PCR that targets a 254 bp 12S rRNA fragment. FZ, *E. granulosus* (G1); 11-67, *E. granulosus* (G1); MJ, negative; LM, *E. granulosus* (G1); 3, *E. granulosus* G1 positive control; SZ, negative; NC, negative control; Marker, 5000 bp DNA marker.

3.5.4 Sequencing of PCR products

A total of ten samples was sent for sequencing and all PCR results were confirmed (Table 3.2). Five samples belonged to *E. granulosus* G1 genotype, four to *E. canadensis* (G6/7) and one to *E. ortleppi* (G5) (Appendix O and P). Of the four G6/7 genotypes, two samples were genotyped as G7 (GenBank accession number: AY462128.1) and the other two samples (SZ and MR) could not be assigned to either G6 or G7, as the NCBI-BLAST search found similar sequence homology to both genotypes (GenBank accession numbers AB235847.1 and AB208063.1). These two samples' genotypes were thus designated as *E. canadensis* (G6/7).

Table 3.2 Comparison of PCR and sequencing results

**E. ortleppi* (G5) not conclusive by PCR; **Sequences regarded as *E. canadensis* G6/7 as they showed 99% homology to both G6 and G7 genotypes.

#	Sample	PCR results	Sequencing results	Maximum sequence identity	GenBank sequence accession number
1	AP	G1	G1	100%	EF367308.1
2	BJ	G6/7	G7	98%	AY462128.1
3	JB	G1	G1	94%	HM988991.1
4	LMax1	G1	G1	100%	HM988991.1
5	MJ	G5?*	G5	99%	AB235846.1
6	MT	G6/7	G7	100%	AY462128.1
7	SA	G1	G1	99%	EF367308.1
8	SZ	G6/7	G6/7**	99%	AB235847.1 and AB208063.1
9	MR	G6/7	G6/7**	99%	AB235847.1 and AB208063.1
10	NT	G1	G1	100%	EF367302.1

3.6 Microscopic diagnosis

3.6.1 Fertility status of cysts

The majority of cyst samples analyzed were fertile (75%), as demonstrated by the presence of hydatid hooklets and/or protoscolices (Table 3.3). Most cysts from the G1 genotype group

(73%) were fertile and only a few were infertile (27%). All cyst samples with the G6/7 genotype were also fertile. The single *E. ortleppi* sample was infertile. There was no significant association between genotype and fertility of cysts when comparing the G1 and G6/7 ($p = 0.6$) or the G1 and G5 genotypes ($p = 0.3$).

Table 3.3 Fertility status of *Echinococcus* genotypes

Genotype	Fertile (%)	Infertile (%)	Total
G1	19 (73)	7 (27)	26
G6/7	5 (100)	0	5
G5	0	1 (100)	1
Total	24 (75)	8 (25)	32

3.6.2 Viability investigation

A total of 20 human samples was received from the Eastern Cape for the cyst viability study, and of these, 15 (75%) were fertile, with three samples found fertile and viable.

3.6.3 Cattle isolate

The single cyst obtained from a cow in Gauteng Province was identified as *E. ortleppi* (confirmed by sequencing). It was pulmonary in origin and also fertile and viable (Figure 3.9).

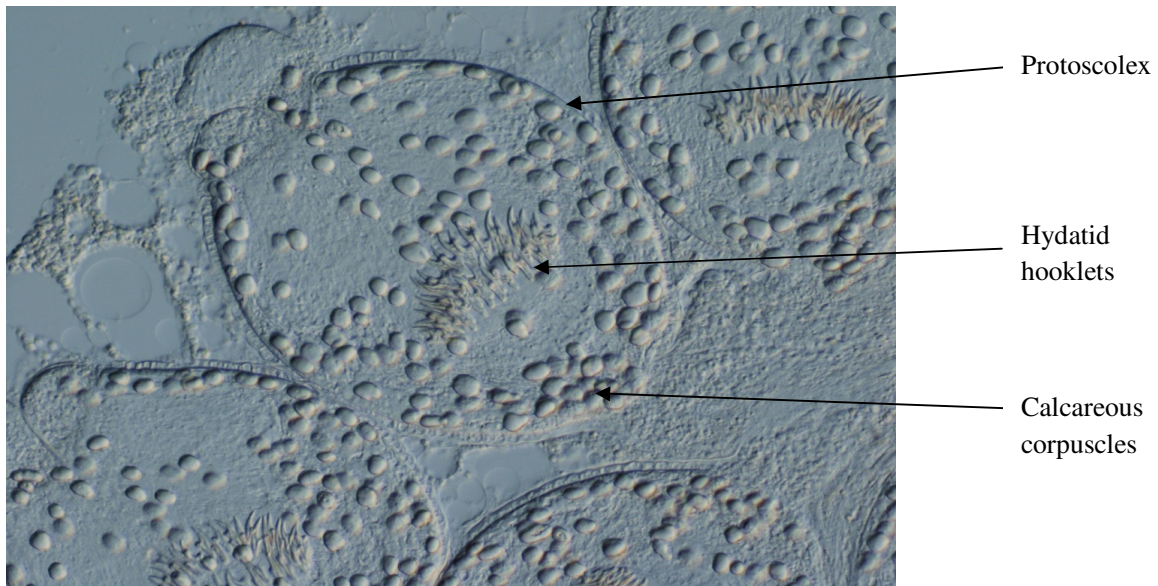


Figure 3.9 Microscopic image of viable *Echinococcus ortleppi* protoscolices from a cow sample (400 x magnification).

CHAPTER 4 - DISCUSSION

Cystic echinococcosis is a zoonosis of veterinary and public health importance. Insufficient attention has been given to the socio-economic and public health impact of CE; however, the World Health Organization (WHO) has recognized this disease as one of the Neglected Zoonosis subgroups for its 2008 to 2015 strategic plan for the control of neglected tropical diseases (NTDs) (32;110;111).

The most important advance in the control of CE is the use of molecular epidemiological methods as it allows public health officials and policy makers to design interventions that are appropriate for the type of *Echinococcus* strains circulating in a particular geographic area. Historically, control measures such as the timing of dog deworming programs have been standardized worldwide, but some species/genotypes have a shorter maturation period than the common sheep strain, thus rendering such deworming programs inadequate for non-*E. granulosus* species/genotypes (10;16;18;24;85). It was also shown that vaccines used to control the spread of the infection were designed on the presumption of the sheep strain (G1) being the primary cause of CE, and that such vaccines were ineffective against *E. canadensis* (G6/7 strains) (68;112).

To our knowledge, this is the first molecular epidemiological report on *Echinococcus* genotypes in South Africa, as available published data is only on clinical case studies (104). The research will hopefully serve to ignite interest on this neglected zoonotic disease in the country and to help to better understand lifecycles implicated in transmission of the infection to humans, in order to enable public health policy makers to design more targeted control programs.

4.1 GENOTYPE CHARACTERISTICS

In 2005, there were about 13.8 million cattle, 25.3 million sheep, 6.4 million goats and 1.7 million pigs in South Africa; and agriculture accounted for 3.2% of GDP and 7% of exports (113).

4.1.1 *E. granulosus* (G1)

The most frequent genotype found in our study was the common sheep strain, *Echinococcus granulosus* (G1). The predominance of this strain was unsurprising as it is the most common genotype worldwide (8;39;73-75). Sheep are thus presumably the main likely intermediate reservoirs of the infection in the country, although other intermediate hosts such as cattle, goats and camels can also harbour (albeit poorly) the G1 genotype (Figure 1.6).

4.1.2 *E. canadensis* (G6/7)

Five samples were genotyped as *E. canadensis* (G6/7). Two of them were homologous to the G7 strain. The failure to identify the other isolates as either G6 or G7 by DNA sequencing highlights the extent to which these two genotypes are closely related, and might in future not even warrant distinction (114;115).

The G6 genotype has previously been reported elsewhere in Africa (7;27;89;116), and is known to primarily use camels as intermediate hosts. Although camels are not utilized as livestock in South Africa, there are other intermediate hosts (mainly goats) that are known to be well adapted to the camel strain and are the most likely candidates as intermediate hosts for this genotype in the country. The presence of G6 in goats was recently described from southern Kenya, where camels are also absent (117).

The presence of the pig strain (G7) was unexpected, because as reflected by the literature reviewed, this genotype has not previously been reported from a human patient in Africa. Although literature reports show that human infectivity of this genotype was initially in doubt, it was later shown to be infective to humans and has been found in Europe, Asia, South America and Australia (8;16;25;28;90;118). The strain appears to be well adapted to pigs as intermediate hosts, but is also widespread in goats, e.g. in Greece (5;8;18;119). The relatively large number of pigs present in South Africa (~1.7 million) could possibly explain the presence of this strain, and a previous study at abattoirs around the country found pigs frequently affected by CE, further supporting their role in transmission of the disease (102). However, a study conducted in eastern Africa using samples of human, camel, cattle, sheep, goat and pig origin only detected G1, G6 and *E. ortleppi* but not the G7 strain (89).

4.1.3 *E. ortleppi*

There was only one human sample from the 32 study samples (3%) that was genotyped as *E. ortleppi*; the other *E. ortleppi* sample was from a cow. The isolation of this species from a human patient was quite interesting, since *E. ortleppi* is thought to have low pathogenicity for humans (8). Although first described in South Africa (81), only two instances of human infection could be found in the reviewed literature, namely, in Holland and Brazil (8;83;86). Therefore, our first record of this species in a human patient in Africa further supports the zoonotic potential of this species. The finding that the one human *E. ortleppi* species in the study was infertile (no hooks or protoscolices) strengthens the observation regarding its poor infectivity to humans.

A review by Thompson *et al* (1984) found that cysts of *E. ortleppi* were often infertile in cattle from Egypt, Italy, Argentina, Great Britain, Kenya, Nigeria, Iraq, Chile, Australia and Bangladesh, with fertility rates ranging from less than 1% to 15% (10). However, a few countries were reported to have high fertility rates, such as South Africa (96.8%), Belgium (94.2%) and Sri Lanka (79%) (10). Another study in Sudan also found that cysts collected from cattle and camels were much more likely to be fertile than those from sheep (35). Unsurprisingly, the cow sample in our study was also fertile and viable. The genotyped *E. ortleppi* human sample, as well as this abattoir sample from a cow, were both from Gauteng Province, showing to some extent that cattle play a role as reservoirs for human infection.

4.1.4 *E. equinus*

E. equinus was not found in this study, supporting reports that the species is not infective to humans (1;85). *E. equinus* species is, however, known to occur in equines in South Africa (8).

4.2 Genotype vs. organ of cyst localization

Although the liver has been infected in up to 70% of all CE cases, and the lungs account for around 20% of cases (12;33), we did not find any significant association between cyst localization and either of the two organs when comparing the G1 and G6/7 genotypes; the lungs were actually slightly more frequently affected than the liver. The G6/7 genotypes and *E. ortleppi* were also both mainly isolated from the lungs, supporting reports from other parts of the world, which tentatively associate *E. canadensis* with a predominance of pulmonary CE. It is also interesting to observe that research conducted in South Africa by Verster *et al*

(1966) also found that the lungs of cattle were the main organs affected by CE (102). The findings are however not conclusive, due to a small sample size, but the general inference from our data is that the cyst localization in the liver is not more predominant than the lungs in South Africa, and that the inverse might hold true.

Our retrospective study of case records from three academic hospitals in Gauteng Province found that pulmonary disease accounted for 64% of cases, with the liver only at 9% (104), but the patient selection was somewhat biased towards those treated in a pulmonary medicine department. This retrospective study hypothesized that genotypes involved in South Africa possibly played a role in influencing these unusual cyst localizations (104), but that hypothesis is clearly not supported by genotype results in this research.

These unexpected findings are difficult to interpret; it is not clear whether there are variants of *Echinococcus* genotypes in South Africa that exhibit different developmental characteristics in intermediate hosts or whether lung surgery is just more common than liver surgery. Arising from discussions with some of the surgeons involved in our study, the hypothesis is that surgeons are probably more comfortable performing lung surgical operations, with liver CE more likely to be treated by non-invasive methods, but this has not been investigated scientifically. The possible influence of HIV and cellular immunity status on the manifestation of the disease should also be investigated (120), to hopefully provide more insights on other epidemiological factors responsible for the biological characteristics of hydatid cysts in South African patients.

4.3 Genotype distribution by province

Samples were predominantly received from the Eastern Cape Province, with very few received from the other two contributing provinces. There was a statistically significant association found between genotypes G1 and G6/7 on prevalence per province, suggesting that G1 was more likely to be found in the Eastern Cape and G6/7 in Gauteng Province. However, this conclusion should be interpreted cautiously due to a small sample size. There was only one sample from the Free State Province and as such, it was not included in the statistical analysis.

4.4 Diagnostic methods

4.4.1 Microscopy

The majority of cyst samples received was fertile (75%), with *E. granulosus* (G1) having a high fertility rate of 73% and all *E. canadensis* (G6/7) samples fertile. The genotypes for the latter species were analyzed together instead of separately as G6 and G7, as they belong to the same species, and also because of a small sample size.

The fertility status of cysts from intermediate hosts can be an indicator of the epidemiological role that such intermediate hosts play in the perpetuation of the lifecycle (10), and the high fertility rates in our study shows the infectivity potential of these genotypes in the transmission of infection. Humans are, however, the dead-end for the lifecycle as definitive hosts have to feed on raw infected offal to contract the infection.

4.4.2 Comparison of *nadI* PCR/RFLP to 12S rRNA PCR method

The *nadI* PCR/RFLP method was found to be useful as it involved fewer PCR steps when compared to the 12S rRNA method, thus leading to a more rapid result and limiting chances for contamination. The method also had the advantages of being able to genotype all known genotypes known to occur in Africa, and to differentiate *E. granulosus* sensu stricto (G1-G3) from a closely related species, *E. felidis*, whereas the 12S rRNA was unable to differentiate the two species (92).

The major shortcoming with this *nadI* PCR/RFLP method for our study design was that we had no control over the sample condition upon receipt. Some samples received were preserved in formalin, which is known to degrade DNA (28), thus making amplification of a 1073-1078 bp *nadI* gene difficult. Three formalin-fixed study samples (MB, 1-67 and MT) failed to amplify by *nadI* PCR, but were successfully genotyped as *E. granulosus* G1 (MB and 1-67) and *E. canadensis* G6/7 by the 12S rRNA method that targets a shorter DNA fragment of 254 bp.

The other shortcoming for both methods was the inability to differentiate closely-related genotypes of *E. granulosus* sensu stricto (G1, G2 and G3), and the G6 and G7 *E. canadensis* genotypes. However, all *E. granulosus* sensu stricto samples that were sent for sequencing were confirmed as the common sheep strain, *E. granulosus* G1. Although this result was unsurprising as genotype G2 (Tasmanian sheep strain) is only known to occur in Australia and Argentina and the G3 genotype (buffalo strain) is not known to infect humans, the unexpected genotypes found in our study that were previously either not known to occur in

Africa (G7 genotype) or for which only two other human infection had previously been reported (*E. oritleppi*), suggests that South Africa has a variety of genotypes. It is important to always confirm random PCR results by sequencing until nationwide, long-term molecular epidemiological work has been done to conclusively characterize all genotypes from the nine South African provinces.

CHAPTER 5 – CONCLUSIONS

For the first time in South Africa, we successfully shed light on, and further characterized, the diversity of *Echinococcus* genotypes responsible for cystic echinococcosis in the country.

This is one of the largest case series ever undertaken on CE from an African country, second only to studies in Kenya (105).

The G1 strain (sheep strain) was the commonly isolated genotype that may be responsible for the majority of human disease in the country, a finding which is consistent with the trends reported in most parts of the world. The majority of cyst samples belonging to this genotype were also fertile, demonstrating its high pathogenicity to humans. Our first records of *E. canadensis* G7 (pig strain) and *E. ortleppi* (cattle strain) in humans are interesting and are also cause for concern.

The presence of these species has potentially serious control implications, since the timing of dog deworming programs worldwide is primarily based on the (long) prepatency period of the sheep strain (G1) in dogs. It has been reported that other genotypes reach patency much faster in the definitive hosts (5), thus potentially rendering control measures inefficient in areas where non-G1 strains circulate.

Although there is no systematic dog deworming program in South Africa, it is still important that any future control program should be administered with the knowledge of genotypes prevalent in a given area. In addition to this, the recognition of lifecycles will be important to control transmission of CE. Based on the first insights gained during this study, transmission

in the Eastern Cape might be rooted in a sheep-dog cycle, which is the basic lifecycle of *E. granulosus* G1. In contrast, the situation is likely to be more complex in Gauteng Province, where, apart from sheep, it certainly also involves goats and cattle, which are the most likely intermediate hosts for *E. canadensis* and *E. ortleppi*. Additional molecular surveys which include the collection of isolates from livestock, and covering other provinces of South Africa, are required.

The South African health system is under serious strain due to the high burden of HIV/AIDS and TB, with most of the priority of policy makers focusing on these immediate public health threats. However, there is no doubt that a lot more needs to be done to accurately estimate the prevalence of CE in the country, and to accordingly introduce appropriate deworming and educational programs as part of broader efforts to improve public health and in order to align with the WHO 2008 to 2015 strategic plan to control NTDs.

The study had some limitations, the most obvious being a small absolute number of samples received. Thus, some conclusions should be treated cautiously as wider, ongoing molecular epidemiological work is needed for a more comprehensive epidemiological picture on the South African situation.

The geographic areas covered were also not representative of the entire country, as samples were only received from three of the nine provinces. It is however, known from our previous retrospective study that CE is present across most parts of the country, with an estimation of at least 137 patients per year presenting with the disease (104). Another limitation was that although some patients from Gauteng Province had resided there for a long time, most were

originally from the Eastern Cape Province, confounding the designation of likely geographic site of infection.

The study however, successfully attained its primary objective of characterizing genotypes prevalent in the country and the sample size was also substantial relative to other publications relating to samples collected from humans. The study will hopefully serve as a good foundation for any prospective molecular epidemiological work that might follow in the future.

CHAPTER 6 – RECOMMENDED FUTURE WORK

There are a number of reasons that make a strong case for continual epidemiological work on cystic echinococcosis in South Africa. It is a well-known fact that most South African cultures practice informal slaughtering, where offal is often fed to free-roaming dogs. Most households in South African townships are not properly fenced to control movement of dogs, and these factors are the hallmarks for how transmission is facilitated in communities.

It will also be interesting to establish if future, larger studies will be able to replicate the findings from this study and if more genotypes can be identified. Future studies should also ideally involve collaborative work between clinicians, veterinarians and laboratory scientists to hopefully perform large-scale community screening by ultrasound, particularly in high risk provinces like the Eastern Cape and to collect more samples from both humans and animals in order to reach more definitive conclusions about genotypes and intermediate animal hosts that serve as reservoirs for the infection. The finding of the strain that has so far only been reported to occur in the wild transmission cycles in Africa, *E. felidis*, also warrants vigilant monitoring as its infectivity to humans has not been excluded and it is not known how pathogenic it is, should human infection be possible.

Appendix A - Informed consent form

Informed Consent

Study:

A Prospective Study into the Epidemiology and Clinical Aspects of Cystic Echinococcosis in South Africa (ECHISA-V2)

Institution: Faculty of Health, University of Witwatersrand, Johannesburg

I hereby confirm, that I have been informed by.....
about the nature, conduct, benefits and risks of the clinical study „Prospective Study into the Epidemiology and Clinical Aspects of Cystic Echinococcosis in South Africa“.

I have also received, read and understood the written information document (Participant Information) regarding the study.

I am aware that the results of the study, including personal details regarding my sex, age, date of birth, initials and diagnosis will be anonymously processed into a study report and published in a medical journal.

In view of the requirements of research, I agree that the data collected during this study can be processed in a computerised system.

I may, at any stage, without prejudice, withdraw my consent and participation in the study.

I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.

Participant (>18 years)/legal guardian:

Printed Name	Signature/Mark or Thumbprint	Date and Time
--------------	------------------------------	---------------

I,, herewith confirm that the above participant has been fully informed about the nature, conduct and risks of the above study

Study doctor:

Printed Name	Signature	Date and Time
--------------	-----------	---------------

Translator/Other person explaining informed consent.....(Designation)

Printed Name	Signature	Date and Time
--------------	-----------	---------------

In cases of routine medical care involves surgery:

I,..... confirm, that I have read and understood the information document about collection and storage of parasite material removed during surgery. I had the opportunity to ask questions and they have been answered.

I agree out of free will to collection of parasite material during surgery.

I agree out of free will to the storage of this material and analysis at a later stage.

Participant/Legal Guardian:

Printed Name

Signature

Date and Time

Appendix B - Assent form

Assent Form (if participant is <18 years of age)

Study:

A Prospective Study into the Epidemiology and Clinical Aspects of Cystic Echinococcosis in South Africa (ECHISA-V2)

Institution: Faculty of Health, University of Witwatersrand, Johannesburg

I hereby confirm, that I have been informed by.....
about the nature, conduct, benefits and risks of the clinical study „Prospective Study into the Epidemiology and Clinical Aspects of Cystic Echinococcosis in South Africa“.

I have also received, read and understood the written information document (Participant Information) regarding the study.

I am aware that the results of the study, including personal details regarding my sex, age, date of birth, initials and diagnosis will be anonymously processed into a study report and published in a medical journal.

In view of the requirements of research, I agree that the data collected during this study can be processed in a computerised system.

I may, at any stage, without prejudice, withdraw my consent and participation in the study.

I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.

Participant (<18 years):

Printed Name	Signature/Mark or Thumbprint	Date and Time
--------------	------------------------------	---------------

I,, herewith confirm that the above participant has been fully informed about the nature, conduct and risks of the above study

Study doctor:

Printed Name	Signature	Date and Time
--------------	-----------	---------------

Translator/Other person explaining informed consent.....(Designation)

Printed Name

Signature

Date and Time

In cases of routine medical care involves surgery:

I,..... confirm, that I have read and understood the information document about collection and storage of parasite material removed during surgery. I had the opportunity to ask questions and they have been answered.

I agree out of free will to collection of parasite material during surgery.

I agree out of free will to the storage of this material and analysis at a later stage.

Participant:

Printed Name

Signature

Date and Time

Appendix C - Patient questionnaire form

A Prospective Study into the Epidemiology and Clinical Aspects of Cystic Echinococcosis in South Africa (ECHISA-V2)

Data Capture Sheet

Patient-ID _____

1. Demographic Data:

Age:

Sex: ☐ female ☐ male

Place of Birth

Place of Residence

Occupation:

Farming Activities:

☐ unknown

☐ no

☐ yes, state what kind

Contact to dogs:

☐ unknown

☐ no

☐ yes

pet dog, mainly in the house:

☐ yes

☐ no

watch dog, mainly outside

☐ yes

☐ no

regular deworming

☐ yes

☐ no

free-roaming dog

☐ yes

☐ no

Livestock:

☐ unknown

☐ no

☐ cattle

☐ sheep

☐ goats

☐ other, please specify:

Homeslaughtering:

☐ unknown

☐ no

☐ yes

☐ raw offal offered to dogs

Family members with hydatid disease:

☐ unknown

☐ no

☐ yes

Additional Information:

2. Clinical Data:

a. At Diagnosis

Organs involved: ☐ lung ☐ liver

☐ other, state which

No of cysts:

Size of cysts: cm

Presenting Symptoms:

Complications of Cyst:

Imaging technique used: ☐ CXR ☐ CT

☐ Ultrasound ☐ other

☐ unknown

Serology: ☐ unknown ☐ positive, Titre:

☐ negative

HIV ☐ unknown ☐ no

☐ yes

CD4-cellcount

On ARVs

Regimen

TB ☐ current ☐ in past

On Tx?

Regimen

HBV ☐ unknown ☐ no

☐ yes

Other diseases

Additional Comments:

b. Treatment:

Treatment: ☐ unknown ☐ Surgery

State Procedure:

☐ PAIR

☐ Albendazole

☐ none

Complications of Treatment: ☐ unknown ☐ no

☐ yes, please state which:

Additional comments:

c. Follow-up and Course of Disease:

Follow-up available:

☐ no

☐ yes

Follow-up 1

Months after initial diagnosis:

Course of disease

☐ cure

☐ progress

☐ relapse

Additional comments:

Follow-up 2

Months after initial diagnosis:

Course of disease

☐ cure

☐ progress

☐ relapse

Additional comments:

Follow-up 3

Months after initial diagnosis:

Course of disease

☐ cure

☐ progress

☐ relapse

Additional comments:

Follow-up 4

Months after initial diagnosis:

Course of disease

☐ cure

☐ progress

☐ relapse

Additional comments:

3. PCR:

☐ available

☐ not available

Strain isolated: _____

Appendix D - Ethics clearance certificate for the collaborative study.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Colin Menezes

CLEARANCE CERTIFICATE

M10719

PROJECT

A Prospective Study into the Epidemiology
and Clinical Cystic Echinococcosis in South
Africa

INVESTIGATORS

Dr Colin Menezes.

DEPARTMENT

Internal Medicine/Infectious Disease

DATE CONSIDERED

30/07/2010

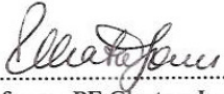
DECISION OF THE COMMITTEE*

Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 16/08/2010

CHAIRPERSON


(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Prof M Grobusch

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.

I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

Appendix E - Ethics clearance certificate for this M.Sc. project

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Mr Kgaile Benjamin Mogoye

CLEARANCE CERTIFICATE M110805

PROJECT Human cystic Echinococcosis in South Africa

INVESTIGATORS Mr Kgaile Benjamin Mogoye.

DEPARTMENT Division of Virology/School of Pathology

DATE CONSIDERED 26/08/2011

M110805DECISION OF THE COMMITTEE* Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 07/10/2011

CHAIRPERSON



(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Professor John Freaan

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.

I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

Appendix F - Correspondence with Animal Ethics Screening Committee

From: Gavin Norton [Gavin.Norton@wits.ac.za]
Sent: 03 03 2011 08:33 AM
To: Benjamin Mogoye
Cc: Iain Burns
Subject: RE: Request to use animal samples

Dear Benjamin,

Thank you for sending us this letter. The Animal Ethics Screening Committee appreciates you keeping us informed of work performed on animal samples. As this is work where tissue samples will be obtained from animals killed at an abattoir and hence, these animals are not killed for the purposes of the study, you do not require animal ethics clearance for this study.

Best wishes,

Gavin Norton (Chair, Animal Ethics Screening Committee, University of the Witwatersrand)

From: Benjamin Mogoye [mailto:kgailem@nicd.ac.za]
Sent: 02 March 2011 04:24 PM
To: Gavin Norton
Cc: Iain Burns
Subject: Request to use animal samples

Dear Prof,

As advised earlier, kindly receive the attached letter in request for use of animal tissue for research purposes.

Kind regards,

Benjamin Mogoye

Medical Scientist
Parasitology Reference Unit
National Institute for Communicable Diseases
A Division of the National Health Laboratory Service
Office: 011 555 0304
Fax : 011 555 0446
www.nicd.ac.za

Appendix G - DNA extraction protocol using the MagNA Pure compact

A. SPECIMEN SELECTION:

1. Select specimens that need to be extracted. Fill in the specimen log with date extracted and initial. There is no need to fill any other paperwork as the robot prints run reports, with all relevant information.

B. PRE-EXTRACTION PREPARATIONS:

1. Thaw specimens plus 1 positive and 1 negative control. Remember that both controls are to be treated in exactly the same way as the specimens. Ensure that there is 100µl of sample volume in all the Starstedt tubes.

B. DNA ROBOT EXTRACTION

2. Switch the DNA robot on. Wait for it to complete its checks.
3. From the menu select "RUN".
4. The next screen can be bypassed by pressing the 'next' icon (>>) as there is no password.
5. From the current kit take out cartridges as needed. Cut open packet and remove cartridge, hold the cartridges on the barcode area and the opposite side only. Do not touch the sealing foil. Check that the sealing foil is not damaged and that mix the content of the cartridge by inverting the cartridge a few times. If the cartridge is unsatisfactory – do not use it; keep it away to give to Roche. Pull the lever on the side of the cartridge rack to raise it; the rack can then be removed for easier use. Scan the barcode on the side of the cartridge, after the code appears in channel 1 on the screen; insert the cartridge (2 empty wells at the back) into channel 1 ensuring it clicks into place. Continue loading the cartridges from the left. Push the cartridge back down. Confirm "CARTRIDGES INSERTED" and select next (>>).
6. Select the following setting:
 - a. Protocol: DNA Blood V3
 - b. Sample Volume: 100µl
 - c. Elution Volume: 100µl
 - d. Sample Material: Blood
 - e. Internal Control Volume: None
7. Insert tip trays (from kit) accordingly. Ensure all the tips and the piercing tools are present; the piercing tool should be in front. Confirm "TIP TRAYS INSERTED" and select next (>>).

8. Type in the sample ID using the keyboard icon on the bottom left of the screen, select next (>>) to enter the next sample ID. Remove the specimen rack and insert specimens (in our Starstedt tubes-do not use tubes provided) in the sample row (S). Confirm "SAMPLES INSERTED" and select next (>>).
9. Scan elution tubes (from kit) and insert in the elution tube row (E). Confirm "ELUTION TUBES INSERTED" and select next (>>). Remove specimen tubes' caps and discard, place specimen rack back into robot.
10. Check data and settings chosen "CONFIRM DATA", ensure that the drop catcher is present "DROP CATCHER PRESENT", ensure lids are removed, close the door and select "START".
11. The robot will display the remaining time and process it is currently undergoing. A run takes ± 28 minutes. Fill in DNA robot log. Make labels with the LabPal, using BAN and small font size [line 1 "specimen number", line 2 "extraction date"].
12. When complete, an orange light under the touch screen will flash indicating "RUN COMPLETED".
13. Print results by selecting the print icon on the bottom of the screen. Open the lid; carefully cap eluted samples, with the caps provided in the kit and remove. Label these with the labels made according to the report, ensuring the correct label goes on the correct elution tube.
14. Throw away the sample tubes, tip trays and cartridges into the biohazardous waste. Clean robot with dH₂O (not alcohol). Go back to the main screen and select "Maintenance" and then "UV Decontamination", select time duration (minimum 1 hour). Complete DNA robot log.
15. On the report, highlight the date, sign at signature and fill in storage box numbers under comments.

Appendix H - DNA extraction protocol from a fluid sample (QIAGEN)

1. Pipette 20 μ l **Proteinase K** into the bottom of a 1.5 ml microcentrifuge tube.
2. Add 200 μ l of sample to the microcentrifuge tube. If the sample is less than 200 μ l, add the appropriate volume of PBS.
3. Add 200 μ l **Buffer AL** to the sample, mix by pulse-vortexing for 15 s, and incubate at 56°C for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.
4. Add 200 μ l **ethanol** (96-100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.
5. Carefully apply all the mixture (including the precipitate) to QIAamp spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 8000 rpm for 1 min. Place the QIAamp spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
6. Carefully open the QIAamp spin column and add 500 μ l **Buffer AW1** without wetting the rim. Close the cap, and centrifuge at 8000 rpm for 1 min. Place the QIAamp spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.
7. Carefully open the QIAamp spin column and add 500 μ l **Buffer AW2** (blue tip) without wetting the rim. Close the cap and centrifuge at full speed 14,000 rpm for 3 min.
8. Place the QIAamp spin column in a new 2 ml collection tube (not provided, use 1.5 ml tube with lid removed) and discard the collection tube containing the filtrate. Centrifuge at full speed for 1 min.
9. Place the QIAamp spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp spin column and add 100 μ l **Buffer AE**. Incubate at room temperature for 5 minutes and then centrifuge at 8000 rpm for 5 min.
10. Carefully open the QIAamp spin column and add another 100 μ l **Buffer AE**. Incubate at room temperature for 5 minutes and then centrifuge at 8000 rpm for 5 min. Discard the column and store at 4°C or -20°C for a longer term. Clean up, switch off mini-spin, put away timer kit, fill in logs & freezer box sheets etc. and put on UV for 30mins.

Appendix I - DNA extraction protocol from a tissue sample (QIAGEN)

1. Excise the tissue sample or remove it from storage and place in a 1.5 ml microcentrifuge tube (Do not use more than 25 mg).
 2. Add 180 µl of Buffer ATL. (It is important to cut tissue into small pieces to decrease lysis time.
 3. Add 20 µl **Proteinase K** using filter tips, mix by vortexing, and incubate at 56°C until the tissue is lysed. Vortex occasionally during incubation to disperse the sample.
 4. Quick spin the 1.5 ml microcentrifuge tubes to remove drops from the inside of the lid.
 5. Add 200 µl **Buffer AL** to the sample, mix by pulse-vortexing for 15 s, and incubate at 70°C for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.
 6. Add 200 µl **ethanol** (96-100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.
 7. Carefully apply all the mixture (including the precipitate) to QIAamp spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 8000 rpm for 1 min. Place the QIAamp spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
 8. Carefully open the QIAamp spin column and add 500 µl **Buffer AW1** without wetting the rim. Close the cap, and centrifuge at 8000 rpm for 1 min. Place the QIAamp spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.
 9. Carefully open the QIAamp spin column and add 500 µl **Buffer AW2** (blue tip) without wetting the rim. Close the cap and centrifuge at full speed 14,000 rpm for 3 min.
 10. Place the QIAamp spin column in a new 2 ml collection tube (not provided, use 1.5 ml tube with lid removed) and discard the collection tube containing the filtrate. Centrifuge at full speed for 1 min.
 11. Place the QIAamp spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp spin column and add 100 µl **Buffer AE**. Incubate at room temperature for 5 minutes and then centrifuge at 8000 rpm for 5 min.
 12. Carefully open the QIAamp spin column and add another 100 µl **Buffer AE**. Incubate at room temperature for 5 minutes and then centrifuge at 8000 rpm for 5 min. Discard the column and store at 4°C or -20°C for a longer term. Clean up, switch off mini-spin, put away timer kit, fill in logs & freezer box sheets etc. and put on UV for 30mins.
-

Appendix J - Reagent preparations (agarose gel)

QUICK REFERENCE GUIDE:

GEL CASTING:

1.5% SMALL AGAROSE GEL:	13 (12+1) wells
1X TAE buffer	30ml
Agarose	0.45g
SYBR safe	3 µl

Total 1X TAE buffer needed = 500ml

1.5% MEDIUM AGAROSE GEL:	27 (26+1) wells
1X TAE buffer	80ml
Agarose	1.2g
SYBR safe	8 µl

Total 1X TAE buffer needed = 1000ml (1 L)

1.5% LARGE AGAROSE GEL:	52 (50+2) wells
1X TAE buffer	125ml
Agarose	1.88g
SYBR safe	12.5 µl

Total 1X TAE buffer needed = 2000ml (2 L)

GEL LOADING:

4 µl 1X TAE buffer
1 µl DNA loading dye
5 µl DNA

Run gel from black (-ve) to red (+ve)
110 volts/ 30 minutes

Appendix K - Reagent preparations (Krebs-Ringer buffer solution)



KREBS-RINGER BICARBONATE BUFFER

With 1800 mg/L Glucose, Without Calcium Chloride and Sodium Bicarbonate
Product Number **K4002**

Product Description

Although there have been many modifications to the original formulas in efforts to produce fully defined media, salt solutions still play an important role in tissue culture. A salt solution's basic function, to maintain the pH and osmotic balance in the medium and to provide the cells with water and essential inorganic ions, is as valuable today as when it was first developed a century ago

Components	g/L
D-Glucose	1.8
Magnesium Chloride [Anhydrous]	0.0468
Potassium Chloride	0.34
Sodium Chloride	7.0
Sodium Phosphate Dibasic [Anhydrous]	0.1
Sodium Phosphate Monobasic [Anhydrous]	0.18

Precautions and Disclaimer

REAGENT

For R&D use only. Not for drug, household or other uses.

Preparation Instructions

Powdered salts are hygroscopic and should be protected from moisture. The entire contents of each package should be used immediately after opening. Preparing a concentrated salt solution is not recommended as precipitates may form. Supplements can be added prior to filtration or introduced aseptically to sterile salt solution.

1. Measure out 90% of final required volume of water. Water temperature should be 15-20 °C.
2. While gently stirring the water, add the powdered medium. Stir until dissolved. Do NOT heat.
3. Rinse original package with a small amount of water to remove all traces of powder. Add to solution in step 2.
4. To the solution in step 3, add 1.26 g sodium bicarbonate or 16.8 ml of sodium bicarbonate solution [7.5%w/v] for each liter of final volume of medium being prepared. Stir until dissolved.

5. While stirring, adjust the pH of the medium to 0.1-0.3 pH units below the desired pH since it may rise during filtration. The use of 1N HCl or 1N NaOH is recommended.
6. Add additional water to bring the solution to final volume.
7. Sterilize immediately by filtration using a membrane with a porosity of 0.22 microns.
8. Aseptically dispense medium into sterile container.

Storage and Stability

Store the dry powdered salts at 2-8 °C under dry conditions and liquid medium at 2-8 °C in the dark. Deterioration of the powdered medium may be recognized by any or all of the following: [1] color change, [2] granulation/clumping, [3] insolubility. Deterioration of the liquid medium may be recognized by any or all of the following: [1] pH change, [2] precipitate or particulates, [3] cloudy appearance [4] color change. The nature of supplements added may affect storage conditions and shelf life of the medium. Product label bears expiration date.

Procedure

Materials Required but Not Provided

Water for tissue culture [W3500]

Sodium Bicarbonate [S5761] or

Sodium Bicarbonate Solution, 7.5% [S8761]

1N Hydrochloric Acid [H9892]

1N Sodium Hydroxide [S2770]

Medium additives as required

Reference

1. Krebs, H. A. and Henseleit, K. (1932). Untersuchungen über die Harnstoffbildung im Tierkörper in Hoppe-Seyler's Zeitschrift für Physiol. Chemie, 210, 33-66.

Revised: June 2007

Sigma-Aldrich, Inc. warrants that its products conform to the information contained in this and other Sigma-Aldrich publications. Purchaser must determine the suitability of the product(s) for their particular use. Additional terms and conditions may apply. Please see reverse side of the invoice or packing slip.

Appendix L - South African department of agriculture, forestry and fisheries disease databases*

Date (1993-2011)	Province	State Vet	District	Disease	Species	Outbreaks	Cases	Dead	Killed
1996/05	EASTERN CAPE	PORT ELIZABETH	NELSON MANDELA	ECHINOCOCCOSIS/HYDATIDOSIS	OVINE	2	14	0	14
1996/07	EASTERN CAPE	PORT ELIZABETH	NELSON MANDELA	ECHINOCOCCOSIS/HYDATIDOSIS	OVINE	2	10	0	10
1996/07	EASTERN CAPE	UMZIMKULU	UMZIMKULU	ECHINOCOCCOSIS/HYDATIDOSIS	BOVINE	2	2	2	0
1997/04	EASTERN CAPE	UMTATA	KING SABATA DALINDY EBO	ECHINOCOCCOSIS/HYDATIDOSIS	CANINE	2	2	2	0
1998/05	EASTERN CAPE	EAST LONDON	BUFFALO CITY	ECHINOCOCCOSIS/HYDATIDOSIS	BOVINE	2	2	0	2
1999/01	MPUMALANGA	KWAMHLANGA	THEMBISILE	ECHINOCOCCOSIS/HYDATIDOSIS	BOVINE	2	2	0	2
1999/09	NORTHERN CAPE	KIMBERLEY	HAY	ECHINOCOCCOSIS/HYDATIDOSIS	BAT-EARED FOX	0	2	2	0
1999/09	NORTHERN CAPE	KIMBERLEY	KIMBERLEY	ECHINOCOCCOSIS/HYDATIDOSIS	YELLOW MONGOOSE	0	2	2	0
2000/06	EASTERN CAPE	ELLIOT	SAKHISIZWE	ECHINOCOCCOSIS/HYDATIDOSIS	OVINE	2	14	14	0
2001/08	MPUMALANGA	PIET RETIEF	SEME	ECHINOCOCCOSIS/HYDATIDOSIS	OVINE	2	10	10	0
2001/08	MPUMALANGA	PIET RETIEF	SEME	ECHINOCOCCOSIS/HYDATIDOSIS	OVINE	2	14	14	0

*Query ran from January 1993 to December 2011. <http://www.nda.agric.za>. [Accessed 12 July 2012].

Appendix M - DNA purification protocol (Zymo research sequencing clean-up kit)

Buffer Preparation

- ✓ **Before starting:** Add 24 ml 100% ethanol (26 ml 95% ethanol) to the 6 ml **DNA Wash Buffer** concentrate. Add 96 ml 100% ethanol (104 ml 95% ethanol) to the 24 ml **DNA Wash Buffer** concentrate.

Protocol

All centrifugation steps should be performed between 10,000 - 16,000 x g.

1. Excise the DNA fragment¹ from the agarose gel using a razor blade, scalpel or other device and transfer it into a 1.5 ml microcentrifuge tube.
2. Add 3 volumes of **ADB** to each volume of agarose excised from the gel (e.g. for 100 µl (mg) of agarose gel slice add 300 µl of **ADB**).
3. Incubate at 37-55 °C for 5-10 minutes until the gel slice is completely dissolved².

For DNA fragments > 8 kb, following the incubation step, add one additional volume (equal to that of the gel slice) of water to the mixture for better DNA recovery (e.g., 100 µl agarose, 300 µl **ADB**, and 100 µl water).

4. Transfer the melted agarose solution to a **Zymo-Spin™ Column** in a **Collection Tube**.
5. Centrifuge for 30-60 seconds. Discard the flow-through³.
6. Add 200 µl of **DNA Wash Buffer** to the column and centrifuge for 30 seconds. Discard the flow-through. Repeat the wash step.
7. Add ≥ 6 µl **DNA Elution Buffer**⁴ or water⁵ directly to the column matrix. Place column into a 1.5 ml tube and centrifuge for 30-60 seconds to elute DNA.

Ultra-pure DNA is now ready for use.

Notes:

¹ The amount of agarose excised from the gel should be as small as possible.

² Do not incubate above 60°C. It is important that the gel slice dissolve completely. This can be facilitated by gentle mixing during the incubation.

³ Remove the flow-through by aspiration. Avoid contamination of the collection tube rim.

⁴ **DNA Elution Buffer:** 10 mM Tris-HCl, pH 8.5, 0.1 mM EDTA.

⁵ Elution of DNA from the column is dependent on pH and temperature. If water is used, make sure the pH is >6.0. Waiting 1 minute prior to elution may improve the yield of larger (> 6 kb) DNA. For even larger DNA (> 10 kb), the total yield may be improved by eluting the DNA with 60-70 °C **DNA Elution Buffer**.

Appendix N - Sequencing protocol (BigDye ver3.1)

Perform cycle sequencing

Quantity of PCR product to use

The minimum quantity of PCR product to use for sequencing is 20 ng, as checked by running on an agarose gel.

Prepare and run the cycle sequencing reactions

IMPORTANT! You need to use the BigDye® Direct M13 forward or reverse primers in your BigDye® Direct cycle sequencing reactions.

1. Prepare a forward or reverse sequencing reaction mix in a tube on ice:

Components	Volume for each reaction
BigDye® Direct Sequencing Master Mix	2.0 µL
One sequencing primer: • BigDye® Direct M13 Fwd Primer or • BigDye® Direct M13 Rev Primer	1.0 µL
Total volume for each reaction	3.0 µL

2. For each sequencing reaction, add 3 µL of the sequencing reaction mix to the appropriate well in the respective forward or reverse reaction plate.
3. Seal the reaction plate with adhesive film or caps, then spin the plate briefly.
4. Run the reactions in a thermal cycler:

Stage	Veriti® thermal cyclers		9700 thermal cycler	
	Temp	Time	Temp	Time
Hold	37°C	15 min	37°C	15 min
Hold	80°C	2 min	80°C	2 min
Hold	96°C	1 min	96°C	1 min
Cycle (25 cycles)	96°C	10 sec	96°C	10 sec
	50°C	5 sec	50°C	5 sec
	60°C	75 sec	60°C	4 min
Hold	4°C	∞	4°C	∞

5. After the cycle sequencing reactions are complete, spin the plate briefly.

STOPPING POINT. (Optional) Store the reaction plate at 4°C overnight or at -15°C or -25°C for long-term storage.

Appendix O - NCBI-BLAST result showing alignment of sample MT with a G7 reference genotype (AY462128.1) deposited in GenBank

|AY462128.1| *Echinococcus canadensis*, 12S ribosomal RNA gene, partial sequence; mitochondrial; genotype G7
Length=335

Score = 329 bits (178), Expect = 3e-87
Identities = 178/178 (100%), Gaps = 0/178 (0%)
Strand=Plus/Minus

MT	3	TGGCTTAACTAAACTACACAACTCAAACCATTAAACGATAATATCAAACCAGATATAC	62
AY462128.1	186	TGGCTTAACTAAACTACACAACTCAAACCATTAAACGATAATATCAAACCAGATATAC	127
MT	63	ACCAACATAACAAGAGTGAAATAATAGGTGGACCATCCTTTACTATGCATCTTCCCCTAA	122
AY462128.1	126	ACCAACATAACAAGAGTGAAATAATAGGTGGACCATCCTTTACTATGCATCTTCCCCTAA	67
MT	123	CAAGAATCGCTCACTGCCAAACCATTTTAGTTACAAAACAGGACGAACATTTTAAAA	180
AY462128.1	66	CAAGAATCGCTCACTGCCAAACCATTTTAGTTACAAAACAGGACGAACATTTTAAAA	9

Appendix P - NCBI-BLAST result showing alignment of sample MJ with *E. ortleppi* (G5) reference genotype (AB235846.1) deposited in GenBank (page 79-80)

```
|AB235846.1| Echinococcus ortleppi mitochondrial DNA, complete genome, genotype
G5
Length=13717

Score = 1775 bits (961), Expect = 0.0
Identities = 969/972 (99%), Gaps = 3/972 (0%)
Strand=Plus/Minus

Query* 1      CAAATTTAATTCACAATAACAAAAATCCTAAAATCAGCACACACAGACAAACCTCTCAG  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct**8372   CAAATTTAATTCACAATAACAAAAATCCTAAAATCAGCACACACAGACAAACCTCTCAG  8313

Query  61      AAAAAATTTAACAAAACAATCATAACGAACGCGTGGTAACGTCGCTCGAGCCACATAAAA  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  8312     AAAAAATTTAACAAAACAATCATAACGAACGCGTGGTAACGTCGCTCGAGCCACATAAAA  8253

Query  121     AACAAATAAATTAAacatcaacataaacataaccaacaataaccaccaccaaccattaatacc  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  8252     AACAAATAAATTAAACATCAACATAAACATACCAACAATACCACCACCAACCATTAAATACC  8193

Query  181     accaccaaccaTGAAAACATGTATATAACGATATACTCGCAAGCAAATAAACACGTAAAA  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  8192     ACCACCAACCATGAAAACATGTATATAACGATATACTCGCAAGCAAATAAACACGTAAAA  8133

Query  241     TATATACCACTATACTCAACATTAAATCCTCTAACCAATTCACCTTCAGCCTCCCCATAA  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  8132     TATATACCACTATACTCAACATTAAATCCTCTAACCAATTCACCTTCAGCCTCCCCATAA  8073

Query  301     TCAAATGGAGTACGATTAGTCTCACACAACACGCACACCAAGAATAACCCATAAATTAAT  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  8072     TCAAATGGAGTACGATTAGTCTCACACAACACGCACACCAAGAATAACCCATAAATTAAT  8013

Query  361     GGAAATAATAACCAACTCCACCAGTAACTATGataaaaaatcaattaaattatacctacaa  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  8012     GGAAATAATAACCAACTCCACCAGTAACTATGATAAAAAATCAATTAAATTATACCTACAA  7953

Query  421     caacacaaaagcacaaaaaataaccacacacataaaaacaagcctcaaacctaacagatcca  480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7952     CAACACAAAGCACAAAAAATAACCACACACATAAAACAAGCCTCAAACCTAACAGATCCA  7893
```

```

Query 481   aaaGCACATCGAACCGACCTTAAAAAGAATAACTATTATAACTACCCCAACCTGCGCAC 540
          |||
Sbjct 7892  AAAGCACATCGAACCGACCTTAAAAAGAATAACTATTATAACTACCCCAACCTGCGCAC 7833

Query 541   AACAAAGAATATCTAGAAATTCTAGCAGCAGCTAAAAATCATAATACAGAAAGACCACTA 600
          |||
Sbjct 7832  AACAAAGAATATCTAGAAATTCTAGCAGCAGCTAAAAATCATAATACAGAAAGACCACTA 7773

Query 601   TACCTAACTCTATAATATCTACCataaacaacgaatatatcacaacaaaaactattaac 660
          |||
Sbjct 7772  TACCTAACTCTATAATATCTACCATAAACAAACGAATATATCACAACCAAAACTATTAAC 7713

Query 661   aacaaaacaccaaataaaccaatataCCTACGACTTTGGAAGTAAAAATTCTTAACTTA 720
          |||
Sbjct 7712  AACAAAACACCAAATAAACCAATATACCTACGACTTTGGAAGTAAAAATTCTTAACTTA 7653

Query 721   ATTACCAACTTCAACAAGTCAGCAAACCTCTGAAGCAAACCAATTATACCAACCTTATTA 780
          |||
Sbjct 7652  ATTACCAACTTCAACAAGTCAGCAAACCTCTGAAGCAAACCAATTATACCAACCTTATTA 7593

Query 781   GGGGCCCTTACGAAATTGAGAATACCCCAAAACCTTACGTTACCCAAAACaaaaaaagc 840
          |||
Sbjct 7592  GGG-CCCTTACGAAATTGAGAATACCCCAAAACCTTACGTTACCCAAAACAAAAAAGC 7534

Query 841   aattattaacaaactaatcaacaaacaaaaacccagaaaccagcccaaaaacaaccaT 900
          |||
Sbjct 7533  AATTATTAACAACTAATCAACAAACCAAAACCCAGAAACCAGCCCAAAAACAACCAT 7474

Query 901   TAGAGCAACCTAATCA-CAAAATCACCATCAATGAGACAAATTGACATTCTAAATAAACT 959
          |||
Sbjct 7473  TAGAGCAACCTAATCAGCAAAATCACCATCAATGAGACAAATTGACATTCTAAATAAACT 7414

Query 960   AAGGTCAC-AAC 970
          |||
Sbjct 7413  AAGGTCATAAC 7402

```

Keys: Query* = Sample MJ; Sbjct** = GenBank reference strain AB235846.1

REFERENCES

- (1) Thompson RCA. Biology and Systematics of *Echinococcus*. In: Thompson RCA, Lymbery AJ, editors. *Echinococcus* and hydatid disease. Wallingford, United Kingdom: CAB International; 1995. p. 1-50.
- (2) Ash L, Orihel T. Atlas of Human Parasitology. 4th ed. Chicago: Am J Clin Pathol; 1997.
- (3) Soulsby EJJ. Helminths, Arthropods and Protozoa of Domesticated Animals. 6th ed. London: Bailliere, Tindall and Cassell Ltd; 1968.
- (4) Budke CM, Deplazes P, Torgerson PR. Global socioeconomic impact of cystic echinococcosis. *Emerg Infect Dis* 2006 Feb;12(2):296-303.
- (5) Eckert J, Gemmell MA, Meslin FX, Pawlowski ZS. WHO/OIE Manual on Echinococcosis in Humans and Animals: a Public Health Problem of Global Concern. Paris, France: World Health Organization; 2001.
- (6) Eckert J, Deplazes P. Biological, epidemiological, and clinical aspects of echinococcosis, a zoonosis of increasing concern. *Clin Microbiol Rev* 2004 Jan;17(1):107-35.
- (7) Hüttner M, Romig T. *Echinococcus* species in African wildlife. *Parasitology* 2009 Sep;136(10):1089-95.
- (8) Jenkins DJ, Romig T, Thompson RC. Emergence/re-emergence of *Echinococcus* spp. A global update. *Int J Parasitol* 2005 Oct;35(11-12):1205-19.
- (9) Thompson RC. The taxonomy, phylogeny and transmission of *Echinococcus*. *Exp Parasitol* 2008 Aug;119(4):439-46.
- (10) Thompson RC, Kumaratilake LM, Eckert J. Observations on *Echinococcus granulosus* of cattle origin in Switzerland. *Int J Parasitol* 1984 Jun;14(3):283-91.
- (11) Nakao M, McManus DP, Schantz PM, Craig PS, Ito A. A molecular phylogeny of the genus *Echinococcus* inferred from complete mitochondrial genomes. *Parasitology* 2007 May;134(Pt 5):713-22.
- (12) Gottstein B. Major Tropical Syndromes by Body System: Systemic Infections. 2nd ed. Edinburgh: Infectious Diseases; 2003.
- (13) Permin A, Hansen JW. Review of echinococcosis/hydatidosis: a zoonotic parasitic disease. FAO 1994 September 5. <www.fao.org/docrep/t1300t/t1300T0m.htm> [Accessed 2012 Aug 27].
- (14) Lawson JR, Gemmell MA. The potential role of blowflies in the transmission of taeniid tapeworm eggs. *Parasitology* 1985 Aug;91(Pt 1):129-43.

- (15) Centers for Disease Control and Prevention. Echinococcosis, 2012.
<<http://www.dpd.cdc.gov/dpdx/html/echinococcosis.htm>>[Accessed 2012 May 10].
- (16) Eckert J, Thompson RC. Intraspecific variation of *Echinococcus granulosus* and related species with emphasis on their infectivity to humans. *Acta Trop* 1997 Apr 1;64(1-2):19-34.
- (17) Thompson RC, Lymbery AJ, Constantine CC. Variation in *Echinococcus*: towards a taxonomic revision of the genus. *Adv Parasitol* 1995;35:145-76.
- (18) Thompson RC, McManus DP. Towards a taxonomic revision of the genus *Echinococcus*. *Trends Parasitol* 2002 Oct;18(10):452-7.
- (19) Ortlepp JR. South African helminths. *Onderstepoort J Vet Sci* 1937;9:311-36.
- (20) Verster AJ. Review of *Echinococcus* species in South Africa. *Onderstepoort J Vet Res* 1965 Jun;32(1):7-118.
- (21) Rausch RL. A consideration of infraspecific categories in the genus *Echinococcus* Rudolphi, 1801 (Cestoda: Taeniidae). *J Parasitol* 1967 Jun;53(3):484-91.
- (22) Harandi MF, Hobbs RP, Adams PJ, Mobedi I, Morgan-Ryan UM, Thompson RC. Molecular and morphological characterization of *Echinococcus granulosus* of human and animal origin in Iran. *Parasitology* 2002 Oct;125(Pt 4):367-73.
- (23) Thompson RC, Lymbery AJ. The nature, extent and significance of variation within the genus *Echinococcus*. *Adv Parasitol* 1988;27:209-58.
- (24) Hüttner M, Nakao M, Wassermann T, Siefert L, Boomker JD, Dinkel A, et al. Genetic characterization and phylogenetic position of *Echinococcus felidis* (Cestoda: Taeniidae) from the African lion. *Int J Parasitol* 2008 Jun;38(7):861-8.
- (25) Schneider R, Gollackner B, Schindl M, Tucek G, Auer H. *Echinococcus canadensis* G7 (pig strain): an underestimated cause of cystic echinococcosis in Austria. *Am J Trop Med Hyg* 2010 May;82(5):871-4.
- (26) McManus DP. The molecular epidemiology of *Echinococcus granulosus* and cystic hydatid disease. *Trans R Soc Trop Med Hyg* 2002 Apr;96 Suppl 1:S151-S157.
- (27) McManus DP, Thompson RC. Molecular epidemiology of cystic echinococcosis. *Parasitology* 2003;127 Suppl:S37-S51.
- (28) Schneider R, Gollackner B, Edel B, Schmid K, Wrba F, Tucek G, et al. Development of a new PCR protocol for the detection of species and genotypes (strains) of *Echinococcus* in formalin-fixed, paraffin-embedded tissues. *Int J Parasitol* 2008 Jul;38(8-9):1065-71.
- (29) Romig T, Dinkel A, Mackenstedt U. The present situation of echinococcosis in Europe. *Parasitol Int* 2006;55 Suppl:S187-S191.
- (30) Schantz PM. The burden of echinococcosis. *Am J Trop Med Hyg* 2005 Jul;73(1):1.

- (31) Wahlers K, Menezes CN, Wong ML, Zeyhle E, Ahmed ME, Ocaido M, et al. Cystic echinococcosis in sub-Saharan Africa. *Lancet Infect Dis* 2012 Nov;12(11):871-80.
- (32) Budke CM, White AC, Jr., Garcia HH. Zoonotic larval cestode infections: neglected, neglected tropical diseases? *PLoS Negl Trop Dis* 2009;3(2):e319.
- (33) Pawlowski ZS, Eckert J, Vuitton DA, Ammann RW, Kern P, Craig PS, et al. Echinococcosis in humans: clinical aspects, diagnosis and treatment. In: Eckert J, Gemmell MA, Meslin FX, Pawlowski ZS, editors. *WHO/OIE Manual on Echinococcosis in Humans and Animals: a Public Health Problem of Global Concern*. Paris, France: World Health Organization; 2001. p. 20-66.
- (34) Siracusano A, Delunardo F, Teggi A, Ortona E. Host-parasite relationship in cystic echinococcosis: an evolving story. *Clin Dev Immunol* 2012;2012:639362.
- (35) Elmahdi IE, Ali QM, Magzoub MM, Ibrahim AM, Saad MB, Romig T. Cystic echinococcosis of livestock and humans in central Sudan. *Ann Trop Med Parasitol* 2004 Jul;98(5):473-9.
- (36) Kebede W, Hagos A, Girma Z, Lobago F. Echinococcosis/hydatidosis: its prevalence, economic and public health significance in Tigray region, northern Ethiopia. *Trop Anim Health Prod* 2009 Aug;41(6):865-71.
- (37) Tashani OA, Zhang LH, Boufana B, Jegi A, McManus DP. Epidemiology and strain characteristics of *Echinococcus granulosus* in the Benghazi area of eastern Libya. *Ann Trop Med Parasitol* 2002 Jun;96(4):369-81.
- (38) Bouree P. Hydatidosis: dynamics of transmission. *World J Surg* 2001 Jan;25(1):4-9.
- (39) Osman MA, Aradaib IE, Ashmaig KA, Gameel AA. Detection and differentiation of *Echinococcus granulosus*-complex using a simple PCR-based assay. *Int J Trop Med* 2009;4:21-6.
- (40) Bowles J, McManus DP. Rapid discrimination of *Echinococcus* species and strains using a polymerase chain reaction-based RFLP method. *Mol Biochem Parasitol* 1993 Feb;57(2):231-9.
- (41) Eckert J, Thompson RC, Lymbery AJ, Pawlowski Z, Gottstein B, Morgan UM. Further evidence for the occurrence of a distinct strain of *Echinococcus granulosus* in European pigs. *Parasitol Res* 1993;79(1):42-8.
- (42) McManus DP, Zhang L, Castrodale LJ, Le TH, Pearson M, Blair D. Short report: molecular genetic characterization of an unusually severe case of hydatid disease in Alaska caused by the cervid strain of *Echinococcus granulosus*. *Am J Trop Med Hyg* 2002 Sep;67(3):296-8.
- (43) Kakrani AL, Chowdhary VR, Bapat VM. Disseminated pulmonary hydatid disease presenting as multiple cannon ball shadows in human immunodeficiency virus infection. *J Assoc Physicians India* 2000 Dec;48(12):1208-9.

- (44) Macpherson CN, Bartholomot B, Frider B. Application of ultrasound in diagnosis, treatment, epidemiology, public health and control of *Echinococcus granulosus* and *E. multilocularis*. Parasitology 2003;127 Suppl:S21-S35.
- (45) Shaw JM, Bornman PC, Krige JE. Hydatid disease of the liver. S Afr J Surg 2006 May;44(2):70-7.
- (46) Craig PS, McManus DP, Lightowlers MW, Chabalgoity JA, Garcia HH, Gavidia CM, et al. Prevention and control of cystic echinococcosis. Lancet Infect Dis 2007 Jun;7(6):385-94.
- (47) Buishi I, Njoroge E, Zeyhle E, Rogan MT, Craig PS. Canine echinococcosis in Turkana (north-western Kenya): a coproantigen survey in the previous hydatid-control area and an analysis of risk factors. Ann Trop Med Parasitol 2006 Oct;100(7):601-10.
- (48) Craig PS, Zeyhle E, Romig T. Hydatid disease: research and control in Turkana. II. The role of immunological techniques for the diagnosis of hydatid disease. Trans R Soc Trop Med Hyg 1986;80(2):183-92.
- (49) French CM, Ingera WE. Hydatid disease in the Turkana District of Kenya. V. Problems of interpretation of data from a mass serological survey. Ann Trop Med Parasitol 1984 Jun;78(3):213-8.
- (50) Chemtai AK, Bowry TR, Ahmad Z. Evaluation of five immunodiagnostic techniques in echinococcosis patients. Bull World Health Organ 1981;59(5):767-72.
- (51) Craig PS, Nelson GS. The detection of circulating antigen in human hydatid disease. Ann Trop Med Parasitol 1984 Jun;78(3):219-27.
- (52) World Health Organization (WHO). Guidelines for treatment of cystic and alveolar echinococcosis. WHO Informal Working Group on Echinococcosis. Bulletin of World Health Organization (WHO) 1996;74:231-42.
- (53) Krige JE, Millar AJ, Rode H, Knobel D. Fatal hypernatraemia after hypertonic saline irrigation of hepatic hydatid cysts. Pediatr Surg Int 2002 Jan;18(1):64-5.
- (54) Aggarwal AR, Garg RL. Formalin toxicity in hydatid liver disease. Anaesthesia 1983 Jul;38(7):662-5.
- (55) Belghiti J, Benhamou JP, Houry S, Grenier P, Huguier M, Fekete F. Caustic sclerosing cholangitis. A complication of the surgical treatment of hydatid disease of the liver. Arch Surg 1986 Oct;121(10):1162-5.
- (56) Nunnari G, Pinzone MR, Gruttadauria S, Celesia BM, Madeddu G, Malaguarnera G, et al. Hepatic echinococcosis: clinical and therapeutic aspects. World J Gastroenterol 2012 Apr 7;18(13):1448-58.
- (57) Division of Clinical Pharmacology, University of Cape Town. South African Medicines Formulary. 6th ed. Cape Town: Health and Medical Publishing Group; 2005.

- (58) Beard TC, Bramble JA, Middleton MJ. Eradication in Our Lifetime: A Log Book of the Tasmanian Hydatid Control Programs. Tasmania: Department of Primary Industry, Water, and Environment; 1996.
- (59) Pharo HJ. New Zealand declares provisional freedom from hydatids. *Surveillance* 2002;29:3-7.
- (60) Dowling PM, bo-Shehada MN, Torgerson PR. Risk factors associated with human cystic echinococcosis in Jordan: results of a case-control study. *Ann Trop Med Parasitol* 2000 Jan;94(1):69-75.
- (61) Irvin AD. Hydatidosis in human patients from Turkana. *Trop Geogr Med* 1974 Jun;26(2):157-9.
- (62) Nelson GS, Rauch RL. Echinococcosis infection in man and animals in Kenya. *Ann Trop Med Parasitol* 1963;57:136-49.
- (63) French CM. The age and sex distribution of hydatid disease in Turkana. *East Afr Med J* 1980 Nov;57(11):791-4.
- (64) Gathura PB, Kamiya M. Echinococcosis in Kenya: transmission characteristics, incidence and control measures. *Jpn J Vet Res* 1990 Dec;38(3-4):107-16.
- (65) Thompson RC, Allsopp CE. Hydatidosis: Veterinary Perspectives and Annotated Bibliography. Wallingford, United Kingdom: C.A.B. International; 1988.
- (66) Omer RA, Dinkel A, Romig T, Mackenstedt U, Elnahas AA, Aradaib IE, et al. A molecular survey of cystic echinococcosis in Sudan. *Vet Parasitol* 2010 May 11;169(3-4):340-6.
- (67) Osman MA. Pathological and biomolecular study on hydatid disease in camels, cattle and sheep. University of Khartoum; 2007.
- (68) Lightowlers MW, Lawrence SB, Gauci CG, Young J, Ralston MJ, Maas D, et al. Vaccination against hydatidosis using a defined recombinant antigen. *Parasite Immunol* 1996 Sep;18(9):457-62.
- (69) Al-Saimary EI, Al-Shemari MN, Al-Fayadh MMA. Molecular characterization of antigens extracted from hydatid cysts of human and other intermediate hosts. *Medical Practice and Review* 2010;1(2):19-25.
- (70) Lightowlers MW, Flisser A, Gauci CG, Heath DD, Jensen O, Rolfe R. Vaccination against cysticercosis and hydatid disease. *Parasitol Today* 2000 May;16(5):191-6.
- (71) Bartlett JM, Stirling D. A short history of the polymerase chain reaction. *Methods Mol Biol* 2003;226:3-6.
- (72) Bowles J, Blair D, McManus DP. A molecular phylogeny of the genus *Echinococcus*. *Parasitology* 1995 Apr;110(Pt 3):317-28.
- (73) Bart JM, Morariu S, Knapp J, Ilie MS, Pitulescu M, Anghel A, et al. Genetic typing of *Echinococcus granulosus* in Romania. *Parasitol Res* 2006 Jan;98(2):130-7.

- (74) Ergin S, Saribas S, Yuksel P, Zengin K, Midilli K, Adas G, et al. Genotypic characterisation of *Echinococcus granulosus* isolated from human in Turkey. *Afr J Microbiol Res* 2010;4(7):551-5.
- (75) Breyer I, Georgieva D, Kurdova R, Gottstein B. *Echinococcus granulosus* strain typing in Bulgaria: the G1 genotype is predominant in intermediate and definitive wild hosts. *Parasitol Res* 2004 Jun;93(2):127-30.
- (76) McManus DP, Ding Z, Bowles J. A molecular genetic survey indicates the presence of a single, homogeneous strain of *Echinococcus granulosus* in north-western China. *Acta Trop* 1994 Feb;56(1):7-14.
- (77) McManus DP, Bryant C. Biochemistry, physiology and molecular biology of *Echinococcus*. In: Thompson RCA, Lymbery AJ, editors. *Echinococcus* and hydatid disease. Wallingford, United Kingdom: CAB International; 1995. p. 135-81.
- (78) Kamenetzky L, Gutierrez AM, Canova SG, Haag KL, Guarnera EA, Parra A, et al. Several strains of *Echinococcus granulosus* infect livestock and humans in Argentina. *Infect Genet Evol* 2002 Dec;2(2):129-36.
- (79) Haag KL, Ayala FJ, Kamenetzky L, Gutierrez AM, Rosenzvit M. Livestock trade history, geography, and parasite strains: the mitochondrial genetic structure of *Echinococcus granulosus* in Argentina. *J Parasitol* 2004 Apr;90(2):234-9.
- (80) Rosenzvit MC, Zhang LH, Kamenetzky L, Canova SG, Guarnera EA, McManus DP. Genetic variation and epidemiology of *Echinococcus granulosus* in Argentina. *Parasitology* 1999 May;118(Pt 5):523-30.
- (81) Ortlepp JR. *Echinococcus* in dogs from Pretoria and vicinity. *Onderstepoort J Vet Sci* 1934;3:97-108.
- (82) Bowles J, Blair D, McManus DP. Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. *Mol Biochem Parasitol* 1992 Sep;54(2):165-73.
- (83) Bowles J, van Knapen F, McManus D. Cattle strain of *Echinococcus granulosus* and human infection. *Lancet* 1992 May 30;339(8805):1358.
- (84) McManus DP, Rishi AK. Genetic heterogeneity within *Echinococcus granulosus*: isolates from different hosts and geographical areas characterized with DNA probes. *Parasitology* 1989 Aug;99(Pt 1):17-29.
- (85) Thompson RCA, McManus DP. Aetiology: parasites and lifecycles. In: Eckert J, Gemmell MA, Meslin FX, Pawlowski ZS, editors. *WHO/OIE Manual on Echinococcosis in Humans and Animals: a Public Health Problem of Global Concern*. Paris, France: World Health Organization; 2001. p. 1-19.
- (86) de la Rue ML, Takano K, Brochado JF, Costa CV, Soares AG, Yamano K, et al. Infection of humans and animals with *Echinococcus granulosus* (G1 and G3 strains) and *E. ortleppi* in southern Brazil. *Vet Parasitol* 2011 Apr 19;177(1-2):97-103.

- (87) Lavikainen A, Lehtinen MJ, Laaksonen S, Agren E, Oksanen A, Meri S. Molecular characterization of *Echinococcus* isolates of cervid origin from Finland and Sweden. *Parasitology* 2006 Nov;133(Pt 5):565-70.
- (88) Bardonnet K, Piarroux R, Dia L, Schneegans F, Beurdeley A, Godot V, et al. Combined eco-epidemiological and molecular biology approaches to assess *Echinococcus granulosus* transmission to humans in Mauritania: occurrence of the 'camel' strain and human cystic echinococcosis. *Trans R Soc Trop Med Hyg* 2002 Jul;96(4):383-6.
- (89) Dinkel A, Njoroge EM, Zimmermann A, Walz M, Zeyhle E, Elmahdi IE, et al. A PCR system for detection of species and genotypes of the *Echinococcus granulosus*-complex, with reference to the epidemiological situation in eastern Africa. *Int J Parasitol* 2004 Apr;34(5):645-53.
- (90) Pawlowski Z, Mrozewicz B, Stefaniak J, Schantz PM, Wilson M, Eckert J, et al. *Echinococcus granulosus* pig strain from Poland has low infectivity to humans. *Am J Trop Med Hyg* 1993;49:342-3.
- (91) Scott JC, Stefaniak J, Pawlowski ZS, McManus DP. Molecular genetic analysis of human cystic hydatid cases from Poland: identification of a new genotypic group (G9) of *Echinococcus granulosus*. *Parasitology* 1997 Jan;114(Pt 1):37-43.
- (92) Hüttner M, Siefert L, Mackenstedt U, Romig T. A survey of *Echinococcus* species in wild carnivores and livestock in East Africa. *Int J Parasitol* 2009 Sep;39(11):1269-76.
- (93) Graber M, Thal J. Echinococcosis in Central African artiodactyla: probable lion/wart-hog cycle. *Rev Elev Med Vet Pays Trop* 1980;33(1):51-9.
- (94) Bowles J, McManus DP. Molecular variation in *Echinococcus*. *Acta Trop* 1993 May;53(3-4):291-305.
- (95) Santivaney SJ, Gutierrez AM, Rosenzvit MC, Muzulin PM, Rodriguez ML, Vasquez JC, et al. Human hydatid disease in Peru is basically restricted to *Echinococcus granulosus* genotype G1. *Am J Trop Med Hyg* 2008 Jul;79(1):89-92.
- (96) da Silva CM, Henrique, Ferreira B, Picon M, Gorfinkiel N, Ehrlich R, et al. Molecular cloning and characterization of actin genes from *Echinococcus granulosus*. *Mol Biochem Parasitol* 1993 Aug;60(2):209-19.
- (97) Haag KL, Araujo AM, Gottstein B, Siles-Lucas M, Thompson RCA, Zaha A. Breeding systems in *Echinococcus granulosus* (Cestoda; Taeniidae): selfing or outcrossing? *Parasitology* 1999 Jan;118(Pt 1):63-71.
- (98) Frosch P, Hartmann M, Muhlschlegel F, Frosch M. Sequence heterogeneity of the echinococcal antigen B. *Mol Biochem Parasitol* 1994 Mar;64(1):171-5.
- (99) Bartholomei-Santos ML, Heinzelmann LS, Oliveira RP, Chemale G, Gutierrez AM, Kamenetzky L, et al. Isolation and characterization of microsatellites from the tapeworm *Echinococcus granulosus*. *Parasitology* 2003 Jun;126(Pt 6):599-605.

- (100) Dinkel A, von Nickisch-Rosenegk M, Bilger B, Merli M, Lucius R, Romig T. Detection of *Echinococcus multilocularis* in the definitive host: coprodiagnosis by PCR as an alternative to necropsy. J Clin Microbiol 1998 Jul;36(7):1871-6.
- (101) Magambo J, Njoroge E, Zeyhle E. Epidemiology and control of echinococcosis in sub-Saharan Africa. Parasitol Int 2006;55 Suppl:S193-S195.
- (102) Verster A, Collins M. The incidence of hydatidosis in the Republic of South Africa. Onderstepoort J Vet Res 1966 Jun;33(1):49-72.
- (103) Conradie A. The prevalence of helminths in warthogs, bushpigs and some antelope species in Limpopo Province, South Africa [dissertation]. Pretoria: Univ. of Pretoria, 2008.
- (104) Wahlers K, Menezes CN, Wong M, Mogoye B, Frean J, Romig T, et al. Human cystic echinococcosis in South Africa. Acta Trop 2011 Dec;120(3):179-84.
- (105) Romig T, Omer RA, Zeyhle E, Hüttner M, Dinkel A, Siefert L, et al. Echinococcosis in sub-Saharan Africa: emerging complexity. Vet Parasitol 2011;181:43-7.
- (106) Yones DA, Taher GA, Ibraheim ZZ. In vitro effects of some herbs used in Egyptian traditional medicine on viability of protoscoleces of hydatid cysts. Korean J Parasitol 2011;49(3):255-63.
- (107) Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium 1999;41:95-8.
- (108) Chase MR, Etter RJ, Rex MA, Quattro JM. Extraction and amplification of mitochondrial DNA from formalin-fixed deep-sea mollusks. Biotechniques 1998 Feb;24(2):243-7.
- (109) Shi SR, Cote RJ, Wu L, Liu C, Datar R, Shi Y, et al. DNA extraction from archival formalin-fixed, paraffin-embedded tissues: heat-induced retrieval in alkaline solution. J Histochem Cytochem 2002;50(8):1005-11.
- (110) World Health Organization. Global plan to combat neglected tropical diseases 2008-2015. <http://whqlibdoc.int/hq/2007/who_cds> [Accessed 2012 Aug 18].
- (111) World Health Organization. The control of neglected zoonotic diseases: a route to poverty alleviation. Geneva: World Health Organization; 2006.
- (112) Chow C, Gauci CG, Vural G, Jenkins DJ, Health DD, Saarma U, et al. *Echinococcus granulosus*: variability of the host-protective EG95 vaccine antigen in G6 and G7 genotypic variants. Exp Parasitol 2008 Aug;119(4):499-505.
- (113) Palmer T, Ainslie A. County pasture/forage resource profiles: South Africa, 2006. <<http://www.fao.org/ag/AGP/AGPC/doc/Counprof>> [Accessed 2012 Jun 6].
- (114) Knapp J, Nakao M, Yanagida T, Okamoto M, Saarma U, Lavikainen A, et al. Phylogenetic relationships within *Echinococcus* and *Taenia* tapeworms (Cestoda:

Taeniidae): An inference from nuclear protein-coding genes. *Mol Phylogenet Evol* 2011;61:628-38.

- (115) Nakao M, Yanagida T, Okamoto M, Knapp J, Nkouawa A, Sako Y, et al. *Echinococcus* and *Taenia*: Phylogenetic taxonomy of human-pathogenic tapeworms and its application to molecular diagnostics. *Infect Genet Evol* 2010;10:444-52.
- (116) Wachira TM, Bowles J, Zeyhle E, McManus DP. Molecular examination of sympatric existence and distribution of sheep and camel strains of *Echinococcus granulosus*. *Am J Trop Med Hyg* 1993;48:473-9.
- (117) Addy F, Alakonya A, Wamae N, Magambo J, Mbae C, Mulinge E, et al. Prevalence and diversity of cystic echinococcosis in livestock in Maasailand, Kenya. *Parasitol Res* 2012 Dec;111(6):2289-94.
- (118) Snabel V, Altintas N, D'Amelio S, Nakao M, Romig T, Yolasigmaz A, et al. Cystic echinococcosis in Turkey: genetic variability and first record of the pig strain (G7) in the country. *Parasitol Res* 2009 Jul;105(1):145-54.
- (119) Vercasia A, Canu S, Kogkos A, Kogkos A, Pipia A, Scala A, et al. Molecular characterization of *Echinococcus granulosus* in sheep and goats of Peloponnesus, Greece. *Parasitol Res* 2007;101:1135-9.
- (120) Chopdat N, Menezes CN, John MA, Mahomed N, Grobusch MP. A gardener who coughed up blood. *Lancet* 2007 Oct 27;370(9597):1520.