

Detection of
Echinococcus granulosus and *Echinococcus*
equinus in Dogs and Epidemiology of
Canine Echinococcosis in the UK

W A I S A N L E T T

A thesis submitted for the partial requirement
of the degree of Doctor of Philosophy (Ph.D.)

University of Salford

School of Environment and Life Sciences

2013

Abstract

Echinococcus granulosus is a canid cestode species that causes hydatid disease or cystic echinococcosis (CE) in domestic animals or humans. *Echinococcus equinus* formerly recognised as the 'horse strain' (*E.granulosus* genotype G4) is not known to be zoonotic and predominantly involves equines as its intermediate host. The domestic dog is the main definitive host for both species, which are also both endemic in the UK but data is lacking especially for *E.equinus*. An *E.equinus*-specific PCR assay was designed to amplify a 299bp product within the ND2 gene and expressed 100% specificity against a panel of 14 other cestode species and showed detection sensitivity up to 48.8pg (approx. 6 eggs). Horse hydatid cyst isolates (n = 54) were obtained from 14 infected horse livers collected from an abattoir in Nantwich, Cheshire and hydatid cyst tissue was amplified using the ND2 PCR primers to confirm the presence of *E.equinus* and used to experimentally infect dogs in Tunisia from which serial post-infection faecal samples were collected for coproanalysis, and indicated *Echinococcus* coproantigen and *E.equinus* DNA was present in faeces by 7 and 10 days post infection, respectively. Canine echinococcosis due to *E.granulosus* appears to have re-emerged in South Powys (Wales) and in order to determine the prevalence of canine echinococcosis a coproantigen survey was undertaken. The Welsh Assembly Government also funded a 2 year hydatid disease eradication campaign (2008-10) as a preventative public health measure and faecal samples were tested from farm dogs in the control area. In addition 8 foxhound packs (5 from Wales and 3 from England) were sampled and screened for echinococcosis infection using an *Echinococcus* genus-specific coproantigen ELISA that was optimised against a panel of known *Echinococcus* and control faecal samples. Farm dogs and foxhounds were also screened using two coproPCR assays (predominantly *E.granulosus* G1 or *E.equinus* G4 specific). In the Welsh farm dog study, 609 dog faecal samples were collected at baseline (pre-treatment) of which 10.8% (66/609) were found to be coproantigen positive, 5.1% (31/609) were G1 *E.granulosus* coproPCR positive and 1.8% (12/609) were *E.equinus* ND2 coproPCR positive. A total of 742 farm dog samples were tested after 3 quarterly deworming treatments and showed a coproantigen decrease to 0.7% (5/742). One year after the last dosing round 4.2% (45/1076) of farm dogs were found to be coproantigen positive; of these only 123 were tested with the G1 primers of which 15.4% (19/123) were positive for *E.granulosus* DNA. Of 8 foxhound packs screened by the *Echinococcus* genus specific coproantigen ELISA and by the two coproPCR tests (*E.granulosus*, *E.equinus*) 3 of the 4 Welsh hunts had copropositive dogs (hunt prevalence 30.9%, 9.7%, 61.2%) and 2 of the 3 English hunts (hunt prevalence 17.5%, 44.5%). Hounds in 6 of the 8 hunts were coproPCR positive for *E.granulosus* DNA and 2 of the 8 hunts were positive for *E.equinus* coproDNA. Additional foxhound data was collected in the form of a survey questionnaire to hunt staff which suggested that there may be a link between increased *Echinococcus* coproantigen prevalence and inadequate worming protocols and unsafe feeding practices. The study showed that canine echinococcosis due to *E.granulosus* and *E.equinus* occurred in farm dogs and foxhounds in Wales and England and that an intervention programme in mid-Wales reduced canine echinococcosis in farm dogs after four dosing rounds, but copro-prevalence increased by 12 months after cessation of dosing. The data are discussed with reference to potential human infection, risk factors and optimal intervention approaches. The study showed that the distribution of canine echinococcosis in farm dogs and foxhounds was not homogenous and also confirms the continued presence of both *E.granulosus* and *E.equinus* in foxhounds in England and Wales.

TABLE OF CONTENTS

Abstract.....	i
Table of contents.....	ii
List of tables.....	v
List of figures.....	vii
Acknowledgements.....	x
Dedication.....	xi
Declaration.....	xii
Abbreviations.....	xiii

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1	Global significance, history and background.....	1
1.2	Life-cycle and transmission dynamics of <i>Echinococcus granulosus</i>	4
1.3	Taxonomy.....	10
1.3.1	<i>Echinococcus vogeli</i>	15
1.3.2	<i>Echinococcus oligarthrus</i>	15
1.3.3	<i>Echinococcus multilocularis</i>	16
1.3.4	<i>Echinococcus granulosus</i>	17
1.3.5	<i>Echinococcus equinus</i>	17
1.3.6	<i>E.ortleppi</i>	19
1.3.7	<i>E.canadensis</i> and <i>E.felidis</i>	19
1.4	<i>Echinococcus granulosus</i> distribution.....	20
1.5	<i>Echinococcus equinus</i> distribution.....	21
1.6	Aetiology of echinococcosis in humans.....	22
1.7	Growth and pathology of <i>Echinococcus granulosus</i>	23
1.7.1	Cystic echinococcosis.....	23
1.7.2	Canine echinococcosis.....	24
1.8	Growth and pathology of <i>Echinococcus equinus</i>	25
1.8.1	Equine echinococcosis.....	25
1.9	Diagnosis and treatment of CE in humans.....	26
1.9.1	Imaging.....	26
1.9.2	Serological detection	27
1.9.3	Treatment.....	27
1.10	Diagnosis in livestock CE.....	27
1.11	Diagnosis and detection of canine echinococcosis.....	28
1.11.1	Direct parasitological observation.....	28
1.11.2	Necropsy.....	29
1.11.3	Immunofluorescent detection of eggs	29
1.11.4	Coproantigen detection by ELISA.....	29
1.11.5	CoproDNA detection by PCR.....	31
1.12	Treatment and control of canine echinococcosis.....	35
1.13	The role of dogs in the epidemiology of equine echinococcosis in the UK.....	37
1.14	Aims and objectives.....	39
1.14.1	Aims of the current epidemiological study.....	39
1.14.2	Objectives.....	40

CHAPTER TWO: STANDARDISATION OF COPROANTIGEN ELISAs FOR DETECTION OF CANINE ECHINOCOCCOSIS

2.1	Introduction.....	41
2.2	Aims of the study.....	45
2.3	Research approach.....	46
	2.3.1 Checkerboard/titration protocols.....	46
	2.3.2 Panel of defined faecal samples.....	47
2.4	‘Allan’ coproELISA test.....	49
2.5	‘Huang/Heath’ coproELISA test.....	50
2.6	‘Hybrid’ coproELISA test.....	51
2.7	<i>Echinococcus</i> antigen and antibody products.....	51
2.8	Results.....	52
	2.8.1 ‘Huang/Heath’ test optimisation.....	52
	2.8.2 ‘Hybrid’ test optimisation (‘Huang/Heath’/‘Allan’).	58
2.9	Discussion.....	62
2.10	Summary.....	65

CHAPTER THREE: DEVELOPMENT OF A NOVEL COPRODNA PCR ASSAY TO DETECT ECHINOCOCCUS EQUINUS IN DOGS

3.1	Introduction.....	66
3.2	Aims of the study.....	68
3.3	Research Approach.....	69
	3.3.1 Development of the <i>E.equinus</i> G4-specific coproDNA PCR.....	67
	3.3.2 Collection of horse hydatid cyst material.....	73
	3.3.3 Observation of live protoscoleces.....	75
	3.3.4 Necropsy.....	78
	3.3.5 Archived horse hydatid cyst isolates.....	80
3.4	Tissue and coproPCR.....	80
	3.4.1 DNA sequencing analysis.....	81
3.5	Time-course experimental infections of dogs with <i>E.equinus</i> (Tunisia).....	82
3.6	Results.....	85
	3.6.1 Optimisation of <i>E.equinus</i> G4-specific coproDNA PCR.....	85
	3.6.2 PCR results for horse hydatid isolates.....	90
	3.6.3 Time-course experimental infections.....	93
3.7	Discussion.....	97
3.8	Summary.....	99

CHAPTER FOUR: AN EPIDEMIOLOGICAL AND INTERVENTION STUDY OF CANINE ECHINOCOCCOSIS IN FARM DOGS IN SOUTH POWYS, WALES

4.1	Introduction.....	100
4.2	Aims and hypotheses.....	102
	4.2.1 Aims of the study.....	103
	4.2.2 Hypotheses.....	104
4.3	Materials and methods.....	104
	4.3.1 Location and description of the Welsh study area.....	104
	4.3.2 Farm dog faecal sampling.....	107
	4.3.3 Questionnaire.....	109
	4.3.4 Coproantigen detection.....	110
	4.3.5 CoproDNA detection.....	110

4.4	Copro-Polymerase Chain Reaction (PCR).....	111
4.4.1	Faecal DNA extraction.....	111
4.4.2	CoproDNA amplification.....	111
4.4.3	Ethanol precipitation and sample dilution.....	112
4.5	Determination of sensitivity and specificity.....	113
4.6	Results.....	115
4.6.1	Coproantigen ELISA results.....	115
4.6.2	CoproDNA PCR results.....	117
4.7	Discussion.....	123
4.8	Summary.....	125

CHAPTER FIVE: AN EPIDEMIOLOGICAL STUDY OF CANINE ECHINOCOCCOSIS IN FOXHOUND PACKS IN ENGLAND AND WALES

5.1	Introduction.....	126
5.2	Aims and hypotheses.....	129
5.2.1	Aims of the study.....	129
5.2.2	Hypotheses.....	130
5.3	Study design and protocol.....	131
5.3.1	Field logistics and foxhound faecal sampling.....	131
5.3.2	Collection of samples and processing.....	134
5.3.3	Coproantigen detection.....	134
5.3.4	CoproDNA detection.....	134
5.4	Results.....	135
5.4.1	Coproantigen ELISA results.....	136
5.4.2	CoproDNA PCR results.....	138
5.4.3	Questionnaire data.....	139
5.5	Discussion.....	142
5.6	Summary.....	146

CHAPTER SIX: GENERAL DISCUSSION

6.1	General overview.....	147
6.2	Conclusions and recommendations.....	147

References.....	153
------------------------	------------

Appendices.....	185
------------------------	------------

Appendix 1: Letter to Director of the Masters of Fox Hounds Association.....	185
-------------------------------------------------------------------------------------	------------

Appendix 2: Letter to foxhound kennelmen.....	186
------------------------------------------------------	------------

Appendix 3: Foxhunt kennel questionnaire.....	187
------------------------------------------------------	------------

Appendix 4: Equipment list for foxhound faecal sampling.....	189
---------------------------------------------------------------------	------------

Appendix 5: Welsh Hydatid Study farm dog questionnaire.....	190
--------------------------------------------------------------------	------------

Appendix 6: Constituents of reagents used in 'Huang/Heath' test.....	191
-----------------------------------------------------------------------------	------------

Appendix 7: Constituents of reagents used in 'Allan' test.....	192
-----------------------------------------------------------------------	------------

Appendix 8: Constituents of reagents for Mastermix for PCR protocols.....	193
----------------------------------------------------------------------------------	------------

LIST OF TABLES

<i>Table 1.1 Current taxonomy of Echinococcus. Adapted from Thompson (2008); Thompson et al. (1995); Thompson and McManus (2001); McManus and Thompson (2003); Jenkins et al. (2005); Nakao et al., (2007) and Huttner et al., (2008)</i>	14
<i>Table 1.2 Comparison of the diagnostic sensitivity and specificity of three assessed PCR assays and cross-reacting species of cestodes according to Boufana et al., (2008) including diagnostic sensitivity and specificity of a coproantigen ELISA developed to target E.granulosus coproantigens (Allan et al., 1992) and targeted genes/antigen</i>	34
<i>Table 2.1 Panel of infected faecal samples and their source of origin</i>	48
<i>Table 2.2 Mean OD values show that there is non-specific binding between the commercial anti-sheep IgG and the 'Heath/Huang' capture rabbit IgG anti-Eg freeze-thaw tegumental antigen antibody (RF/T)</i>	54
<i>Table 2.3 Subpanel results 'Hybrid test' compared to 'Allan test' Red indicates positive results (≥ 0.095); green indicates negative results (< 0.095)</i>	60
<i>Table 2.4 Subpanel results of time-course experimental infections with E.equinus using the 'Allan test'. Red indicates positive results (≥ 0.095); green indicates negative results (< 0.095)</i>	61
<i>Table 3.1 Time-course Experimental Infections 1-4, sample used to infect dogs, number of infected dogs, number of days post infection</i>	82
<i>Table 3.2 Horse information and PCR results with E.equinus G4-specific primers ('U' indicates information unavailable, 'X' indicates did not amplify; '✓' indicates PCR amplification but sample not sequenced; 'Ee' indicates PCR amplification and confirmed as E.equinus with genetic sequencing)</i>	91
<i>Table 3.3 Horse information and PCR results from 'cestode-specific' primers ('U' indicates information unavailable, 'X' indicates did not amplify; 'Ee' indicates PCR amplification and confirmed as E.equinus with genetic sequencing)</i>	92
<i>Table 3.4 E.equinus time-course experimental infections, horse data, estimated viability of protoscoleces (PSC), coproantigen ELISA results and coproDNA PCR results using optimised E.equinus primers red indicates positive coproantigen ELISA results (≥ 0.095); green indicates negative coproantigen ELISA results (< 0.095)</i>	95
<i>Table 4.1 Illustrates the formulae used to determine the parameters for determining sensitivity and sensitivity</i>	113
<i>Table 4.2 Number of coproantigen ELISA positive farm dogs from south Powys, mid-Wales against total number of samples tested and percentage</i>	116
<i>Table 4.3 CoproDNA PCR results and coproantigen ELISA testing of samples for E.granulosus sensu lato</i>	119
<i>Table 4.4 Comparison of 'Abbasi' coproDNA PCR positive dogs with coproantigen ELISA OD values result. Red indicates positive results (≥ 0.095); green indicates negative results (< 0.095)</i>	120
<i>Table 4.5 Comparison of optimised E.equinus G4-specific coproDNA PCR positive dogs with coproantigen ELISA OD values result. Red indicates positive results (≥ 0.095); green indicates negative results (< 0.095)</i>	121
<i>Table 4.6 Coproantigen ELISA prevalence of farm dogs from 1993 to current study</i>	121

<i>Table 5. 1 Foxhound coproDNA PCR results for E.granulosus sensu lato and E.equinus-specific DNA.....</i>	<i>138</i>
-------------------------------------------------------------------------------------------------------------	------------

<i>Table 5.2 Foxhound pack coproantigen and coproDNA and corresponding questionnaire data.....</i>	<i>141</i>
----------------------------------------------------------------------------------------------------	------------

LIST OF FIGURES

<i>Figure 1.1 Life-cycle of Echinococcus granulosus (Modified from http://www.dpd.cdc.gov/dpdx/html/frames/af/echinococcosis/body_Echinococcosis_page1.htm)...</i>	9
<i>Figure 1.2 Measurements carried out on rostellar hooks. Total length: A to F, and B to E. Ventral Blade length: D to E. Dorsal Blade length: C to E. Handle length: B to C (Verster, 1965). Porcine hydatid cysts were used to infect dogs and jackals and results gave rise to a catalogue of large hook and small hook drawings.....</i>	11
<i>Figure 2.1 Rabbit IgG anti-Eg freeze thaw tegumental antigen (RF/T) vs. EgWWE in buffer BCB (neat).....</i>	55
<i>Figure 2.2 Rabbit IgG anti-Eg freeze-thaw tegumental antigen (RF/T) vs. Eg WWE spiked in negative diluent.....</i>	56
<i>Figure 2.3 Sheep IgG anti-Eg freeze-thaw excretory-secretory antigen (SE/S) vs. EgWWE in negative diluent.....</i>	57
<i>Figure 2.4 A 3-D graph that shows the checkerboard titration of capture rabbit IgG anti-Eg freeze-thaw tegumental antigen (RF/T) vs. conjugate rabbit anti-Eg excretory-secretory antigen (RE/S7) Signal:noise ratio is shown (=positive/negative).....</i>	59
<i>Figure 3.1 Diagram of the circular mitochondrial genome of E. granulosus, G1 genotype (McManus et al., 2004).....</i>	69
<i>Figure 3.2 Hydatid cysts in horse liver collected from Red Lion Abattoir Nantwich, Cheshire.....</i>	74
<i>Figure 3.3 E.equinus protoscoleces (PSC) liberated from a hydatid cyst and immediately viewed (100x magnification).....</i>	77
<i>Figure 3.4 Dissected dog intestines washed repeatedly in fresh saline solution.....</i>	79
<i>Figure 3.5 Dissected dog intestines laid out on metal trays for visual inspection for adult worms.....</i>	79
<i>Figure 3.6 PCR amplification of 373bp product using 'cestode-specific' primers against panel of specific cestodes (Dinkel et al., 1998). Lane M, position of the size marker bands; lane 1 E granulosus (G1), lane 2 T.ovis, lane 3 T.hydatigena, lane 4 T.pisiformis, lane 5 D.caninum, lane 6 T.crassiceps, lane 7 T.multiceps, lane 8 E.granulosus (G3), lane 9 E.granulosus G7; lane N shows negative control.....</i>	87
<i>3.7 PCR amplification of G4 primers at temperature gradient. Lane M, positions of the size marker bands; lanes 1-6 show amplified 299bp product at 59-64 °C.....</i>	87
<i>Figure 3.8 PCR amplification of E.equinus G4-specific primers at temperature gradient. Lane M, positions of the size marker bands; lanes 1-6 show amplified 299bp product at 65-70 °C.....</i>	87

Figure 3.9 PCR amplification of 299bp product using optimised *E.equinus* G4-specific primers against panel of cestodes. Lane M, position of the size marker bands; lane 1 *E.equinus* positive control, lane 2 *Taenia pisiformis*, lane 3 *Taenia ovis*, lane 4 *Taenia hydatigena*, lane 5 *Taenia multiceps*, lane 6 *Dipylidium caninum*, lane 7 *E.granulosus* (G1), lane 8 *E. granulosus* (G3), lane 9 *E.granulosus* (G7), lane 10 *Taenia crassiceps*, lane 11 *E.multilocularis*, lane 12 *E.granulosus* (G5), lane 13 shows negative control, lane 16 *E.granulosus* (G6), lane 17 *E.granulosus* (G8), lane 18 *E.granulosus* (G10), lane 19 *E.granulosus* (G7).....88

Figure 3.10 PCR amplification of twofold serial dilutions of *E.equinus* tissue DNA at 50,000-6.1pg; lowest detectable concentration was shown to be 4.88pg (equivalent to less than one *Echinococcus* egg).....88

Figure 3.11 CoproDNA PCR detection of negative faecal sample spiked with 0.1, 1, 10 and 100ng/μl of *E.equinus* tissue DNA. Lane M, position of the size marker bands; lane 1 *E.equinus* (G4) positive control, lanes 2-5 100-0.1ng/μl ethanol precipitated samples, lanes 6-9 double dilution of ethanol precipitated samples, negative control included but not shown.....89

Figure 3.12 Experimental Infection 2, photo A mature *E.equinus* worm with gravid posterior proglottid (20x), photo B immature eggs (40x), photo C immature eggs (100x), photo D egg (400x), images courtesy of Professor Samia Lahmar.....96

Figure 4.1 Map of Wales; the control area was identified by the northernmost limits of the ancient districts of Brecknockshire and Radnorshire within the county of Powys as identified from Welsh Assembly Government (WAG) records and from speaking with farmers (Mastin et al., 2011).....106.

Figure 4.2 Praziquantel drug tablet administered orally using bread and butter.....108

Figure 4.3 Farmer assisting with the oral administering of the supervised dog worming treatment.....108

Figure 4.5 Coproantigen prevalence (%) of Welsh farm dogs tested from Quarter 1-8 of the Welsh Hydatid Study 2008-2010. Year 1, 2008-2009 (Q1-Q4); Year 2, 2009-2010 (Q5-Q8).....117

Figure 4.6 PCR amplification of coproDNA using 'Abbasi' primers for *E.granulosus*. Lane M, 100-bp molecular DNA ladder; lanes 1 and 2, faecal samples from WHS Quarter 2; lanes 3 & 4, positive and negative controls respectively.....122

Figure 4.7 PCR amplification of coproDNA using optimised *E.equinus* G4-specific primers. Lane M, 100-bp molecular DNA ladder; lane 1, 2, 5 & 7, faecal samples WHS Quarter 1; lanes 2 & 3 tissue and copro positive controls respectively and lane 20 negative control.....122

Figure 5.1 Hunt No. 3 Foxhound Pack.....133

Figure 5.2 Hunt No. 5 Foxhound Pack.....133

Figure 5.3 Coproantigen prevalence (%) of foxhound packs in Wales and England 2010.....137

Figure 5.4 PCR amplification of coproDNA using ‘Abbasi’ primers for E.granulosus sensu lato. Lane M, DNA marker, lanes 1 and 2, G4 and G1 positive controls respectively; lanes 9 and 12, Hunt No. 3 foxhound coproDNA from WHS Quarter 1 (pre-treatment); lane 13, negative control.....140

Figure 5.5 PCR amplification of coproDNA using ‘Abbasi’ primers for E.granulosus sensu lato. Lane M, DNA marker, lanes 1 and 2, G4 and G1 positive controls respectively; lanes 12, Hunt No. 3 foxhound coproDNA from WHS Quarter 2 (post-treatment); lane 13, negative control.....140

Figure 5.6 PCR amplification of coproDNA using a novel E.equinus G4-specific coproDNA PCR for E.equinus. Lane M, DNA marker, lanes 1 and 2, G4 tissue and G4 copro positive controls respectively; lanes 3-5, Hunt No. 3 foxhound coproDNA from WHS Quarter 1 (pre-treatment); lane 6, Brecon & Talybont foxhound coproDNA from WHS Quarter 2 (post-treatment); lane 7, negative control.....140

ACKNOWLEDGEMENTS

There are numerous people I would like to acknowledge for their assistance and support during this study. First and foremost I would like to thank Professor Philip Craig who allowed me to experience first hand *Echinococcus* fieldwork in the Tibetan Plateau while I was an MSc student and later accepted me as his PhD student. His guidance and patience has been invaluable and for this I am extremely grateful.

Others who have been there for me during this research process including fellow post-graduate students Nicole Dodd, Rob Coles, Sameena Haq and Ebiye Okiti for their much-needed sense of humour and camaraderie. I would like to thank past students of Salford University for their contribution to the laboratory investigations Holly Adams, Lauren Cushnie, Mohamed Omari Mbogah and Mei Pheon Li. I would also like to extend my gratitude to the members of the Cestode Zoonoses Research Group at Salford University including Helen Bradshaw, Tony Bodell, Freya van Kesteren and Alex Mastin for their input and contribution during the laboratory investigations.

I would like to thank Professor Samia Lahmar who assisted in the experimental infections at the Ecole Nationale de Médecine Vétérinaire in Sidi Thabet, Tunisia. Thank you to her family who looked after me during my stay in their home. I would also like to thank LVI Locums Limited Operatives for collecting the Welsh samples and also David Godfrey for his advice and support. Thank-you to all those huntsmen and senior meat inspectors who kindly agreed to participate in the project without whom, this study would not be possible. This work would have never been accomplished without the financial assistance of Welsh Assembly Government and the support of the University of Salford Biomedical Research Institute.

Special thanks are given to my mentor Dr. Belgees Boufana at Salford University whose time, input, help and support was invaluable throughout the whole study. Above all I would like to express my gratitude for her friendship and trust.

And finally a huge thank-you to my dear husband David for his support and endless patience throughout the project.

DEDICATIONS

This thesis is dedicated to two very special people who sadly lost their battle to cancer during my Ph.D study. To my Uncle Guy Li who always had me in his heart even when we spent years apart, you are very much missed. To Brian Lett who welcomed me into his heart, I could not have asked for a better father-in-law.

DECLARATION

This is my original work and has not been presented for a degree in any other University. All photographs and illustrations, unless acknowledged, were taken developed and printed by the author. This thesis includes data from field epidemiological surveys, post-treatment follow-up study and laboratory work.

ABBREVIATIONS

AE	Alveolar Echinococcosis
Ag	Antigen
BCB	Bicarbonate Coating Buffer
°C	Degrees Centigrade
CE	Cystic Echinococcosis
ddNTPs	Dideoxynucleotide Triphosphates
DNA	Deoxyribonucleic Acid
dpi	Days Post Infection
Eg	Echinococcus Granulosus
ELISA	Enzyme Linked Immunosorbent Assay
ES	Excretory/Secretory
FT	Freeze Thaw
g	Gram
h	Hour
HI FCS	Heat Inactivated Foetal Calf Serum
HPA	Health Protection Agency
HRP	Horseradish Peroxidase
IgG	Immunoglobulin G
Kg	Kilogram
Km	Kilometre
Mab	Monoclonal Antibody
mg	Milligram
min	Minute(s)
ml	Milliliter

mm	Millimole
mm	Millimeter
ng	Nanogram
nm	Nanometer
OD	Optical Density
ONC	Oncosphere
PAIR	Puncture Aspiration, Injection and Re-aspiration
PBS _t 20	Phosphate-Buffered Saline with Tween 20
PCR	Polymerase Chain Reaction
PE	Polycystic Echinococcosis
PNPP	P-nitrophenylphosphate
PSC	Protoscoleces
PZQ	Praziquantel
RT	Room Temperature
Spp.	Species
SD	Standard Deviations
TMB	Tetramethylbenzidine
UK	United Kingdom
μl	Microlitre
US	Ultrasound
Wb	Worm Burden
WWE	Whole Worm Extract
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Global significance, history and background

Echinococcus granulosus is of pathogenic and economic significance in intermediate and aberrant intermediate hosts (Torgerson and Budke, 2003) and infections with cystic echinococcosis (CE) cause major health and economic problems in many areas of the world (Larrieu *et al.*, 1999). The global impact of livestock production losses (liver condemnation, decreased: carcass weight, milk production, hide value and fecundity) due to cystic *echinococcosis* is \$125,000 billion (US dollars) annually (Budke, 2006). This is an actual figure, not taking into account for under-reporting, thus highlighting the need for more accurate reporting on a global scale. Due to the lack of identification and reporting in both humans and livestock globally, prevalence figures are inaccurate if present on a large scale. The prevalence of hydatid disease is always difficult to determine because of the large number of cases that are asymptomatic however it may be possible to provide a rough estimate of the incidence of new cases by looking at the number of new cases tested at referenced laboratories. Hydatid disease is not a notifiable infectious disease therefore studies investigating incidence and prevalence rely on other sources of information such as hospital admissions data and information from laboratories testing for hydatid disease. Although this means that data has to be collated from a number of different sources it appears that this provides a more accurate estimate of incidence and prevalence than notification. A study carried out in New Zealand, where hydatid disease was a notifiable disease found that notification underestimated the incidence and prevalence (Isaacs *et al.*, 1985). The study used hospital discharge data, pathology and radiology records and a diagnostic index from a chest medical unit to calculate the number of patients presenting with hydatid disease between 1967 and 1982 in the Auckland area (Isaacs *et al.*, 1985). The number of disability-adjusted life-years (DALYs) lost globally due to echinococcosis is 3.6 million and estimate numbers of human cases of echinococcosis caused by *E.granulosus* that occur in 2 large endemic zones are North Africa/Middle East and China/Central Asia indicate >423,000 and >484,000 cases respectively (Craig *et al.*, 2007a).

E.granulosus causes CE a chronic cyst-forming disease in humans with the dog acting as the major definitive host to transmit infection to humans and agricultural animals such as sheep and cattle. The most cosmopolitan form of *E.granulosus* is the sheep strain - genotype 1 (G1) and it is most commonly associated with human infections (Moro and Schantz, 2009). Human CE occurs predominantly in pastoral communities that raise agricultural livestock and keep dogs for guarding and/or herding animals (Craig *et al.*, 2007b). Echinococcosis is of an increasing public health concern and is considered to be emerging or re-emerging (McManus *et al.*, 2003b). It is also considered a neglected zoonotic disease (NZD) and urgent attention is required to reduce morbidity in humans by reducing or eliminating the parasite in domestic and/or wildlife populations (Craig *et al.*, 2007a; Brown, 2004).

Echinococcosis, also known as hydatidosis, was recognised as far back as four centuries BC by ancient scholar Hippocrates who compared human hydatid cysts with ‘water-filled tumours’ he observed in post-mortem examination of livestock (Eckert *et al.*, 2001). Other ancient scholars such as Arataeus in the first century AD and Galenus of Pergamon of 129 to 200 AD also knew of these bladder-like cysts (Foster, 1965). Despite the awareness of echinococcosis over the last two thousand years, it was not until the 17th century when Francisco Redi demonstrated the parasitic nature of these cysts and that they were of animal origin (Redi, 1684). Over a century later the German clinician and natural historian Pierre Simon Pallas hypothesised that these cysts were larval stages of tapeworms (Pallas, 1766). Shortly after in 1782, Goeze accurately described the cysts and the tapeworm heads; and in 1786, an accurate description of *E.granulosus* was produced by Batsch who also renamed it *Hydatigera granulosa* (Grove, 1990; Rausch, 1995). In 1853, Carl Von Siebold demonstrated through a series of experiments that cysts from sheep caused adult tapeworms in dogs and therefore demonstrated the life cycle and link between larval and adult stages (Von Siebold, 1853). A decade later a different species called *Echinococcus multilocularis* was identified by Rudolf Leuckhart (1863) when he differentiated between the unilocular type caused by *E.granulosus* and multivesicular characteristics of other cysts. Around the same time, Bernhard Naunyn found that adult tapeworms in dogs directly developed when fed with hydatid cysts from a human (Naunyn, 1863). Further understanding of the clinical features of this disease came about in the late 1800’s with more researchers like Virchow (1856) who recognised

that colloid carcinoma was of parasitic origin. However the taxonomic status of *E.multilocularis* remained in doubt for around a hundred years. Rausch and Schiller (1956) described alveolar hydatid in a tundra vole. Then, Vogel (1957) successfully completed the life cycle in the laboratory and drew significance to the distinct features of multivesicular larval stage and its occurrence in rodents. The disease caused by *E.multilocularis* is known as alveolar echinococcosis (AE).

Although it had been widely known that *E.granulosus* and *E.multilocularis* were both linked to human echinococcosis around the 20th century, it wasn't until the 1900's that neotropical species *Echinococcus oligarthrus* and *Echinococcus vogeli* were also identified as being causes of human polycystic echinococcosis (PE) (Tappe *et al.*, 2008). *E.oligarthrus* had first been observed in the 1800's by Johannes Natterer who had collected a helminth from Brazil taken from the small intestine of a puma - *Felis concolor* however it wasn't until the 1900s that there was a link between this species and PE (Tappe *et al.*, 2008). In contrast to *E.granulosus* and *E.multilocularis*, felids are the major definitive host of *E.oligarthrus*, though mainly through sylvatic transmission cycles (Sousa and Thatcher, 1969). Natural infections of *E.oligarthrus* have been found in seven species of wild felid (Tappe *et al.*, 2008). *E.vogeli* was quite recently recognised as a species and later confirmed as being the causative agent for human PE not long after it was first (Rausch and Bernstein, 1972; Rausch *et al.*, 1978). It was first described as being morphologically different from known *Echinococcus* species when it was examined after being expelled from a captured Ecuadorian bush dog (Rausch and Bernstein, 1972). The life cycle of *E.vogeli* involves the definitive host - bush dog (*Speothos venaticus*) and the rodent intermediate host - paca (*Cuniculus paca*), both species originating from the neotropical zone. The feeding practice of local paca hunters who have admitted to rewarding their dogs with viscera that may harbour viable cysts could be a risk factor for zoonotic human infection (Rausch and D'Alessandro, 2002). Over one hundred human cases of PE have been reported from twelve South and Central American countries (Tappe *et al.*, 2008).

Even more recently in 2005, a new species called as *Echinococcus shiquicus* was identified from specimens collected from Tibetan foxes (*Vulpes ferrilata*) and plateau pika (*Ochotona curzoniae*), which share a predator-prey relationship and are both

considered to be natural hosts of this species (Xiao *et al.*, 2005). Shiqu County situated in the Tibetan Plateau is considered to be highly endemic for human cases of CE and AE. So far no human cases of *E.shiquicus* have been detected (Li *et al.*, 2008). Theories as to why human infection has not been detected are that humans are not susceptible; limited human exposure to transmission cycles; small Tibetan fox populations may account for reduced risk to human exposure and also limitations to current diagnostic methods may not distinguish between *E.shiquicus* infection and CE or AE (Xiao *et al.*, 2005).

Another species within the genus *Echinococcus* that has so far not been associated with human infection is *E.granulosus* genotype G4 formerly known as the ‘horse strain’ or recently recommended as being named ‘*Echinococcus equinus*’ (Tappe *et al.*, 2010). The term ‘*Echinococcus granulosus equinus*’ was first coined by Williams and Sweatman (1963) who made observations during experimental and natural infections in the UK and New Zealand. *E.equinus* appears to be poorly or non-infective to humans and involves equine species as its primary intermediate host (Thompson and Smyth, 1975; Thompson and McManus, 2002). In the current study the name *Echinococcus equinus* will be used.

1.2 Life-cycle and transmission dynamics of *Echinococcus granulosus*

Dogs (*Canis familiaris*) and other suitable carnivores are the usual definitive hosts of *Echinococcus granulosus*, whilst a large number of mammalian species can be intermediate hosts, including domestic ungulates and humans (Torgerson and Budke, 2003). The intermediate hosts harbour the metacestode stage of the parasite, which may develop into sterile or fertile hydatid cysts that may contain thousands of protoscoleces (PSCs). Dogs become infected after ingesting the offal of an intermediate host that is contaminated with hydatid cysts containing viable protoscoleces. When the PSCs have been ingested by a suitable definitive host, they evaginate in the upper duodenum following exposure to pepsin in the stomach, bile and an increase in temperature (Thompson and McManus, 2001). A sexually mature adult worm can develop from each protoscolex (Thompson and McManus, 2001). Depending on the species and strain (genotype) and on the susceptibility of the host, the adult tapeworm reaches sexual maturity approximately four to six weeks after infection (Thompson and McManus, 2001). The adults can grow up to 7mm in length and comprise of 2-6 segments, known as proglottids and a scolex that has two rows of hooks and four suckers. The penultimate

proglottid becomes mature and gravid depending on which species or strain. For example the cattle strain (G5 or *E.ortleppi*) has a shorter pre-patent period (33 to 35 days) than that of the common sheep strain (42 days) (Thompson, 1995). In comparison to the common sheep strain, the pre-patent period of the horse strain (G4, *E.equinus*) was found to be about 70 days (Cook, 1989). The terminal segment maybe passed with faces or disintegrates in the intestine so that either eggs only or proglottids and eggs may occur in faeces (Soulsby, 1982; Craig *et al.*, 2003).

The biotic potential of *E.granulosus* is a major contribution to the transmission dynamics of this parasite. The biotic potential can be defined as ‘the potential number of viable cysts which can be established in an intermediate host by an individual definitive host per day,’ (Gemmell, *et al.*, 2001). The average worm burden in dogs varies considerably between endemic areas (Macpherson and Craig, 2000). Most cases of canine echinococcosis from developed countries reported a mean worm burden for *E.granulosus* of about 200-400 (Gemmell, *et al.*, 1987). However a heavily infected dog may harbour over 50,000 adult worms, which completely covers the entire length of the small intestine (Macpherson *et al.*, 1983). In drier parts of the world much heavier worm burdens have been reported, which indicates that biotic potential varies widely in different ecological situations and climatic zones (Macpherson and Craig, 2000). It has been suggested that this environment may increase biotic potential of the parasite by providing favourable conditions for egg survival (Wachira *et al.*, 1991). High worm burdens have been recorded in some highly endemic regions, for example in Tunisia 21% of stray dogs necropsied were infected with a mean worm burden of 2,543 adult worms (Lahmar *et al.*, 2001). In Australia, one wild dog (domestic dog and dingo hybrid) was found to have over 300,000 worms (Jenkins and Morris, 1991). Dingoes and other wild canids are considered to be highly susceptible animals and generally tend to harbour high worm burdens (Macpherson and Craig, 2000; Jenkins *et al.*, 2000). In the Turkana district of Kenya, the prevalence of canine echinococcosis was found to be 39.4% (274/695), out of which 35.8% of those harboured heavy worm burdens of over 1000 worms (Macpherson *et al.*, 1985). Once the worms reach maturity, the gravid proglottid may shed about 1000 eggs every 2 weeks (Schantz *et al.*, 1995). The breed of dog may also affect worm development as shown by a study carried out by Clarkson and Walters (1991). The study

showed that Border collie dogs were efficient hosts but Beagle dogs and foxes were shown to be poor hosts (Clarkson and Walters, 1991).

Gravid proglottids or eggs are released in the faeces and contaminate surrounding grazing vegetation. Herbivores are exposed to infection from the pasture or from the water supply which may be contaminated by direct access of infected carnivores. It has been observed in a study in the highly endemic region of Turkana, northern Kenya that the local people and their livestock share drinking water supplies from water holes that are accessible to dogs and/or wild carnivores. It has also been observed in the dry season that dogs cool themselves in these water holes (Macpherson *et al.*, 1985) and contamination with *E.granulosus* eggs occurs (Craig *et al.*, 1988)

Humans can become infected orally through contact with infected dogs, particularly in the course of playful and intimate contact between children and dogs. *E.granulosus* eggs may adhere to hairs around the infected dog's anus and are also found on the muzzle and paws (Matoff and Kolev, 1964; Nelson, 1972; Torgerson and Heath, 2003). Indirect means of contact may also play a part, for example via drinking water and/or ingestion of contaminated fruit and vegetables (Carmona *et al.*, 1998); possibly through bird carriers (Silverman and Griffiths, 1955); blow flies and coprophagic flies (Heinz and Brauns, 1955; Lawson and Gemmell, 1990); beetles, ants and other arthropods (Bily *et al.*, 1978; Torgerson *et al.*, 1995), or inhaled in dust (Soulsby, 1968), resulting in human infection. The potential for transmission of taeniid eggs over a dispersed distance has been shown by the discovery of *T. hydatigena* cysts in a population of feral sheep on a remote Scottish island (St. Kilda) despite the apparent complete absence of dogs on or visiting the island and the fact that the nearest definitive hosts were located at a distance of 40km away (Torgerson and Heath, 2003). Exposure to *Echinococcus* eggs may also be affected by occupational risk factors and human behaviour plays a crucial role in the perpetuation of many parasitic zoonoses, including *E.granulosus* (Macpherson and Craig, 2000). Risk factors such as poor hygienic practices, limited education/knowledge of the parasite life-cycle transmission routes and close association of humans with their domestic animals may also play a part, especially in rural areas of developing countries and may collectively contribute to provide suitable environmental conditions for potential transfer of this parasite from definitive hosts to accidentally infect humans. Using participant

questionnaires in the Tibetan counties of Qinghai, other significant risks factors were identified, these included herding occupation, water source, offal disposal practices and dog care (Schantz *et al.*, 2003).

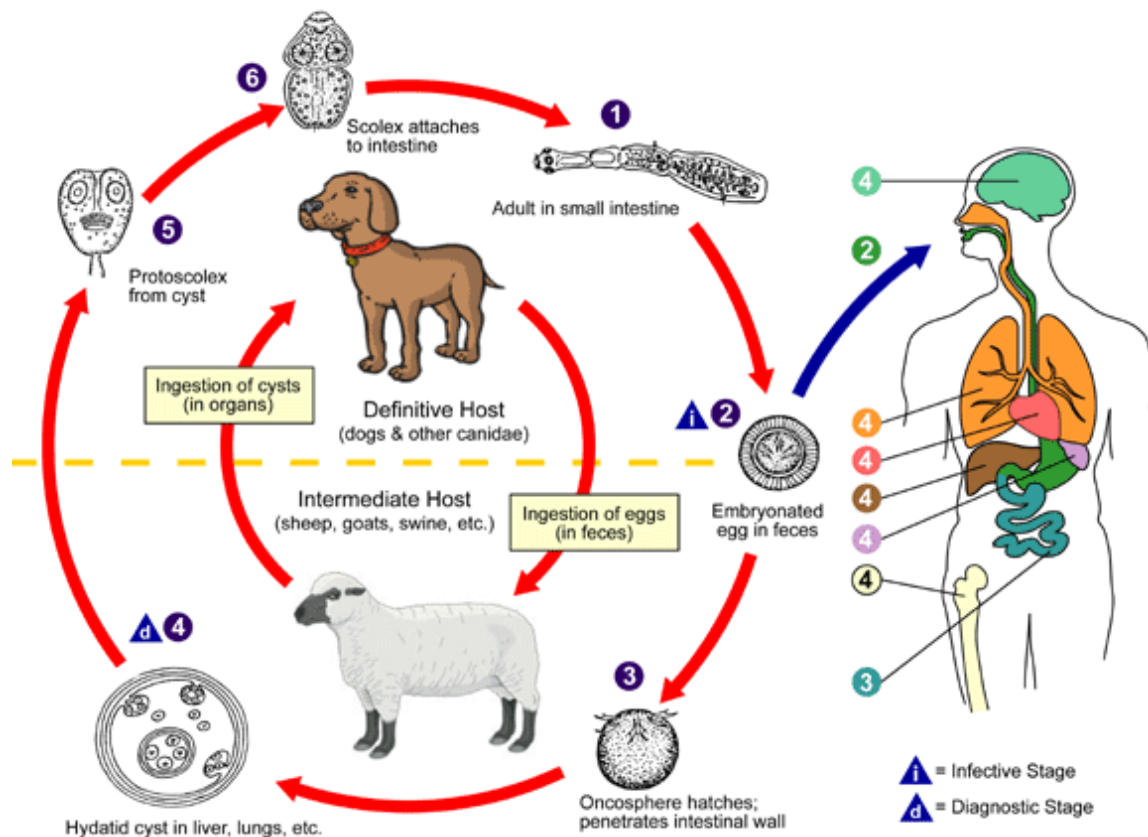
E.granulosus eggs are highly resistant to physical factors and can remain infective for a long period in a suitable environment (Nelson, 1972). Their survival is dependent on temperature and relative humidity; *E.granulosus* eggs are capable of surviving snow and freezing conditions remaining viable for at least a year. A study on egg survival under natural conditions of arid climate demonstrated that *E.granulosus* eggs were still viable after 41 months (Thevenet *et al.*, 2005). On pasture at 7°C and 21°C, eggs survived for over 200 days and 50 days respectively (Gemmell, 1977). Increasing the temperature gradually decreases the life-span of the egg to 2-14 days at 37-39°C and likewise, lower humidity decreases the life-span dramatically (Torgerson and Heath, 2003). Eggs are susceptible to desiccation and will become incapable of hatching after only a few hours when exposed to direct intense sunshine but survival maybe prolonged if the eggs are in water (Wachira *et al.*, 1991).

Eggs become ingested by ungulate intermediate hosts at which point the oncosphere larva is released from the egg. Three pairs of hooks in the region opposite to the nuclei are equipped with a complex muscle system designed to cut tissue for penetration (Holcman and Heath, 1997). In this way an oncosphere larva is liberated from its surrounding envelopes within the thick-walled egg and can reach the lamina propria within 30-120 minutes after hatching. The larvae then penetrate into the lamina propria and are transported passively through the blood or lymph to the target organs (Zhang *et al.*, 2003), where the oncosphere larvae develop slowly into hydatid cysts. Hydatid cysts comprise of two parasite-derived layers: an inner nucleated germinal layer from which brood capsules and protoscoleces bud and an outer acellular laminated layer surrounded by a host-derived fibrous capsule (McManus *et al.*, 2003). Protoscoleces develop in brood capsules derived from the germinal layer and may vesiculate to produce daughter cysts (Rogan *et al.*, 2006).

Humans have been described as ‘dead-end’ hosts for the parasite, since the life cycle usually relies on carnivores eating infected herbivores (McManus *et al.*, 2003, Zhang *et*

al., 2003). There may be rare circumstances however, for example in the Turkana region of northwest Kenya, where humans do not bury their dead, dogs and wild carnivores are able to scavenge on human remains. Under these circumstances, if the corpses harbour viable cysts humans could also serve to complete the life cycle of *E.granulosus* (Macpherson *et al.*, 1983).

Figure 1.1 Life-cycle of *Echinococcus granulosus*
(Modified from http://www.dpd.cdc.gov/dpdx/html/frames/af/echinococcosis/body_Echinococcosis_page1.htm)



- 1** The adult *Echinococcus granulosus* (3 to 6 mm long) resides in the small bowel of the definitive hosts, dogs or other canids.
- 2** Gravid proglottids release eggs that are passed in the feces.
- 3** After ingestion by a suitable intermediate host (under natural conditions: sheep, goat, swine, cattle, horses, camel), the egg hatches in the small bowel and releases an oncosphere that penetrates the intestinal wall and migrates through the circulatory system into various organs, especially the liver and lungs.
- 4** In these organs, the oncosphere develops into a cyst that enlarges gradually, producing protoscoleces and daughter cysts that fill the cyst interior.
- 5** The definitive host becomes infected by ingesting the cyst-containing organs of the infected intermediate host.
- 6** After ingestion, the protoscolices evaginate, attach to the intestinal mucosa, and develop into adult stages in 32 to 80 days.
- 2** Humans become infected by ingesting eggs, with resulting release of oncospheres **3** in the intestine and the development of cysts **4** in various organs.

1.3 Taxonomy

Echinococcosis is a term used to describe the parasitic and zoonotic disease caused by adult or larval (metacestode) stages of cestode species belonging to the genus *Echinococcus* (Rudolphi, 1801), which is a member of the family Taeniidae (Ludwig, 1886), in the order Cyclophyllidea (Schmidt, 1982), subclass Eucestoda (Southwell, 1930), class Cestoda and phylum Platyhelminthes. Rudolphi (1801) named the genus *Echinococcus* in which the name for the parasitic organism *Echinococcus granulosus* has since been coined. There have been many taxonomic revisions of the genus *Echinococcus* over the years, the earlier reviews being based on morphological and biological observations of natural and experimental infections (Kumaratilake and Thompson, 1982). Following a simplifying revision by Rausch and Nelson (1963) in which the species were grouped, the arrangement of the genus *Echinococcus* in the family Taeniidae was erected as:

Family Taeniidae Ludwig, 1886

Genus *Echinococcus* Rudolphi, 1801

E. granulosus Batsch, 1786

E. multilocularis Leuckhart, 1863

E. oligarthrus Diesing, 1863

E. vogeli Rausch and Bernstein, 1972

However currently 5 other species have been considered valid (*E. equinus*, *E. ortleppi*, *E. canadensis*, *E. shiquicus* and *E. felidis*) (Nakao *et al.*, 2007; Huttner *et al.*, 2008). The species name *E. granulosus* comes from early descriptions of hydatid cysts in sheep (Batsch, 1786). Despite attempts to clarify its taxonomy, many researchers would agree that the classification and nomenclature of *Echinococcus* has been a controversial issue for a long time (Thompson and Lymbery, 1988). Reasons for this controversy are because organisms from this genus have a very small number of phenotypic characteristics and because of taxonomic descriptions that were insufficient (Nakao *et al.*, 2007). The species and subspecies of *Echinococcus* were originally described based mainly on host-parasite specificity characteristics (Ortlepp, 1937; Williams and Sweatman, 1963; Verster, 1965).

Morphological taxonomic reviews have been carried out; most significantly by Robert Rausch in 1953, (Rausch and Nelson 1963; Rausch, 1967 and 1968. Before the

development of modern techniques, morphological characteristics were used to differentiate between species. To name but a few, these characteristics include; the number of segments, the size and shape of rostellar hooks; and the number and distribution of testes (Rausch, 1953, Rausch and Nelson, 1963, Verster, 1965). In a review by Verster (1965) the length of the blade as oppose to the total length of the hook was considered as a possibility for taxonomic purposes.

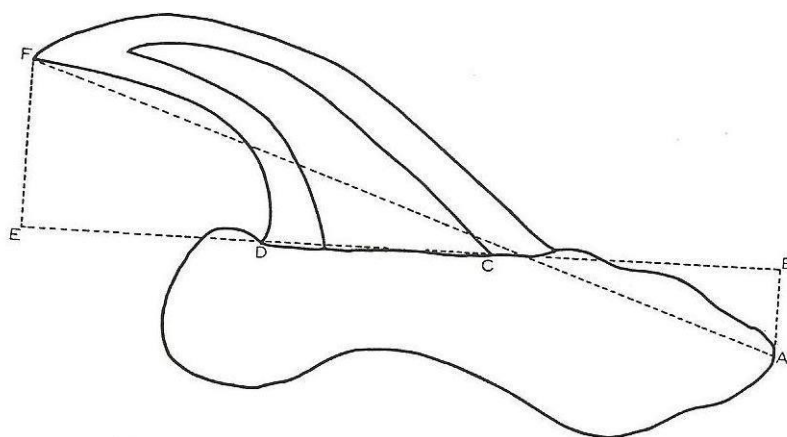


Figure 1.2 Measurements carried out on rostellar hooks. Total length: A to F, and B to E. Ventral Blade length: D to E. Dorsal Blade length: C to E. Handle length: B to C (Verster, 1965). Porcine hydatid cysts were used to infect dogs and jackals and results gave rise to a catalogue of large hook and small hook drawings.

In the Rausch review (1953), certain morphological characteristics that were once used to distinguish different species of *Echinococcus* were considered and to some degree they were disregarded as forms of recognition. For example, the number of rostellar hooks and egg size were considered as having little value in differentiating species. Rostellar hook length and number of segments (subjective to geographical variation) remained important aspects to consider, whilst minor differences in hook shape were not significant, rather their characteristic pattern.

A decade later, it seems that morphological characterisation alone was not enough to determine species status of *Echinococcus*. Out of the eleven samples of species only three could be distinguished, six were considered to be very similar to *E.granulosus* and the status of the other two remained uncertain Rausch and Nelson (1963). This led to the suggestion that applying different populations as designated informal strains might be

more appropriate (Rausch (1967). This review conflicted with the newly coined Williams and Sweatman (1963) *E.g.equinus* subspecies. It proposed that this subspecies be invalidated because it occupied the same geographical location as well as the same definitive host as the other subspecies *E.granulosus* (Le *et al.*, 2002) and it could therefore potentially interbreed where the two cycles interact (Thompson 2008). More recently, due to the ability to sequence complete mitochondrial genomes, there has been overwhelming evidence to reinstate *E.equinus* to its own species level (Le *et al.*, 2002). The results of genetic sequencing has shown that the *E.granulosus* G1 genotype differs to the G4 genotype by 12.4% in terms of nucleotides and 11.6% of amino acids, a level similar to that shown between these two genotypes and *E.multilocularis* (Le *et al.*, 2002).

Rausch (1968) attempted to define taxonomic characters in order to distinguish between the *Echinococcus* species. It was explained that through the domestication of various animals and the introduction of livestock from Europe, the domestic dog had replaced the wolf (the definitive host under natural conditions at higher latitudes), domestic livestock have replaced wild prey and *E.granulosus* has become more widespread in distribution (Rausch, 1968). Morphological characteristics of the larval forms may change depending on the host in which they developed, therefore it was suggested that taxonomic identification should be based on specimens from the respective natural hosts, which could be identified by ecological studies in endemic areas and experimental infection studies (Rausch, 1968).

Experimental infections gave rise to adult worms, which were distinguished morphologically in the black-backed jackal, the lion and the Cape hunting dog (Verster, 1965). The Cape hunting dog is an interesting group of dogs because they are more susceptible to infestation than the domestic dog (Lapage, 1956). In the UK foxhound packs are frequently fed or have access to raw livestock offal, suggesting that this group of dogs are also more susceptible to harbour worms. Foxhound packs as a selected study population will be discussed in more detail in due course. Verster reported that the domestic dog was the most important source of infestation of domestic livestock in South Africa.

It has been advised that a formal taxonomic nomenclature is needed for effective communication at all levels and that a sound classification system will not only identify but will also provide stability and predictive value about the characteristics of a particular species (Thompson & McManus, 2002). This has also been highlighted to be an important requirement in order to address the issues of surveillance and control of hydatid disease (Kumaratilake and Thompson, 1982). The concept of a 'strain' was accepted as a reference to 'intraspecific variants' of uncertain taxonomic status, making both phrases interchangeable (Kumaratilake and Thompson, 1982, Thompson and Lymbery, 1988). The current generally accepted taxonomic classification of the genus *Echinococcus* is shown in Table 1.1.

Table 1.1 Current taxonomy of *Echinococcus*. Adapted from Thompson (2008); Thompson et al. (1995); Thompson and McManus (2001); McManus and Thompson (2003); Jenkins et al. (2005); Nakao et al., (2007) and Huttner et al., (2008).

Species	Strain/genotype	Known intermediate hosts	Known definitive hosts	Zoonotic
<i>Echinococcus granulosus sensu stricto</i>	Common sheep/G1	Sheep (cattle, pigs, camels, goats,	Dog, fox, dingo, jackal and	Yes
	Tasmanian	macropods)	hyena	
	sheep/G2	Sheep (cattle?)	Dog, fox	
	Buffalo/G3	Buffalo (cattle?)	Dog, fox?	
<i>Echinococcus equinus</i>	Horse/G4	Horses and other equines	Dog	Unknown
<i>Echinococcus ortleppi</i>	Cattle/G5	Cattle	Dog	Yes
<i>Echinococcus canadensis</i>	Camel/G6, Pig/G7, Cervid/G8, G9, G10	Cattle	Wolf	Yes
<i>Echinococcus multilocularis</i>	Some isolate variation	Rodents, domestic and wild pig, dog, monkey, (horse?)	Fox, dog, cat, wolf, racoon dog, coyote	Yes
<i>Echinococcus shiquicus?</i>	?	Pika and ?	Tibetan fox and ?	Unknown
<i>Echinococcus vogeli</i>	None reported	Rodents	Bush dog	Yes
<i>Echinococcus oligarthrus</i>	None reported	Rodents	Wild felid	Yes
<i>Echinococcus felidis</i>	Lion	Zebra, wildebeest, warthog, bushpig, buffalo, various antelope, giraffe? Hippopotamus?	Lion	Unknown

1.3.1 *Echinococcus vogeli*

Echinococcus vogeli was first identified when it was examined after being expelled from a captured Ecuadorian bush dog, *Speothos venaticus* (Rausch and Bernstein, 1972). This species of cestode has been reported in Central and South America (D'Alessandro, 1996). *E.vogeli* has been reported in humans and other animal intermediate hosts in Costa Rica, Panama, Columbia, Equador, Brazil and Bolivia (Sousa and Thatcher, 1969; Rausch, 1986). Compared with other species of *Echinococcus*, *E.vogeli* is least studied and detailed information about its development in the definitive host is limited because the bush dog is elusive and is listed as a vulnerable species by the International Union for Conservation of Nature and Natural Resources (IUCN) (Matsuo *et al.*, 2000; MacDonald, 1984). The life cycle of *E.vogeli* involves the definitive host - bush dog and the rodent intermediate host - paca (*Cuniculus paca*), both species originating from the neotropical zone. The feeding practice of local paca hunters who have admitted to rewarding their dogs with viscera that may harbour viable cysts could be a risk factor for zoonotic human infection (Rausch and D'Alessandro, 2002).

1.3.2 *Echinococcus oligarthrus*

The only natural definitive host of *Echinococcus oligarthrus* is the wild felid. Natural infections have been shown in the puma (*Felis concolor*), the jaguar (*Panthera onca*), the ocelot (*Felis pardalis*) and the Pampas cat (*Leopardus pajeros*) (Thakur, 1999). One of the natural intermediate hosts for *Echinococcus oligarthrus* is the Brazilian rodent, the agouti (*Dasyprocta leporina*) (Tappe *et al.*, 2008). Experimental infections carried out on climbing rats and spiny rats showed that they too were suitable intermediate hosts and further experiments suggested that the domestic house cat may play an important role as the definitive host and therefore contribute to zoonotic human infection (Tappe *et al.*, 2008). Adult and larval stages of *Echinococcus oligarthrus* have been reported throughout a wide range of Central and South America including Costa Rica and Argentina (Thakur, 1999; Guarnera *et al.*, 2004). Polycystic echinococcosis, the disease caused by *E.oligarthrus* has been reported in Venezuela, Brazil and Surinam (D'Alessandro, 1996).

1.3.3 *Echinococcus multilocularis*

Echinococcus multilocularis, commonly known as the fox tapeworm, is widespread in the northern hemisphere and can be found in areas of central and northern Europe, Russia, Turkey, northern and central Asia, including Japan and in particular western China representing major endemic areas such as Tibet and parts of North America. Cases of alveolar echinococcosis in humans and animals have also been reported in other countries such as Slovenia, Hungary, Bosnia, Bulgaria, Greece and Romania (Schantz *et al.*, 1995; Sreter *et al.*, 2004; Barabasi *et al.*, 2010). In central Europe, the life-cycle of *E.multilocularis* is primarily perpetuated by the sylvatic cycle of the predator-prey interactions between wild definitive hosts such as the red fox (*Vulpes vulpes*) and intermediate hosts such as the common vole (*Microtus arvalis*), the water vole (*Arvicola terrestris*) and the muskrat (*Ondatra zibethica*). In some parts of Europe, the sylvatic cycle of *E.multilocularis* is not restricted to rural regions but also occurs in urban areas; studies have shown that successful vaccination campaigns against rabies have driven fox populations into urban areas (Chautan *et al.*, 2000; Gloor *et al.*, 2001). This suggests that the ecological barrier between foxes infected with *E.multilocularis* and the human populations may be low (Deplazes *et al.*, 2002).

As well as the red fox, the arctic fox (*Alopex lagopus*), the coyote (*Canis latrans*), the wolf (*Canis lupus*), the raccoon-dog (*Nyctereutes procyonoides*), the sand fox (*Vulpes corsac*), and the Tibetan fox (*Vulpes ferrilata*) are all known definitive hosts, depending on geographic location (Torgerson and Budke, 2003). Domestic dogs and cats can also serve as definitive hosts of *E.multilocularis* if they become infected through the ingestion of rodents infected with metacestodes, thus perpetuating a synanthropic cycle (Petavy *et al.*, 1991; Craig *et al.*, 2000). Cats act as poor final definitive hosts with low or negligible egg excretion (Goodfellow *et al.* 2006). Both foxes and domestic carnivores are considered to be potential sources of human infection (Leiby and Kritsky, 1972).

Various small microtine mammal intermediate hosts have been recorded as susceptible species of *E.multilocularis* including small mammals from the families *Sciuridae*, *Cricetidae* and *Muridae* to name but a few (Vuitton *et al.*, 2003). Lagomorphs of the family *Ochotonidae* are frequently infected in parts of China (Torgerson and Budke, 2003); the two main species of Tibetan plateau pika being *Ochotona curzoniae* (black-

lipped pika) and *Ochotona thibetana* (Tibetan pika) (Giraudoux *et al.*, 2006). There have also been occasional reports of infections in insectivores such as the *Soricidae* and *Talpida* (Torgerson and Budke, 2003).

1.3.4 *Echinococcus granulosus*

There have been 9 or 10 main genotypes identified within the species of *E.granulosus* based on morphological distinction, biochemical and molecular biological characterisation (G1-G10): two sheep strains (G1, G2), two bovid strains (G3, G5), a horse strain (G4), a camel strain (G6), a pig strain (G7) and the cervid strain (G8) (Bowles *et al.*, 1992a; Pearson *et al.*, 2002; Craig *et al.*, 2003; Mwambete *et al.*, 2004) as shown in Table 1.1 G9 has been described for *E.granulosus* isolates from pigs in Poland (McManus and Thompson, 2003) and in Argentina (Guarnera *et al.*, 2004), while G10 has been found in cervid hosts in Eurasia (Lavikainen *et al.* 2003, 2006; Moks *et al.* 2006). Strains G1-3 are grouped together to form the species *E.granulosus* ‘sensu stricto’ and taxonomic revision has grouped G6-8 to form the species *E.granulosus* ‘sensu lato’ (Nakao *et al.*, 2007). In the current study the term ‘sensu lato’ refers to *E.granulosus* genotypes (or species) not associated with genotypes G1-3.

1.3.5 *Echinococcus equinus*

A comprehensive study by Williams and Sweatman (1963) coined a new subspecies; ‘*E.granulosus equinus*’, today recognised as the species *E.equinus*, which was formerly known as the G4 strain of *E.granulosus* or the ‘horse strain’ (Thompson, 2008, Tappe *et al.*, 2010). Previous to the 1960s, it was generally considered that equine echinococcosis resulted from infection arising from a predominantly sheep/dog cycle and that infected horses played little part in the maintenance of the cycle (Hatch, 1975).

E.equinus was recognised as distinct from the sheep strain and promoted to a subspecies (*E.granulosus equinus*) by Williams and Sweatman (1963). Rausch (1967) dismissed this as the sheep and horse strain exist sympatrically. However, the epidemiological evidence, particularly host specificity, supports a separate taxonomic status. Recent molecular evidence, implied that *E.granulosus* (G4) strain is at least as distinct from the sheep strain (G1) as either is from *E.multilocularis*, strongly supported the taxonomic status as a separate species *E.equinus* (Le *et al.*, 2002; McManus, 2002; Thompson and

McManus, 2002). The parasite seems to have the dog as the only known definitive host and all infections in horses seem to be exclusive to *E.equinus* (Jenkins *et al.*, 2005). The cycle of *E.equinus* appears to be maintained by the feeding of cheap horse and donkey meat to hounds bought in locally and slaughtered at the kennels, often the meat is not cooked (Hatch, 1967). In Ireland, the sale of raw horse flesh from knacker's yards was exposed for sale as dog food in pet shops in Belfast (Swann, 1957). An epidemiological survey carried out in the UK in 1975 found that out of twenty one foxhound packs examined, eleven of the packs (52%) harboured *E.equinus* infected dogs (Thompson and Smyth, 1975). Due to economic pressures and lack of labour forces, the dietary practices of foxhound have changed leading to an increase in the feeding of raw flesh and offal.

Williams and Sweatman (1963) describe cases of hydatid infections in horses from 1932 to 1962. They summarise data and cases that have been reported over this 30 year period. These include reports from England, Ireland and Wales, as well as across Europe, eastern and southern Australia, an isolated case in Venezuela and North America (imported horse from England) and Canada. They found that out of 709 horses inspected in Doncaster, England over a 6-month period in 1960, 12.8% (91/709) had light infections and 2.3% (16/709) had heavy infections. None of the infections were pulmonary. The majority of horses were from Lincolnshire and Yorkshire, one from Wales and one from the Isle of Man. They suggested that this incidence level was not only high but also widespread.

The zoonotic transmission potential of *E.equinus* is unknown however it has been suggested, based on epidemiological grounds as having low or no infectivity to humans (Thompson and Smyth, 1975). *E.equinus* does not appear to be zoonotic it is almost always reported to date from equines however, Boufana *et al.*, (2012) recently described a viable *E.equinus* infection in primate intermediate host - a captive born and bred red ruffed lemur (*Varecia rubra*) in the UK. It has been well documented that human echinococcosis can remain undiagnosed until adulthood because of the asymptomatic and slow growing and nature of the cysts (Torgerson and Budke, 2003; Moro and Schantz, 2009). Human CE is rare in the UK and official figures from the last century show an annual average of just 0.3 human cases per million, although this rate was double (0.6 p.m.) in Wales (Thompson and Smyth, 1975). It was reported that 15 people die from the disease in the UK every year (Forbes and Cook, 1963). In a short communication

published in 1974, Thompson and Smyth suggested that, although there had not been any reports of the equine subspecies being infective to humans, cases may emerge in the future due to laboratory observations of its slower development compared with that of the sheep strain.

1.3.6 *Echinococcus orteppi*

Likewise with G4, it has been suggested that the G5 cattle strain also deserves its own taxonomic status of species, therefore it has been designated *Echinococcus orteppi* (Thompson and McManus, 2002; Jenkins *et al.* 2005; Nakao *et al.*, 2007). Other molecular studies however consider the status of *E.orteppi* as unresolved, based on phylogenetic relationships (Lavikainen *et al.*, 2006; Moks *et al.*, 2008). The cattle strain has been observed in Europe, India, Sri Lanka, Africa, and South America (Eckert *et al.*, 2001; Dinkel *et al.*, 2004; de la Rue, 2011). Cystic echinococcosis caused by G5 in humans is extremely rare with only a few cases being confirmed. One case in the Netherlands, two cases in Argentina and one case in Mexico (Bowles *et al.*, 1992b; Kamenetzky *et al.*, 2002; Guarnera *et al.*, 2004; Maravilla *et al.*, 2004). The CE isolate from the Netherlands was found to be genetically indistinguishable from the cattle strain observed in Switzerland (Thompson *et al.*, 1984). Results have been produced to show that the naturally infected adult worm of *E.orteppi* is morphologically distinguishable from that of the G1 genotype of *E.granulosus* (de la Rue *et al.*, 2011). A more recent study suggested that there may be a possible increase over time in the proportion of *E.orteppi* (G5) loads in cattle from different localities of the Rio Grande do Sul state in Southern Brazil (Balbinotti *et al.*, 2012). The study identified the frequencies of G1 and G5 genotypes in fertile and infertile cysts in cattle isolates; concluding from statistical analysis that G5 occurrence was increasing although it was not possible to predict whether this trend would continue.

1.3.7 *E.canadensis* and *E.felidis*

The proposed taxonomic proposal for unifying the following strains; camel (G6), pig (G7), cervid (G8), Polish pig (G9) and the Fennoscandian cervid (G10) into a single species called *Echinococcus canadensis* is supported by various molecular studies (Lavikainen *et al.*, 2006; Nakao *et al.*, 2007; Xiao *et al.*, 2005 and Thompson *et al.*, 1995). *Echinococcus granulosus* in cervids is maintained by a predator-prey cycle,

involving mostly wolf and large cervids such as moose, *Alces alces*; elk, *Cervus elaphus* and reindeer, *Rangifer tarandus* in Eurasia and North America (Rausch, 1967). In a molecular study carried out by Nakao *et al.*, (2007), complete mitochondrial DNA sequences were obtained for *E.granulosus* genotype G6 (Kazakhstan), *E.granulosus* genotype G7 (Poland) and *E.granulosus* genotype G8 (USA). Nakao *et al.*, (2007) suggested that the group G6-G10 be named *E.canadensis* and considered as a single species.

Echinococcus felidis was previously described as the ‘lion strain’ by Ortlepp (1937) from the lion, *Panthera leo* in South Africa, based on morphological observations. According to Rausch and Nelson (1963), this taxon was considered to belong to the same species as *Echinococcus granulosus*. Further morphological studies suggested that it should be assigned to subspecies status as *Echinococcus granulosus felidis* (Verster, 1965), however Rausch (1967) considered this to be invalid because of the sympatric occurrence of various such ‘subspecies’ of *E. granulosus* in southern Africa. Recently molecular studies have been carried out to investigate the taxonomic position amongst the present classification system (Huttner *et al.*, 2008). Up until this study, the ‘lion strain’ was considered as a form of *E.granulosus* of uncertain taxonomic status that was known to be transmitted between lions and large wild herbivores in Africa (Macpherson and Wachira, 1997). Molecular techniques involving the use of mitochondrial genes used by Huttner *et al.* (2008) formed the proposal that *E.felidis* is positioned as a sister taxon of *E.granulosus sensu stricto*. Further studies revealed that *E.felidis* possibly also occurs in hyenas (Huttner *et al.*, 2009).

1.4 *Echinococcus granulosus* distribution

Echinococcus granulosus has a worldwide geographic distribution and occurs on all continents. There are not many countries in the world where CE has not been recorded (Macpherson and Craig, 2000). The highest prevalences are found in parts of Eurasia, (Mediterranean region, Russia, adjacent independent states and China) northern and eastern Africa, Australia and South America (Eckert *et al.*, 2001). Domestic cycles of *E.granulosus* are supported in all types of pastoral regions such as arid, temperate, mountain and plateau, where predominantly sheep and other livestock occur, as a result produces the risk of human infection (Craig *et al.*, 2007a).

In the United Kingdom, *Echinococcus granulosus* is limited in distribution, being primarily restricted to mid and south Wales (Torgerson and Budke, 2003). *E.granulosus* has also been reported in northern and southwest England and northwest Scotland (Craig *et al.*, 1996). In contrast to Wales, *E.granulosus* appears not to have become established in Ireland despite the free movement of animals between the UK and Ireland, reasons for this are unknown (Torgerson and Budke, 2003). It has been hypothesised that this is because of the relatively low sheep population in Ireland, the sheep sector accounting for only 4% of agricultural output in 2005, compared with 28.5% of agricultural output for cattle (European Commission, 2008). In Wales, the sheep sector accounted for 21.5% agricultural output in 2006 with 8.5 million sheep accounting for 26% of the UK total in 2008 (IWA, 2011). However, there may be an increasing risk of introducing *E.granulosus* as there has been an increase in the sheep population in recent times (Torgerson and Budke, 2003).

1.5 *Echinococcus equinus* distribution

Equine echinococcosis is not uncommon throughout the world and it has been recognised on every continent (Thompson, 1975). *Echinococcus equinus* (*E.granulosus* G4/horse strain) is present in many areas where *E.granulosus* is found (Torgerson and Budke, 2003). In contrast to the situation in many European countries, equine echinococcosis was rare in Great Britain before the Second World War (Southwell, 1927). After the Second World War, from the 1950s onwards, many more cases of echinococcosis in horses were reported in the literature (Thompson, 1975). These reports included; a recording of a 1.8% prevalence of equine echinococcosis (Miller and Poynter, 1956), accounts that abattoir workers and butchers at the time knew of a cystic condition in horse livers (Sinclair, 1956) and that there were concerns from the Animal Health Trust Annual Report of 1960 that hydatid cysts in horses were being observed more and frequently (Anon, 1960). The prevalence of equine echinococcosis seemed to increase up to 7% according to two reports on large scale horse slaughter (Hay, 1962; Edwards, 1962). From the early 1960s onwards, it was reported that equine echinococcosis reached high epidemic proportions of up to 61.7% (Thompson and Smyth, 1974; 1975; Dixon, 1973). According to Thompson and Smyth (1975), the increase was due to a major change in the way that packs of hunting dogs were fed. Before the Second World War, hunting dogs were primarily fed on boiled horse flesh mixed with oatmeal

(Higginson, 1984). Due to the expense of fuel and labour costs after the war, hunt kennelmen fed their hunting packs raw horse and sheep flesh, resulting in an accelerated increase of equine echinococcosis (Smyth, 1976). The distribution of equine echinococcosis does not appear to be localised, data shows that the infection may be widespread because the origins of the slaughtered horses are spread widely over Great Britain (Thompson, 1975).

In contrast to *E.granulosus*, only the horse/dog cycle seemed to exist in Northern Ireland as it was observed regularly at a horse abattoir in Ulster, whereas the sheep/dog cycle did not appear to exist or was at least very rarely seen in sheep (Logan, 1971). *E.equinus* is reported to be widespread in Ireland (Hatch, 1970) but zoonotic strains of *E.granulosus* appear absent and no autochthonous cases of human CE have been reported (Torgerson and Budke, 2003). The situation was reported as having a low incidence of human hydatidosis and only two reported cases at The Royal Victoria Hospital in Belfast (Logan, 1971) but human isolates have not been genotyped. Several recommendations were put forward to maintain this low incidence such as not to allow dogs access to the slaughter house area; no dog should be given uncooked meat or offal; and no dog should have access to pasture where horses graze unless the dog is free from the parasite (Logan, 1971).

1.6 Aetiology of echinococcosis in humans

All *Echinococcus* spp. are zoonotic, except *E.equinus* and there is no information on *E.shiquicus* and *E.felidis*. There are 4 species of *Echinococcus* that are of public health concern; *E.granulosus*, *E.multilocularis*, *E.vogeli*, *E.oligarthrus* and *E.canadensis*. *E.granulosus* forms a unilocular cyst(s) and causes cystic echinococcosis (CE) the form that is most frequently encountered in the liver and lungs. The common sheep strain (G1) is the form that is mostly associated with human infection. *E.multilocularis* forms multivesicular cysts that are poorly margined and are more invasive in growth with greater ability to spread, known as alveolar echinococcosis (AE) (Scherer *et al.*, 1978; Macpherson *et al.*, 2003). Although not as common (around 100 recorded cases), *E.vogeli* causes polycystic echinococcosis (PE) (D'Alessandro, 1996; Rausch and D'Alessandro, 2002).

1.7 Growth and pathology of *Echinococcus granulosus*

1.7.1 Cystic echinococcosis

Cystic echinococcosis (CE) is caused by *Echinococcus granulosus*. CE is also known as hydatid disease, hydatidosis, cystic hydatidosis, hydatid cyst and *Echinococcus* metacestodiasis (Schantz and Schwabe, 1969; Schantz, 1982; Filice *et al.*, 1991). The terms hydatidosis or hydatid disease refers to infection with the metacestode, whereas echinococcosis is applied in a general way to both adult and larval infection stages. In the current study the term canine echinococcosis will be used to refer to the infection in the definitive host, namely the dog, while the term cystic echinococcosis (CE) will be used to refer to the infection in the intermediate mammalian hosts including humans.

Human CE results when a person ingests eggs, which have been shed in the faeces of the definitive host. Human CE presents non-specific symptoms, making it difficult to diagnose as clinical signs may take months to years to develop and only become apparent as the metacestode grows. The beginning of primary infection is always without symptoms and it has been reported that up to 60% of all CE cases may be asymptomatic (Pawlowski *et al.*, 2001). After an undefined period of several months to years, the infection may become symptomatic as a space-occupying lesion. Studies show that patients presenting symptoms were mostly aged between 4 and 15 years of age and infection rates are fairly similar in both males and females (Utrilla *et al.*, 1991; Menghebat *et al.*, 1993). It is uncertain how long the incubation period of CE is, however it has been suggested that it may be from many months to years (McManus *et al.* 2003). Cysts may form in any organ of the body and can affect single or multiple organs. Over 90% of cysts occur in the liver or lungs, or both (McManus *et al.* 2003). Between 50-77% of human CE is hepatic and 8.5-43% is pulmonary and although rare, cerebral CE can occur with approximately 2% of cases (Tuzun *et al.*, 2002). CE due to other 'genotypes', apart from G1 may vary in cyst development, pathology and site.

The body can become distended if the metacestode increases in size and may lead to signs of disorders related to disruption of the infected organ functions. Clinical features are also dependent on; their position within the organ; the whole effect within the organ and surrounding structures. The most serious consequence is if the hydatid cyst ruptures and causes an anaphylactic reaction, which may be fatal. The development of

immunological responses may be as a result of cyst leakage, rupture or dissemination. As cited by McManus *et al.* (2003), one study revealed that ‘anaphylaxis complicated 10% of all intraperitoneal ruptures.’ Not only can damaged cysts cause immediate hypersensitivity/ anaphylaxis but also secondary infection caused by daughter cyst PSCs can spill into the body forming more cysts. Another factor that influences daughter cyst development is the release of vesiculating PSCs after surgical endocystectomy. Cerebral hydatid disease can cause clinical symptoms such as headache, nausea, seizures and vomiting and may occur as single or multiple lesions.

E.granulosus metacestodes can be found in a large number of intermediate hosts, including sheep, cattle, horses, pigs, camels, giraffes, hippos, elephants, primates, marsupials and cervids. Similarly with human infection, animals infected with *E.granulosus* cysts may not show symptoms for a long period of time or even during the whole life-span of the host. However, it has been suggested that symptoms experienced by humans infected with hydatid cysts may also occur in infected animals and that knowledge based on human CE cases can be assumed for animals (Pawlowski *et al.*, 2001). In particular the development of pathological changes is related to various factors such as, which organ(s) is/are involved, cyst size and number and adjacent structure interaction.

1.7.2 Canine echinococcosis

E.granulosus is infective to various species of carnivore however the domestic dog (*Canis familiaris*) is the predominant definitive host causing the intestinal form of echinococcosis. Canine echinococcosis is comparatively harmless and does not induce any major ill effects to the definitive host, even in those with heavy infection (Eckert, *et al.*, 2001). In heavy infections in young dogs may be a pot-bellied in appearance and obstruction in the small intestine may rarely occur (Soulsby, 1982). A large number of mammalian species can be intermediate hosts, including domestic ungulates and humans (Torgerson and Budke, 2003). Dogs become infected after ingesting offal contaminated with hydatid cysts containing viable protoscoleces (PSCs) (McManus *et al.*, 2003). After ingestion, the PSCs evaginate, attach to the canine intestinal mucosa and develop into adult stages 4-5 weeks later. The adult tapeworm reaches sexual maturity and gravid

proglottids or eggs are released in the faeces and contaminate surrounding grazing vegetation.

The parasite penetrates deeply between the villi of the epithelium within the small intestine but does not cause significant pathology. Small occurrences happen such as local flattening of epithelial cells, slight cellular infiltration of the mucosa and increased mucus production. Circulatory antibodies may be produced due to excretory/secretory products being released from the scolex. It is rare that dogs and cats become intermediate hosts for *E.granulosus* metacestodes (Pawlowski *et al.*, 2001) however concurrent infection of the dog as both the definitive and the intermediate host has been reported (Torgerson and Budke, 2003). Abdominal enlargement, ascites and hyper-c-globulinaemia were all clinical signs found in rare cases of dogs with metacestode infection of the liver and or peritoneum (Haller *et al.*, 1998).

1.8 Growth and pathology of *Echinococcus equinus*

1.8.1 Equine echinococcosis

Echinococcosis of equine origin has been reported in Europe, the Middle East, New Zealand, Asia and America (Rezabek *et al.*, 1993; Mukbel *et al.*, 2000; Chiou *et al.*, 2001; Varcasia *et al.*, 2008; Blutke *et al.*, 2010). The disease in horses (and other equids such as donkeys and zebras) has been well recognised for many years in Great Britain (Williams and Sweatman, 1963). In 1989 B.R. Cook published a controversial paper that described experiments that spanned 13 years (Cook, 1989). His overall conclusions from his experimental infections were that only one subspecies of *E.granulosus* of horse origin naturally occurred in Great Britain. It is now recognised that there are distinct horse/dog and sheep/dog forms of *E.granulosus* in the UK and that these differ in a widely in terms of biological and biochemical criteria, including morphology, metabolism and developmental biology (McManus *et al.*, 1989, Thompson and Lymbery, 1988). There have been comparative reports carried out on the equine form in England, Ireland, Scotland, Belgium, Switzerland, South Africa and New Zealand and have shown the uniformity and widespread geographical distribution of the horse strain of *E.granulosus* (Kumaratilake *et al.*, 1986).

In Ireland *E.equinus* is widespread (Hatch, 1970) and earlier cases were reported of horses infected with hepatic hydatid cysts (Baxter *et al.*, 1956). Data generated from three surveys conducted on horses slaughtered in Irish abattoirs showed that 25% (7/28) of horses were infected in one survey (Baxter *et al.*, 1956); 55.4% (496/896) in one survey (Gracey, 1962) and 22% (94/426) in another (Hatch, 1972). To date there have not been any cases of *E.equinus* being infective to domestic animals such as cattle or pigs (Smyth, 1976). *Echinococcus equinus* appears to use equines only as intermediate hosts (Jenkins *et al.*, 2005). Although there are no cases of the equine strain being infective to humans, cases may emerge in the future due to its slower development under laboratory conditions, (Thompson, 1974).

1.9 Diagnosis and treatment of CE in humans

Cystic echinococcosis (CE) in the intermediate host can be asymptomatic for many years before the onset of clinical signs associated with the pressure from the enlarging cyst(s) or tissue fibrosis/necrosis in the affected organs – primarily liver and other abdominal viscera but also lungs, brain, bones and other areas (Craig *et al.*, 2007a).

1.9.1 Imaging

The WHO-Infomal Working Group on Echinococcosis (WHO-IWGE) identified that the best approach for human CE treatment should be image-based and stage-specific, which is helpful for choosing one of the following options: (1) percutaneous treatment, (2) surgery, (3) anti-infective drug treatment or (4) watch and wait (Brunetti *et al.*, 2010). Diagnosis of human CE is largely based on imaging techniques such as ultrasound (US), computed axial tomography (CT) scans, magnetic resonance imaging (MRI), X-ray, angiography (AG) cholangiography (CAG), endoscopic retrograde cholangiography (ERC), percutaneous transhepatic cholangiography (PTC) and MRI-cholangiography (MRIC) (Craig *et al.*, 2003; McManus *et al.*, 2003; Pawlowski *et al.*, 2001). X-ray imaging is particularly useful for detecting upward diaphragm displacement indicative of hepatic cyst(s) and it may also detect asymmetry of the heart outline, which may be a sign of a hydatid cyst of the heart (Pawlowski *et al.*, 2001). According to Rogan *et al.*, (1990), characteristic cyst structures do not present or are absent in many cases.

1.9.2 Serological detection

Detection of circulating antigens may be relevant as a method for post-surgical follow-up of patients and for monitoring the growth dynamics and activity of cysts (Craig, 1986). Current serological tests for antibodies that use purified lipoprotein antigen B in ELISA or detect antigen B in immunoblots are the most specific, though sensitivity varies with clinical presentation, site, number of cysts and pathology (Rogan *et al.*, 1991; Craig, 1997; Rogan and Craig, 2002). At present there is no global standard, highly sensitive, and specific test available for antibody detection for human CE (Pawlowski *et al.*, 2001; Craig *et al.*, 2003). Detection of antibodies against native or recombinant antigen B remains the gold standard for serology at present (Ito, 2002). Due to the residual nature of serum antibodies coupled with the problem of non- or low responders, serum antibody detection may not be the best approach as false positives may be produced (Craig, 1997).

1.9.3 Treatment

By way of the PAIR technique (Puncture, aspiration, injection and re-aspiration), microscopy may be used to identify the presence of protoscoleces (PSC) in the aspirated cyst fluid to confirm the diagnosis, in some cases parasitic material can also be removed (Smego *et al.*, 2003). Chemotherapy treatment with drugs, such as albendazole or mebendazole are used to soften the cysts and reduce internal pressure so that surgeons are able to remove the cyst more easily (Pawlowski *et al.*, 2001).

1.10 Diagnosis in livestock CE

In the intermediate host, CE has usually been detected at post-mortem by examination of the viscera. This can provide important epidemiological data, which can be used to define the likely infection pressure (Cabrera *et al.*, 1996; Ming *et al.*, 1992). Examination of the liver and lungs of the abattoir animals remains the only practical method for diagnosis. Smaller lesions in the mesentery and liver may not always be easy to distinguish from other parasitic helminths such as *Taenia hydatigena*, therefore further histopathological confirmation may be required (Maxson *et al.*, 1996; Lloyd *et al.*, 1991). Ultrasound has been found to be reasonably sensitive (>70%), however specificity was a problem (Njoroje *et al.*, 2000; Eduardo *et al.*, 2001). It is a particularly useful tool, particularly in developing regions with poor medical facilities (Macpherson *et al.*, 2003). It can be used as a portable method for detecting hydatid cysts in livestock; investigations in Kenya

showed positive predictive values (PPV) of 80.6-82% (Maxson *et al.*, 1996; Njoroje *et al.*, 2000). These studies provide a useful way of detecting CE in livestock when mass slaughter is not feasible due to the dependence of the Turkana people on the livestock milk and blood (Maxson *et al.*, 1996). This technique provides information about the number, size, site and condition of the cysts (Njoroje *et al.*, 2000).

1.11 Diagnosis and detection of canine echinococcosis

Canine echinococcosis is difficult to detect compared to other gastrointestinal helminth infections in dogs (Craig, 1997). Despite this, a number of parasitological diagnostic techniques have been developed to detect *E.granulosus* in domestic and wild canids, as well as indirect immunodiagnostic approaches such as detection of serum antibody and parasite antigens in faeces (coproantigens) as well as molecular approaches for amplification of parasite DNA (coproDNA) (Craig *et al.*, 2003).

1.11.1 Direct parasitological observation - purgation

The 'gold standard' method of detecting echinococcosis infection in dogs is the use of arecoline salts such as arecoline hydrobromide (2mg/kg) or arecoline acetarsol (3mg/kg) given to dogs antemortem (Craig, 1997) causing purgation of the entire intestinal contents. The dogs should ideally be starved for 12 hr prior to dosing and usually produce purge within 30 min to 1 hr (Craig, 1997). The arecoline paralyses tapeworms which can then be collected and identified (Torgerson and Budke, 2003). Arecoline purgation is time consuming, can be hazardous to the operator and occasionally produces severe reactions in the dogs (Torgerson and Budke, 2003). Although the technique is 100% specific, it has low sensitivity as not every dog will purge (up to 25%), and a significant number of carriers are not detected (Craig, 1997; Schantz *et al.*, 1995). Purged material is examined using a magnifying glass, although further examination with a dissecting microscope is recommended (Craig, 1997). If any dogs are found to be *Echinococcus*-positive they should be treated with praziquantel or in Africa and Middle Eastern countries it is recommended they are to be destroyed (Craig, 1997). Purgation remains the only quantitative technique that can be used in the living dog and continues to play an important role in epidemiological studies (Torgerson *et al.*, 2003).

1.11.2 Necropsy

The most reliable means of diagnosis of canine echinococcosis is by necropsy, as the worm burden can be estimated and parasites collected for identification (Craig, 1997; Eckert, 1997). Straightforward coprological examination may reveal the presence of taeniid eggs but will not distinguish infection with *Echinococcus* spp. and *Taenia* spp. Scotch tape perianal swab techniques followed by diagnoses using light microscopy have also been implemented (Craig *et al.* 1988). The problem with techniques such as these is that they are not species-specific and lack sensitivity (Cabrera *et al.*, 2002). Consequently immunological and molecular approaches have been developed.

1.11.3 Immunofluorescent detection of eggs

A method known as indirect fluorescent antibody test (IFAT) was used in the identification of *E.granulosus*, *Taenia hydatigena* and *Taenia pisiformis* eggs (Craig, 1983). The test involves using immunoabsorbed polyclonal antibodies or *Echinococcus* oncosphere specific monoclonal antibody (Craig, 1983; Craig *et al.*, 1986). Eggs were hatched or activated from perianal scotch-tape swabs taken from naturally infected dogs in Kenya and results showed 100% specificity and 73% sensitivity for *E.granulosus* (Craig *et al.*, 1988). Despite the test being highly specific, it has been described as cumbersome and impractical for testing large numbers of dogs and it relies on perianal contamination and egg hatchability (Craig, 1997).

1.11.4 Coproantigen detection by ELISA

The detection of parasite antigens in body excretions e.g. faeces (coproantigens) is considered to be the diagnostic test with the most potential to replace the traditional method of arecoline purgation as a method for antemortem diagnosis (Craig, 1997). Coproantigens can be detected using an enzyme-linked immunosorbent assay (ELISA); providing data that can be quantified to indicate levels of infection. The history of ELISAs and other immunological tools such as radioimmunoassays (RIA) and enzyme immunoassays (EIA) is described by Lequin (2005). ELISAs were developed in the 1960s and were then used commercially in the 1970s and 1980s. First reports of coproantigen detection in dogs produced cross-reaction with antigens in human *Taenia*-infected faeces. In the 1980s specificity was increased by using raised antibodies in hyperimmunised rabbits to surface antigens.

The ELISA is a biochemical technique used to detect the presence of a specific antibody or an antigen in a sample and involves the use of a capture and a detection antibody. The test relies on the parasite releasing metabolic products into the intestine that can be useful to immunological detection and if these antigens are not directly related to parasite reproduction they should be present when eggs are absent from the faeces (Allan and Craig, 2006), suggesting that the metabolic products should disappear after successful treatment. This theory provides a good basis for detecting parasite antigens during the pre-patent period when eggs are absent from the faeces and it was first demonstrated when Babos and Nemeth, (1962) detected *E.granulosus* antigen in canine faeces prior to the onset of egg production.

The specificity of a diagnostic test refers to how the assay detects the targeted antigen in the sample, whereas the sensitivity refers to how sensitive the assay is in detecting the antigen if present in the sample. To set up a diagnostic ELISA assay, polyclonal antibodies are raised in an experimental mammal i.e. a rabbit, by injecting it intramuscularly with parasite derived material mixed with an adjuvant. The antibodies that are subsequently raised are then processed to produce a capture antibody and a detection antibody (Allan *et al.*, 1992). The detection antibody is usually conjugated to an enzyme such as horse radish peroxidase (HRP). HRP reacts to a substrate solution i.e. tetramethylbenzidine (TMB) giving a detectable colour which can then be read by a plate reader to give numerical data in the form of optical density (OD) values.

Various coproantigen ELISA tests have been modified for the surveillance of canine echinococcosis. Huang *et al.*, (2007) describe a double-sandwich coproantigen ELISA assay that has been designed whereby the capture and detection antibodies were produced to recognise specifically the carbohydrate portion of the parasite tegument. Previous studies carried out in order to characterise the biochemical elements of the antigenic material revealed the important components of carbohydrate-rich *E.granulosus* adult antigens in infected dog faecal samples (Elayoubi *et al.*, 2003). Huang *et al.*, (2007) reported that the assay was used in the surveillance of Tibetan dogs and that it detected both *E.granulosus* and *E.multilocularis* coproantigens alike but was unable to distinguish between the species. The assay also does not take into account the recently discovered species *Echinococcus shiquicus* (Xiao *et al.*, 2005), which is also endemic in this part of

the world. The diagnostic specificity and sensitivity of coproantigen ELISA tests do vary according to different studies. A coproantigen ELISA assay was developed to target *E.granulosus* and *E.multilocularis* coproantigens in dogs, dingoes and foxes showed diagnostic specificity and sensitivity of 98% and 87% respectively for animals harbouring ≥ 200 worms (Deplazes *et al.*, 1992). The diagnostic sensitivity for the detection of *E.granulosus* was shown to be 46% in dingoes and 56% in dogs; the overall diagnostic sensitivity was 42% for *E.multilocularis* in foxes (Deplazes *et al.*, 1992). In another study into the development of a coproantigen ELISA, the authors report an unexpectedly low diagnostic probability of 37.5% in the detection of canine echinococcosis in 59 dogs in Uruguay (Sakai *et al.*, 1995). It was suggested that it may be due to excess antigen presentation in the host or there could be antigenic differences of genetic variants of the parasite in Uruguay (Sakai *et al.*, 1995). In the current study, several coproantigen ELISA tests are investigated for their variance in diagnostic sensitivity and specificity with reference to the currently used assay developed by Allan *et al.*, (1992). Table 1.2 outlines its diagnostic sensitivity and specificity capabilities in comparison with other diagnostic tests.

1.11.5 CoproDNA detection by PCR

Another approach to parasite detection is the polymerase chain reaction (PCR), a molecular method used for amplifying DNA molecules using only a small amount of original DNA so that it can be easily visualised against known DNA markers. The scientific community were first introduced to the concept of PCR in 1984 by Kary B. Mullis (Mullis, 1990). Today PCR is an invaluable molecular method used to synthesize many copies of matching sequences of target DNA by using a pair of PCR primers known as a template. In brief, a PCR involves two oligonucleotide primers that flank the DNA fragment to be amplified and repeated cycles of heat denaturation of the DNA at 94°C, annealing of the primers to their complementary sequences at 40°C, and extension of the strands by DNA polymerase at 72°C.

The coproDNA PCR method follows a relatively simple DNA extraction procedure from faecal samples. The term ‘copro’ refers to samples that have derived from faecal origin. Parasite DNA is excreted alongside eggs and other matter (Mathis and Deplazes, 2006). In contrast to blood or tissue samples, because the embryo of an *Echinococcus* egg is

surrounded by the embryophore layer, which cannot be extracted as easily with lysis chemicals and enzymes, a different procedure to extract from faeces has been developed for coproDNA PCR (Bretagne *et al.*, 1993). Extraction of coproDNA from eggs in faeces was first attempted using a lysis step adapted from a sperm lysis technique (Cui *et al.*, 1989).

Currently the coproDNA PCR technique has been developed and is only available for a limited number of species or genotypes in particular *E.multilocularis* and *E.granulosus* sheep strain (Craig *et al.*, 2003; Mathis and Deplazes, 2006). Bretagne *et al.*, (2003) first developed a species-specific coproDNA PCR for *E.multilocularis* that showed 100% specificity and sensitivity up to 1 egg per 4g of fox faeces. The specificity of such tests can be as high as 100% however the sensitivity can vary depending on worm burden and maturity of worms (Mathis and Deplazes, 2006). The sensitivity was found to be improved by concentrating the eggs using a process of sequential sieving and zinc chloride flotation (Mathis *et al.*, 1996). A PCR test was developed by Cabrera *et al.* (2002) and showed high levels of specificity and sensitivity for the identification of *E.granulosus* eggs from a contaminated environment. Cabrera *et al.*, (2002) pointed out that the primer set did not cross-react with *E.multilocularis* but shared similar genetic sequences to other *Echinococcus* species such as *E.oligarthrus* and *E.vogeli* therefore they were only considered to be species-specific in samples from countries other than South or Central America. A coproDNA PCR assay developed by Stefanic *et al.*, (2004) to detect *E.granulosus* sheep strain (G1) showed 100% specificity against other *Echinococcus* spp. including *E.multilocularis* and *E.vogeli*. A tissue DNA PCR was originally developed by Dinkel *et al.*, (1998) to detect *Echinococcus multilocularis* DNA by amplifying a target region within the mitochondrial 12S rRNA gene (Dinkel *et al.*, 1998; von Nickisch-Roseneck *et al.*, 1999; Dinkel *et al.*, 2004).

Another coproDNA PCR test was developed to amplify a tandem repeat sequence of *E.granulosus* sheep strain and detect *E.granulosus* eggs in dog faecal samples; likewise with the 'Stefanic' test, this assay did not cross-react with *E.multilocularis* (Abbasi *et al.*, 2003). The test was reported to be 100% specific and 100% (34/34) sensitive (Abbasi *et al.*, 2003). It is important to point out however that the test was later found to cross react with horse, camel, cattle and goat hydatid isolates (Abbasi *et al.*, 2003). Therefore for the

purpose of the current study, the ‘Abbasi’ primers are described as amplifying *E.granulosus* ‘sensu lato’ DNA (G6, G7 & G10) rather than solely *E.granulosus* sheep strain (G1) DNA. In addition, the ‘Abbasi’ primers also amplify *E.equinus* (formerly G4) DNA and *E.ortleppi* (formerly G5). Furthermore, a comprehensive study carried out by Boufana *et al.*, (2008) evaluated the ‘Abbasi’, ‘Stefanic’ and ‘Dinkel’ primers to assess their capabilities and limitations for detecting *E.granulosus* sheep strain (G1). Boufana *et al.*, (2008) reported that the ‘Abbasi’ test was not shown to be *E.granulosus* G1 strain-specific however it was highly species-specific and therefore considered to be the optimum assay to use for confirmation of *E.granulosus* infection in dogs. The assessment also found varying degrees of sensitivity and specificity in the hands of the authors (see Table 1.2). According to Boufana *et al.*, (2008) the ‘Stefanic’ primers did reproduce the same level of specificity as cited in the original study and it was also shown that this test was the most sensitive of the three PCR assays. Table 1.2 shows which other species of cestodes cross-reacted in the hands of Boufana *et al.*, (2008).

Table 1.2 Comparison of the diagnostic sensitivity and specificity of three assessed PCR assays and cross-reacting species of cestodes according to Boufana et al., (2008), including diagnostic sensitivity and specificity of a coproantigen ELISA developed to target *E.granulosus* coproantigens (Allan et al., 1992) and targeted genes/antigen.

	‘Abbasi’ primers (repeat sequence EG1 <i>Hae</i> III gene)	‘Dinkel’ primers (12S rRNA gene)	‘Stefanic’ primers (12S rRNA gene)	‘Allan’ coproantigen ELISA (genus-specific)
Sensitivity	52.6%	73.7%	100%	~80%
Specificity of tissue DNA (metacestode/adult)	90.9%	63.6%	27.3%	N/A
Specificity of coproDNA (egg)/coproantigen	75%	100%	25%	>95%
Cross-reacting species of cestodes		<i>E.granulosus</i>	<i>E.granulosus</i>	
		<i>T.hydatigena</i>	<i>E.multilocularis</i>	
	<i>E.granulosus</i>	<i>T.hydatigena</i>	<i>E.shiquicus</i>	
	<i>E.shiquicus</i>	<i>T.ovis</i>	<i>E.vogeli</i>	
	<i>E.equinus</i>	<i>T.pisiformis</i>	<i>T.multiceps</i>	<i>Echinococcus</i>
	<i>E.ortleppi</i> (G5) <i>E.canadensis</i> (G6)	<i>T.multiceps</i> (from tissue not infected dog faeces)	<i>T.hydatigena</i> <i>T.ovis</i> <i>T.pisiformis</i> <i>D.caninum</i> <i>T.solium</i>	spp.

Currently there are no commercial coproDNA PCR kits available, therefore it is largely used for confirming coproantigen-positive samples or for differentiating between *Echinococcus* and *Taenia* eggs recovered from faecal specimens or from environment samples (Mathis and Deplazes, 2006). In the current study, the ‘Abbasi’ coproDNA PCR assay is used to amplify *Echinococcus* DNA that has been extracted from canine faecal samples.

The ‘Dinkel’ PCR assay (referred to as the ‘cestode-specific’ primers) is used to amplify hydatid cyst tissue both to confirm which species are involved in transmission cycles within the UK.

1.12 Treatment and control of canine echinococcosis

There is a wide variety of anthelmintic drugs that have been used for the treatment of canine echinococcosis. Anthelmintic drugs generally kill the tapeworm by facilitating the expulsion from the canine intestines or disrupting metabolic pathways. The original drugs contained agents that temporarily paralysed the adult tapeworms causing them to lose their attachment to the gastrointestinal tract. Re-attachment of a number of tapeworms was likely to occur even when these drugs contained purgative properties or were given with harsh laxatives (McCurnin, 1998).

Praziquantel (PZQ) is currently the drug of choice after it was commercially available for veterinary use (Droncit, Bayer) and replaced most other anti-cestode drugs for the treatment of *Echinococcus* infections because of its high efficacy, limited or no toxicity and wide margin of safety (Thakur *et al.*, 1978; Rausch *et al.*, 1990; Macpherson and Craig, 2000). A PZQ dose of 5mg/kg is administered to the dog and it works by disrupting calcium pathways in cestodes. The prepatent period of *E.granulosus* is approximately 6 weeks and therefore this is usually the recommended treatment interval with praziquantel being the most effective anthelmintic treatment to do this (Torgerson and Budke, 2003). Praziquantel is quickly absorbed from the gastrointestinal tract and its distribution throughout the body makes it extremely effective against various stages of tapeworm development (Thakur *et al.*, 1978; McCurnin, 1998). In some countries including the UK, praziquantel is the only drug licensed and recommended against *E.granulosus* infection (Lloyd *et al.*, 1998).

Control programmes in some countries have been successful and have managed to eradicate the disease altogether, whereas others have failed and continue to fail. Successful epidemiological studies and surveillance of hydatid control programmes rely on the identification of *E.granulosus* in the canine definitive host (Gemmell *et al.*, 1987). A hydatid control programme can be divided into 4 phases; preparatory/planning, attack, consolidation and maintenance of eradication (Gemmell and Schantz, 1997). The preparatory phase involves collecting base-line data, conducting field trials into dog-dosing and analysing costs and benefits to determine the duration of the attack phase. The attack phase involves control measures that are applied nondiscriminately to the entire host populations at risk (Gemmell and Schantz, 1997).

Island control programmes have been the most successful in reducing dog-human transmission and dog-livestock transmission namely New Zealand, Cyprus and Tasmania (Gemmell and Schantz, 1997). Australasian control programmes have been successful on both islands of New Zealand and Tasmania, with *E.granulosus* infection being driven from endemic towards extinction status (Gemmell, 1990). The Tasmanian CE control programme carried out by the State Department of Agriculture started in 1964 took approximately 8 years to reduce transmission between dogs and humans and a total of 33 years to reduce transmission between dogs and livestock. All phases were successfully carried out to greatly reduce the transmission of the parasite.

The Cyprus control programme started in 1971 with existing base-line data that showed *Echinococcus* was present in 40-100% of adult sheep (Economides *et al.*, 1998). The Cyprus attack phase focussed heavily on dog control; stray dogs were shot on sight, infected dogs were put down and owners were obliged to register and test their dogs every 3 months with arecoline hydrobromide; bitches were spayed and those that weren't, high fines were issued to owners (Economides *et al.*, 1998). As well as dog control, the Cyprus Department of Veterinary Services put in place a public health programme and introduced tight regulations in abattoirs (Economides *et al.*, 1998). The programme ended in 1985, when transmission was reduced so much that it was thought that eradication had been achieved however after the control programme had ended it emerged that the parasite had not been eradicated, as new cases were reported (Economides *et al.*, 1998). These resulted from illegal transportation of animals between regulated and non-regulated areas. The Department of Veterinary Services, therefore introduced the consolidation phase, whereby targeted areas were dog dosed with praziquantel and infected livestock areas were quarantined (Economides *et al.*, 1998). Livestock in quarantined areas were monitored for 3 years and only released after there were no signs of *E.granulosus* or *T.hydatigena*; in addition food animals could only be sold to official abattoirs (Economides *et al.*, 1998).

In the current study, as part of the Welsh Hydatid Study (a pilot control programme) the control measures that were applied as part of the attack phase included mass dog-dosing under supervision and an educational campaign to regulate dog-feeding practices. A baseline surveillance (pre-treatment) and a 2-year follow-up study was implemented

based on testing farm dog faecal samples by coproantigen ELISA and coproDNA PCR. Prior to this, the last dog surveillance was undertaken in 2002 in the same region of mid-Wales (Buishi *et al.*, 2005a).

1.13 The role of dogs in the epidemiology of equine echinococcosis in the UK

The dog has been confirmed experimentally as the most likely definitive host of equine echinococcosis in Great Britain (Williams and Sweatman, 1963; Thompson, 1974). It has been suggested that the role of dogs in the epidemiology of equine echinococcosis in the UK changed dramatically since the end of the Second World War (Thompson and Smyth, 1974). In Ireland cases of '*E.granulosus equinus*' were reported in Irish hounds that were fed raw horse liver and lungs (Hatch, 1970). The short communication put forward a recommendation to remove liver and lungs from carcasses before feeding meat to the hounds. In another short communication, equine hydatidosis was reported to have increased dramatically during a period when foxhound authorities fed the hounds raw flesh due to economic pressure after the end of the Second World War (Thompson and Smyth, 1974). It was suggested that hunting packs were the major definitive host of *E.equinus* from survey evidence that revealed that they were fed uninspected horse flesh and offal (Thompson and Smyth, 1975). The survey showed that over half of the hunting packs harboured worms identified as equine origin (Thompson and Smyth, 1975). A study carried out in Dyfed, Wales also found that 29% of hounds sampled from 8 foxhound packs were infected with *E.granulosus* (Williams, 1976a). The recommended procedure for sterilising offal is outlined by Fastier, (1949) and involves immersing offal in boiling water for at least 40 minutes. This process leads to scolex death in fertile cysts and the study showed that death occurred after 70 minutes at 50°C or 30 minutes at 55°C (Fastier, 1949). In the study carried out by Thompson and Smyth (1975), inspection of the boiling equipment revealed that it was often inadequate because it was inoperable, unsuitable for cooking large quantities of meat or the water wasn't the correct temperature and the meat wasn't completely submerged. In 2007 the Council of Hunting Associations Code of Practice for the Welfare of Hounds in Hunt Kennels made the several recommendations. Some hunt packs have acquired approval from their local Animal Health Officer to become 'collection centres' for fallen stock to be fed to the hounds. The Hunting Associations Code of Practice points out that some raw flesh from fallen stock may contain infectious tapeworm cysts especially sheep stock, therefore

appropriate evisceration should be carried out. A flesh house is a building used for the handling of fallen stock and it is recommended that if a kennel has one, it is hosed down thoroughly after use. The Hunting Association's Code of Practice 2007 recommends regular treatment with praziquantel at least twice a year, at the start and end of the hunting season, to eliminate tapeworms.

The situation in Ireland was not much different. In 1975 there were 90 packs of hounds including beagles, harriers, foxhounds and staghounds (Allen, 1974), currently there are 115 (Baily's Hunting Directory online). Unpublished data showed that 10 hounds that were inspected post-mortem from 5 packs were diagnosed with having *E.equinus* infections (Hatch, 1975). It was suggested that if hound husbandry was not robust, they could be largely responsible for the maintenance of *E.equinus* in Ireland (Hatch, 1975). Several recommendations were made by Hatch, (1975) to break the hound/horse cycle; removal of liver and lungs from all carcasses before being fed to the hounds; liver and lungs should be disposed of appropriately so that hounds do not have access to them; hounds that are fed liver and lungs should be treated for *Echinococcus* infection.

The role of foxes as a potential definitive host and contributor to the epidemiology of equine echinococcosis is unclear. Experimental infections of foxes have shown contradicting results; Thompson, (1974) showed that they were poor hosts and Dailey and Sweatman (1965) found that foxes did not become infected with hydatid material from Lebanese donkeys, which was considered to be the same subspecies as that occurring in British horses (Williams and Sweatman, 1963). In contrast Howkins *et al.*, (1965) showed that protoscoleces from British horses grew at the same rate in three foxes as in dogs. It has been suggested that foxes may not scavenge on dead horse carcasses in the UK (Thompson and Smyth, 1975). A study in Northern Ireland where *E.equinus* is prevalent but where *E.granulosus* is not established showed that none of the 569 red foxes examined were infected with *Echinococcus* (Ross and Fairley, 1969).

1.14 Aims and objectives

1.14.1 Aims of the current epidemiological study

Currently there is no information on the prevalence of equine hydatidosis in the UK. Thompson (2008) suggested that a decline in horse infection may follow the foxhunting ban in 2004 because this may reduce the widespread contamination of grazing land due to infected hunting dogs covering wide areas of countryside during hunts (Thompson and Smyth, 1975). However, when it became an offence to hunt wild mammals with dogs to include hare coursing as well as foxhunting (Hunting Act, 2004), to comply with the new legislation, huntsmen adapted by trailing an artificial scent along the countryside ahead of the hunt or beforehand, this practice is known as drag or trail hunting. One of the aims of the current study is to investigate whether the foxhunting ban does have any significance in the prevalence of infection.

The change in foxhound husbandry since the Second World War may have had a huge impact on the prevalence of *E.equinus* (Thompson and Smyth, 1974). The widespread dissemination of eggs in dog faeces throughout the countryside may have implications for stablehands, kennelmen, riders and the general public as the situation may put them at risk (Smyth, 1976). The zoonotic potential of *E.equinus* remains unclear and earlier attempts to infect rhesus monkeys (*Macaca mulatta*) with *E.equinus* did fail (Thompson and Smyth, 1976), however with the recent finding of a captive UK born and bred red ruffed lemur (*Varecia rubra*) with *E.equinus* (Boufana *et al.*, 2012), the major question is; which species of *Echinococcus* is affecting domestic cycles in the UK and what is its likely impact on human health? A key aim was to determine the prevalence rates of *E.granulosus* and *E.equinus* in farm dogs in mid-Wales and foxhound packs in England and Wales.

1.14.2 Objectives

The main objectives of the study were:

1. To develop primers, which are G4 genotype-specific for *E.equinus* and to standardise a genotype-specific coproDNA PCR assay.
2. To apply coproantigen ELISA and coproPCR to investigate the epidemiology of *E.granulosus* and *E.equinus* in farm dogs in mid-Wales, UK.
3. To compare prevalence rates of canine echinococcosis between different foxhound packs and to consider how different risk factors including foxhound husbandry might affect the transmission of *Echinococcus spp.* and risk of human CE.
4. To experimentally infect dogs with horse hydatid cysts collected from a horse abattoir.
5. To standardise and compare existing coproELISA tests for diagnosis of canine echinococcosis caused by *E.granulosus* and *E.equinus*.

CHAPTER TWO

STANDARDISATION OF COPROANTIGEN ELISAs FOR DETECTION OF CANINE ECHINOCOCCOSIS

2.1 Introduction

Accurate diagnosis of echinococcosis in the definitive host plays a potentially important role in the surveillance of hydatid control programmes. Surveillance of canine echinococcosis is useful for establishing baseline data at the beginning of a control programme and for monitoring progress in control. It can also provide an indicator for the potential risk to humans of being infected with cystic echinococcosis (CE) (Huang *et al.*, 2007). Indirect diagnosis of *E.granulosus* infection in dogs by detection of specific antigens in dog faecal samples has proved to be a useful and reliable alternative to parasitological examination that relies on direct detection of the tapeworm either by necropsy or purgation (Craig, 1997).

The principle basis of coproantigen diagnosis relies on the fact that the parasite releases metabolic products into the intestinal lumen. This material could be scolex and/or proglottid derived excretory-secretory products, tegumental turnover material and/or degeneration products from detached proglottids and possibly from egg-derived antigens, though the latter appear not to be the case (Allan *et al.*, 1992; Elayoubi *et al.*, 2003; Elayoubi and Craig; 2004). Detection of coproantigens in comparison to antibody detection in serum was shown to be two and a half times more sensitive (Craig *et al.*, 1995; Walters and Craig, 1992).

Faecal antigen detection in canine echinococcosis was first reported in 1962 using a gel precipitation test with rabbit anti-hydatid cyst fluid antibodies (Babos, 1962; Babos and Nemeth, 1962). Coproantigen detection assays usually rely on the use of specific polyclonal and/or monoclonal antibody as a capture layer in a sensitive solid-phase assay such as ELISA (Craig, 1997). Detection of specific coproantigens in faecal specimens of infected hosts has been used for immunodiagnosis to detect a broad range of infections such as intestinal protozoan infections (Grundy, 1982; Goldine *et al.*, 1990), bacterial infections (Jackson *et al.*, 1985), viral infections (Yolkens *et al.*, 1977; Ellens and de Leeuw, 1977), and for a number of helminthic infections such as fascioliasis (Youssef *et*

al., 1991), opisthorchiasis (Sirisinha *et al.*, 1991), strongyloidiasis (Nageswaran *et al.*, 1992), human and canine taeniasis (Maass *et al.*, 1991) as well as echinococcosis (Deplazes *et al.*, 1990; 1992; Allan *et al.*, 1992).

The first development of an *Echinococcus* coproantigen ELISA to detect specific antigens in faecal supernatants was achieved using immunoglobulin G (IgG) purified from rabbit antisera raised against *Echinococcus* proglottid somatic antigens or against excretory-secretory (E/S) antigens (Allan *et al.*, 1992; Deplazes *et al.*, 1992; Craig *et al.*, 1995). The coproantigen ELISA was used with faecal supernatants from dogs experimentally infected with *E.granulosus* (worm burdens of >10,000) and coproantigen was detected in the prepatent period at 10 days post-infection (dpi), the assay was repeated on supernatants of dogs infected with *E.multilocularis*, and coproantigens were detectable by ELISA at 5dpi (Deplazes *et al.*, 1992).

Coproantigens could be specific for the parasite either at genus or species level and therefore can be used for diagnosis based on coproantigen detection (Craig, 1997). First reports of parasite-specific detection in dogs produced cross-reaction with coproantigens in human faeces (Babos and Nemeth, 1962). In the late 1980s specificity was increased by using raised antibodies in hyperimmunised rabbits of surface antigens (Machnicka and Krawczuk, 1988; Allan and Craig, 1989). Since then rabbit polyclonal antibodies, chicken egg yolk-derived antibodies and mouse monoclonal antibodies (MAbs) have been used in ELISA-based techniques to detect antigen in detergent solubilised faecal samples. It is a technique that is based on MAbs or polyclonal antibodies raised against adult tapeworm antigens (Allan and Craig, 2006). This method has provided more than 95% genus-specificity; parasite-specific antigens can be detected in faeces weeks before patency (period of time that adults releases eggs), coproantigens are independent of egg output; coproantigen stays stable for days at temperatures ranging from -80°C to 35°C; they can also stay stable for several months when fixed in formalin and levels of coproantigen rapidly decrease following successful treatment, suggesting that they can be examined at a much later date (Allan and Craig, 2006). However, coproantigens are not detectable in faeces treated with organic solvents and are not preserved well in ethanol (J.C. Allan, PhD Thesis, 1990).

There are several advantages to coproantigen-based detection of *Echinococcus* coproantigens. One of the advantages is that the sensitivity of the test was found to be far superior to serum antibody detection (Craig *et al.*, 1995). Coproantigens have been shown to disappear after 1 week after successful treatment (Allan *et al.*, 1992; Allan and Craig, 2006). In other studies coproantigen ELISA OD values rapidly dropped and disappeared within 2-5 days post treatment (Deplazes *et al.*, 1999; Jenkins *et al.*, 2000). This suggests that the ELISA provides rapid assessment of treatment effectiveness as there is a close link of faeces antigens and active infection (Allan and Craig, 2006). Various degrees of sensitivity and specificity for the *Echinococcus* coproantigen test have been reported with sensitivity levels ranging from 50-87.5% (Allan *et al.*, 1992; Craig *et al.*, 1995; Moro *et al.*, 1999). The specificity has been reported to be consistently high, varying between 76.9% and 96.5% (Allan *et al.*, 1992; Deplazes *et al.*, 1999; Craig *et al.*, 2003). In one study, a maximum of 73% sensitivity was achieved using *E.granulosus* protoscolex or oncosphere antigens to detect serum antibody in infected dogs (Gasser *et al.*, 1988). The sensitivity of the coproantigen ELISA test is known to be broadly dependent on the worm burden and/or concentrations levels of coproantigens in faecal material (Allan *et al.*, 1992; Fraser *et al.*, 2002).

One of the disadvantages of the ELISA is the possibility of the presence of proteases within the faecal supernatants, which could act as inhibitory factors and interfere with the results (Hanvanich *et al.*, 1985; Craig *et al.*, 2003). Much research has shown that there is a link between coproantigen test sensitivity and worm burden, when more than 50 worms were present, which is a possible explanation for the wide range of sensitivity (Allan and Craig, 2006). Craig *et al.*, (1995) reported a positive correlation ($r = 0.65$) between coproantigen ELISA OD values and purged worm count when more than 20 worms were present. Buishi *et al.*, (2005b) also reported a positive correlation ($r = 0.087$) between coproantigen ELISA OD values and worm burden in necropsied dogs. Despite these findings, it has been suggested that this technique may not be sensitive enough for detecting coproantigens in faecal samples taken from animals harbouring low worm burdens of around 50 worms or less (Craig, 1997). Even so, its sensitivity level is similar to that of highly biohazardous purgation techniques. Coproantigen testing may provide a semi-quantitative assessment of the typical worm burden in a specific location by comparing data shown by purgation/necropsy (Buishi *et al.*, 2005a; Lahmar *et al.*, 2007).

When the above is taken into consideration, coproantigen ELISA is currently the most accurate diagnostic method available for ante-mortem detection of canine echinococcosis.

Huang *et al.*, (2007) used a capture antibody against *E.granulosus* excretory-secretory and freeze-thaw antigen and detection antibody of rabbit anti-*E.granulosus* excretory-secretory and freeze-thaw antigen for improved sensitivity and specificity of *Echinococcus* coproantigen detection. Up to now there has been no comparison of the coproantigen test of Huang *et al.*, (2007) versus that of Allan *et al.*, (1992); neither whether a combination of reagents might result in a better test. The current study investigates the potential of these tests for the detection of canine echinococcosis and makes recommendations for future development. In the current study, the Allan *et al.*, (1992) ELISA will be referred to as the ‘Allan’ test and the Huang *et al.*, (2007) ELISA will be referred to as the ‘Huang/Heath’ test.

It was deemed necessary to investigate a novel ELISA test because there are limited supplies and the ‘Allan’ reagents will not last indefinitely i.e. rabbit 47 (R47) capture and rabbit 47 (R47) conjugate antibodies and an optimised assay may provide a suitable replacement if and when these reagents run out.

A third coproantigen ELISA format was investigated by considering the capture antibody of the ‘Huang/Heath test’ with a conjugated detection antibody produced by Allan *et al.*, (1992). In the current study this assay is referred to as the ‘Hybrid test’ (‘Huang/Heath’/‘Allan’).

2.2 Aims of the study

The current investigation had two main aims:

6. To standardise and compare existing coproantigen ELISA assays developed by Allan *et al.*, (1992) and Huang *et al.*, (2007) to detect canine echinococcosis caused by *Echinococcus* spp.
7. To investigate the potential for a 'Hybrid' ELISA assay using reagents from various sources.
8. To determine whether any of the optimised ELISA tests could detect dogs infected with *Echinococcus equinus*.

2.3 Research approach

The current *Echinococcus* coproantigen ELISA adapted from Allan *et al.*, (1992) test involves a sandwich-ELISA protocol that has been standardised for diagnostic use in the Cestode Zoonoses lab at Salford University. The antibodies that were used were an anti-*E.granulosus* polyclonal antibody raised against somatic adult worm extracts in a rabbit (R47) and subsequently processed to produce the capture antibody (primary Ab) and a conjugated antibody (secondary Ab). The secondary Ab was conjugated to horseradish peroxidase (HRP), which catalyses the tetramethylbenzidine (TMB) substrate to produce a blue colour that can be quantified using a plate reader. The sensitivity of this test has previously been shown to be >80% and *Echinococcus* genus specificity is > 95% (Allan and Craig, 2006).

In order to standardise and compare existing coproantigen ELISA formats for diagnosis of *E.granulosus* in dogs, reagents derived from Huang *et al.* (2007) were kindly provided by Dr. David Heath and investigated to assess efficacy in Salford. Unlike the ‘Allan’ test, the protocol of the ‘Huang/Heath’ test used a double-sandwich ELISA format, which comprised 3 antibodies. The 2 primary antibodies were rabbit immunoglobulin G anti-*E.granulosus* freeze-thaw tegumental antigen antibody (RF/T) and rabbit immunoglobulin G anti-*E.granulosus* excretory-secretory antigen antibody (RE/S). The secondary antibody or as described by Huang *et al.*, (2007) as the ‘detection’ antibody, was a sheep immunoglobulin G anti-*E.granulosus* freeze-thaw and excretory-secretory antigen antibody (SE/S). The tertiary antibody that was used in the study of Huang *et al.*, (2007) was a commercial product i.e. anti-sheep IgG horseradish peroxidase (HRP) conjugate (Sigma), however this was substituted in our lab with anti-sheep immunoglobulin G alkaline phosphatase (AP) conjugate (Sigma) and a corresponding p-nitrophenylphosphate (PNPP) substrate was used to produce a quantifiable colour change.

2.3.1 Checkerboard/titration protocols.

Before the specificity of ‘Huang/Heath’ test could be carried out by testing a panel of defined faecal samples, all the reagents were investigated to determine whether they would successfully bind to their corresponding reagent. This was done by carrying out either a checkerboard ELISA or titration assay in order to define the optimum concentrations of each reagent, maximising the use available yet giving a clear definitive

signal vs. noise results. Two 'blank' wells were left untreated until the substrate stage to determine overall background noise. Firstly the commercial anti-sheep IgG alkaline phosphatase conjugate (Sigma) was tested against the p-nitrophenylphosphate (PNPP) substrate. The capture antibody (RF/T) was tested against *E.granulosus* whole worm extract (EgWWE) antigen diluted in BCB and also diluted into negative faecal supernatant derived from a known negative dog. Then the detection antibody (SE/S) was tested against EgWWE antigen in the same way. Another checkerboard assay using serial dilutions of SE/S vs. serial dilutions of the anti-sheep IgG (AP) was carried out. In all these assays binding was observed. Finally a ELISA test was carried out to determine the specificity of the reagents.

The positive-negative cut-off level was calculated by the mean optical density (OD) value plus three standard deviations of the negative controls (Craig *et al.*, 1995). A positive result was judged to be the OD value 3 SDs above a control mean. A positive coproantigen result was not taken as final, if the OD value was just above (borderline) the cut-off margin, then the assay was repeated to confirm the initial result. The cut-off point was used for the ELISA testing only.

2.3.2 Panel of defined faecal samples

A panel of parasitically defined faecal samples were used to test the specificity of the tests. The faecal samples were obtained from several different origins e.g. Australia, China and the UK. The samples infected with *E.granulosus* from Australia were selected from an experimentally infected dog sample at 31 days post infection (dpi) and a combined pool of naturally infected dogs. Faecal samples infected with *E.granulosus* and *E.multilocularis* from China were purged with arecoline salts and kindly supplied by Dr. Christine Budke. The worm burdens for these samples are shown in Table 2.1. Negative faecal samples were obtained from routinely wormed UK domestic dogs (Leigh Cat and Dogs Home, Manchester Dogs Home) and were used for negative control subjects.

Table 2.1 Panel of infected faecal samples and their source of origin.

Faecal sample & worm burden (Wb)	Origin	Lab ID
<i>Echinococcus granulosus</i>	Experimental infection - Australia	(31dpi)
<i>Echinococcus granulosus</i>	Natural infection dog pool - Australia	(Aus pool)
<i>Echinococcus granulosus</i> (800Wb)	Purged dog - China	(SS39 B7)
<i>Echinococcus granulosus</i> (58Wb)	Purged dog - China	(SS39 FY)
<i>Echinococcus multilocularis</i> (0Wb)	Purged fox - China	(53D)
<i>Echinococcus multilocularis</i> (1165Wb)	Purged fox - China	(28J)
<i>Taenia multiceps</i>	Experimental infection – Tunisia	(Tm)
<i>Taenia hydatigena</i>	Experimental infection – Tunisia	(Th)

2.4 'Allan' coproELISA test

Constituents of reagents for the 'Allan' test are shown in Appendix 7. Immulon 4HBX plates (Thermo Electron Corporation) were coated with a working dilution of capture rabbit (R47) anti Eg WWE IgG (whole worm extract) antibody at a 1:8000 (1µg/ml) dilution in bicarbonate coating buffer (BCB), 100µl per well. Wells G12 and H12 were left uncoated to act as blank controls for the plate (these wells should not have antibody, faeces or conjugate solution added to them). The plate was then covered with clingfilm and incubated at 4°C overnight.

The following day the faecal supernatants were taken out of -20°C storage and allowed to thaw. The plate was washed three times with 0.1% phosphate buffered saline-Tween 20 (PBSt20) on an automated plate washer (Tecan Columbus/Columbus Pro Washer). The plate was loaded with 100µl of blocking buffer (0.3% PBSt20) and re-sealed with clingfilm to be incubated for one hour at room temperature. The blocking buffer was discarded and the plate was patted dry onto a paper towel. To each well (except blanks) was added 50µl of heat inactivated foetal calf serum (HI FCS), followed by the addition of 50µl per well of faecal supernatant in duplicate. The HI FCS is a reagent that protects the coated antibody and prevents it from being damaged by any proteases present in the faecal sample. A selection of positive and negative controls was used during each assay to confirm assay viability. The negative controls consisted of dog faecal samples taken from wormed dogs at Leigh Cat and Dogs Home in Greater Manchester, UK. The positive samples consisted of a supernatant pool of Australian positive faecal samples from natural infections. The plate was covered in clingfilm and incubated at room temperature for one hour on an orbital shaker.

A working dilution of rabbit anti-EgWWE-IgG linked to horseradish peroxidase (HRP) enzyme conjugate at 1:2000 in 0.3% PBSt20 was prepared. The plate contents were discarded into a 5% Virkon solution and washed three times with 0.1% PBSt20 as before. To all wells (except the blanks) was added 100µl of conjugate solution. The plate was incubated for one hour at room temperature on an orbital shaker. At this point in the assay, tetra-methyl benzidine (Pharmingen, TMB substrate) substrate was removed from the fridge and allowed to warm to room temperature.

The conjugate solution was discarded and the plate was washed three times with 0.1% PBSt20 as before. The plate was patted dry onto a paper towel. To each well (including the blanks) was added 100µl of the TMB substrate solution and the plate was finally incubated in a dark cupboard for 20 minutes. The plate was read on a microplate ELISA reader using wavelength 630nm. The colour of the substrate turned from colourless to blue.

2.5 ‘Huang/Heath’ coproELISA test

Constituents of reagents for the ‘Huang/Heath’ test are shown in Appendix 6. The following information outlines the procedure described by Huang *et al.* (2007) and includes changes made in attempt to optimise the protocol in our laboratory. Using a needle and syringe, 10ml of distilled water was added to a vial of freeze-dried capture antibody (RF/T). The contents were mixed well until dissolved. The mixture was then aliquoted and stored at -80°C. The same was carried out for the freeze-dried detection antibody (SE/S) except it was reconstituted in 8ml of distilled water.

The following outlines the ‘Huang/Heath’ test ELISA procedure: An Immulon 4HBX plate (Thermo Electron Corporation) was coated with a 1:300 dilution of capture IgG RF/T in bicarbonate coating buffer (BCB). 100µl was added to each well, the plate was sealed using clingfilm and placed at 4°C overnight.

The following day the faecal supernatants were taken out of -20°C storage and allowed to thaw. The plate was washed three times with 0.1% PBSt20 (phosphate buffered saline-Tween 20) on an automated plate washer (Tecan Columbus/Columbus Pro Washer). The plate was then loaded with 350µl/well blocking solution (5% skimmed milk powder); the plate was covered with a plastic guard and left for one hour on the bench at room temperature (RT). The blocking solution was poured off and plate washed three times on the plate washer. 100µl of dog faecal extract was added to each well and plates were incubated at RT for 2h. Plates were then washed manually three times and 100µl/well of detection antibody sheep IgG (SE/S) was added at a dilution of 1:2000 in 5% milk. The plates were incubated at RT for 1h. Plates were washed three times on the plate washer, 100µl of anti-sheep IgG alkaline phosphatase conjugate (Sigma) at a dilution of 1:4000 in 5% milk was added and the plate were left for 1h at RT. The plate was washed three

times on a plate washer and 100µl of prepared diethanolamine buffer with PNPP substrate was added to each well. Plates were incubated at RT on the bench for 20 min. The plate was then read at 405nm for optical density (OD) using an automated ELISA plate reader (Ascent).

2.6 ‘Hybrid’ coproELISA test

A hybrid ELISA was designed to incorporate a mixture of different capture and conjugate antibodies from the different antibodies available. In this case the ‘Huang/Heath’ capture (RF/T) and Salford produced conjugated detection rabbit excretory-secretory (RE/S7) antibody was investigated to see whether they could be optimised to be used as an improved ELISA. Firstly a checkerboard assay was carried out to determine the optimum working concentrations of each reagent. As with a checkerboard titration, the plate was divided in half, one side was allocated for a positive pool of *E.granulosus* faecal supernatant samples and the other side was allocated for a negative pool of *E.granulosus* faecal supernatant samples. Two ‘blank’ wells are left untreated until the substrate stage to determine overall background noise. Both sides of the plate are treated identically throughout.

2.7 *Echinococcus* antigen and antibody products

The antigen extracts that were used to investigate the ELISA tests. *Echinococcus granulosus* whole worm extract (EgWWE) which is a pure antigen was used to test the efficacy of the ELISA reagents. *Echinococcus granulosus* excretory-secretory (EgES) products were cultured from *E.granulosus* worms under controlled conditions. This serum was processed by the protein A IgG purification method resulting in purified IgG which was concentrated and processed to produce the sandwich ELISA component reagents i.e. capture and conjugate. In the current study rabbit excretory-secretory 7 (RE/S7) antibody was used to develop the ‘Hybrid’ ELISA test.

2.8 Results

All of the experiments were repeated three times and the results are representative of all of the results.

2.8.1 'Huang/Heath' test optimisation

Commercial anti-sheep IgG alkaline phosphatase (Sigma) vs. p-nitrophenylphosphate substrate (PNPP). The commercial anti-sheep IgG alkaline phosphatase (Sigma) was tested against the PNPP substrate by titrating the reagents across the plate. It was evident from the observations that there was appropriate binding between the reagents. The commercial anti-sheep IgG (AP) was titrated against the PNPP substrate and produced a yellow colour development, which was confirmed by visualising by eye.

Capture rabbit IgG anti-Eg freeze-thaw tegumental antigen antibody (RF/T) vs. adult *E.granulosus* whole worm extract (EgWWE) in buffer bicarbonate coating buffer (BCB). The capture antibody (RF/T) has shown that it binds to EgWWE in BCB (see Fig. 2.1). These initial results show that the optimal working dilution for RF/T antibodies was 1:300 as stated in the original study by Huang *et al.*, (2007). This gave a maximum OD value of 4.13 when titrated against a 1:200 EgWWE sample (see Fig. 2.1). In this experiment the highest working dilution (1:75) did not produce the highest mean OD value unlike the next assay (RF/T vs. EgWWE).

Capture rabbit IgG anti-Eg freeze-thaw tegumental antigen antibody (RF/T) vs. adult *E.granulosus* whole worm extract (EgWWE) spiked in negative faecal diluent. The capture antibody (RF/T) has shown that it binds to EgWWE spiked in negative faecal diluent (see Fig. 2.2). These results show that the optimal working dilution for RF/T was 1:75 giving an OD value of 1.73. However the results suggest that there may be some unwanted background noise with the negative faecal diluent sample. This can be seen from the lack of titration curve that has been produced as the concentration of EgWWE decreases two-fold along the graph, yet the signal remains high until the 1:51200 EgWWE dilution at which point it decreases sharply for all concentrations of the capture antibody (see Fig. 2.2).

Detection sheep IgG anti-Eg freeze-thaw excretory-secretory antigen antibody (SE/S) vs. adult *E.granulosus* whole worm extract (EgWWE) spiked in buffer BCB. The detection antibody (SE/S) vs. EgWWE spiked in BCB showed binding. A working dilution for IgG SE/S of 1:250 gave a maximum OD value of 1.69 when titrated against a 1:100 EgWWE sample (figure not shown).

Capture sheep IgG anti-Eg freeze thaw excretory-secretory antigen antibody (SE/S) vs. adult *E.granulosus* whole worm extract (EgWWE) spiked in negative diluent. SE/S vs. EgWWE spiked in negative faecal diluent showed binding. According to Figure 2.3 the optimal working dilution for SE/S vs. EgWWE in negative diluent is also 1:250. This gave a maximum OD value of 2.51 when titrated against a 1:400 EgWWE sample.

The detection antibody (SE/S) has also shown that it binds to EgWWE in both neat BCB and negative faecal diluents. Again Figure 2.3 suggests that there may be some unwanted background noise in the negative faecal diluent sample. This can be seen from the lack of titration curve that has been produced as the concentration of EgWWE decreases two-fold along the graph. Background noise in a titration assay suggests that there is some unspecific binding occurring between the reagent and the negative diluent. This is not a desired outcome as this may produce inaccurate results when running a panel of samples.

Checkerboard assay of SE/S IgG vs. anti-sheep IgG alkaline phosphatase (AP). The checkerboard experiments were repeated three times and the results are representative of all of the results.

The checkerboard assay was carried out according to the modified 'Huang/Heath' test. The SE/S IgG titrations went from 1:2000 to 1:256000 and the anti-sheep IgG (AP) titrations went from 1:2000 to 1:4096000. From this experiment it was shown that there was high background noise produced in the negative samples.

An ELISA was carried out to determine the specificity of the anti-sheep IgG (AP) vs. RF/T IgG however it showed non-specific binding. The wells were coated with the suggested working dilution of 1:300 RF/T. The following day the anti-sheep IgG (AP)

was titrated across the plate starting from 1:2000 to 1:32000. The results in Table 2.2 show that there was non-specific binding between the conjugated sheep antibody and the capture rabbit antibody.

Due to the non-specific binding between the capture rabbit antibody and the conjugated sheep antibody in addition to the non-specific binding of the detection antibody (SE/S) with the negative faecal diluent, it was decided that a specificity test using the panel of defined faecal samples would not produce any conclusive data, and may produce false-positive results.

Table 2.2 Mean OD values show that there is non-specific binding between the commercial anti-sheep IgG and the 'Heath/Huang' capture rabbit IgG anti-Eg freeze-thaw tegumental antigen antibody (RF/T).

Anti-sheep IgG (AP) dilution	1:2000	1:4000	1:8000	1:16000	1:32000
Mean OD values	0.76	0.51	0.32	0.16	0.10

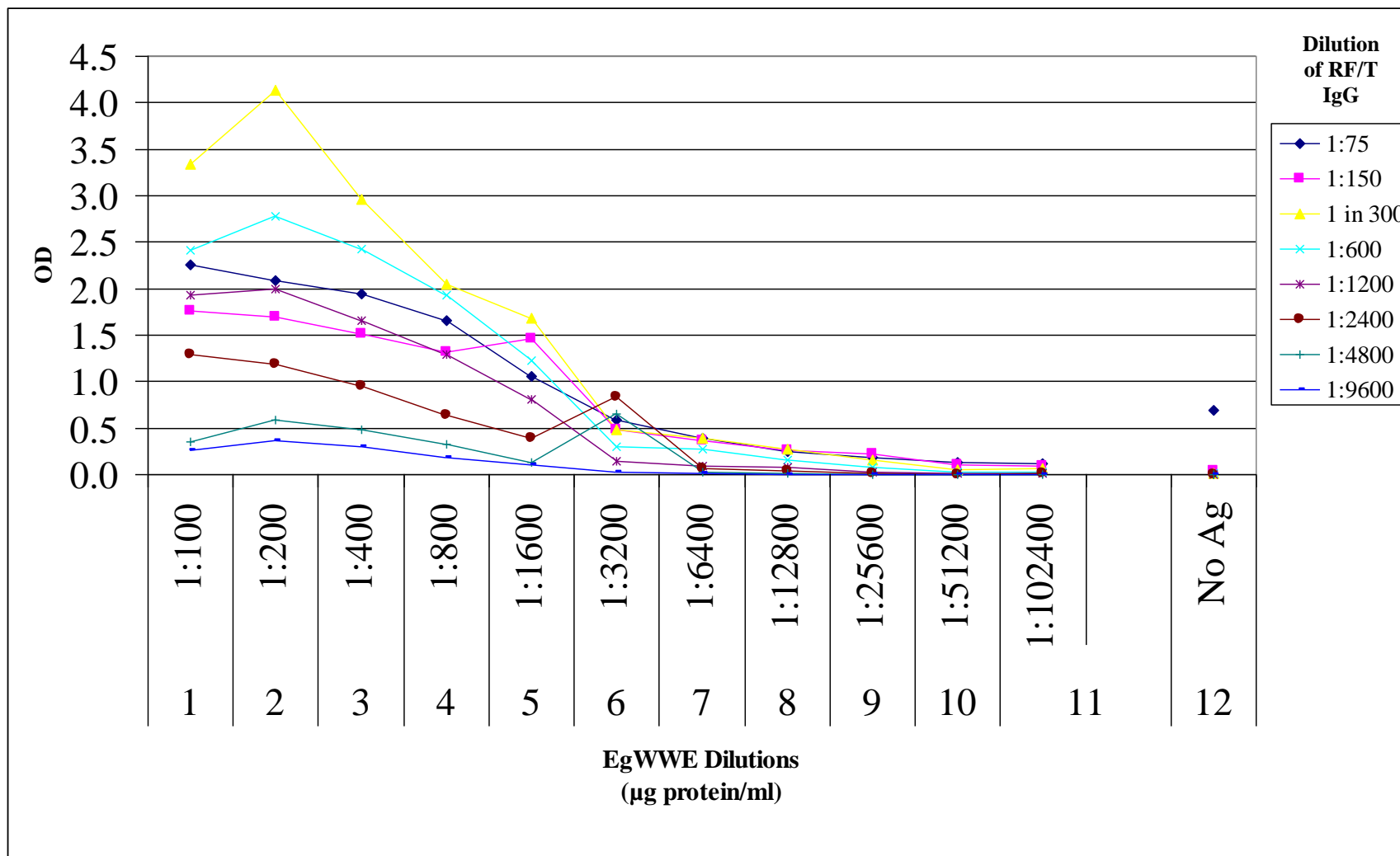


Figure 2.1 Rabbit IgG anti-Eg freeze thaw tegumental antigen (RF/T) vs. EgWWE in buffer BCB (neat).

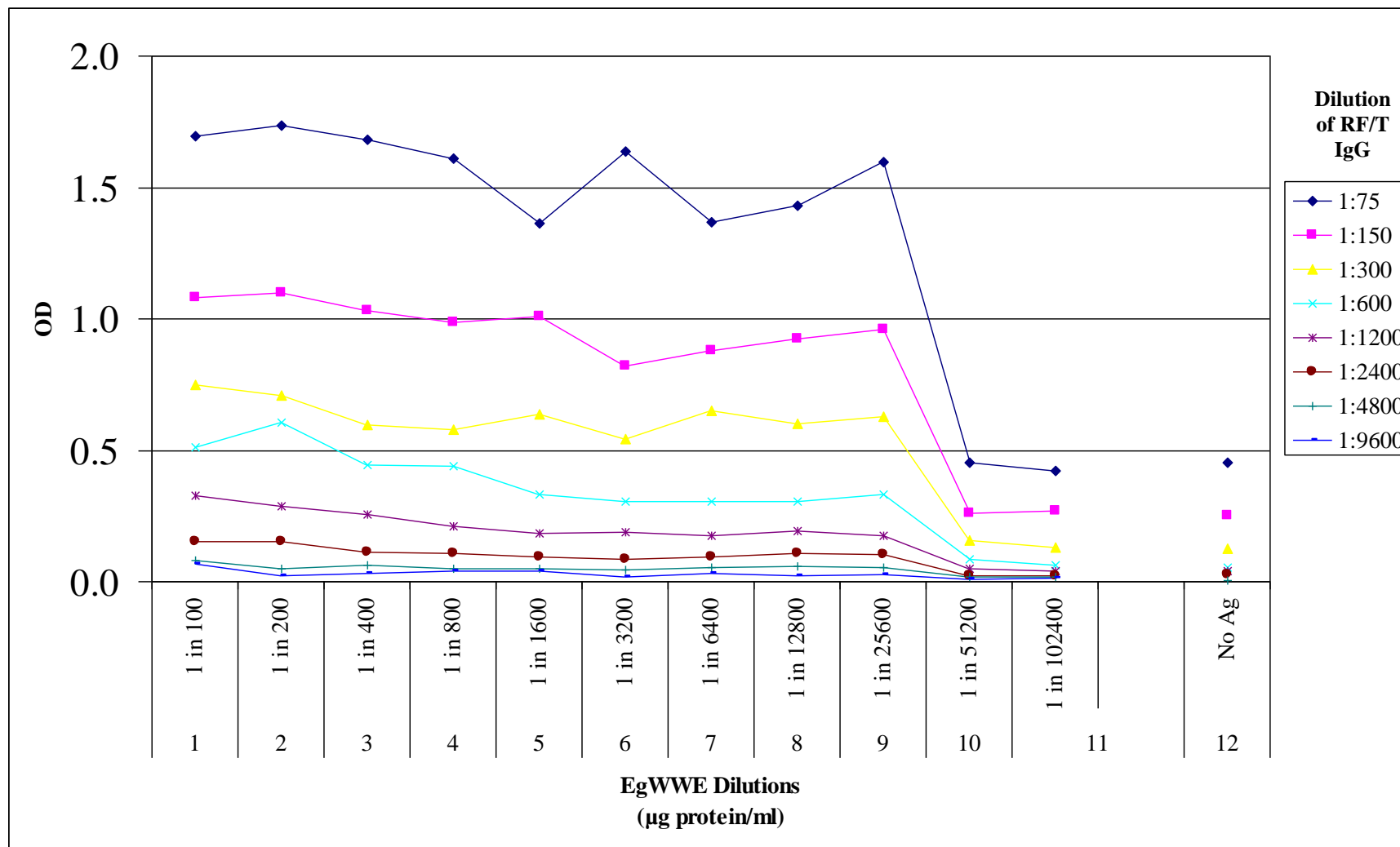


Figure 2.2 Rabbit IgG anti-Eg freeze-thaw tegumental antigen (RF/T) vs. Eg WWE spiked in negative diluent.

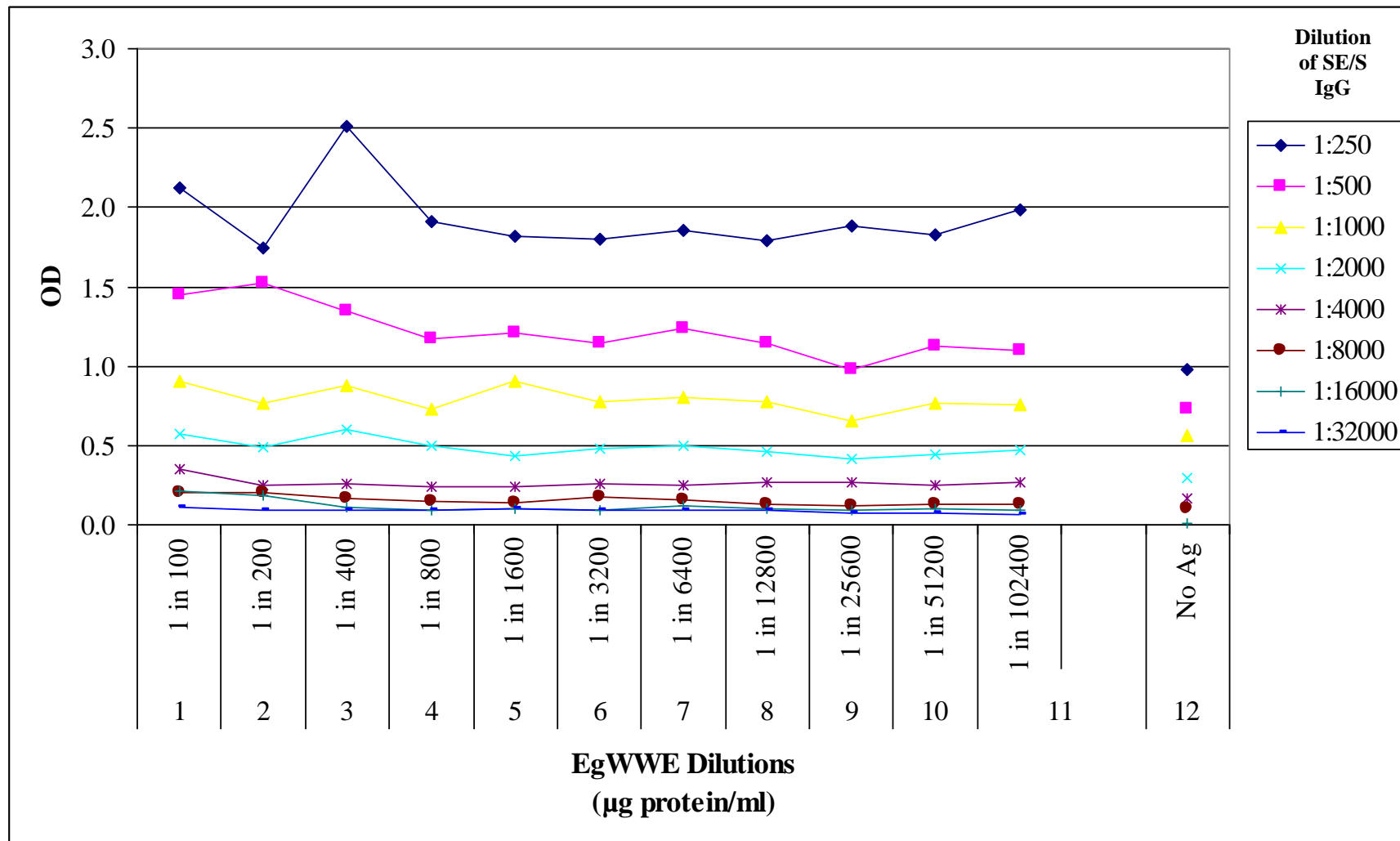


Figure 2.3 Sheep IgG anti-Eg freeze-thaw excretory-secretory antigen (SE/S) vs. EgWWE in negative faecal diluent.

2.8.2 Hybrid test optimisation ('Heath/Huang'/'Allan')

For the optimisation of the Hybrid test the capture rabbit IgG anti-Eg freeze-thaw tegumental antigen (RF/T) from the 'Huang/Heath' test was coupled with a conjugate antibody chosen from a Salford University stock developed by Allan *et al.*, (1992). The conjugate antibody was rabbit anti-Eg excretory-secretory antigen 7 (RE/S7) and was conjugated to horseradish peroxidase (HRP).

RF/T vs. RE/S7 checkerboard. The RF/T antibody was titrated two-fold down the plate starting from a 1:75 dilution down to a 1:9600 dilution. The RE/S7 conjugated antibody was titrated across the plate starting from a 1:250 dilution to a 1:8000 dilution. Figure 2.5 shows a 3-D graphical interpretation of this checkerboard titration, which gave fairly good signal: noise ratios of 7.60 at 1:300 (capture) 9.38 at 1:600 (capture) both at 1:1000 (conjugate).

A panel of parasitically defined samples was used to test the working dilution of capture antibody 1:500 against a detection conjugate antibody 1:1000. The results are shown in Table 2.3, which compares the mean OD values of the 'Hybrid' test with those of the 'Allan' test.

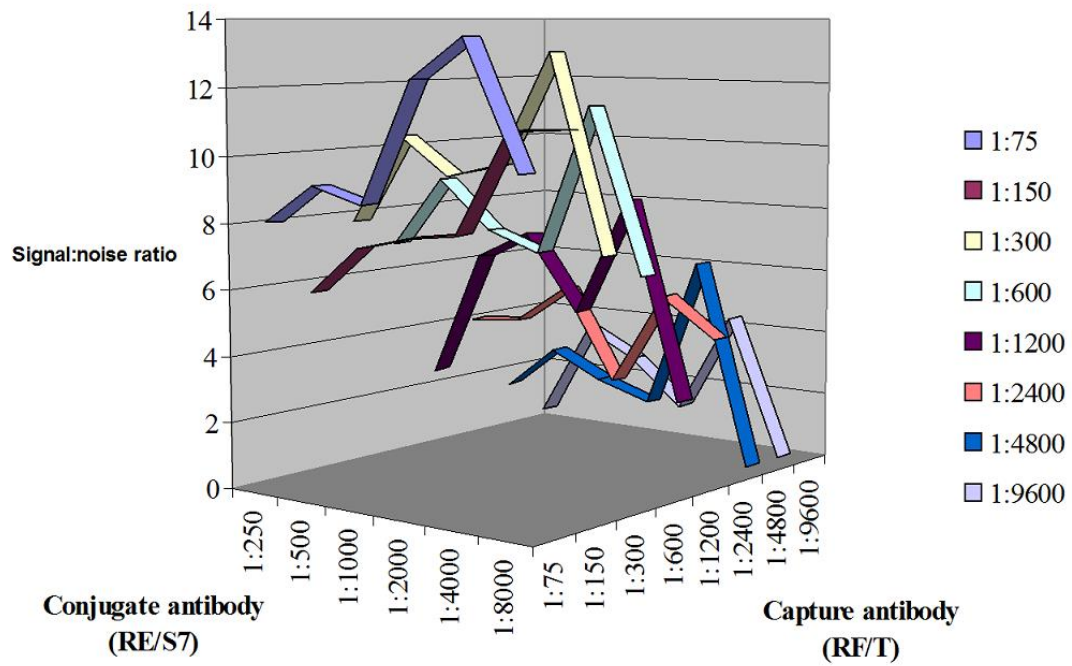


Figure 2.4 A 3-D graph that shows the checkerboard titration of capture rabbit IgG anti-Eg freeze-thaw tegumental antigen (RF/T) vs. conjugate rabbit anti-Eg excretory-secretory antigen (RE/S7) Signal:noise ratio is shown (=positive/negative).

Table 2.3 Subpanel results 'Hybrid test' compared to 'Allan test'. Red indicates positive results (≥ 0.095); green indicates negative results (< 0.095).

Faecal sample	Hybrid test mean OD values	Allan test mean OD values
<i>E.granulosus</i> experimental infection 31dpi	0.41	1.03
<i>E.granulosus</i> experimental infections Australian pool	0.36	1.11
<i>E.granulosus</i> 800Wb (SS39 B7)	0.19	0.28
<i>E.granulosus</i> 58Wb (SS39 FY)	0.22	0.26
<i>E.multilocularis</i> 0Wb (53D)	0.06	0.08
<i>E.multilocularis</i> 1165Wb (28J)	0.04	0.06
<i>Taenia multiceps</i> (Tm)	-	0.03
<i>Taenia hydatigena</i> (Th)	-	0.03

Table 2.4 Subpanel results of time-course experimental infections with *E.equinus* using the 'Allan test'. Red indicates positive results (≥ 0.095); green indicates negative results (< 0.095).

Experimental infection dog faecal samples		Allan test mean OD values
Experimental Infection 1	Dogs #16 and #27	All negative (> 0.095)
Experimental Infection 2	Dog #09 (3dpi)	0.05
	Dog #09 (7dpi)	0.31
	Dog #09 (10dpi)	0.59
	Dog #09 (14dpi)	0.03
	Dog #09 (21dpi)	0.14
	Dog #09 (28dpi)	0.55
	Dog #09 (34dpi)	0.41
	Dog #09 (50dpi)	0.15
	Dog #15 (3dpi)	0.04
	Dog #15 (7dpi)	0.23
	Dog #15 (10dpi)	0.09
	Dog #15 (14dpi)	0.15
	Dog #15 (21dpi)	0.53
	Dog #15 (28dpi)	0.25
	Dog #15 (34dpi)	0.15
	Dog #15 (50dpi)	0.35

2.8 Discussion

A coproantigen ELISA test for canine echinococcosis that was developed by Allan *et al.*, (1992) has been applied in many studies over the years (Allan and Craig, 2006). However its sensitivity is around 80% and is primarily genus specific (>95%), thus improvement is still possible especially for sensitivity. In this study a comparison was made between the ‘Allan’ test and a newer version of the ‘Huang/Heath’ test (Huang *et al.*, 2007). Furthermore a combination test (Hybrid test) using anti-somatic and excretory-secretory reagents were investigated to assess whether a better assay could be developed. An improved coproantigen ELISA was developed for human *Taenia solium* taeniasis by combining an anti-somatic capture IgG with an anti-ES detection antibody (Guezala *et al.*, 2009). The purpose of the current study was to standardise and compare existing coproantigen ELISA assays developed by Allan *et al.*, (1992) and Huang *et al.*, (2007) for diagnosis of canine echinococcosis caused by *E.granulosus*. Various panels were included in the development of the ELISA assays, including a panel of time-course experimentally infected dog faecal samples with *E.equinus*.

Due to the non-specific binding between the ‘Huang/Heath’ reagents in particular the capture RF/T with the conjugated sheep antibody and also the non-specific binding of the detection antibody (SE/S) with the negative faecal diluent it was not feasible to use the test as it stands due to the possibility of producing false positive results. The preliminary results of these experiments showed that a commercial conjugate antibody (anti-sheep IgGAP) was not suitable for use with the ‘Huang/Heath’ reagents, particularly because it bound non-specifically to the capture rabbit antibody. Future work could involve using an alternative commercial conjugate antibody that is more specific for anti-sheep reagents and that has been tested for specificity against other mammals, for example a monoclonal antibody (MAb). The ‘Huang/Heath’ test differs from many ELISA assays in that it incorporates a double-sandwich format whereby the detection antibody was not labelled with an enzyme such as horseradish peroxidase. According to the authors (Huang *et al.*, 2007), this was to improve the robustness of the test and the secondary was detected using a commercial horseradish peroxidase labelled antisera (anti-sheep IgG HRP conjugate, Sigma). On reflection, the fact that a third antibody is required to produce a quantifiable signal suggests that perhaps the non-conjugation of the detection antibody to the enzyme produces an issue with non-specific binding. The author recommends that

future production of detection antibodies should include a conjugation process so that it eliminates the need to involve a generic commercial product that has had no bearing on the original production of ELISA antibodies.

The panel results for the Hybrid test did not perform as well as the Allan test. For example, the *E.granulosus* experimental and natural infections produced lower OD values compared with those from the Allan test (see Table 2.3). This may be due to several factors; the working dilutions that were selected were not the optimal working dilutions for this combination of reagents and/or the reagents were not as sensitive as the Allan reagents. Despite these lower OD values, the Hybrid test results did produce positive results albeit much lower than those of the Allan test. In addition, the Hybrid results did not detect *Echinococcus multilocularis* antigens from both panel samples, interestingly neither did the Allan test in fact both tests produced similar negative OD values. The Hybrid test results indicate that the test is specific to *E.granulosus* because *E.multilocularis* antigens were not detected. The reagents were originally raised against *E.granulosus* antigens therefore there is a high probability that the antibodies only recognise *E.granulosus* antigens. This has the potential of being used as a valid test to be investigated further for its diagnosis potential. Future work could involve using a 1:500 dilution for the capture antibody (R/FT) and a 1:700 dilution for the conjugate antibody (RE/S7).

A further aspect of the current study was to determine whether the ELISA tests could detect dogs infected with *Echinococcus equinus*. The panel samples included naturally infected foxhound samples that were confirmed with having *E.equinus* infections using an optimised *E.equinus* G4-specific coproDNA PCR assay (the details of the coproDNA PCR are shown in Chapter Three). The infected samples were confirmed as having *E.equinus* DNA present using samples collected ante-mortem therefore it was not possible to speculate on the sensitivity aspect of the test in relation to worm burden. At the time of the Hybrid test study, the *E.equinus* foxhound samples were not available to be tested therefore it is suggested that for future work these sample should be tested using the Hybrid assay. The panel also included the dog faecal samples from time-course experimental infections. For Experimental Infection 1 all of the faecal samples for both dogs tested negative for *Echinococcus* infection using the Allan test. For Experimental

Infection 2, both dogs tested positive for Echinococcus infection using the Allan test (see Table 2.4). At the time of the Hybrid test study, these samples were not available to be included therefore it is suggested that for future work these samples should be tested using the Hybrid assay.

2.10 Summary

The results for the optimisation of the 'Huang/Heath' test showed currently it is not effective in detecting *Echinococcus granulosus* coproantigens in canine faecal samples. The issue are that the reagents cross-react with each other and the reagent that was used as the detection antibody showed non-specific binding to the confirmed negative faecal control. With these conclusions in mind the test would potentially produce false positive OD values if it were to be adopted as a canine echinococcosis screening tool.

The results for the optimisation of the Hybrid test look promising. Similar results were produced when tested against a panel of defined faecal samples compared with those of the Allan test. The Hybrid test should be developed further to improve its sensitivity.

In the current study, the Allan test was selected as the most sensitive and specific assay to screen canine faecal samples for *Echinococcus* spp. (see Chapter Four and Chapter Five).

CHAPTER THREE

DEVELOPMENT OF A NOVEL COPRODNA PCR ASSAY TO DETECT ECHINOCOCCUS EQUINUS IN DOGS

3.1 Introduction

It is a well accepted phenomenon that there is variation within the *Echinococcus* genus. In the UK *Echinococcus granulosus* (G1 genotype or sheep strain) and *Echinococcus equinus* (G4 genotype or horse strain) occur sympatrically. Human cystic echinococcosis (CE) is associated with *E.granulosus* sensu stricto and to date no human infection with CE has been associated with *Echinococcus equinus*. The zoonotic potential of *E.equinus* is unknown however a viable *E.equinus* infection in a non-human primate was recently discovered (Boufana *et al.*, 2012). Over recent years molecular genetic approaches have contributed significantly towards genetic variation and phylogeny of the *Echinococcus* spp. (Bowles and McManus, 1993). Previous studies focused on morphology, developmental biology and in vitro experiments (Smyth and Davies, 1974; Smyth, 1982; Thompson and Lymberry, 1990). Phenotypic traits may not have been significant enough to be recognized as strain differences and yet their genetic differences may have been detected with molecular techniques (Thompson and Lymberry, 1990). Such studies are crucial as genetic diversity may have an impact on infectivity, especially to humans, with important implications for the epidemiology and control of hydatid disease (Bowles *et al.*, 1992a; Bowles and McManus, 1993).

There have been a number of published molecular protocols for the detection of various *Echinococcus* spp. for example *E.granulosus* (Cabrera *et al.*, 2002; Stefanic *et al.*, 2004; Abbasi *et al.*, 2003) and *Echinococcus multilocularis* (van der Giessen *et al.*, 1999; Dinkel *et al.*, 2004). The assays showed varying levels of sensitivity and specificity and a recent comprehensive assessment carried out by Boufana *et al.*, (2008) confirmed the validity of three PCR assays that were developed for the identification of *E.granulosus* (G1 genotype sheep strain). The study carried out by Boufana *et al.*, (2008) investigated 3 published and established PCR assays; Abbasi *et al.*, (2003); Stefanic *et al.*, (2004) and Dinkel *et al.*, (2004). Boufana *et al.*, (2008) found that the PCR assays cross-reacted with other species and/or subspecies other than those published in the original studies. During

the assessment of the specificity of the PCR assays they were all found to cross-react with *E.equinus* (Boufana *et al.*, 2008). In another study that utilised the ‘Stefanic’ primers to detect canine echinococcosis in Kyrgyzstan it was found that the primers picked up ‘*E.granulosus* G4 genotype’, thus compounding the evidence that these primers cross-react with *E.equinus*. For this reason the need for a uniplex coproDNA PCR to detect *E.equinus* was identified. Due to the fact that *E.granulosus* and *E.equinus* have been found to be co-endemic in parts of the UK, further studies on transmission ecology and epidemiology and the development of species-specific diagnostic assays for the detection of these *Echinococcus* species within both the intermediate and definitive hosts are needed. In response to this a novel coproDNA PCR has been developed to detect *E.equinus* and in the current study it shall be referred to as the ‘*E.equinus* G4-specific coproDNA PCR’. An *E.equinus* G4-specific coproDNA PCR would be beneficial to screen UK dogs for canine echinococcosis associated with *E.equinus* especially farm dogs and foxhound packs.

In addition to the development of a new test, experimental infections of Tunisian dogs were carried out to investigate the development of *E.equinus* originating from the UK. The pre-patent period for *E.equinus* has been reported as being longer (70 days post infection) than that of *E.granulosus*, which is approximately 42 days post infection (Cook, 1989). In the current study time-course experimental infections were carried out under controlled conditions to investigate the development of *E.equinus* infections in dogs. Faecal samples are collected intermittently during the time-course of the experiment and various immunological and molecular techniques are applied to monitor coproantigen levels and detect the presence of coproDNA. Time-course infections of *Echinococcus* spp. have been used previously to investigate parasite development, worm burden, determination of pre-patent periods and treatment efficacy (Nonaka *et al.*, 1996; Malgor *et al.*, 1997; Jenkins *et al.*, 2000).

3.2 Aims of the study

The current study has 4 main aims:

- 1 To use molecular genotyping to find out which *Echinococcus* spp. are involved in Welsh farm dogs and foxhound packs in the UK by developing a novel *E.equinus* G4-specific coproDNA PCR assay.
- 2 To analyse horse hydatid cyst material collected from a UK abattoir using an optimised *E.equinus* G4-specific coproDNA PCR assay.
- 3 To use molecular genotyping to analyse archived horse hydatid wax-embedded cyst isolates from The Royal Veterinary College, Hatfield, UK using ‘cestode-specific’ primers to amplify a targeted region within the 12S rRNA gene.
- 4 To infect dogs under controlled experimental conditions using fertile horse hydatid cysts to investigate time-course infections of *Echinococcus equinus*.

3.3.1 Development of the *E. equinus* G4-specific coproDNA PCR

Echinococcus granulosus (G1)
mt genome
13588 bp

Preliminary identity of a genetic sequence or region was assigned by comparison with corresponding cestode sequences obtained from the availability in the GenBank database (<http://www.ncbi.nlm.nih.gov/nucleotide>). For each gene that was investigated the following cestodes were identified and transferred into a ClustalW program, which compared nucleotides by multiple sequence alignment; *E.granulosus*, *E.mutilocularis*, *E.equinus*, *E.shiquicus*, *E.canadensis*, *E.ortleppi*, *E.vogeli*, *E.oligarthus*, *T.taeniaeformis*, *T.hydatigena*, *T.crassiceps*, *T.multiceps*, *T.polyacantha*, *T.serialis*, *T.pisiformis* and *T.ovis*. In order to identify suitable primers to amplify *E.equinus* G4-specific DNA, each gene was compared amongst the Platyhelminthes and a set of primers located within the NADH (nicotinamide adenine dinucleotide) dehydrogenase subunit 2 (ND2) gene was

selected. Only *E.granulosus*, *E.mutilocularis*, *E.equinus*, *E.shiquicus*, *E.canadensis*, *E.ortleppi*, *E.vogeli*, *E.oligarthus*, *T.hydatigena*, *T.crassiceps*, and *T.multiceps* were found to have the ND2 gene sequence availability on the GenBank database.

Parasite tissue DNA extraction. Tissue DNA extraction was carried out using the DNeasy Blood and Tissue Kit (Qiagen House, West Sussex, UK). The DNA extracted from adults of *E.equinus* (Bristol abattoir, Bristol, UK), protoscoleces of *E.granulosus* (G1 sheep genotype, Tunisia) was verified by sequencing and used as controls in this study. The following information outlines the procedure involved according to the manufacturer's instructions and including any changes made to optimise the process.

- 1 1x open tube and 1x 1.5ml microcentrifuge tube for each sample were autoclaved so that the procedure could be carried out under aseptic conditions. The ethanol from each sample was removed and approximately 1cm³ of protoscoleces (PSC) material was obtained or 1cm² of germinal layer (GL) was cut out. To each sample 180µl of Buffer ATL and 20µl Proteinase K stock solution was added. The mixture was immediately vortexed vigorously and further vortexed occasionally during incubation. The sample was incubated at 55°C overnight.
- 2 The samples were removed from the water bath and were vortexed for 15s. To each sample 200µl Buffer AL was added and the mixture was vortexed thoroughly. To each sample 200µl of 100% ethanol was added and the mixture was vortexed thoroughly.
- 3 The mixture was pipetted into a DNeasy Mini spin column placed in a 2ml collection tube. The samples were centrifuged at 8000rpm for 1 min. The flow-through and collection tubes were discarded and the spin columns were placed into new collection tubes.
- 4 To each sample 500µl of Buffer AW1 was added and centrifuged at 8000rpm for 1min. The flow-through and collection tubes were discarded and placed into new collection tubes.

- 5 To each sample 500µl of Buffer AW2 was added and centrifuged at 14000rpm for 3min. The flow-through and collection tubes were discarded and placed into new autoclaved collection tubes.
- 6 To each sample 200µl of Buffer AE was added and the samples were allowed to incubate at room temperature for at least 1min. The samples were centrifuged at 8000rpm for 1min.
- 7 The samples were transferred to autoclaved 1.5ml microcentrifuge tubes and stored at 4°C until required.

Faecal DNA extraction. CoproDNA was extracted from farm dog and foxhound faecal samples using the QIAamp DNA Mini Stool Kit (Qiagen House, West Sussex, UK) implementing the procedure recommended to process 2g of faeces with adjustment of lysis buffer volume. The suspension was heated in a water bath for ~25-30 minutes at >90°C and then processed according to the manufacturer's instructions. The following information outlines the procedure involved with this protocol including any changes made to optimise the process.

1. 1x 2ml microcentrifuge tube, 3x 1.5ml microcentrifuge tubes and 1x open tube for each sample was autoclaved so that the procedure could be carried out under aseptic conditions. The samples were taken out of -20°C storage and allowed to thaw. They were then stirred as much as possible (to distribute any eggs within the sample) and 2g of each sample were weighed out and 10 volumes of Buffer ASL were added. The mixture was vortexed vigorously for 1 minute or until the sample was thoroughly homogenised.
2. The suspension was then heated at >90°C for 10 minutes. According to the protocol handbook, this step increases total DNA yield 3 to 5-fold and helps to lyse bacteria and parasites. The samples were vortexed for 15 seconds and centrifuged at full speed for 3 minutes to pellet faecal particles and 1.2ml of the supernatant was pipette into a new autoclaved 2ml microcentrifuge tube, the pellet was discarded. An InhibitEX tablet was added to each sample and vortexed immediately and

continuously for 1 minute or until the tablet was completely suspended in order to remove any inhibitors.

3. The suspension was incubated for 1 minute at room temperature to allow inhibitors to absorb into the InhibitEX matrix. The sample was then centrifuged at full speed for 3 minutes to pellet any inhibitors bound to InhibitEX, all the supernatant was pipette into a new autoclaved 1.5ml microcentrifuge tube and centrifuged at full speed for 3 minutes.
4. 15µl of Proteinase K was pipetted into a new autoclaved 1.5ml microcentrifuge tube, to which 200µl of the sample supernatant was pipette into. 200µl of Buffer AL was then added and vortexed for 15 seconds. Direct contact between Proteinase K and lysis buffer (Buffer AL) was not allowed.
5. The samples were then incubated at 70°C for 10 minutes. 200µl of 100% ethanol was added to the lysate and mixed by vortexing. The samples were centrifuged briefly to remove drops from inside the tube lid. The lid of a QIAmp spin column was labelled and the complete lysate was carefully placed into the spin column without moistening the rim. The cap was then closed and centrifuged at full speed for 1 minute. The spin column was placed in a new collection tube and the tube containing the filtrate was discarded. The spin column was carefully opened and 500µl of Buffer AW1 was added and the sample was centrifuged at full speed for 1 minute.
6. The spin column was then placed in a new collection tube, the collection tube containing the filtrate was discarded and the spin column was carefully opened 500µl of Buffer AW2 was added. Buffers AW1 and AW2 were used to wash the DNA that was collected on the spin column membrane. The sample was centrifuged at full speed for 3 minutes, the collection tube containing the filtrate was discarded and the spin column was placed into a new collection tube and centrifuged for 1 minute.

7. The collection tube containing the filtrate was discarded and the spin column was transferred into an autoclaved open tube and 200µl of Buffer AE was pipette directly onto the QIAmp membrane.
8. The sample was incubated for 1 minute at room temperature and then centrifuged at full speed for 1 minute to elute the DNA. Once the DNA had been eluted, it was transferred to a new, labelled, autoclaved 1.5ml microcentrifuge tube and stored in a refrigerator at 4°C until required.

Evaluation of PCR detection sensitivity. Detection sensitivity for the *E.equinus* PCR assay was determined by using twofold serial dilutions (2,500-0.6pg) of tissue DNA extracted from *E.equinus*. In addition, the detection limit of the *E.equinus* G4-specific coproDNA PCR was evaluated using a negative faecal sample (1 gram) spiked with 0.1, 1, 10 and 100ng/µl *E.equinus* tissue DNA. *E.equinus* DNA extracted from faeces collected 3-50 days post infection (dpi) from experimentally infected Tunisian dogs was also used to determine copro-sensitivity and to investigate prepatent DNA detection.

3.3.2 Collection of horse hydatid material

A panel of hydatid cyst material of horse origin was used to test the optimised *E.equinus* PCR assay. With the help and support of senior meat inspectors at a local abattoir in Cheshire, UK, infected horse livers were collected and analysed at Salford University. In total, 69 horse hydatid cysts were obtained from a total of 14 infected horse livers collected over a period of 12 months between 2010 and 2011. Horse passport documentation was collected for most cases. Wherever possible, the horse hydatid material was collected on the same day as the slaughter and processed accordingly, however if this was not possible, the livers were frozen on site for later collection. In most cases the horse passports were obtained to collate horse data such as age, sex and in particular last known place of residence.



Figure 3.2 Hydatid cysts in horse liver collected from Red Lion Abattoir Nantwich, Cheshire.

Fresh samples were immediately dissected, whereas frozen samples were allowed to thaw and then dissected for hydatid material. Removal of parasitic material from the liver was carried out as described by Smyth and Davies (1974).

- 1 Hydatid cyst fluid (HCF), protoscoleces (PSC) and cyst walls containing germinal layer (GL) were obtained aseptically from viable cysts. 70% ethanol was poured 2-3 times over the exposed surface of cyst and allowed to dry.
- 2 Fluid was aspirated from cysts using a sterile 20cm³ hyperdermic syringe and a 1.2 x 40mm needle and transferred into sterile 50cm³ screw cap tubes.
- 3 A small flap was cut into the cyst with a sterile blade and with a sterile Pasteur pipette some of the cyst fluid was taken up and PSCs and brood capsules were knocked off from the wall of the cyst, this was repeated several times.
- 4 All PSCs and brood capsules were transferred into a new sterile 50cm³ screw cap tube. Live PSCs were allowed to settle by gravity and washed 3 times in sterile 1% PBS, any dead PSCs and PBS buffer were discarded.

Thawed PSC material was centrifuged at 8000rpm for 5 minutes to sediment the protoscoleces, washed three times in sterile 1% PBS and preserved in 70% ethanol. HCF was transferred to sterile 50cm³ screw cap tubes and frozen at -20°C. GL was preserved in 70% ethanol and stored at room temperature until required. The DNA from the PSC and GL material was extracted using the DNeasy Blood and Tissue Kit (Qiagen House, West Sussex, UK).

3.3.3 Observation of live protoscoleces

Where fresh horse hydatid cysts were obtained it was possible to observe the protoscoleces for viability. Approximately 600µl of PSC sediment was transferred into a sterile 25cm³ culture flask with a vented cap with 10ml Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% glucose and penicillin/streptomycin Amphotericin B (100U/ml/100µg/ml- Sigma). The PSCs were incubated at 37°C with 5% CO₂. After 14 hours the culture medium was transferred to a sterile 50cm³ screw cap

tube and stored at -80 °C until required. The culture medium was replaced with new supplemented DMEM. The PSCs were stained with either 0.2% aqueous eosin or 0.5% typan blue and viewed with a Nikon TE 2000 microscope (Eclipse fluorescent) after the first 14 hours of incubation to check for parasite viability. The procedure was repeated every 12-14hours until the PSCs started to perish.



Figure 3.3 E.equinus protoscoleces (PSC) liberated from a hydatid cyst and immediately viewed (100x magnification).

3.3.4 Necropsy

Dogs were euthanised between 32-50dpi as a precaution against environmental egg contamination. The dogs were euthanised intravenously with sodium thiopental. Immediately after euthanasia, the intestines were dissected and placed in a tray. A final faecal sample was obtained and stored for future analysis before the intestines were opened up using dissecting scissors and forceps. The exposed intestines were placed in a tray of saline and washed systematically, transferring from one tray of fresh saline to another (see Figure 3.4). The contents of the first tray were put through a sieve to remove faecal material. The wash-through was saved in large plastic containers and later examined with a magnifying glass in glass trays placed over black plastic sheeting. The intestines were laid out on metal trays and scraped for any parasitic material and inspected for any inflammation of the intestinal wall, which may indicate the presence of the parasite (see Figure 3.5).

Any suspected parasitic material was examined under a light microscope. Any parasites that were retrieved were stored in 70% ethanol in plastic screw cap microtubes and stored for later analysis. The intestines and cadavers were incinerated after inspection.



Figure 3.4 Dissected dog intestines washed repeatedly in fresh saline solution.



Figure 3.5 Dissected dog intestines laid out on metal trays for visual inspection for adult worms.

3.3.5 Archived horse hydatid cyst isolates

Archived horse hydatid cyst material from The Royal Veterinary College, Hatfield, UK (kindly supplied by Elizabeth Browne) was also included in the study. These samples (n = 20) were wax embedded for preservation and extracted at the vet college using a standard DNA extraction kit. The samples were also supplied with some horse information i.e. age, sex, breed, location of hydatid cyst, place of last residence and time of ownership. The DNA was sent to the University of Salford and stored at 4°C until required. The samples were ethanol precipitated and analysis using cestode-specific primers and genetically sequenced for species confirmation.

3.4 Tissue and coproPCR

Cestode-specific PCR. Established and previously assessed primers were used to amplify tissue DNA of horse hydatid cyst material and archived wax-embedded samples used (von Nickisch-Rosenegk *et al.*, 1999; Dinkel *et al.*, 1998; Dinkel *et al.*, 2004; Boufana *et al.*, 2008). The primers known as ‘cestode-specific’ primers in the current study (P60F 5-TTAAGATATATGTGGTACAGGATTAGATACCC-3 and P375R 5-GGTACACACCGCCCGTCACCCTCGGTT-3) amplifying a targeted region within the mitochondrial 12S rRNA gene were used implementing reagent modifications described by Boufana *et al.* (2008). The diagnostic fragment generated by these primers is 373bp. The constituents of the Mastermix for the ‘cestode-specific’ test, is given in Appendix 8. A total of 5µl of DNA was added to a 100µl reaction mixture containing 20mM Tris-HCl (pH 8.5), 50mM KCl, 0.2 mM MgCl₂, 0.2 mM dNTPs, 40pmol of each of the amplification primers and 2 units of *Taq* DNA polymerase (HotstartTaq, Promega, UK). The Mastermix fluid was covered with a layer of mineral oil to prevent evaporation. Thermal cycling was performed in a Strategene® Robocycler 96 (La Jolla, CA) for 40 cycles and represents denaturation for 30s at 94°C, annealing for 1min at 55°C and elongation for 30s at 72°C.

***Echinococcus equinus* G4-specific coproPCR.** For the amplification of *E.equinus* DNA, 0.25 µM of each of the amplification primers (F, 5'-GGT TTT GAG ATA CAT AAT AAT GTC CGG AC-3' and R, 3'-CTC ACA CCA AGC ACC TAC ACA TAA ATA TAG TT-5') was used to target a 299bp diagnostic fragment within the NADH (nicotinamide adenine dinucleotide) dehydrogenase subunit 2 (ND2) gene. A total of 5µl of DNA was added to a 50µl reaction mixture containing a 2 x reaction buffer of 10 mM

Tris-HCl (pH 8.3), 50mM KCl, 1.5mM MgCl₂, 0.2 mM dNTPs and 2.5 units of *Taq* DNA polymerase (HotstartTaq, Promega, UK). The mastermix was overlaid with mineral oil and the cycling profile was as follows: 5 min at 94 °C for 1 cycle, and then 35 cycles each consisting of 30s at 94°C, 30s at 58°C, and 30s at 72°C. The diagnostic product was 299bp in size.

The PCR procedures were carried out in fully equipped molecular laboratories using dedicated equipment to prevent amplification of extraneous DNA. Negative controls (PCR grade water) were included in all experiments to monitor contamination. A Stratagene (La Jolla, CA) Robocycler was used for all cycling profiles. The PCR products were resolved on a 1.5% (w/v) agarose gel (Bioline) in 1 + Tris-Borate-EDTA buffer (Severn Biotech, Kidderminster, UK) at 110V, stained with gel red DNA dye (Cambridge BioSciences, Cambridge, UK), and visualized using Syngene G:Box gel documentation system (Cambridge Biosciences). Validation of the PCR test was made against defined panels of parasite tissue-derived DNA and using DNA extracted from infected canid faecal samples as described.

3.4.1 DNA sequencing analysis

Sequencing was carried out to further confirm the genetic makeup of diagnostic fragments. For the ‘Abbasi’ PCR products, genetic sequencing could not be adequately analysed because at the time of amplification there were no Accession sequences deposited into the GenBank database specific for *E.equinus*, only *E.granulosus* sensu lato, which would not determine categorically the specific species. Since the cestode-specific primers are specific for all cestode species, it was important to distinguish which species DNA was amplified during the PCR assay. For the optimised *E.equinus* G4-specific diagnostic fragments, it was important to determine whether the primers did amplify the targeted region, especially as it is a novel test which has not been published to date.

Bands of amplified products were cut out under UV light and gel purified using PurLink quick gel purification Kit (Invitrogen, Paisley, UK). Purified PCR products were commercially sequenced (Beckman Coulter, Essex, UK). Nucleotide sequences were analysed using FinchTV software package (Geospiza, Seattle, WA, USA) and compared

with those deposited on GenBank database through the use of BLAST (Basic Local Alignment Search Tool, <http://www.ncbi.nlm.nih.gov/BLAST/>).

3.5 Time-course experimental infections of dogs with *E.equinus* (Tunisia)

Live horse hydatid cysts were subjected to time-course infections by being fed to experimental dogs in a controlled environment. The infected horse livers were immediately dissected on arrival at Salford University; the cyst material was packaged appropriately and then sent via courier to Professor Samia Lahmar at the Ecole Nationale de Médecine Vétérinaire in Sidi Thabet, Tunisia. On arrival to the vet school in Tunisia, the hydatid cysts were dissected and parasite viability and fertility were estimated before feeding to the dogs. The protoscoleces (PSC) and germinal layer of each hydatid cyst was preserved in 100% ethanol and sent to the University of Salford for analysis with a cestode-specific PCR assay.

In total there were four attempts to infect dogs with *E.equinus*; these were set up and named Experimental Infections 1-4 as outlined in Table 3.1. The experimental dogs were of a mixed breed and were purchased from local kennels in Tunisia. The dogs were pre-treated with anthelmintics including praziquantel and maintained under a controlled diet. Faecal samples were collected every 3 days and frozen until required. At the end of the time-course infection, all faecal samples were sent to the University of Salford for analysis with a coproantigen ELISA test.

Table 3.1 Experimental Infections 1-4, sample used to infect dogs, number of infected dogs, number of days post infection.

Experimental Infection Study Number	Infective sample	No dogs infected	Time course (days post infection)
Experimental Infection 1	Pooled PSCs	2	32dpi
Experimental Infection 2	Cysts 1-3, whole cysts	3	50dpi
Experimental Infection 3	Cysts 1-6, whole cysts	2	35dpi
Experimental Infection 4	Cysts 1-8, whole cysts	4	34dpi

In Experimental Infection 1, the liver of an infected horse was dissected and 5 fertile cysts were obtained. The protoscoleces (PSC) from 5 fertile cysts were processed for DNA extraction using the procedure as outlined in Chapter Two; the remaining PSCs

were pooled together and transferred to a 50 cm³ screw cap tube. The pooled PSCs were sent via international courier to the Ecole Nationale de Médecine Vétérinaire in Sidi Thabet, Tunisia and were kept chilled on route using packed ice packs and ice blocks. On arrival, the PSCs were examined under a light microscope and their viability was estimated before being fed to 2 experimental dogs. Experimental Infection 1 was conducted over 32 days post infection (dpi), faecal samples were collected from each dog approximately every 3 days.

In Experimental Infection 2, the liver of an infected horse was dissected and 5 fertile cysts were obtained. Cyst 1 was dissected and some PSCs were observed for viability and kept alive for observation, the culture details are outlined in Chapter Two. The remaining PSCs and germinal layer were dissected and stored appropriately to be processed for DNA extraction. The remaining cysts 2-5 were left in tact, packaged with ice packs and ice blocks and sent via international courier to Tunisia. On arrival, the cysts were dissected and the PSCs were examined and their viability was estimated. Some of the PSCs from each cyst were couriered to Salford University before the rest cysts 2, 3 & 4 were fed to 3 experimental dogs. Experimental Infection 2 was conducted over 50dpi; faecal samples were collected from each experimental dog approximately every 3-4 days.

In Experimental Infection 3, the liver of an infected horse was dissected and 7 fertile cysts were obtained. Cysts 1-6 were left in tact and couriered to Tunisia as before. The same procedures were carried as in Experimental Infection 2 except that the time-course infection was terminated at 35dpi and only 2 experimental dogs were infected. Only cysts 2 and 5 were used to infect the experimental dogs. Faecal samples were collected from each experimental dog at 27dpi, 30dpi, 32dpi and 5dpi. Cyst 7 was dissected and some PSCs were observed for viability and kept alive for observation, the culture details are outlined in Chapter Two.

In Experimental Infection 4, the liver of an infected horse was dissected and 9 fertile cysts were obtained. Cyst 9 was dissected and some PSCs were observed for viability and kept alive for observation, the culture details are outlined in Chapter Two. Cysts 1-8 were left in tact and couriered to Tunisia as before. The same procedures were carried as

in Experimental Infection 2 and 3 except that the time-course infection was terminated at 34dpi and 4 experimental dogs were infected. Only cysts 3, 6, 7 and 8 were used to infect the experimental dogs. At 14dpi the experimental dog that was infected with cyst 8 was found dead. This dog was autopsied on the same day; faecal samples were obtained for 1dpi, 3 dpi, 5dpi, 10dpi and 14dpi. Faecal samples were collected from each of the remaining experimental dog at 1dpi, 3 dpi, 5dpi, 10dpi, 12dpi, 17dpi, 20dpi, 24dpi, 28dpi, 31dpi and 34dpi.

All horse hydatid cyst material that was used for the experimental infections was extracted using a tissue DNA extraction kit and was subsequently tested using the 'cestode-specific' primers (Dinkel *et al.*, 1998) as described in detail in Chapter Two. All of the experimental dogs were terminated on the last day of the time-course infection using an intravenous injection of sodium thiopental (1g). Immediately after the dose was administered, a stethoscope was used to confirm that the animal was dead. The intestines were immediately dissected and inspected (see Chapter Two). At the end of every time-course infection, all dog faecal samples were sent via courier to Salford University.

On arrival, the faecal samples were frozen at - 80°C for a minimum of 3 days to kill off any infective worms. The samples were then processed and tested for the presence of coproantigens using the 'Allan' ELISA (see Chapter Two for procedure and Chapter Three for coproantigen ELISA results). The dog faecal samples were also processed using the Qiagen QIAmp® DNA Stool Mini Kit (Qiagen House, West Sussex, UK) as outlined in Chapter Two. The extracted coproDNA was then tested for the presence of *E.granulosus* sensu lato using established and previously assessed 'Abbasi' primers (Boufana *et al.*, 2008). The coproDNA was also tested for the presence of *E.equinus* coproDNA using the optimised *E.equinus* G4-specific primers.

3.6 Results

3.6.1 Optimisation of *E.equinus* G4-specific coproDNA PCR

DNA extraction from parasite tissue. A panel of cestode tissue DNA was put together to test the specificity of the optimised primers. DNA from parasite tissue was extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. The 'cestode-specific' primers were used to detect the presence of DNA i.e. to ascertain whether the DNA extraction process was successful. These primers were designed to amplify a target region within the mitochondrial 12S rRNA gene (Dinkel *et al.*, 1998). The 'cestode-specific' primers (Dinkel *et al.*, 1998) amplified the target 373bp DNA sequence for all of the cestode species in the panel indicating that all cestode DNA was successfully extracted (Figure 3.2).

Optimum annealing temperature for *E.equinus* G4-specific primers. A temperature gradient PCR was carried out to determine the optimal temperature for producing the most yield of the desired product. The PCR was conducted using a temperature range of 53-70°C. The primers amplified target DNA across the entire temperature range (Figures 3.4 and 3.5). The brightest band was at 60°C (Figure 3.3) therefore this temperature was selected to be used as a starting point in the PCR design to produce the best product yield.

Evaluation of *E.equinus* G4-specific PCR specificity. A panel was used to evaluate the specificity of the G4 primers. PCR specificity of the *E.equinus* G4 genotype was checked using tissue derived DNA from the following cestodes of canids (stage and place of origin): (*Dipylidium caninum* (adult, Wales, U.K.), *Taenia crassiceps* (cysts, experimental mice, Belfast, U.K.), *Taenia hydatigena* (adult, Wales), *Taenia multiceps* (adult, Wales), *Taenia ovis* (adult, Wales), *Taenia pisiformis* (adult, Wales). Strain specificity was tested using DNA extracted from protoscoleces or the germinal layer from hydatid cysts of various *E. granulosus* genotypes, namely, the sheep - G1 (PSCs, Libya), buffalo – G3 (Italy) and the pig - G7 (germinal layer, Slovak Republic). In addition to these panel samples the following cestodes were included; *E.equinus* (positive control), *E.multilocularis*, *E.granulosus* (G5), *E.granulosus* (G6), *E.granulosus* (G8), *E.granulosus* (G10) and *E.granulosus* (G7). Amplification of the target 299bp product using the *E.equinus* G4-specific primers showed specificity against the cestodes in the panel. The test showed 100% specificity against the whole cestode panel (see Fig. 3.9).

***E.equinus* G4-specific PCR detection sensitivity.** A panel of twofold serial dilutions of *E.equinus* tissue DNA was used to test the assay. The assay was shown to have a detection sensitivity of up to 4.88pg, which is equivalent to approximately less than one *Echinococcus* egg (see Figure 3.10). A panel of negative faecal samples spiked with *E.equinus* DNA; 0.1, 1, 10 and 100ng/μl representing 12.5, 125, 1,250 and 12,500 eggs respectively was used to test the detection sensitivity of coproDNA samples based on the finding that a single taeniid egg contains 8pg of DNA (Rishi and McManus, 1987). The samples tested negative initially, however after the samples had been ethanol precipitated detection was shown at 0.1ng/μl. The samples were diluted down with PCR water twofold and tested positive at 1ng/μl (see Figure 3.11).

All PCR products were run on a 1.5% agarose gel, containing a molecular weight marker (HyperLadder I or HyperLadder II, Bioline, London, England) was included on each gel for confirmation of amplicon sizes. Positive controls to monitor PCR success and negative controls to check for false-positive results that may have arisen from carry-over contamination were also included. From this temperature gradient 60°C showed the optimal temperature for gaining the best yield of PCR product.

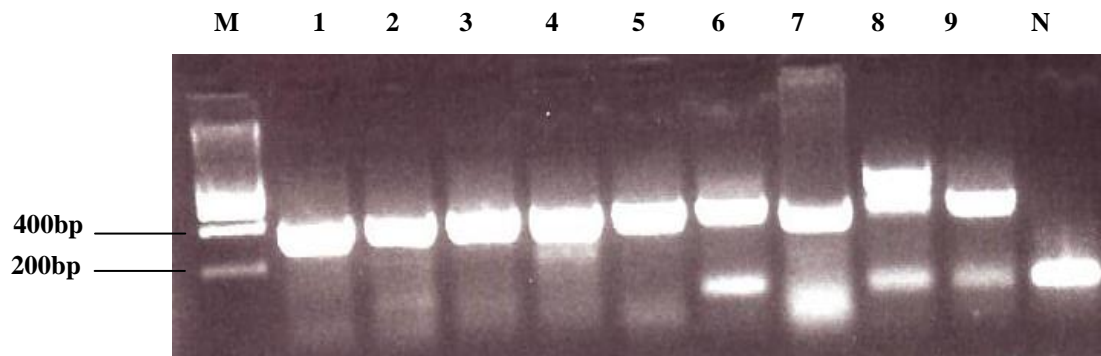


Figure 3.6 PCR amplification of 373bp product using 'cestode-specific' primers against panel of specific cestodes (Dinkel et al., 1998). Lane M, position of the size marker bands; lane 1 *E. granulosus* (G1), lane 2 *T. ovis*, lane 3 *T. hydatigena*, lane 4 *T. pisiformis*, lane 5 *D. caninum*, lane 6 *T. crassiceps*, lane 7 *T. multiceps*, lane 8 *E. granulosus* (G3), lane 9 *E. granulosus* G7; lane N shows negative control.

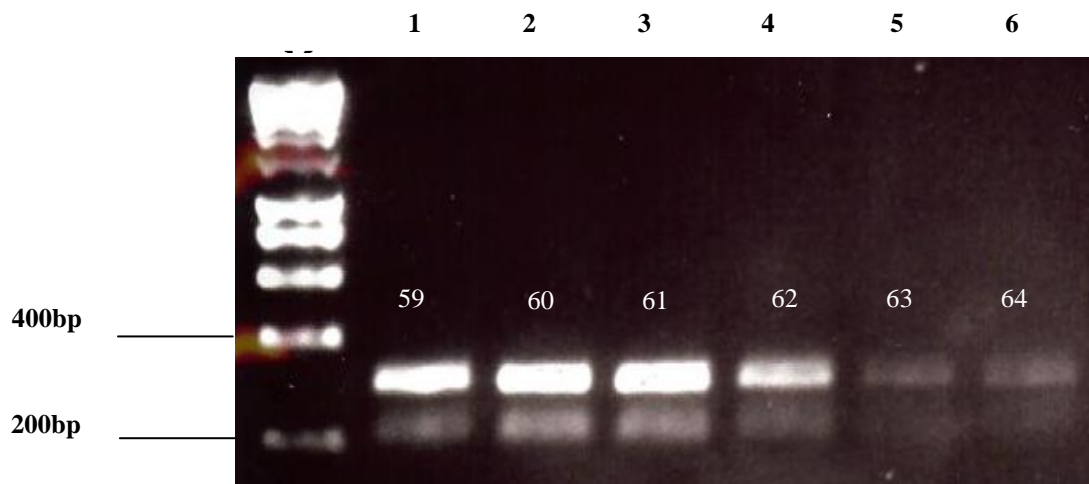


Figure 3.7 PCR amplification of G4 primers at temperature gradient. Lane M, positions of the size marker bands; lanes 1-6 show amplified 299bp product at 59-64 °C.

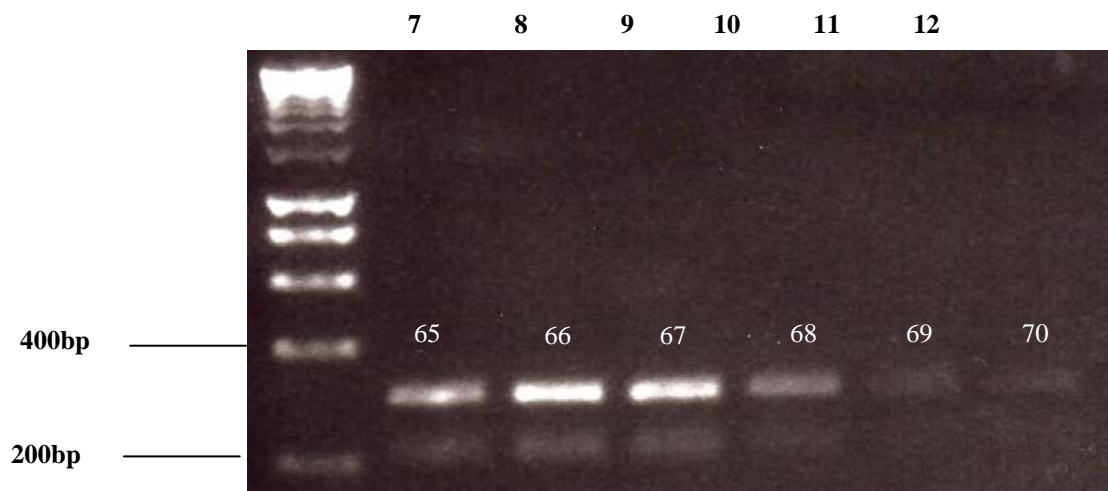


Figure 3.8 PCR amplification of *E. equinus* G4-specific primers at temperature gradient. Lane M, positions of the size marker bands; lanes 1-6 show amplified 299bp product at 65-70 °C.

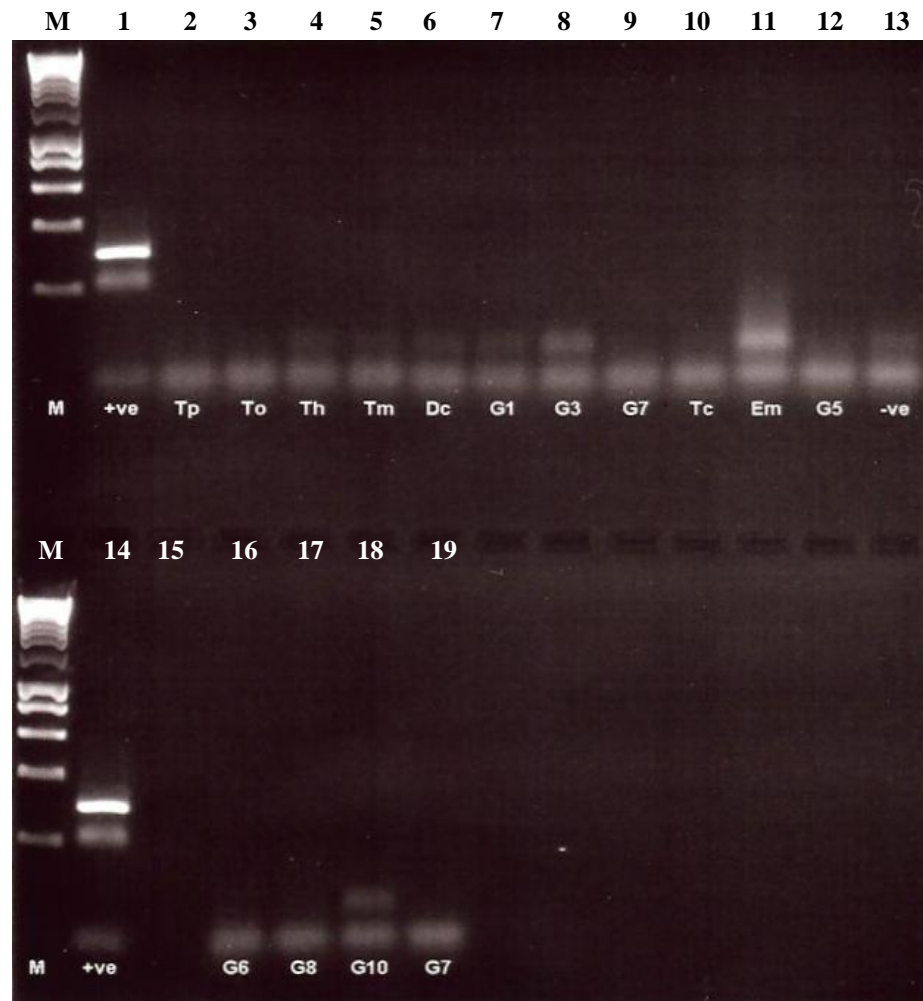


Figure 3.9 PCR amplification of 299bp product using optimised *E.equinus* G4-specific primers against panel of cestodes. Lane M, position of the size marker bands; lane 1 *E.equinus* positive control, lane 2 *Taenia pisiformis*, lane 3 *Taenia ovis*, lane 4 *Taenia hydatigena*, lane 5 *Taenia multiceps*, lane 6 *Dipylidium caninum*, lane 7 *E.granulosus* (G1), lane 8 *E. granulosus* (G3), lane 9 *E.granulosus* (G7), lane 10 *Taenia crassiceps*, lane 11 *E.multilocularis*, lane 12 *E.granulosus* (G5), lane 13 shows negative control, lane 16 *E.granulosus* (G6), lane 17 *E.granulosus* (G8), lane 18 *E.granulosus* (G10), lane 19 *E.granulosus* (G7).

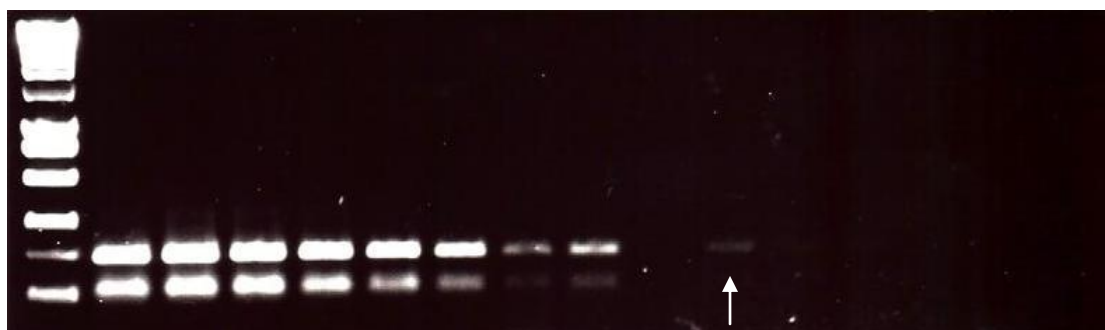


Figure 3.10 PCR amplification of twofold serial dilutions of *E.equinus* tissue DNA at 50,000-6.1pg; lowest detectable concentration was shown to be 4.88pg (equivalent to less than one *Echinococcus* egg).

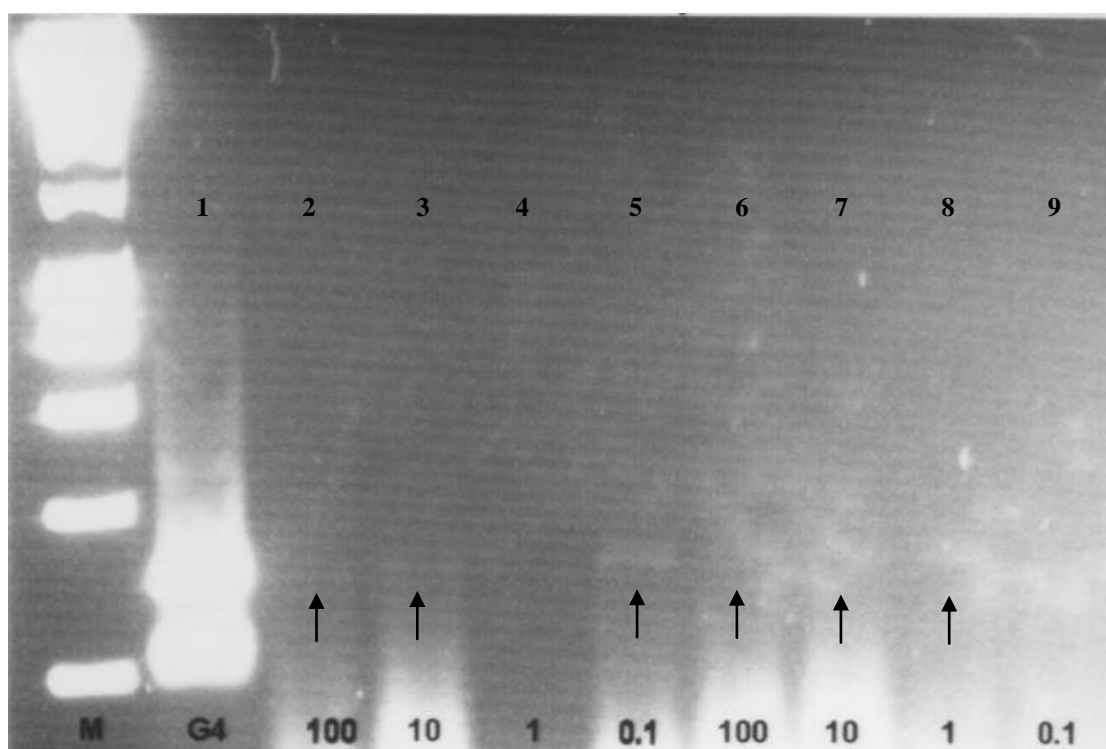


Figure 3.11 CoproDNA PCR detection of spiked negative faecal sample with 0.1, 1, 10 and 100ng/μl of *E.equinus* tissue DNA. Lane M, position of the size marker bands; lane 1 *E.equinus* (G4) positive control, lanes 2-5 100-0.1ng/μl ethanol precipitated samples, lanes 6-9 double dilution of ethanol precipitated samples, negative control included but not shown.

3.6.2 PCR results for horse hydatid isolates

A panel of 41 horse hydatid cyst isolates obtained from 10 infected horse livers were tested using the optimised *E.equinus* G4-specific PCR assay. Out of the 41 horse hydatid cyst samples, 22 amplified a 299bp diagnostic fragment within the target gene. From these samples, 14 diagnostic PCR products were sequenced and all were confirmed as *E.equinus* (GenBank accession no. AB786665). According to the information available on the horse passports that were provided, the place of last residence for some of the horses were; Gwent, Ceredigion and Swansea (Wales) and West Sussex, Shropshire, Yorkshire, Hampshire and Worcestershire (England) (see Table 3.2).

A panel of archived horse hydatid wax-embedded samples from The Royal Veterinary School, Hatfield, UK were tested using the ‘cestode-specific’ primers (Dinkel *et al.*, 2004). Out of the 20 samples, 14 samples tested positive for cestode DNA and the diagnostic PCR products were sequenced. All sequences were confirmed as *E.equinus* (GenBank accession no. AB786665). Horse information such as age, sex and last place of residence was recorded (Table 3.3). Figure 3.11 shows a map of the UK with the locations for the last place of residence for the horses found to be infected with hydatid disease.

Table 3.2 Horse information and PCR results with *E.equinus* G4-specific primers ('U' indicates information unavailable, 'X' indicates did not amplify; '✓' indicates PCR amplification but sample not sequenced; 'Ee' indicates PCR amplification and confirmed as *E.equinus* with genetic sequencing).

Sample lab ID number	Horse number	Age	Sex	Breed	Last place of residence	PCR results
#01	H1	8	F	Standardbred	Gwent	X
#02						X
#03						Ee
#04						Ee
#05						Ee
#06						Ee
#07	H2	8	M	Irish Sport Horse	West Sussex	Ee
#08						Ee
#09						Ee
#10						Ee
#11						Ee
#17						X
#18	H3	8	F	Karinga Bay x Sahara Reem (Ire)	Shropshire	Ee
#19						X
#20						Ee
#21						✓
#22	H4	14	F	Appaloosa	Yorkshire	X
#23						Ee
#24						X
#25						Ee
#26	H5	U	U	U	Swansea	X
#27						Ee
#28						✓
#29						Ee
#30	H6	3	M	New Forest Pony	Hampshire	X
#31						Ee
#33						X
#34						X
#35	H7	6	M	Welsh Pony	Ceredigion	X
#36						Ee
#37						X
#38						X
#39	H8	7	F	Cob x	U	X
#40						X
#41						X
#42						X
#43	H9	U	U	U	U	X
#44						X
#45						Ee
#46						Ee
#47	H10	21	F	Piebald Shetland Pony	Worcestershire	✓

Table 3.3 Horse information and PCR results from 'cestode-specific' primers ('U' indicates information unavailable, 'X' indicates did not amplify; 'Ee' indicates PCR amplification and confirmed as *E. equinus* with genetic sequencing).

Lab ID number	Age	Sex	Breed	Last place of residence	PCR results
LB1	30	F	Chestnut	Kings Langley, Hertfordshire	X
LB2	31	F	Chestnut	U	Ee
LB3	U	U	New forest pony	St. Albans, Hertfordshire	X
LB4	29	M	U	Horse Trust, Buckinghamshire	X
LB5	16	F	TB x Welsh	Lower Shelton, Bedfordshire	Ee
LB6	15	F	IS	Hoddesdon, Hertfordshire	X
LB7	12	M	Warmblood	Chipperfield, Hertfordshire	Ee
LB8	U	M	IDH	U	Ee
LB9	26	M	Welsh x	Walkern, Hertfordshire	Ee
LB10	13	F	Selle Francais x Irish Draught	Alsbridge, Hertfordshire	Ee
LB11	20	M	Irish Draught	Flamstead, Hertfordshire	Ee
LB12	20	M	New forest pony	Hendon-on-the-Hill, Essex	X
LB13	28	F	Grey	Compton, Hampshire	Ee
LB14	U	F	Native pony	U	Ee
LB15	U	U	U	U	Ee
LB16	14	M	Welsh D x	Amphill, Bedfordshire	Ee
LB17	U	F	cob	U	Ee
LB18	U	U	U	U	Ee
LB19	10	M	Cob x	Stockenchurch, Buckinghamshire	Ee
LB20	7	M	Clydesdale	U	X

3.6.3 Time-course experimental infections

The fresh horse hydatid cysts (n = 14) that were used to infect the experimental dogs were examined to find out the viability of the protoscoleces (PSC) (see Table 4.1). The horse hydatid cyst samples tested positive for the presence of *Echinococcus* DNA using the previously described ‘cestode-specific’ primers (Dinkel *et al.*, 1998). The PCR products were subsequently sequenced and all except one (Experimental Infection 4 – cyst 6) were confirmed as *E.equinus* (GenBank accession no. AB786665).

Dogs #16 and #27 from Experimental Infection 1 did not harbour any *E.equinus* worms. The dog faecal samples (n =16) tested negative for the presence of coproantigens. The dog faecal samples were tested for the presence of *E.granulosus* sensu lato and *E.equinus* coproDNA using the ‘Abbasi’ and primers and the optimised *E.equinus* G4-specific primers respectively. The dog #27 (10dpi and 27dpi) tested positive for the presence of *E.granulosus* sensu lato using the ‘Abbasi’ primers. All of the dog faecal samples tested negative for the presence of *E.equinus* coproDNA using the *E.equinus* G4-specific primers.

It was observed during Experimental Infection 2 that dog #10 had vomited shortly after infection therefore it was decided that it would be euthanised and omitted from the study. It was estimated that dog #09 had ingested approximately 19,460 viable PSCs and autopsy of the animal at 50dpi showed a large worm burden (approximately 11,000 worms). The worms were very well developed with 3 proglottids and 7mm in length; the gravid proglottid was full of eggs. In contrast, dog #15 which was fed approximately 12,733 viable PSCs, only 25 immature worms were observed at 50dpi. The dog faecal samples for dog #09 (n = 8) tested positive for the presence of *Echinococcus* coproantigens at 7dpi, 10dpi, 21dpi, 28dpi, 34dpi and 50dpi. The dog faecal samples for dog #15 (n = 8) tested positive for the presence of *Echinococcus* coproantigens at 7dpi, 14dpi, 21dpi, 28dpi, 34dpi and 50dpi. The dog faecal samples were tested for the presence of *E.equinus* coproDNA using the optimised *E.equinus* G4-specific primers. Dog #09 (3dpi, 7dpi, 21dpi, 33dpi and 50dpi) tested positive for the presence of *E.equinus* coproDNA. All of the faecal samples for dog #15 tested negative for *E.equinus* coproDNA.

The experimental dogs #01 and #02 for Experimental Infection 3 were infected with fertile horse hydatid cysts. However due to a delay in the courier service, the viability of the cysts were diminished; 6.35% (13,500 viable PSCs) in cyst 2 and 16.45% (38,250 viable PSCs) in cyst 5. Dogs #01 did not harbour any *E.equinus* worms but did harbour 12 *D.caninum* worms and 57 *Uncinaria stenocephala* worms. Dog #02 was found to harbour one *E.equinus* worm that was 4mm in length with developed eggs in the gravid proglottid with a mixed infection of 4 *D.caninum* worms and 6 *Uncinaria stenocephala* worms.

It was observed during Experimental Infection 4 that dog #03 had died at 14dpi. Faecal samples had been collected at 1dpi, 3dpi, 5dpi, 10dpi and 14dpi. The viability of PSCs was estimated at 93.4% with an estimation of 562,250 ingested PSCs. The autopsy of dog #03 showed a large worm burden on 35,000 immature worms and a mixed infection with 54 *Uncinaria stenocephala* worms. Dog #04 had ingested approximately 170,500 PSCs (viability 93.8%) and autopsy showed a worm burden of approximately 16,800 immature worms. Dog #05 had ingested approximately 30,000 PSCs (viability 96.7%) and autopsy showed a worm burden of approximately 1,300 immature worms and a mixed infection with one *Mesocestoides* spp. worm and one *D.caninum* worm. Dog #06 had ingested approximately 52,500 PSCs (viability 90.4%) and autopsy showed a worm burden of approximately 3,000 immature worms and a mixed infection with 6 *D.caninum* worms.

Table 3.4 *E.equinus* time-course experimental infections, horse data, estimated viability of protozoocysts (PSC), coproantigen ELISA results and coproDNA PCR results using optimised *E.equinus* primers, red indicates positive coproantigen ELISA results (≥ 0.095); green indicates negative coproantigen ELISA results (< 0.095).

95

Experimental Infection study	Horse information (including last known address)	Infective sample and estimated viability	Necropsy/faecal samples & coproantigen ELISA OD value/ <i>E.equinus</i> -specific coproDNA PCR results (ELISA negative (green) = >0.095 ELISA positive (red) = <0.095)			
Experimental Infection 1 (32dpi)	2-year-old stallion shire (Hyde, Greater Manchester)	Pooled PSCs from 5 fertile cysts (30%)	Dog #16 (0wb) 10pi (0.05/PCR -ve) 13dpi (0.02/PCR -ve) 16dpi (0.02/PCR -ve) 20dpi (0.03/PCR -ve) 23dpi (0.02/PCR -ve) 27dpi (0.03/PCR -ve) 30dpi (0.02/PCR -ve) 32dpi (0.02/PCR -ve)	Dog #27 (0wb) 10pi (0.08/PCR -ve) 13dpi (0.02/PCR -ve) 16dpi (0.01/PCR -ve) 20dpi (0.01/PCR -ve) 23dpi (0.02/PCR -ve) 27dpi (0.02/PCR -ve) 30dpi (0.03/PCR -ve) 32dpi (0.02/PCR -ve)		
Experimental Infection 2 (50dpi)	3-year-old mare (Ireland)	Whole cysts: Cyst 2 (93%) Cyst 3 (96%) Cyst 4 (76%)	Dog #09 (11,000wb) 3dpi (0.05/PCR +ve) 7dpi (0.31/PCR +ve) 10dpi (0.59/PCR -ve) 14dpi (0.03/PCR -ve) 21dpi (0.14/PCR +ve) 28dpi (0.55/PCR -ve) 34dpi (0.41/PCR +ve) 50dpi (0.15/PCR +ve)	Dog #15 (25wb) 3dpi (0.04/PCR -ve) 7dpi (0.23/PCR -ve) 10dpi (0.09/PCR -ve) 14dpi (0.15/PCR -ve) 21dpi (0.53/PCR -ve) 28dpi (0.25/PCR -ve) 34dpi (0.15/PCR -ve) 50dpi (0.35/PCR -ve)		
Experimental Infection 3 (35dpi)	No data available	Whole cysts: Cyst 2 (63%) Cyst 5 (16%)	Dog #01 (0wb) 27dpi (PCR -ve) 30dpi (PCR -ve) 32dpi (PCR -ve) 30dpi (PCR -ve)	Dog #02 (1wb) 27dpi (PCR -ve) 30dpi (PCR -ve) 32dpi (PCR -ve) 30dpi (PCR -ve)		
Experimental Infection 4 (34dpi)	No data available	Whole cysts: Cyst 3 (91%) Cyst 6 (94%) Cyst 7 (97%) Cyst 8 (94%)	Dog #03 (died at 14dpi) (35,000wb) 1dpi (PCR -ve) 3dpi (PCR -ve) 5dpi (PCR -ve) 10dpi (PCR +ve)	Dog #04 (16,800wb) 1dpi (PCR -ve) 3dpi (PCR -ve) 5dpi (PCR -ve) 10dpi (PCR -ve) 12dpi 17dpi 20dpi 24dpi 28dpi 31dpi 34dpi	Dog #05 (1,300wb) 1dpi (PCR -ve) 3dpi (PCR -ve) 5dpi (PCR -ve) 10dpi (PCR +ve) 12dpi 17dpi 20dpi 24dpi 28dpi 31dpi 34dpi	Dog #06 (3,000wb) 1dpi (PCR -ve) 3dpi (PCR -ve) 5dpi (PCR -ve) 10dpi (PCR -ve) 12dpi 17dpi 20dpi 24dpi 28dpi 31dpi 34dpi

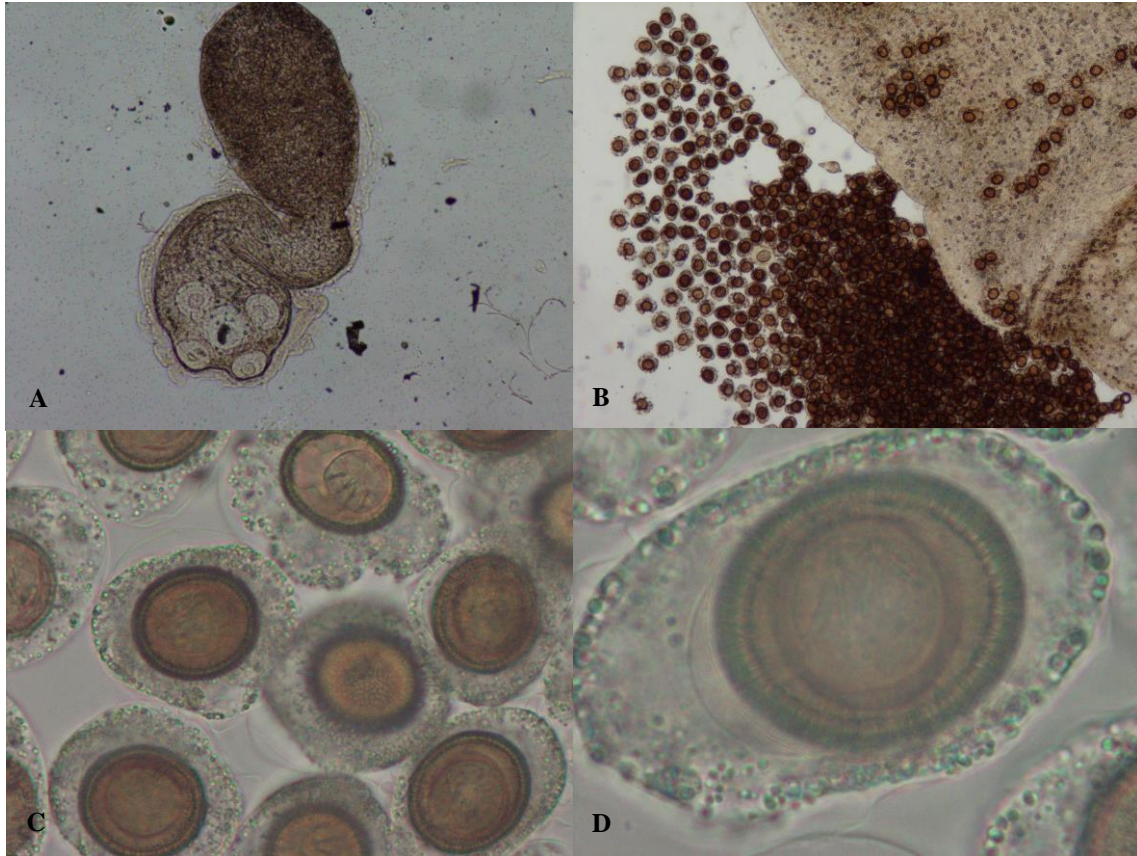


Figure 3.12 Experimental Infection 2, photo A mature *E. equinus* worm with gravid posterior proglottid (20x), photo B immature eggs (40x), photo C immature eggs (100x), photo D egg (400x), images courtesy of Professor Samia Lahmar.

3.7 Discussion

It has been reported that in the UK *Echinococcus granulosus* is limited in distribution, being primarily restricted to mid and south Wales (Torgerson and Budke, 2003). *Echinococcus equinus* (*E.granulosus* G4/horse strain) is present in many areas where *E.granulosus* is found (Torgerson and Budke, 2003). Before the Second World War, equine echinococcosis was rare in Great Britain (Southwell, 1927). After the Second World War, from the 1950s onwards, many more cases of echinococcosis in horses were reported in the literature (Thompson, 1975). It seems that due to the expense of fuel and labour costs after the war, hunt kennelmen fed their hunting packs raw horse and sheep offal, which resulted in an accelerated increase of equine echinococcosis (Smyth, 1976). The distribution of equine echinococcosis does not appear to be localised, data shows that the infection may be widespread because the origins of the slaughtered horses are spread widely over Great Britain (Thompson, 1975).

Human cystic echinococcosis (CE) is caused by the accidental ingestion of *E.granulosus* (G1 genotype sheep strain) eggs and the transmission cycle occurs between the definitive canine host and agricultural animals such as sheep and cattle. Human CE is rare in the UK and official figures from the last century show an annual average of just 0.3 human cases per million, although this rate was double (0.6 p.m.) in Wales (Thompson and Smyth, 1975). Human CE levels are 10 times higher in Wales than they are in England (0.2 cases per million in England and 2 cases per million in Wales) with the highest rates of 5.6 cases per million occurring in south Powys, Wales (Stallbaumer *et al.*, 1986). According to the Health Protection Agency (HPA) figures for human CE there was an average of approximately 8 recorded cases per year from 2000 in England and Wales (HPA, 2013).

The zoonotic transmission potential of *E.equinus* is unknown however it has been suggested, based on epidemiological grounds as having low or no infectivity to humans (Thompson and Smyth, 1975). *E.equinus* does not appear to be zoonotic it is almost always reported to date from equines however, Boufana *et al.*, (2012) recently described an *E.equinus* infection in a primate intermediate host - a captive born and bred red ruffed lemur (*Varecia rubra*) in the UK. This suggests that a non-human primate is able to maintain a viable *E.equinus* infection. Although the infectivity (if any) for *E.equinus* to

humans is unknown the availability of a specific copro-detection test for this species and its differentiation from *E.granulosus* occurring in the UK would be useful for epidemiological studies. Epidemiology in the UK would hinge on having a test that differentiates between the two species. A novel coproDNA PCR assay has been developed to distinguish between G1 genotype sheep strain and G4 genotype horse strain that are known to be co-endemic in the UK.

The *E.equinus* G4-specific coproDNA PCR assay was found to be 100% specific against 15 cestode species and strains these included; *Dipylidium caninum*, *Taenia crassiceps*, *Taenia hydatigena*, *Taenia multiceps*, *Taenia ovis*, *Taenia pisiformis*. In addition to these cestodes, DNA was extracted from *E.multilocularis* and *E.shiquicus* (results not shown). Strain specificity was tested using DNA extracted *E. granulosus* G1, G3, G5, G6, G7, G8 and G10. The most significant result for the purpose of the current study was a non cross reaction with *E.granulosus* (G1 genotype sheep strain). It was deemed important that the assay did not cross-react because both *E.equinus* and *E.granulosus* are both prevalent in the UK. Differentiation between these two species in particular would mean that the test could be a useful diagnostic tool for the detection of canine echinococcosis associated with *E.equinus* in the UK. The optimised assay was shown to have a detection sensitivity of up to 4.88pg, which is equivalent to approximately less than one *Echinococcus* egg. In comparison to the serial dilutions of tissue DNA, the primers were able to detect spiked faecal samples of 0.1, 1, 10 and 100ng/µl *E.equinus* tissue DNA representing 12.5, 125, 1,250 and 12,500 eggs respectively after the samples had been ethanol precipitated and diluted again.

3.8 Summary

A novel coproDNA PCR assay was developed to detect *Echinococcus equinus* DNA. The results from the optimisation process show that it is 100% specific against a panel of 14z cestode species and strains, in particular *Echinococcus granulosus* (G1 genotype sheep strain), which is also prevalent in the UK. The assay was shown to detect a serial dilution of *E.equinus* DNA of up to 4.88pg, which is the equivalent amount of approximately less than one *Echinococcus* egg. The assay was shown to detect spiked faecal samples of up to 0.1ng/μl, which is the equivalent concentration of the DNA in approximately 12.5 *Echinococcus* eggs. The *E.equinus* coproDNA PCR assay detected 22 horse hydatid cyst isolates obtained from 10 infected horse livers. Out of the positive samples, 14 were sequenced and confirmed as *E.equinus* (GenBank accession no. AB786665). Experimental infections of Tunisian dogs with horse hydatid cysts were carried out to investigate whether *E.equinus* of UK origin could be maintained in dogs. The horse hydatid cyst material that was used to infect the experimental dogs were tested using the ‘cestode-specific’ primers and all cysts were confirmed as *E.equinus* (GenBank accession no. AB786665). Archived horse hydatid wax-embedded samples (n = 20) were also tested using the ‘cestode-specific’ primers and the 14 samples that amplified were sequenced and confirmed as *E.equinus* (GenBank accession no. AB786665). The *E.equinus* coproDNA PCR assay was used to detect a panel of coproDNA from the experimental infections of Tunisian dogs. The optimised assay detected *E.equinus* DNA in the experimental infections as early as 10dpi.

CHAPTER FOUR

AN EPIDEMIOLOGICAL AND INTERVENTION STUDY OF CANINE ECHINOCOCCOSIS IN FARM DOGS IN SOUTH POWYS, WALES

4.1 Introduction

It has been well documented that *Echinococcus granulosus* infection transmitted between dogs and livestock has been endemic in parts of Wales and the English border areas for many years (Cook, 1964; Walters, 1977; Howells and Taylor, 1980; Palmer and Biffin, 1987). Historically south Powys is known to have been a hot spot for *E.granulosus* transmission to humans however the real incidence of human cystic echinococcosis (CE) or prevalence of domestic livestock infection in the UK is unclear because it is not a notifiable disease. A study carried out in Powys (mid-Wales) in 1973-1984 showed that up to 37% of sheep were recorded to be infected with hydatid disease (Walters, 1977). In 1975, over an 8-month period, dogs on 114 farms in Powys were arecoline purged and 25.2% were found to be positive for *E. granulosus* tapeworms (Walters and Clarkson, 1980). As well as these findings, 9% of purged foxhounds and 7% of red foxes (post-mortem) in mid-Wales were found to harbour adult *E.granulosus* worms (Walters, 1984). In the last 75 years, reported human CE cases in the UK appear to be largely restricted to two main sheep farming foci, one in mid-Wales and a smaller focus in the Scottish Hebrides (Howell, 1940; Walters, 1977; Chisholm *et al.*, 1983 and Stallbaumer *et al.*, 1986). Between 1927 and 1936, a total of 144 cases of human CE were recorded in all Welsh hospitals (Howell, 1940). Between 1964 and 1974, there were 77 deaths reported due to human CE in England and Wales (Walters, 1977). Analysis of national hospital records showed that the incidence of human CE was 0.2 per million in England and 2 cases per million in Wales with highest rates of 5.6 cases per million occurring in south Powys (Stallbaumer *et al.*, 1986). Due to the relatively high levels of infection, a voluntary hydatid control programme was introduced in mid-Wales between 1983 and 1989 to reduce the incidence of human CE and was primarily based on supervised 6 weekly dog dosing with praziquantel (Walters, 1984; 1986; Palmer *et al.*, 1996). The control programme however was terminated prematurely because financial resources were withdrawn and it was replaced with a health education programme (Lloyd *et al.*, 1991; Craig *et al.*, 1996; Buishi *et al.*, 2005a). The replacement health education

programme did not appear to be as effective, which was shown in 1995-1996, whereby a follow-up abattoir and dog coproantigen survey indicated that *E.granulosus* infection had re-emerged in sheep and dogs in the previous hydatid-control intervention areas (Palmer *et al.*, 1996). Between 1993 and 2002, the coproantigen prevalence in farm dogs in south Powys, Wales had more than doubled from 3.4% to 8.1%, following the policy changes favouring health education over the supervised dosing of dogs (Buishi *et al.*, 2005a).

In part, as a result of the study of Buishi *et al.* (2005a), the Welsh Assembly Government (WAG) introduced a 1 year pilot intervention for canine echinococcosis in a region of south Powys starting in May, 2008 (Edwards *et al.*, 2005; Anon, 2008; Brouwer and Willson, 2009). Farms were randomly selected and farm dogs from those farms were treated under supervision with praziquantel 4 times per year. It was agreed that dog infection would be monitored using the coproantigen ELISA at the Cestode Zoonoses Research Group, University of Salford and as utilised by Buishi *et al.*, (2005a). A baseline surveillance and 2-year follow-up study was implemented based on testing dog faecal samples by coproantigen ELISA. Prior to this the last dog surveillance was undertaken in 2002 in the same region of mid-Wales (Buishi *et al.*, 2005a). This chapter describes the results of the baseline, intervention and follow-up (1 year) studies.

In addition to the application of the coproantigen ELISA, a coproDNA PCR test was used on baseline samples to identify *Echinococcus* species present. No molecular genotyping had previously been undertaken in the mid-Wales endemic area. The presence or not of *E.equinus* DNA in farm dogs has not been previously investigated.

4.2 Aims and hypotheses

4.2.1 Aims of the study

The current survey in mid-Wales had two main aims:

1. To determine the baseline (pre-treatment) prevalence of canine echinococcosis in farms dogs within the Welsh county of Powys using a coproantigen ELISA and compare with the post-treatment data by evaluating the impact and efficacy of the supervised free dog dosing campaign (with praziquantel).
2. To estimate the baseline prevalence of *Echinococcus* spp. infection in the first quarter (pre-treatment) using a coproDNA PCR test to determine presence of *E.granulosus* and possible *E.equinus* in those dogs.

4.2.2 Hypotheses

1. There has been no decrease in the prevalence of canine echinococcosis in south Powys since the last *E.granulosus* dog survey in 2002 (8.1% coproantigen prevalence).
2. A praziquantel dosing frequency of 4 times per year will reduce the coprovalence of canine echinococcosis.

4.3 Materials and methods

The study was carried out between the years of 2008 and 2010. The study formed part of a pilot control programme (Hydatid Disease Eradication Campaign) that was funded by the Welsh Assembly Government (WAG) to reduce the incidence of hydatid infection in targeted areas of Wales. In this study, it will be referred to as the Welsh Hydatid Study (WHS).

4.3.1 Location and description of the Welsh study area

Wales is a country that is part of the United Kingdom of Great Britain and Northern Ireland. It is bordered by England to its east and the Atlantic Ocean and Irish Sea to its west. Wales also includes the island of Anglesey, which is separated from the mainland by the narrow Menai Strait. Wales is bordered on the east by the English counties of Cheshire, Shropshire, Hereford and Worcester, and Gloucester; on the south by the Bristol Channel; and on the west by St. George's Channel; and Cardigan Bay. Wales covers a total area of 20,779km² (8,023 sq miles) of which roughly 80% is devoted to agriculture. Wales has a varied geography with strong contrasts. In the south, a flat coastal plain gives way to valleys, then to ranges of hills; mountains in mid-Wales cover a quarter of the landmass of Wales. About 80% of the land is dedicated to agriculture and livestock rearing. In general the raising of livestock, mainly sheep, beef and dairy cattle, is more important than crop cultivation. Crops include barley, oats, potatoes and hay. Forests cover about 12% of the land and government reforestation programs are gradually increasing the area. The fishing industry is concentrated along the Bristol Channel.

The county of Powys in mid-Wales has the highest density of sheep in Western Europe. According to latest figures released in June 2012 by the Survey of Agriculture and Horticulture in Wales, the total number of sheep and lambs in Wales was nearly 8.9 million, with lamb numbers rising by nearly 5% to 4.6 million and ewe numbers rising slightly by 1.2% to 4.2 million (Wales Online, 2012). According to the National Audit Office, the annual estimation of sheep population in Wales for 2000/2001 was nearly 11.7 million, however the Welsh sheep industry suffered a short-term livestock problem in the form of an outbreak of foot-and-mouth disease (FMD). By early 2001, nearly 600 farms and abattoirs were affected by the FMD outbreak, which led to the death of

400,000 animals. Subsequently nearly six million animals were slaughtered in the UK devastating the livelihoods of thousands of farmers. A study by Buishi *et al.*, (2005a) indicated that the FMD outbreak did not increase the risk of echinococcosis despite large numbers of sheep slaughtered on properties.

The study areas were determined by the Welsh Assembly Government (WAG) and included the county of Powys, which covers the districts of Brecknockshire and Radnorshire. The target sites were those which consisted of all dogs on farms containing livestock (cattle and/or sheep) within the region of South Powys. This was defined within the county of Powys by the northernmost limits of the ancient counties of Brecknockshire and Radnorshire, (as shown in Fig. 4.1) as identified from Welsh Assembly Government (WAG) records and from speaking with farmers (Mastin *et al.*, 2011). A total of 1415 farms were identified, of which 109 farmers declined or were unable to participate, 234 farms were not contactable leaving a total of 1072 target sites.

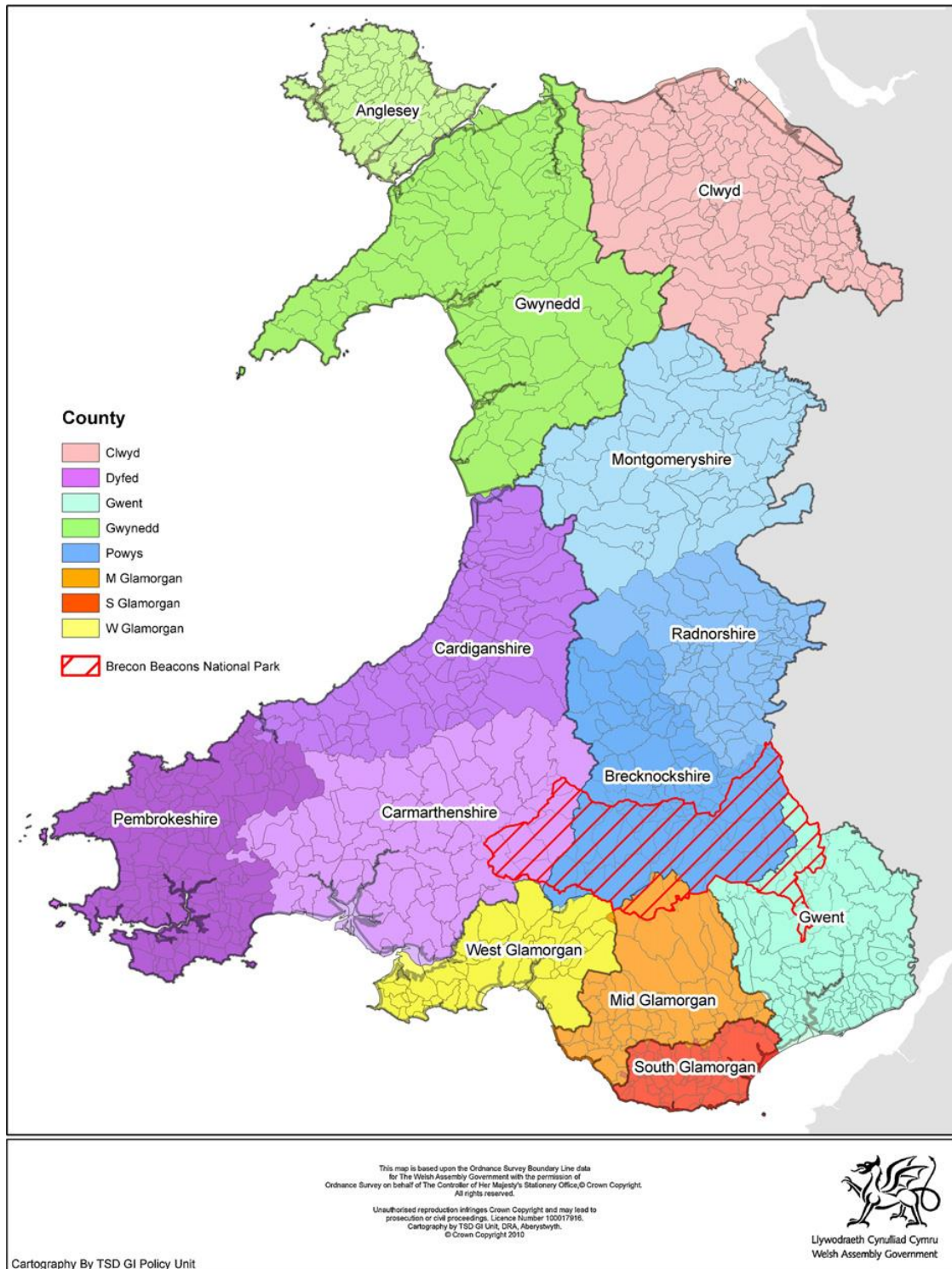


Figure 4.1 Map of Wales; the control area was identified by the northernmost limits of the ancient districts of Brecknockshire and Radnorshire within the county of Powys as identified from Welsh Assembly Government (WAG) records and from speaking with farmers (Mastin et al., 2011).

4.3.2 Farm dog faecal sampling

The WHS farm dog faecal sampling took place over a twenty two month period from May 2008 to July 2010. Each farm target site was allocated a county parish holding (CPH) number. A random sample of the 1072 available farms was selected to undergo laboratory testing using Microsoft Excel to generate block randomisation of target sites according to region, CPH number and random number generation (Mastin *et al.*, 2011). The sample size was dictated by budget constraints and determined by the Welsh Assembly Government (WAG) totalling 2,427 dog faecal samples.

Attempts were made to sample every dog on every selected farm, however as the operatives were not trained to collect faecal samples per rectum, freshly voided faecal samples were collected by the farmer prior to the visit and provided for testing. Inappropriate segregation from other dogs prior to passing faeces or not having passed faeces at the time of sampling may have resulted in samples not being obtained (Mastin *et al.*, 2011). The faecal samples were initially collected in plastic bags and upon arrival the operatives transferred 2-3g of the sample into 50ml screw-capped centrifuge tubes. Each tube was labelled with dog name, date and county parish holding (CPH) number. The samples were placed in pre-labelled polyethylene bags and then transported by road to the pathogen laboratory (OIE/DEFRA licensed) at the University of Salford. The dog weight was either measured or estimated and an appropriate dose (5mg/kg) of praziquantel (Droncit® tablet; Bayer) was administered orally.

Upon arrival to the laboratory, the samples were frozen at -80°C for a minimum of 3 days to kill off any infective eggs. This was to ensure maximum safety to the researcher when handling faecal matter during the processing stage. After 3 days the samples were transferred to -20°C until required for faecal processing and coproantigen ELISA screening.



Figure 4.2 Praziquantel drug tablet administered orally using bread and butter.



Figure 4.3 Farmer assisting with the oral administering of the supervised dog worming treatment.

4.3.3 Questionnaire

For the WHS, veterinary staff administered a short questionnaire relating to each dog on the farm in a face-to-face interview with the farmer (see Appendix 5). Data relating to each dog's age, sex and type (categorised into working dog, retired working dog, pet dog or hound), roaming behaviour and previous worming history (time of last worming, wormer used and dosage) were collected.

On the 19th May 2008, work on farm visits and worming of dogs commenced in south Powys, mid-Wales. The Office of the Chief Veterinary Officer and the Department for Public Health and Health Professions in the Welsh Assembly Government jointly funded a pilot dog worming campaign as a preventative public health measure known as the Welsh Hydatid Study (WHS). The programme was two-fold; (i) an awareness campaign to raise awareness of the disease and communicate good worming practices and hygiene to prevent the spread of the disease, (ii) participating farms were visited quarterly and given free dog worming treatment (praziquantel – Droncit) and a selection of farm dogs were sampled for coproantigen ELISA for canine echinococcosis diagnosis.

Over a period of approximately two years (2008-2010), dog faecal samples were collected by veterinary operatives and sent by road to The University of Salford to be tested using a coproantigen ELISA test. In Year 1, all of the farms in the study area were given free quarterly dog worming treatment however in Year 2, based on the analysis and evaluation of the results in Year 1 by the Welsh Assembly Government, only the participating farms of the dog sampling were given free dog worming treatment. Farmers who had participated in Year 1 of the campaign but not selected for Year 2 were advised to continue worming their own dogs and the importance of this was emphasised.

Faecal samples were delivered in batches 1-8 to the University of Salford for coproantigen testing. Additional samples were collected and were called 'spare samples', which were included in the study in cases whereby chosen samples were deemed unviable i.e. sample had leaked or could not be matched to the sample list. In total 2,427 dog faecal samples were tested by coproantigen ELISA (609 in Q1, 270 in Q2, 252 in Q3, 220 in Q4, 270 in Q5, 300 in Q6, 245 in Q7, 261 in Q8).

As part of the WHS, foxhound faecal samples were also collected from 2 foxhound packs; Hunt 3 and Hunt 2. Both hunts were sampled three times (Q1, Q2 and Q3) over the course of the WHS. The coproantigen ELISA results were uploaded to the secure Welsh Government AFON server for their own analysis/records.

4.3.4 Coproantigen detection

Coproantigen ELISA techniques were applied to the samples. These methods are described in Chapter Two. Prior to testing, faecal supernatants were thawed and mixed by hand shaking. A total of 2,427 faecal supernatants were tested in duplicate for the presence of *Echinococcus* coproantigens using the standardised coproantigen ELISA (Chapter Three) that utilised capture antibody against *E.granulosus* adult somatic antigens, whole worm extract (Allan *et al.*, 1992; Craig *et al.*, 1995; Buishi *et al.*, 2005a). The performance of the coproantigen ELISA test is described in Chapter Two. The cut-off value for coproantigen-negative threshold was determined as >3SD above the mean OD value for 20 control dogs from a local dogs home in the UK. This was carried out as described in Chapter Two.

Faecal supernatant processing. The samples were processed to prepare them for coproantigen ELISA testing. The required samples were defrosted for 1-2 hours at room temperature. Each sample was provided with 1x 5ml bijou and 1x 2ml Eppendorf, which were pre-labelled with lab ID and dog name (if applicable). Once the sample had defrosted, 0.5g-1g of faeces was removed from the original tube and placed into a 5ml Bijou using a clean wooden spatula. The 5ml bijou was topped up with 0.3% phosphate-buffered saline with Tween 20 (PBSt20). The faeces and 0.3% PBSt20 was thoroughly mixed with the wooden spatula. The faeces and 0.3% PBSt20 was sealed and shaken then shaken and centrifuged at 3600g for 5 minutes. The supernatant was tipped into a labelled 2ml Eppendorf. The remaining Bijou and contents were disposed of and the supernatant was frozen at -20°C until required.

4.3.5 CoproDNA detection

A polymerase chain reaction (PCR) assay to amplify *Echinococcus* DNA was applied to those samples that tested coproantigen ELISA positive and others. A coproDNA PCR for *E.granulosus* sensu lato was applied as previously described (Abbasi *et al.*, 2003) to all

of the faecal samples in Quarter 1, baseline (n = 609), all of the ELISA positive samples (n = 120) and also approximately 10% of randomly selected ELISA negative samples (n = 105). In addition a new optimised *E.equinus* G4 PCR (see Chapter Four: Development of coproDNA PCR to detect *Echinococcus equinus* in dogs and PCR for hydatid isolates) was also applied to all of the dog faecal samples in Quarter 1 (n = 609). The performance of the optimised *E.equinus* G4 primers are described in detail in Chapter Three.

4.4 Copro-Polymerase Chain Reaction (PCR)

4.4.1 Faecal DNA extraction.

CoproDNA was extracted from farm dog and foxhound faecal samples using the QIAamp DNA Mini Stool Kit (Qiagen House, West Sussex, UK) as outlined in Chapter Three.

4.4.2 CoproDNA amplification

Primers (Eg2691 5-ACACCACGCATGAGGATTAC-3 and Eg2692 5-ACCGAGCATTTGAAATGTTGC-3) amplifying an *E.granulosus* 133bp fragment of the tandem repeat and larger bands corresponding to size increments of 269bp (the size of the unit repeat) were used (Abbasi *et al.*, 2003), implementing reagent modifications described by Boufana *et al.* (2008). The constituents of the Mastermix for the ‘Abbasi’ test, is given in the Appendix 8. The PCR was performed in a final volume of 25µl containing 10mM Tris-HCl, pH 9.2, 25mM KCl, 1.5 mM MgCl₂, 200 µM (each) dNTPs (Promega, UK), 0.4µM of each of the amplification primers, 2.5 units of *Taq* DNA polymerase (GoTaq, Promega, UK), and target DNA. The Mastermix fluid was covered with a layer of mineral oil to prevent evaporation. Thermal cycling of the amplification mixture was performed in a Strategene® Robocycler 96 (La Jolla, CA) and involved five minutes at 95°C, followed by 35 cycles, each of one minute at 95°C, one minute at 55°C, and one minute at 72°C, and a final elongation step for 10 minutes at 72°C for 40 cycles.

An optimised *E.equinus* G4-specific coproPCR assay was used to detect target DNA in the Welsh Farm dog faecal samples. These primers (forward 5-GGT TTT GAG ATA CAT AAT AAT GTC CGG AC-3 and reverse 3-CTC ACA CCA AGC ACC TAC ACA TAA ATA TAG TT-5) amplified a 299bp diagnostic fragment. The optimisation process of these designed primers is described in detail in Chapter Three.

Amplified products were separated by electrophoresis on a 1.5% TBE agarose gel stained with GelRedTM (Cambridge Biosciences, UK) in Tris-borate EDTA buffer and left to run for approximately 1 hour at around 70V for the small gel and 110V for the large gel. A 1kb plus molecular weight marker (HyperLadderTM 1kb - formerly HyperLadder I, Bioline, London, England) was included on each gel for confirmation of amplicon size. Positive controls to monitor PCR success and negative controls to check for false-positive results that may have arisen from carry-over contamination were also included in all experiments. Gels were visualized under UV illumination using a Syngene G: Box gel documentation system (Geneflow, Cambridge, UK) and a photograph was taken to record the results.

4.4.3 Ethanol precipitation and sample dilution.

If no bands were visible for suspected positive samples, the DNA extractions were ethanol precipitated. Ethanol precipitation is a commonly used technique for making the DNA more concentrated and removing the salt concentration from the nucleic acid. By precipitating the DNA it becomes more concentrated and therefore increases the chance of amplification by PCR. The following ethanol precipitation procedure was carried out according to an optimised protocol.

1. The volume of the DNA stock sample was measured. One tenth of this volume of 3M sodium acetate was added to the DNA stock sample. 2 x of this new volume of chilled 100% ethanol was added. The samples were placed at -20°C overnight.
2. The following day the samples were centrifuged at 3600g for 20 mins after which point a white pellet may be seen. The supernatant was carefully removed from the side of the tube.
3. The samples were centrifuged for 3 mins. The supernatant was carefully removed. The samples were washed with 100µl of 70% ethanol and vortexed briefly.
4. The samples were then centrifuged for 10 mins. The supernatant was carefully removed from the side of the tube.

5. The samples were centrifuged for a further 5 mins and the ethanol was removed with a fine pipette tip.
6. The samples were allowed to air dry for 20 mins and re-suspended in a proportion of starting volume (x10 concentration).
7. The samples were vortexed briefly and stored at 4°C until required.

4.5 Determination of sensitivity and specificity

The sensitivity and specificity of the diagnostic test was calculated using the following formulae from Table 4.1 (Sacket *et al.*, 1985). Determination of sensitivity, specificity and kappa statistic for agreement of different tests was carried out for the coproantigen test and coproDNA PCR.

Table 4.1 Illustrates the formulae used to determine the parameters for determining sensitivity and specificity.

		Diagnosis based on coproantigen ELISA test (Allan <i>et al.</i> , 1992)		
		+	-	Total
CoproDNA PCR test	+	a	b	a+b
	-	c	d	c+d
	Total	a+c	b+d	a+b+c+d=n

$$\text{Sensitivity} = \frac{a}{a+c} \times 100$$

$$\text{Specificity} = \frac{d}{b+d} \times 100$$

$$\text{PPV} = \frac{a}{a+b} \times 100$$

$$\text{NPV} = \frac{d}{c+d} \times 100$$

Agreement between coproDNA PCR test and coproantigen ELISA test

$$\text{Observed agreement} = \frac{a+d}{n}$$

$$\text{Expected agreement due to chance} = \frac{(a+c)(a+b) + (b+d)(c+d)}{n^2}$$

Actual agreement beyond chance = observed agreement – expected agreement

Potential agreement beyond chance = 1.00 – actual agreement

Kappa = actual agreement / potential agreement

Key:

- a Number of samples which were found positive at both coproDNA PCR and coproantigen screening.
- b Number of samples which were positive by coproDNA PCR testing but tested negative for presence of coproantigens.
- c Number of samples found coproantigen ELISA positive but were negative by coproDNA PCR.
- d Number of samples which appeared negative with both coproantigen ELISA and coproDNA PCR.

Kappa statistic method was used when an agreement between categorical assessment were sought and assessed. It is often used when data were ordinal that is when the categories follow a numerical order. It is a way of testing independence, which is testing the null hypothesis that there is no more agreement that might occur by chance given random guessing. As a test statistic, kappa can verify that agreement exceeds chance levels. The kappa statistic varies between 0 (no agreement better than chance) and 1 (perfect agreement). One of the shortcomings of the kappa statistic is that it is dependent on the proportion of subjects in each category (prevalence). This makes it not applicable when comparing different studies. It is worth noting that, as sensitivity and specificity do not depend on prevalence, a high sensitivity/specificity does not necessarily result in a high kappa value.

4.6 Results

4.6.1 Coproantigen ELISA results

Over the twenty two month sample collection period, a total of 2,427 samples were tested using the coproantigen ELISA test. A total of 247 farms were sampled in the first quarter of the study. In Year 1, 1351 samples were tested, of those 609 were collected in Quarter 1 (pre-treatment) and 66 (10.8%) were found to be ELISA positive ($>0.095\text{OD}$). In Quarters 2-4 (post-treatment), 742 samples were tested and 5 were found to be ELISA positive giving a coproantigen prevalence of 0.7%. In Year 2 (after cessation of treatment), 1,076 samples were tested and of those 45 were found to be ELISA positive giving an overall coproantigen prevalence of 4.2% for Year 2. Figure 4.5 shows how the coproantigen prevalence of the farm dogs changes over the course of the WHS control programme.

Table 4.2 Number of coproantigen ELISA positive farm dogs from south Powys, mid-Wales against total number of samples tested and percentage.

WHS Study	Quarter	Number of samples tested	Coproantigen ELISA positive
Year 1	1	609	66 (10.8%)
	2	270	4 (1.5%)
	3	252	1 (0.4%)
	4	220	0 (0%)
Year 2	5	270	26 (9.6%)
	6	300	5 (1.7%)
	7	245	14 (5.7%)
	8	261	0 (0%)

Coproantigen prevalence of Welsh farm dogs from 2008-2010 in target zone of the WHS control programme

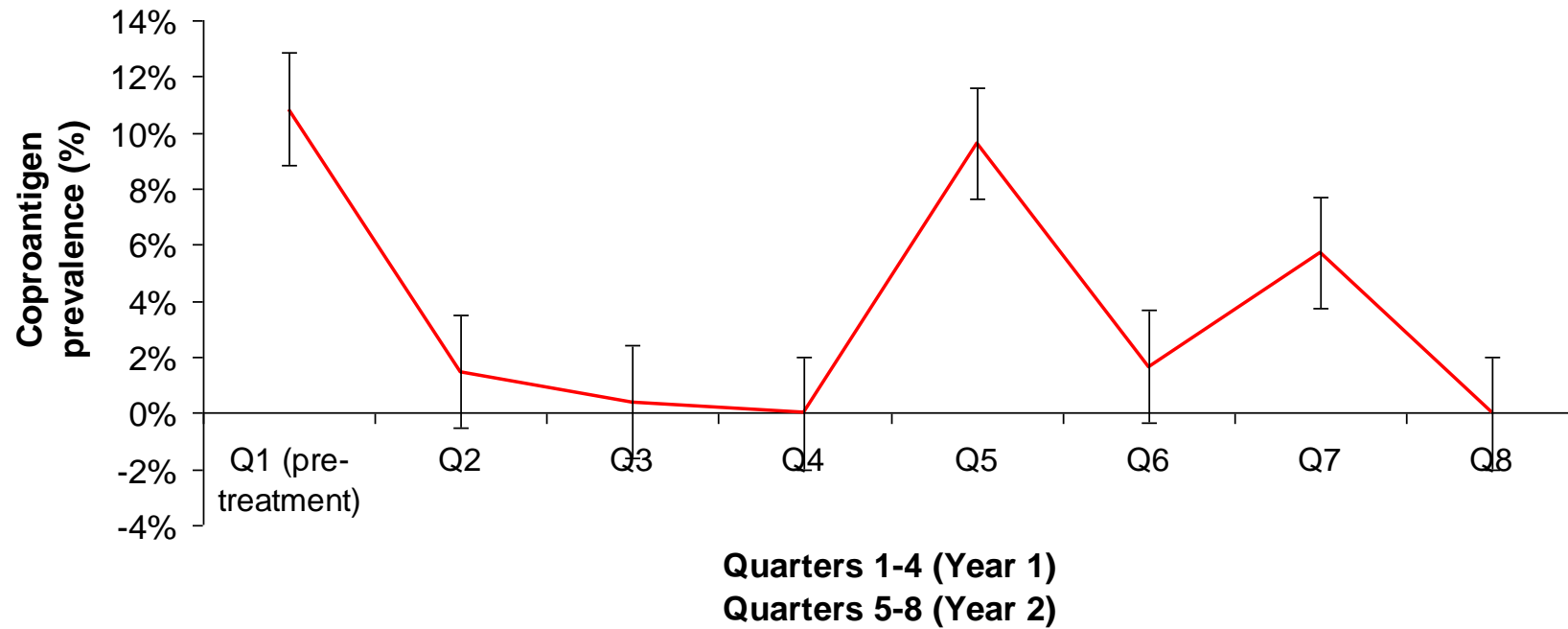


Figure 4.5 Coproantigen prevalence (%) of Welsh farm dogs tested from Quarter 1-8 of the Welsh Hydatid Study 2008-2010. Year 1, 2008-2009 (Q1-Q4); Year 2, 2009-2010 (Q5-Q8)

4.6.2 CoproDNA PCR results

As part of the WHS all of the dog faecal samples that tested coproantigen ELISA positive were tested for *E.granulosus* sensu lato using a coproPCR assay that amplifies a 133bp diagnostic fragment (Abbasi *et al.*, 2003). Table 4.4 shows that in Quarter 1 (pre-treatment), out of the 66 dog faecal samples that were coproantigen ELISA positive, 6 were shown to be coproDNA PCR positive for *E.granulosus* sensu lato (9.1%). A further 18 coproantigen negative samples were randomly selected to be tested with the ‘Abbasi’ coproDNA PCR assay, of which 4 tested coproDNA positive for *E.granulosus* sensu lato (22.2%). Table 4.4 also shows that in Quarters 2-8 (post-treatment), out of the 50 dog faecal samples that were coproantigen ELISA positive, 2 were shown to be coproDNA PCR positive for *E.granulosus* sensu lato (4.0%). A further 167 coproantigen negative samples were randomly selected to be tested with the ‘Abbasi’ coproDNA PCR assay, of which 19 tested coproDNA positive for *E.granulosus* sensu lato (11.4%). In total 31 dog faecal samples tested positive for *E.granulosus* sensu lato using the ‘Abbasi’ primers (Table 4.4). Figure 4.6 shows a representative PCR gel image of 2 farm dog samples from Quarter 2 that tested positive for *E.granulosus* sensu lato.

As part of the current study, dog faecal samples in Quarter 1 (n = 609), baseline (pre-treatment) were tested for *E.equinus*-specific DNA using an optimised coproDNA PCR assay that amplified a 299bp diagnostic fragment (see Chapter Three). These samples (n = 59) included those from the foxhound hunt packs; Hunt 3 and Hunt 2, collected in Q1, Q2 and Q3. The results from the *E.equinus* G4-specific primers showed that 4 faecal samples from Hunt 3 tested positive (3 from Q1 and 1 from Q2) for *E.equinus*. Out of the WHS farm dog samples, 7 showed very faint bands (see Fig. 4.4) however when the coproDNA PCR was repeated, no further amplification occurred even after the process of ethanol precipitation. The coproDNA PCR was repeated using the previously described ‘cestode-specific’ primers (Dinkel *et al.*, 1998) producing 1 out of the 7 samples to produce a very faint band however when the coproDNA PCR assay was repeated, no further amplification occurred even after the process of ethanol precipitation. This PCR product was genetically analysed, however the results are inconclusive and cannot be confirmed whether it is *E.equinus* or not.

In addition to the PCR confirmation of the immunodiagnostic results from the WHS, the ‘Abbasi’ primers were used to screen the 609 samples from Quarter 1 (pre-treatment). The samples included ‘spare’ samples that were collected but were never used in the WHS (n = 51). Out of a total of 660 samples, an additional 24 dog faecal samples tested positive for the presence of *E.granulosus* (3.6%).

Table 4.3 CoproDNA PCR results and coproantigen ELISA testing of samples for *E.granulosus sensu lato*.

		Diagnosis based on coproantigen ELISA test (Allan <i>et al.</i> , 1992)		
		Positive	Negative	Total
CoproDNA PCR test (‘Abbasi’ test)	Positive	6	60	a+b
	Negative	7	524	c+d
	Total	13	584	a+b+c+d = n

Sensitivity = $6/13 \times 100 = 46\%$

Specificity = $524/584 \times 100 = 90\%$

Positive predictive value (PPV) = $6/14 \times 100 = 42.9\%$

Negative predictive value (NPV) = $524/583 \times 100 = 89.9\%$

Agreement between coproDNA PCR and coproantigen ELISA results:

Observed agreement = $(6+524)/75 = 7.0\%$

Expected agreement due to chance = $[(6)(13)+(524)(584)]/75^2 = 9.6$

Actual agreement beyond chance = $7.0 - 9.6 = -2.6$

Potential agreement beyond chance = $1.00 - -2.6 = -1.6$

Kappa = $-2.6/-1.6 = -4.2$

20Table 4.4 Comparison of 'Abbasi' coproDNA PCR positive dogs with coproantigen ELISA OD values result. Red indicates positive results (≥ 0.095); green indicates negative results (< 0.095).

WHS Study	Lab ID number	CoproDNA PCR (<i>E.granulosus sensu lato</i>)	ELISA OD value
Quarter 1 (Pre-treatment) ELISA +ve	#43	POSITIVE	0.27
	#138	POSITIVE	0.13
	#44	POSITIVE	0.11
	#188	POSITIVE	0.09
	#385	POSITIVE	0.11
	#552	POSITIVE	0.12
Quarter 1 Randomly selected ELISA -ve	#47	POSITIVE	0.08
	#536	POSITIVE	0.02
	#546	POSITIVE	0.06
	#567	POSITIVE	0.04
Quarters 2-8 (post-treatment) ELISA +ve	#753	POSITIVE	0.13
	#2116	POSITIVE	0.13
Quarters 2-8 Randomly selected ELISA -ve	#772	POSITIVE	0.06
	#1601	POSITIVE	0.04
	#1784	POSITIVE	0.04
	#1897	POSITIVE	0.04
	#1948	POSITIVE	0.06
	#1984	POSITIVE	0.08
	#1995	POSITIVE	0.07
	#2087	POSITIVE	0.07
	#2091	POSITIVE	0.06
	#2100	POSITIVE	0.07
	#2127	POSITIVE	0.08
	#2135	POSITIVE	0.06
	#2142	POSITIVE	0.06
	#2229	POSITIVE	0.03
	#2235	POSITIVE	0.01
	#2243	POSITIVE	0.02
	#2268	POSITIVE	0.01
	#2295	POSITIVE	0.03
	#2308	POSITIVE	0.02

Table 4.5 Comparison of optimised *E.equinus* G4-specific coproDNA PCR positive dogs with coproantigen ELISA OD values result. Red indicates positive results (≥ 0.095); green indicates negative results (< 0.095).

WHS Study	Lab ID number	CoproDNA PCR (<i>E.equinus</i> G4-specific)	ELISA OD value
Quarter 1 (pre-treatment) Hunt No. 3	#216E	POSITIVE	0.02
	#216H	POSITIVE	0.04
	#216I	POSITIVE	0.03
	#642	POSITIVE	0.03

The results show that coproantigen ELISA prevalence in dogs on farms at pre-treatment baseline was 10.8% (66/609). After 3 treatment quarters (9 months) during the dog worming campaign the coproantigen prevalence had reduced to 0.7% (5/742). Table 4.4 represents the coproantigen ELISA results from various hydatid control programmes from 1993 to the current study. In the current study a total of 2,427 farm dog faecal samples were coproantigen ELISA tested however it is unknown exactly how many dogs are involved as some of the farms may have been repeatedly sampled.

Table 4.6 Coproantigen ELISA prevalence of farm dogs from 1993 to current study.

Year	Prevalence	Dogs sampled	Reference
1993	0%	107 unwormed farm dogs	(Palmer <i>et al.</i> , 1996)
1995-96	6.3%	112 dogs from sheep farms	(Lloyd <i>et al.</i> , 1998)
2002	8.5%	928 dogs on sheep farms, selected according to foot and mouth disease status	(Buishi <i>et al.</i> , 2005)
Year 1 (2008)			
Quarter 1 (pre-treatment)	10.8%	609 farm dogs	current study
Year 1 (2008-2009)			
Quarters 2-4 (post-treatment)	0.7%	742 farm dogs	current study
Year 2 (2009-2010)			
Quarters 5-8 (cessation of treatment)	4.2%	1,077 farm dogs	current study

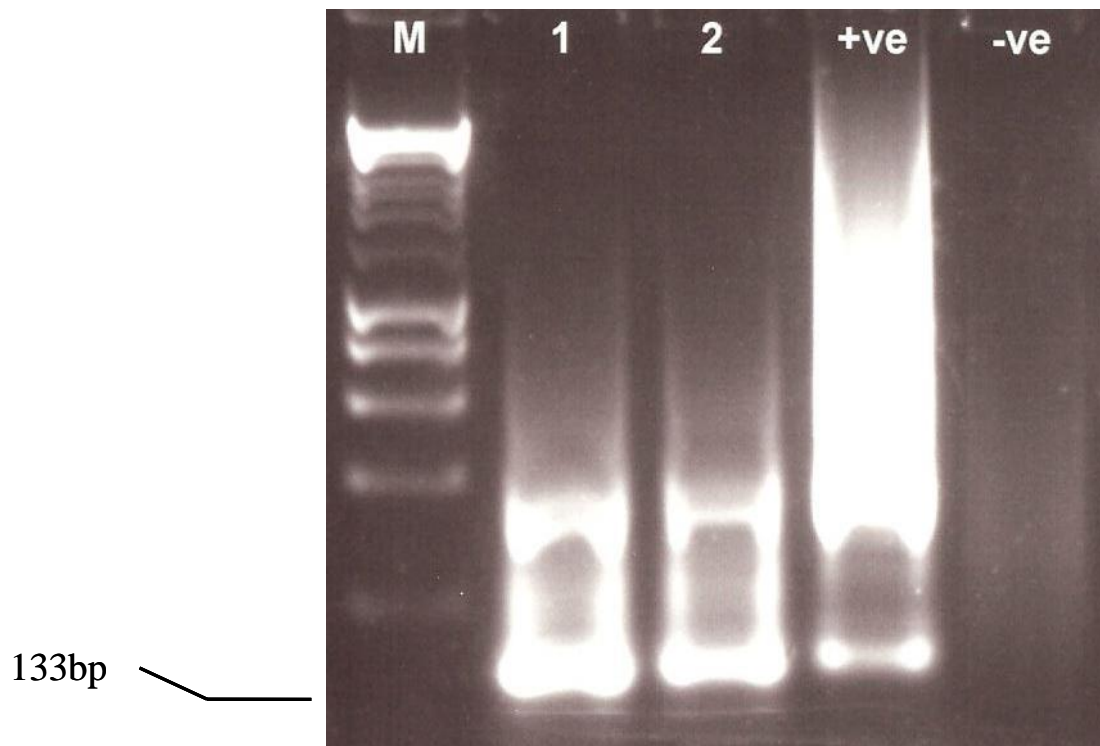


Figure 4.6 PCR amplification of coproDNA using 'Abbasi' primers for *E.granulosus*. Lane M, 100-bp molecular DNA ladder; lanes 1 and 2, faecal samples from WHS Quarter 2; lanes 3 & 4, positive and negative controls respectively.

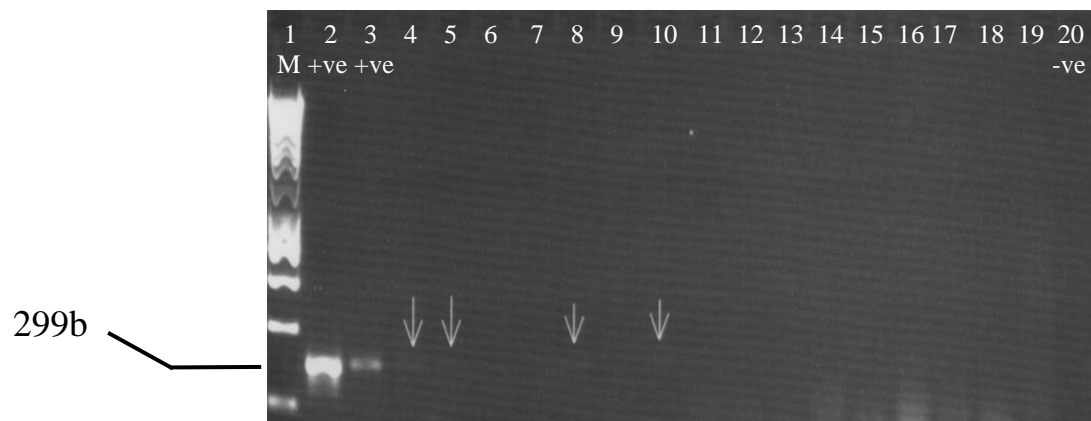


Figure 4.7 PCR amplification of coproDNA using optimised *E.equinus* G4-specific primers. Lane M, 100-bp molecular DNA ladder; lane 1, 2, 5 & 7, faecal samples WHS Quarter 1; lanes 2 & 3 tissue and copro positive controls respectively and lane 20 negative control.

4.7 Discussion

Echinococcus granulosus is endemic in the UK with transmission occurring in England, Wales and Scotland. However, human cystic echinococcosis (CE) cases are predominantly found in two sheep farming regions; Powys county in mid-Wales and the Hebridean Islands in northwest Scotland (Williams, 1976b; Walters, 1978; Chisholm *et al.*, 1983; Stallbaumer *et al.*, 1986). In 1975, 114 farms in Powys, Wales were visited twice, faecal purges were examined and it was found that nearly 60% of farms contained at least one infected dog between the two visits with 25.2% found to be purge positive for *E. granulosus* tapeworms (Walters and Clarkson 1980). The current study used a genus specific coproantigen ELISA test (Craig *et al.*, 1995; Buishi *et al.*, 2005a) to indicate the prevalence of canine echinococcosis. It was shown that coprovalence was 10.8% in this area of mid-Wales. This is higher than the coproantigen prevalence (8.5%) last reported for 2002 (Buishi *et al.*, 2005a) (Table 3.4).

From 1983-1989 the then Ministry of Agriculture Fisheries and Food (MAFF - now name the Department for Environment, Food and Rural Affairs - DEFRA) introduced the South Powys Hydatid Control Scheme, which involved regular worming of farm dogs with anthelmintic treatment praziquantel and health education. The study by Walters (1984) showed that sheep post-mortem infection rates for the south Powys area fell from 37% for the period 1973-1984 to 10% in 1988/89. According to Palmer *et al.* (1996), this was considered to be a direct result of the South Powys Hydatid Control Scheme. Further indication that the control programme was a success was that the incidence of hospital treated human CE in the Powys area fell from 4 per 100,000 to 2.3 per 100,000 for the period 1984-1990 (Palmer *et al.*, 1996). In comparison, the highest annual human CE incidence rate recorded was 7 per 100,000 in the Brecknock district of Powys, Wales in the 1970s/1980s (Palmer and Biffin, 1987).

The Welsh Assembly Government launched a campaign to raise awareness of the tapeworm in dogs, with the aim of preventing any increased risk of infection to humans. The campaign included the provision of free anthelmintic treatment (praziquantel) combined with the collection of data and samples as a pilot over one year to evaluate the efficacy, efficiency and practicability of dog anthelmintic treatment as a public health preventative measure.

The results suggest that mass dosing in quarter 1 and in the three subsequent quarters had significantly reduced coproantigen prevalence amongst dogs on farms and maintained a low prevalence.

In the current study, in the absence of purgation, a further diagnostic test for *E.granulosus* was applied i.e. coproDNA and the PCR results confirmed the presence of *E.granulosus* DNA in a total of 29 dog faecal samples. In Quarter 1 (pre-treatment), 9.1% (6/66) of coproantigen ELISA positive dog faecal samples were confirmed to have *Echinococcus* DNA present. In Quarters 2-8 (post-treatment and after cessation of treatment), 4.0% (2/50) of coproantigen ELISA positive dog faecal samples were confirmed to have *Echinococcus* DNA present. Overall 6.9% (8/116) of coproantigen ELISA positives were confirmed as PCR positive. The ‘Abbasi’ primers have been shown previously to have a sensitivity of 52.6% with dog faecal samples infected with *E.granulosus* (Boufana *et al.*, 2008). The coproantigen ELISA test however indicates the presence of antigen derived from the adult tapeworm independent of egg production (Elayoubi *et al.*, 2003) whereas coproDNA PCR sensitivity depends primarily on successful DNA extraction of parasite eggs in the faeces (Abbasi *et al.*, 2003).

In the current study, the ‘Abbasi’ primers were found to cross-react with other species namely *E.equinus* another species that is endemic in the UK (see Chapter Four). In order to assess if *E.equinus* was present in the south Powys farm dog population, an *E.equinus* G4-specific PCR was developed (see Chapter Four) and applied to Quarter 1, baseline (pre-treatment) samples. It was shown that none of *E.granulosus* DNA positive farm dogs were positive for *E.equinus* DNA, however 7 other farm dog samples produced very faint bands with the *E.equinus* G4-specific primers. This was surprising and suggests that either *E.equinus* may be present in the sheep population or other livestock hosts or that farm dogs had access to horse hydatid cysts (e.g. in offal), which is more likely.

4.8 Summary

The Welsh Assembly Government launched a hydatid control programme (the Welsh Hydatid Study - WHS) in the endemic area of south Powys, Wales (2008-2010) to raise awareness of the disease in the community to determine the current coproantigen prevalence and to measure the effect of dosing dogs. An *Echinococcus* coproantigen ELISA was used to screen a total of 2,427 dog faecal samples on a random selection of farms dogs from a possible 1,072 farms in south Powys, Wales. The baseline coproantigen prevalence (pre-treatment) was 10.8% (66/609) and after 4 x 3-month dosing of farm dogs with praziquantel, coproantigen prevalence fell to 0.7% (5/742) by 1 year post-treatment. The WHS study continued to sample farm dogs for another 12 months after the cessation of free anthelmintic provision to all of the participating farms in the study. In Year 2 (cessation of supervised dosing), the overall coproantigen prevalence increased to 4.2% (45/1,077). The last testing in quarter 8 showed a lower prevalence of 0%. A coproDNA PCR test for *E.granulosus* (sensu lato) confirmed the presence of *E.granulosus* DNA in 29 dog faecal samples – 9.6% (29/301). Further coproDNA PCR analysis of Quarter 1 (pre-treatment) confirmed the presence of *E.granulosus* in an additional 22 dog faecal samples - 3.3% (22/660). *E.equinus* DNA was detected for the first time 11.7% (7/660) in Welsh farm dog faecal samples. The study showed that the baseline prevalence of *E.granulosus* in farm dogs had not declined since 2002 but a 3-monthly dog dosing programme over a year reduced coprovalence significantly.

CHAPTER FIVE

AN EPIDEMIOLOGICAL STUDY OF CANINE ECHINOCOCCOSIS IN FOXHOUND PACKS IN ENGLAND AND WALES

5.1 Introduction

Echinococcus granulosus (G1 genotype/sheep strain) and *Echinococcus equinus* G4 genotype/horse strain) is responsible for canine echinococcosis in parts of the UK. Domestic cycles of *E.granulosus* are supported in all types of pastoral regions such as arid, temperate, mountain and plateau, where predominantly sheep and other livestock occur, as a result produces the risk of human infection (Craig *et al.*, 2007). It would appear that the distribution of *E.granulosus* is restricted to localised areas such as mid and south Wales (Torgerson and Budke, 2003), whereas infection does not seem to appear in Northern Ireland (Logan, 1971). *E.equinus* is present in many areas in the world where *E.granulosus* is found however it has been suggested that *E.equinus* infection in the UK may be more widespread in distribution, due to observations made into the wide-ranging origins of slaughtered horses (Thompson, 1975) and equine echinococcosis has also been regularly observed in Ireland (Hatch, 1970; Logan, 1971). It was reported that after the Second World War levels of equine echinococcosis reached high epidemic proportions (up to 61.7%) due to a major change in the way that hunting dog packs were fed (Thompson and Smyth, 1974; 1975; Dixon, 1973). In the UK, hunting has been practised for many centuries and is part of British rural culture. The Hunting Act 2004 made it illegal for people to hunt some animals such as foxes, hares and deer however drag and trail hunting is still permitted and there are currently around 174 hunting packs in the UK each with as many as 100 - 150 hounds in each pack. This cohort of canine animals makes up a significant group that may be involved in domestic transmission cycles of *E.equinus*.

Williams and Sweatman (1963) described cases of hydatid infections in horses from 1932 to 1962. They summarised data and cases that had been reported over this 30 year period. These included reports from England, Ireland and Wales, as well as across Europe, eastern and southern Australia, an isolated case in Venezuela and North America (imported horse from England) and Canada. They found that out of 709 horses inspected

in Doncaster, England over a 6-month period in 1960, 12.8% (91/709) had light infections and 2.3% (16/709) had heavy infections; none of the infections were pulmonary. The majority of horses were from Lincolnshire and Yorkshire, one from Wales and one from the Isle of Man. They suggested that this incidence level was not only high but also widespread.

Williams and Sweatman (1963) also carried out studies based on biological and morphological comparisons between the horse-dog origin from England and the sheep-dog origin from New Zealand. Horse hydatid cysts from England were fed to dogs to produce eggs, which were then fed to 2 horses and 2 sheep. After 15 months all intermediate hosts were autopsied. They found that one of the horses was heavily infected with pulmonary cysts and 1 sheep was found to have had only 2 small pulmonary cysts. When this procedure was repeated with the sheep-dog origin eggs, neither of the horses became infected, however the sheep became heavily infected with 1048 cysts found in the liver, lungs, kidneys and spleen. These findings strongly suggested distinct biological differences between *E.granulosus* in horses and New Zealand sheep.

It has been recognised that the best approach for investigating the epidemiology of echinococcosis infection is by determining the presence of the parasite in the canine definitive host (Gemmell *et al.*, 1987). Several methods for detection of canine echinococcosis have been developed over the years such as immunofluorescent detection of eggs; serological detection, coproantigen and coproDNA detection. A study carried out in 1995-96 showed that there was a coproantigen prevalence of 6.3% amongst 112 dogs sampled from sheep farms in Wales (Lloyd *et al.*, 1998). Previously the coproantigen prevalence of 107 farms dogs in the same area was found to be 0% (Palmer *et al.*, 1996). Since then the coproantigen prevalence has been shown to have increased to 8.5% after a study carried out on 928 farm dogs sampled from Welsh farms affected by foot and mouth disease (Buishi *et al.*, 2005a).

Since 1975 (R.C.A Thompson PhD Thesis, 1975) there have not been any comprehensive studies carried out on the prevalence of *E.equinus* in foxhound packs in the UK. Based on morphological differentiation as described by Williams and Sweatman, (1963),

Thompson was able to distinguish between *E.granulosus* and *E.equinus* and his findings indicated the presence of both species identified from purged foxhound packs. However at the time *E.equinus* was not considered to be a subspecies because according to Rausch, (1967) *E.granulosus* and *E.equinus* existed sympatrically and therefore no well-defined predator-prey relationships existed to ensure their ecological isolation. In more recent years it has been recommended that *E.equinus* should be known by its own taxonomic status as a separate species (Le *et al.*, 2002; McManus, 2002; Thompson and McManus, 2002).

In the current study a survey questionnaire was designed to find out whether foxhound husbandry may influence the prevalence of *Echinococcus* spp. infection in UK foxhound packs. The survey included questions asking about what was fed to the hounds, i.e. bagged food, fallen stock, cooked or uncooked offal. Another question was about worming practice i.e. how often were the hounds treated and were they treated with effective drugs to eliminate tapeworms. Effective tapeworm drugs include those that contain praziquantel (PZQ) such as Drontal Plus and Milbemax, whereas other drugs are ineffective. Another question that was asked was whether the kennelmen were aware of echinococcosis or hydatid disease and if so were they aware of how humans became infected i.e. from dogs, sheep or other. The prevalence of *Echinococcus* spp. in the UK was further investigated with particular focus on foxhound hunting packs using a coproantigen ELISA protocol developed by Allan *et al.* (1992). For the first time, molecular techniques were used to confirm the presence of *E.equinus* in UK transmission cycles. The presence of coproDNA was investigated using a published and established coproDNA PCR assay (Abbasi *et al.*, 2003). In addition a novel coproDNA PCR assay was used to test for *E.equinus* G4-specific DNA.

5.2 Aims and hypotheses

5.2.1 Aims of the study

The current epidemiological study had 2 main aims:

1. To investigate the prevalence of *Echinococcus* spp. in foxhound packs in the UK and to determine whether *Echinococcus granulosus* or *Echinococcus equinus* are responsible for transmission cycles within UK foxhound packs.
2. To use molecular diagnostic techniques to confirm the presence of *Echinococcus* DNA in foxhounds for the first time.
3. To find out whether foxhound husbandry may influence the prevalence of *Echinococcus* spp. infection in UK foxhound packs.

5.2.2 Hypotheses

1. Canine echinococcosis caused by *Echinococcus equinus* is still prevalent in UK foxhound packs.
2. Foxhound husbandry and practice is not consistent from hunt pack to hunt pack.

5.3 Study design and protocol

The foxhound hydatid study was carried out between 2010 and 2011. With the support of the Director of the Masters of Fox Hounds Association, every foxhound kennel in the UK from the Masters of Fox Hounds Association list of recognised hunts 2008-2009 (of which there were 174) was sent a letter requesting permission from the foxhound huntsmen to sample their pack (see Appendix 2) and a survey questionnaire (Appendix 3). The study was conducted in sites that were determined by the response from foxhound huntsmen who replied to the letters. In total, 8 foxhound packs were sampled, of which 5 were from Wales and 3 were from England. Foxhound faecal samples were collected from the ground in the foxhound penned areas; the following information lists the hunts that were sampled with the number of samples collected against the number of possible samples i.e. total number of foxhounds in the pack. The names of all the hunts have been anonymised for the purpose of the current study. The Welsh hunts include: Hunt No. 1 (n = 71/58); Hunt No. 2 (n = 31/unknown); Hunt No. 3 (n = 60/80); Hunt No. 4 (n = 49/51); Hunt No. 5 (n = 7/64). The English hunts include: Hunt No. 6 (n = 63/70); Hunt No. 7 (n = 57/100) and Hunt No. 8 (n = 36/68). As part of the Welsh Hydatid Study (WHS) (described in Chapter Four) Hunt Nos. 2 and 3 were sampled three times over the course of the Welsh study sampling period. These samples have been included in this study and in addition, Hunt No. 3 was sampled independently from the WHS.

The twenty-three question survey was designed to obtain information regarding foxhound husbandry and feeding practice and hydatid disease awareness. Several foxhound kennelmen returned the completed survey questionnaires however they politely declined their foxhound pack to be sampled (n = 9) these were Hunt Nos. 9-17.

5.3.1 Field logistics and foxhound faecal sampling

A checklist of equipment was produced and completed for every sampling visit to ensure that each visit was sampled properly (Appendix 4). A total of 364 foxhound faecal samples were collected. The huntsmen assisted by segregating the foxhounds away from the ground samples, which were deposited less than 24 hours before. Foxhound faecal samples were collected from the ground where the foxhounds were kept, consequently each sample could not be uniquely identified to an individual foxhound. The samples were collected from the enclosed areas of the pens using pre-labelled 50ml universal

tubes and assigned a unique lab ID number. The tubes were double packaged in polyethylene bags and later stored at -80°C for a minimum of 72 hours. Appropriate biohazard precautions were taken such as double-gloving; wearing Wellington boots and disposable overalls.



Figure 5.1 Hunt No. 3 Foxhound Pack.



Figure 5.2 Hunt No. 5 Foxhound Kennel.

5.3.2 Collection of samples and processing

Foxhound faecal samples were collected from the ground where the foxhounds were kept. Consequently each sample was not uniquely identified to each individual foxhound and also duplicate ground samples may have been taken. Before the collection of the faecal samples, the foxhound kennelman herded the foxhounds away the penned area and into a separate pen for ease of collection. Each sample was placed into a 50ml plastic tube and assigned a unique lab ID number for that hunt.

On arrival to the laboratory, the samples were placed in the -80°C freezer for at least 3 days to kill off any infective eggs. This was to ensure maximum safety to the researcher when handling faecal matter during the processing stage. After 3 days the samples were stored in the minus 20°C freezer until required. The samples were then processed to prepare them coproantigen ELISA testing. The required samples were defrosted for 1-2 hours at room temperature. Each sample was provided with 1x 5ml bijou and 1x 2ml Eppendorf, which were pre-labelled with lab ID and dog name (if applicable). Once the sample had defrosted, 0.5g – 1g of faeces was removed from the original tube and placed into a 5ml Bijou using a clean wooden spatula. The 5ml bijou was topped up with 0.3% phosphate-buffered saline with Tween 20 (PBSt20). The faeces and 0.3% PBSt20 was thoroughly mixed with the wooden spatula. The faeces and 0.3% PBSt20 was sealed and shaken then shaken and centrifuged at 2500rpm for 5 minutes. The supernatant was tipped into a labelled 2ml Eppendorf. The remaining Bijou and contents were disposed of and the supernatant was frozen at -20°C until required.

5.3.3 Coproantigen detection

The ‘Allan test’ ELISA (Allan *et al.*, 1992) was used to detect *Echinococcus* coproantigens in all of the 8 sampled foxhound hunt pack samples. The ‘Allan test’ ELISA is described in detail in Chapter Two.

5.3.4 CoproDNA detection

Published and established primers were used to detect *Echinococcus granulosus* sensu lato DNA (Abbasi *et al.*, 2003). The ‘Abbasi’ test is described in detail in Chapter Four. The optimised *E.equinus* G4-specific primers were used to detect *E.equinus* DNA. This novel coproDNA PCR assay is described in detail in Chapter Three. CoproDNA was

extracted from foxhound faecal samples using the QIAamp DNA Mini Stool Kit (Qiagen House, West Sussex, UK) as outlined in Chapter Three.

5.3.5 Statistical analysis

For statistical analysis the data were subjected to a chi squared test was used to determine whether there was significant correlation between the published coproDNA PCR (Abbasi *et al.*, 2003) and coproDNA PCR (*E.equinus*-specific) prevalence rates. Statistical significant correlation was approximated by odd ratios (OR). Correlation was considered significant at the level of $p < 0.05$. To identify significant correlation between the diagnostic tools used to detect canine echinococcosis, corresponding to p-values and 95% confidence intervals.

5.4 Results

5.4.1 Coproantigen ELISA results

In total 364 foxhound faecal samples were collected and tested using the ‘Allan’ coproantigen ELISA test (Allan *et al.*, 1992) as outlined in Chapter Two. The *Echinococcus* coproantigen ELISA was positive in 25.5% (93/364) of tested foxhounds. Positive coproantigen foxhounds were present in 5 out of 8 sampled packs. Prevalence of positive coproantigen foxhounds was found in 3 Welsh foxhound packs (Hunt Nos. 1, 2 and 4) and 2 English foxhound packs (Hunt Nos. 6 and 7).

The coproantigen prevalence varied from pack to pack from 0 – 61.2% (see Figure 5.3). Figure 5.3 shows that Hunt No. 4 produced the highest coproantigen prevalence (61.2%) compared to the other hunts. Hunt No. 6 produced the second highest coproantigen prevalence of 44.4% whilst Hunt Nos. 3, 5 and 8 all showed coproantigen prevalences of 0%.

Table 5.1 shows the coproantigen prevalence for each hunt with reference to the total number of foxhounds listed for each hunt. For example, Hunt No. 1 kennelman reported that the pack consisted of 58 foxhounds in total however 71 ground foxhound faecal samples were collected from the ground in the penned areas. Hunt No. 3 kennelman reported that the pack consisted of 80 foxhounds in total but only 60 foxhound faecal samples were collected from the ground.

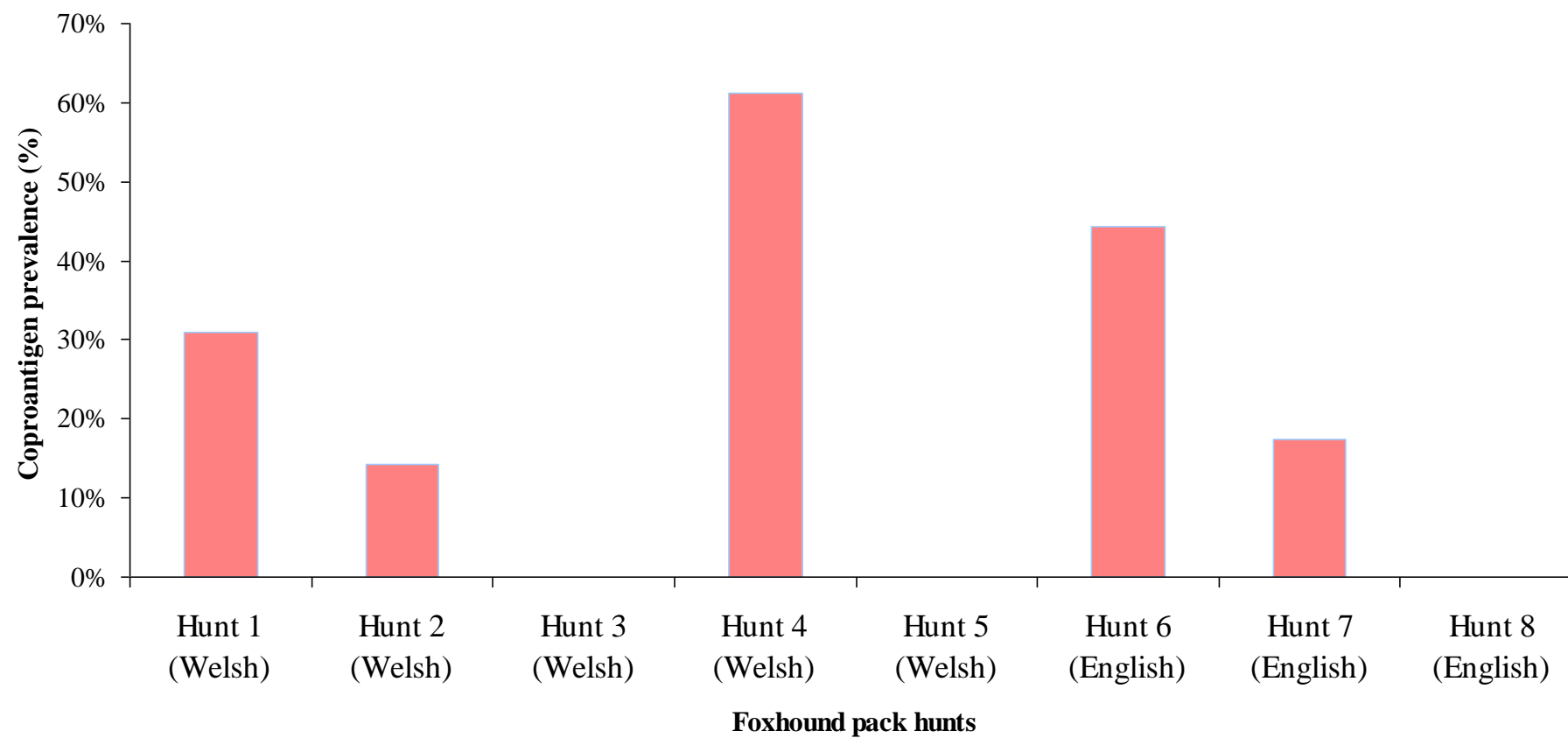


Figure 5.3 Coproantigen prevalence (%) of foxhound packs in Wales and England 2010

5.4.2 CoproDNA PCR results

CoproDNA PCR was carried out on all foxhound faecal samples. A total of 364 foxhound samples from 8 foxhound packs were tested for *Echinococcus granulosus* sensu lato with the ‘Abbasi’ primers (Abbasi *et al.*, 2003) and *Echinococcus equinus* (G4 genotype/horse strain) with a novel *E.equinus* G4-specific coproDNA PCR (W. Lett, unpublished) as described in Chapter Three.

Table 5.1 Foxhound coproDNA PCR results for *E.granulosus* sensu lato and *E.equinus*-specific DNA.

Hunt number	<i>E.granulosus</i> sensu lato (‘Abbasi’ primers)	<i>E.equinus</i> (G4-specific primers)
Hunt 1	1/71	0
Hunt 3	1/60	4/60
Hunt 4	4/49	1/49
Hunt 5	2/7	0
Hunt 6	1/63	0
Hunt 7	1/57	0

The *Echinococcus granulosus* sensu lato coproDNA PCR was positive in 3.8% (14/364) of tested foxhounds. Positive coproantigen foxhounds were present in 7 out of 8 sampled foxhound packs. Prevalence of positive coproDNA PCR foxhounds was found in all 5 Welsh foxhound packs and 2 of the English foxhound packs (Hunt Nos. 6 and 7). A total of 14 faecal samples tested positive for *E.granulosus* sensu lato, these were: Hunt No. 3 (4/60); Hunt No. 2 (1/21); Hunt No. 1 (1/71); Hunt No. 4 (4/49); Hunt No. 5 (2/7); Hunt No. 6 (1/63) and Hunt No. 7 (1/57). Figure 6.2 shows the results of the coproDNA PCR for 2 Hunt No. 3 foxhound samples collected as part of the WHS in Quarter 1 (pre-treatment). For the remaining two coproDNA positive samples from Hunt No. 3, one was also collected in Quarter 1 (data not shown) and the other was taken in Quarter 2 (post-treatment) as shown in Figure 6.3.

The *Echinococcus equinus* coproDNA PCR was positive in 1.4% (5/364) of tested foxhounds. Positive coproantigen foxhounds were present in 2 out of 8 sampled packs. Prevalence of positive coproDNA PCR foxhounds was found in 2 Welsh foxhound packs only, these were: Hunt No. 3 and Hunt No. 4. The results showed that 5 faecal samples tested positive for *E.equinus*: Hunt No. 3 (4/60) and Hunt No. 4 (1/49). Figure 5.6

includes the results of the coproDNA PCR for the Brecon & Talybont foxhound samples collected as part of the WHS, 3 from Quarter 1 (pre-treatment) and 1 from Quarter 2 (post-treatment). The diagnostic products were sequenced and all were confirmed as *E.equinus* (GenBank accession no. AB786665).

5.4.3 Questionnaire data

A survey questionnaire was sent to approximately 174 foxhound pack kennels in the UK. The survey questionnaire was devised of 23 questions about foxhound husbandry, practice and echinococcosis/hydatid disease perception. In total 16 questionnaires were completed. Out of 16 completed questionnaires: 12 kennelmen reported that they fed the hounds raw offal from fallen stock including sheep, lamb, calf, cattle and horse (75.0%). The kennelman from Hunt No. 8 reported that the offal was raw cattle tripe only and that liver and lungs from any livestock was never fed to the hounds. The kennelman from Hunt No. 13 reported that occasionally raw heart or kidney was fed to the hounds. Out of the 16 completed questionnaires: 5 kennelmen reported that they did not know what echinococcosis or hydatid disease was (31.3%); 1 kennelman reported that he did know what it was but reported that humans became infected from sheep and dogs; the remaining 10 kennelmen reported that they knew what echinococcosis/hydatid disease was and that it was infective from the dog. The questionnaire results are shown with the laboratory findings in Table 5.1.

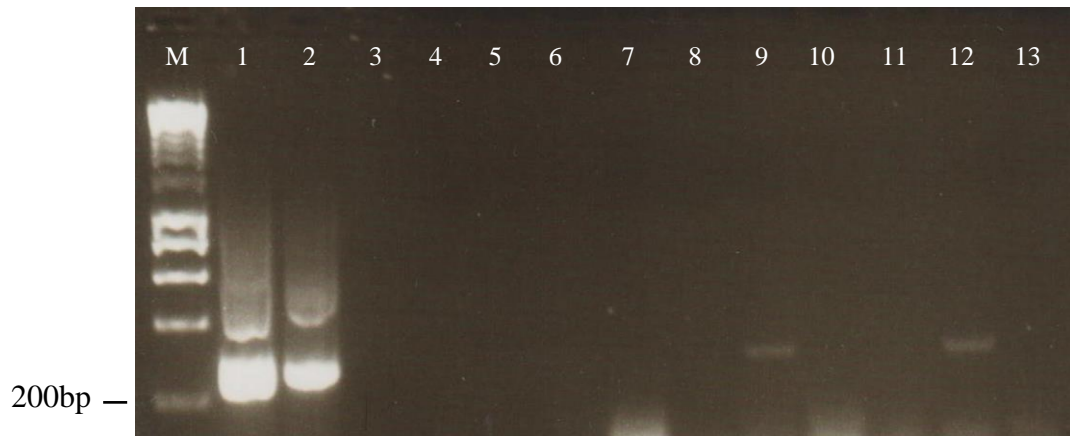


Figure 5.4 PCR amplification of coproDNA using 'Abbasi' primers for *E.granulosus sensu lato*. Lane M, DNA marker, lanes 1 and 2, G4 and G1 positive controls respectively; lanes 9 and 12, Hunt No. 3 foxhound coproDNA from WHS Quarter 1 (pre-treatment); lane 13, negative control.

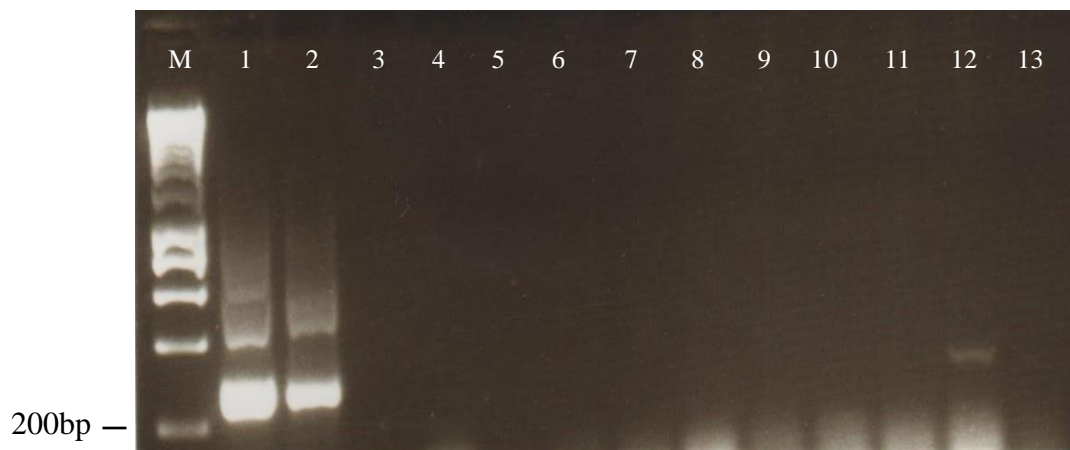


Figure 5.5 PCR amplification of coproDNA using 'Abbasi' primers for *E.granulosus sensu lato*. Lane M, DNA marker, lanes 1 and 2, G4 and G1 positive controls respectively; lanes 12, Hunt No. 3 foxhound coproDNA from WHS Quarter 2 (post-treatment); lane 13, negative control.

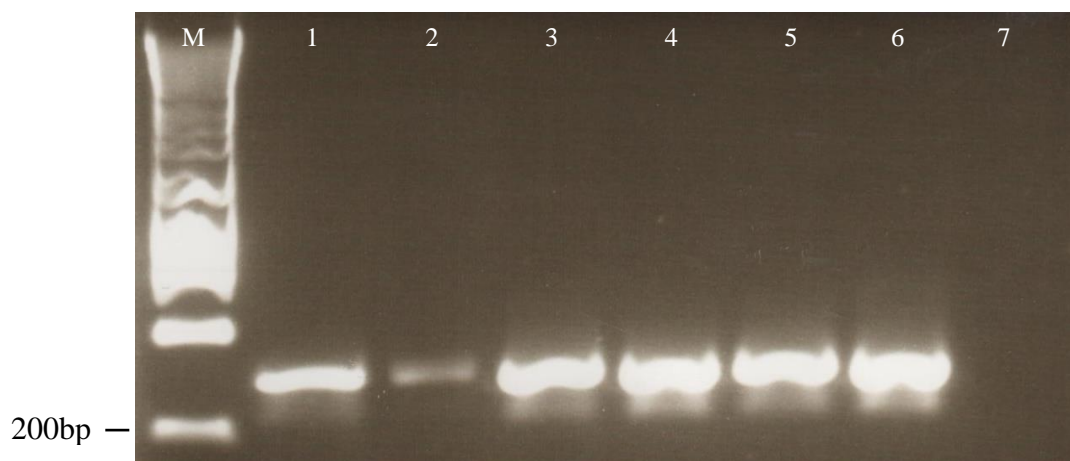


Figure 5.6 PCR amplification of coproDNA using a novel *E.equinus* G4-specific coproDNA PCR for *E.equinus*. Lane M, DNA marker, lanes 1 and 2, G4 tissue and G4 copro positive controls respectively; lanes 3-5, Hunt No. 3 foxhound coproDNA from WHS Quarter 1 (pre-treatment); lane 6, Brecon & Talybont foxhound coproDNA from WHS Quarter 2 (post-treatment); lane 7, negative control.

Table 5.2 Foxhound pack coproantigen and coproDNA and corresponding questionnaire data.

Allocated hunt number and total no. of hounds per pack	Coproantigen prevalence %	<i>E.granulosus</i> sensu lato CoproDNA prevalence %	<i>E.equinus</i> CoproDNA prevalence %	Foxhound diet	Worming practice	Knowledge of CE/hydatid disease
Hunt No. 1 (58)	30.9% (22/71)	1.4% (1/71)	0.0% (0/22)	Bagged meal (fallen stock historically)	Panacur	Yes
Hunt No. 2 - WHS Year 1 Quarter 1 & 3 (?)	14.3% (3/21)	0.0% (0/21)	0.0% (0/21)	Data unavailable	Data unavailable	Data unavailable
Hunt No. 3 - inc. WHS Year 1 Quarter 1 & 3 (80)	0.0% (0/60)	1.7% (1/60)	6.7% (4/60)	100% bagged meal, never fallen stock	Drontal/ Panacur	Yes
Hunt No. 4 (51)	61.2% (30/49)	8.2% (4/49)	2.0% (1/49)	Occasional horse, commercial waste (pies), odd meat from local butchers	Drontal Plus	No
Hunt No. 5 (64)	0.0% (0/7)	28.6% (2/7)	0.0% (0/7)	Raw offal cattle, calf, horse	Panacur	Yes
Hunt No. 6 (70)	44.4% (28/63)	1.6% (1/63)	0.0% (0/63)	Raw offal sheep cattle and horse (tripe and biscuits)	Ivomec Drontal	No
Hunt No. 7 (100)	17.5% (10/57)	1.8% (1/57)	0.0% (0/57)	Raw liver from sheep, lamb, cattle, and horse	Panacur	Yes (stated human infection from sheep)
Hunt No. 8 (68)	0.0% (0/36)	0.0% (0/36)	0.0% (0/36)	Cattle tripe	Equitape Ivomec	Yes
Hunt No. 9 (80)	-	-	-	Raw offal cattle, calf, horse	Panacur	No
Hunt No. 10 (150)	-	-	-	Raw offal sheep, lamb, cattle calf, horse	Panacur	No
Hunt No. 11 (50)	-	-	-	Raw offal sheep, lamb, cattle calf, horse	Cyclactin	No
Hunt No. 12 (58)	-	-	-	Raw offal cattle	Panacur	Yes
Hunt No. 13 (77)	-	-	-	Sheep, lamb, cattle calf, horse meat only	Ivomec	Yes
Hunt No. 14 (20)	-	-	-	Bagged food	Drontal	Yes
Hunt No. 15 (88)	-	-	-	Raw offal sheep, lamb, cattle calf, horse	Milbemax (PZQ)	Yes
Hunt No. 16 (19)	-	-	-	Bagged food	Drontal Plus	Yes
Hunt No. 17 (100)	-	-	-	Bagged food	Drontal Plus	Yes

5.5 Discussion

In the current study the results show that approximately a quarter of all the foxhound samples were coproantigen ELISA positive (25.5%). The coproantigen prevalence was significantly different between individual foxhound packs. Some coproantigen prevalence levels were shown to be particularly high for example, Hunt No. 1 at 30.9% (22/71), Hunt No. 4 at 61.2% (30/49), Hunt No. 6 at 44.4% (28/63) and Hunt No. 7 at 17.5% (10/57). During the sampling visit to Hunt No. 1, the kennelman reported that despite the recommendations made by the Masters of Fox Hounds Association Code of Practice not much had improved regarding the feeding of uncooked livestock offal to the hounds. The kennelman reported that in the past whole horse carcasses had been thrown into the foxhound pens to feed them without removal of any organs. He also reported that worming treatments did not include drugs used to eliminate tapeworms and that he was aware of what echinococcosis/hydatid disease was and that it was transmitted to humans by association with dogs.

During the sampling visit to Hunt No. 4, the kennelman reported that he was not aware of what echinococcosis or hydatid disease was. The kennelman generally fed the hounds with commercial waste such as pies, odd cuts of meat from local butchers as well as the occasional horse. He also reported that in the past he had come across large cysts of horse liver origin and that he did not know what they were and fed them directly to the hounds after bursting one of them. He described the burst cyst as being very watery like a water-filled balloon. However the kennelman reported that he did use Drontal Plus to treat the hounds for worms. The questionnaire data for Hunt No. 4 suggests that its foxhound husbandry and feeding practise is inadequate for eliminating cestode infection.

The kennelman for Hunt No. 6 was not aware of what echinococcosis or hydatid disease was, he used a combination of Ivomec and Drontal to treat for worms and regularly fed the hounds raw liver from fallen stock such as sheep, lamb, cattle and horse. It is not surprising that a high percentage (44.4%) of samples tested positive for *Echinococcus* coproantigens. The kennelman for Hunt No. 7 in Northumberland reported that he used Panacur a worming drug that did not contain praziquantel and he also fed the foxhound pack raw liver and lungs from fallen stock including sheep and horse. The coproantigen results show that 10/57 (17.5%) samples test positive for *Echinococcus* coproantigens.

Interestingly, 31.3% (5/16) of the foxhound kennelmen reported that they did not know what echinococcosis or hydatid disease was. Although the kennelman from Hunt No. 7 reported that he did know what the disease was he stated that humans could become infected from sheep as well as dogs. In a recent case, cystic echinococcosis (CE) was found in a person who used to work as a UK foxhound kennelman (Craig *et al.*, 2012). The results confirmed that the cyst was *E.granulosus* G1 genotype sheep strain and suggests that this profession may be a risk factor for contracting human CE. In light of this it is important that foxhound workers, kennelmen and other hunt staff are made fully aware of the risks of echinococcosis (Craig *et al.*, 2012).

The ‘Allan test’ coproantigen ELISA is genus-specific only and therefore it cannot distinguish between different species i.e. *E.granulosus* and *E.equinus* that are both endemic in the UK. From the coproantigen results it can only be determined that the samples were positive for *Echinococcus* spp. only. One of the aims of the current study was to use molecular diagnostic techniques to confirm the presence of *Echinococcus* DNA in foxhounds for the first time. A published and established coproDNA PCR was used to detect *Echinococcus granulosus* sensu lato DNA (Abbasi *et al.*, 2003). A total of 14 out of 364 foxhound faecal samples tested positive for *E.granulosus* sensu lato using the ‘Abbasi’ primers. The ‘Abbasi’ primers were found to cross-react with *E.equinus* DNA therefore a novel coproDNA PCR assay was developed to detect *E.equinus* G4-specific DNA (see Chapter 4). It was not possible to confirm which species is involved in the ‘Abbasi’ coproDNA PCR positive samples because the appropriate sequences were not deposited on the GenBank database at the time of the development of the primers. It is likely that all 14 samples are *E.granulosus* G1 genotype sheep strain because the same samples tested negative with the *E.equinus* G4-specific primers. A total of 5 out of 364 foxhound faecal samples tested positive for *E.equinus* G4-specific DNA. From these samples 4 came from Hunt No. 3, 3 of which were collected as part of the WHS during Quarter 1 (pre-treatment) and 1 came from the WHS during Quarter 2 (post-treatment). These results suggest that treatment with PZQ worming drugs is an effective approach to reduce canine echinococcosis. It has been recommended that foxhounds are dosed orally with a PZQ-based worming treatment at least four times per year (Craig *et al.*, 2012). Canine echinococcosis of *E.equinus* origin has also been determined in Hunt No. 4 using molecular techniques. This is not

surprising considering the questionnaire and interview responses given by the kennelman at the time of sample collection.

The data from the survey questionnaire suggests that there is a correlation between foxhound husbandry and feeding practise and laboratory results. Of the 8 foxhound packs that were sampled only 7 had corresponding survey questionnaire data that could be associated with the laboratory results. The data indicates that where foxhounds were fed raw offal, there was also a coproantigen or coproDNA prevalence of *Echinococcus* infection. The kennelman from Hunt No. 8 reported that only raw offal from cattle tripe was fed to the hounds (never raw liver or lungs) and the laboratory findings showed a 0% prevalence of coproantigens and 0% prevalence of coproDNA for *E.granulosus* and *E.equinus*. The kennelman from Hunt No. 3 reported that it only ever fed the foxhound pack with commercial bagged meal produced specifically for hounds and results showed that the coproantigen prevalence was 0% (0/60). Despite these findings the PCR results confirmed the presence of *E.granulosus* sensu lato coproDNA in 1.7% (1/60) and DNA sequencing results confirmed the presence of *E.equinus* coproDNA in 6.7% (4/60) of the samples. These results suggest that the foxhounds in this pack have access to infected horse material and also infected sheep material. It is a possibility that whilst drag or trail hunting the foxhounds may come across fallen livestock that hasn't yet been cleared by the farmers in the surrounding fields and countryside and then scavenge on the infected carcasses.

An epidemiological survey carried out in 1975 in the UK, where foxhound hunting packs were examined found that 52% harboured the *E.equinus* infected dogs (Thompson and Smyth, 1975). Due to economic pressures and lack of labour forces, the dietary practices of foxhunt dogs have changed leading to an increase in the feeding of raw flesh and offal. It had been suggested that the foxhunting ban passed in 2004 may play a role in reducing equine hydatidosis in the UK by Thompson (2008) however since foxhunting has been replaced by drag and trail hunting, this theory does not seem to stand as foxhound packs still cover many areas of the countryside during the amended practice and feeding practice is not considered to be a priority with some hunts.

The current study uses various laboratory techniques such as coproantigen ELISA and coproDNA PCR assays to investigate the epidemiology of echinococcosis in foxhound packs in the UK. In comparison with the last comprehensive study carried out on canine echinococcosis in foxhounds in the UK (Thompson and Smyth, 1975), the laboratory findings show that the coproantigen prevalence still remains high in some foxhound packs. For the first time molecular techniques have been used to confirm the presence of *Echinococcus* spp. in foxhound packs in the UK. Further studies would include more foxhound hunts from further a field, for example southern and eastern counties.

5.6 Summary

A total of 8 foxhound packs were sampled for *Echinococcus* spp. coproantigen and coproDNA testing. The foxhound packs were from the UK and ranged from Northumberland in England to Glamorgan in Wales. In the current study approximately a quarter (25.5%) of the foxhound samples were coproantigen ELISA positive (93/364). Coproantigen prevalence for individual foxhound packs ranged from 0% to 61.2% and was shown to be particularly high in some packs. Both *E.granulosus* and *E.equinus* coproDNA was found to be prevalent in foxhound faecal samples, 3.8% and 1.4% respectively and confirmed for the first time using molecular techniques. Out of the 8 foxhound packs that were sampled 7 were found to have a coproantigen and/or a coproDNA prevalence. Questionnaire data suggests that there is a correlation between poor foxhound husbandry and feeding practice and laboratory findings. The data indicates that where raw offal was fed to the foxhounds, there was also a coproantigen or coproDNA prevalence of *Echinococcus* infection. Despite evidence of good foxhound husbandry and feeding practice there is evidence to suggest that foxhounds have access to infected livestock material. Nearly a third of the kennelmen reported that they were not aware of what echinococcosis or hydatid disease was. In light of these findings and the recent case of a foxhound worker diagnosed with human CE (Craig *et al.*, 2012), it is recommended that policies are to be put in place in the Masters of Fox Hound Associations Code of Practice that provide clear guidelines to all kennel staff with close association with foxhounds. It is advised that foxhounds are dosed with a PZQ-based worming treatment at least four times per year; raw livestock products especially liver/lungs not be fed to dogs at all, or only after appropriate cooking.

CHAPTER SIX

GENERAL DISCUSSION

6.1 General Overview

This study incorporated several different approaches to investigate the epidemiology of *Echinococcus granulosus* and *Echinococcus equinus* in dogs in the UK. Areas of research included field-based parasitology, questionnaire analysis, laboratory detection optimisation, time-course experimental infections and application of optimised assays to detect canine echinococcosis in endemic regions within the UK. The findings of these research areas have enabled a better understanding of the prevalence rates of canine echinococcosis in the south Powys region of Wales and foxhound packs in England and Wales. In particular the research focused on which species were involved in UK transmission cycles as well as looking at potential risk factors associated with foxhound husbandry. It is the hope that these insights will aid in the future control of canine echinococcosis in these regions which in turn will help to reduce the impact of human cystic echinococcosis.

6.2 Conclusions and recommendations

Human cystic echinococcosis (CE) is caused by the accidental ingestion of *E.granulosus* (G1 genotype sheep strain) eggs; the transmission cycles occur between the definitive canine host and agricultural animals such as sheep and cattle. Human CE is rare however prevalence levels are 10 times higher in Wales than they are in England, 0.2 cases per million in England and 2 cases per million in Wales (Stallbaumer *et al.*, 1986). According to the Health Protection Agency (HPA) the last reported figures for human CE were in 2011 with 12 recorded cases in England and Wales (HPA, 2013).

E.equinus does not appear to be zoonotic it is almost always reported to date from equines however, Boufana *et al.*, (2012) recently described an *E.equinus* infection in a primate intermediate host - a captive born and bred red ruffed lemur (*Varecia rubra*) in the UK. This suggests that a non-human primate is able to maintain a viable *E.equinus* infection. The availability of a specific copro-detection test for this species and its differentiation from *E.granulosus* would be useful for epidemiological studies in the UK. Successful epidemiological studies and surveillance of hydatid control programmes rely on the identification of *E.granulosus* in the canine definitive host (Gemmell *et al.*, 1987).

In the current study laboratory techniques have been investigated and developed to ascertain the most appropriate method of canine echinococcosis detection in dog faecal samples.

In this study a comparison was made between the 'Allan' test (Allan *et al.*, 1992) and a newer version of the 'Heath' test (Huang *et al.*, 2007). Furthermore a combination test (Hybrid test) using anti-somatic and excretory-secretory reagents were investigated to assess whether a better assay could be developed. The purpose of the current study was to standardise and compare existing coproantigen ELISA assays developed by Allan *et al.*, (1992) and Huang *et al.*, (2007) for diagnosis of canine echinococcosis caused by *E.granulosus*. Various panels were included time-course experimentally infected dog faecal samples with *E.equinus*. Due to the non-specific binding between the Heath reagents it was deemed not feasible to use the test as it currently stands due to the possibility of producing false positive results. Future work could involve using an alternative commercial conjugate antibody that is more specific for anti-sheep reagents and that has been tested for specificity against other mammals, for example a monoclonal antibody (MAb). The Heath test differs from many ELISA assays in that it incorporates a double-sandwich format whereby the detection antibody was not labelled with an enzyme such as horseradish peroxidase. The author recommends that future production of detection antibodies should include a conjugation process so that it eliminates the need to involve a generic commercial product that has had no bearing on the original production of ELISA antibodies. This suggestion would also eliminate a step in the ELISA process, ELISAs are notoriously 'temperamental' and any simplification to the process would enhance the performance of the test. The panel results for the Hybrid test did not perform as well as the Allan test. Despite lower OD values, the Hybrid test results did depict positive results albeit much lower than those of the Allan test. Neither tests detected *Echinococcus multilocularis* antigens producing similar negative OD values. The Hybrid test results indicate that the test is specific to *E.granulosus* because *E.multilocularis* antigens were not detected. The reagents were originally raised against *E.granulosus* antigens therefore there is a high probability that the antibodies only recognise *E.granulosus* antigens. This has the potential of being used as a valid test to be investigated further for its diagnosis potential to detect *E.granulosus* exclusively. Future work could involve using different dilutions for the capture and conjugate antibodies.

Faecal supernatants extracted for Welsh and Foxhound samples were tested for the presence of genus specific *Echinococcus* coproantigens using an established ELISA (IgG) against *E.granulosus* adult somatic antigens – whole worm extract (EgWWE) (Allan *et al.*, 1992; Craig *et al.*, 1995; Jenkins *et al.*, 2000).

A further aspect of the current study was to determine whether the ELISA tests could detect dogs infected with *Echinococcus equinus*. The panel samples included naturally infected foxhound samples that were confirmed with having *E.equinus* infections using an optimised *E.equinus* G4-specific coproDNA PCR assay. The infected samples were confirmed as having *E.equinus* DNA present using samples collected ante-mortem therefore it was not possible to speculate on the sensitivity aspect of the test in relation to worm burden. At the time of the Hybrid test study, the *E.equinus* foxhound samples were not available to be tested therefore it is suggested that for future work these sample should be tested using the Hybrid assay. The panel also included the dog faecal samples from time-course experimental infections. For Experimental Infection 2, both dogs tested positive for Echinococcus infection using the Allan test. At the time of the Hybrid test study, these samples were not available to be included therefore it is suggested that for future work these samples should be tested using the Hybrid assay.

It has been reported that in the UK *Echinococcus granulosus* is limited in distribution, being primarily restricted to mid and south Wales (Torgerson and Budke, 2003). *Echinococcus equinus* (*E.granulosus* G4/horse strain) is present in many areas where *E.granulosus* is found (Torgerson and Budke, 2003). Before the Second World War, equine echinococcosis was rare in Great Britain (Southwell, 1927). After the Second World War, from the 1950s onwards, many more cases of echinococcosis in horses were reported in the literature (Thompson, 1975). It seems that due to the expense of fuel and labour costs after the war, hunt kennelmen fed their hunting packs raw horse and sheep flesh, resulting in an accelerated increase of equine echinococcosis (Smyth, 1976). The distribution of equine echinococcosis does not appear to be localised, data shows that the infection may be widespread because the origins of the slaughtered horses are spread widely over Great Britain (Thompson, 1975). The current study has shown that *E.equinus* is widespread across the UK. Horse passport and archived records show that

horse hydatid cases and canine echinococcosis associated with *E.equinus* are spread as West Sussex to Northumberland.

A novel coproDNA PCR assay has been developed to distinguish between G1 genotype sheep strain and G4 genotype horse strain that are known to be co-endemic in the UK. The *E.equinus* G4-specific coproDNA PCR assay was found to be 100% specific against 15 cestode species and strains these included; *Dipylidium caninum*, *Taenia crassiceps*, *Taenia hydatigena*, *Taenia multiceps*, *Taenia ovis*, *Taenia pisiformis*. In addition to these cestodes, DNA was extracted from *E.multilocularis* and *E.shiquicus* (results not shown). Strain specificity was tested using DNA extracted *E. granulosus* G1, G3, G5, G6, G7, G8 and G10. The most significant result for the purpose of the current study was a non cross reaction with *E.granulosus* (G1 genotype sheep strain). It was deemed important that the assay did not cross-react because both *E.equinus* and *E.granulosus* are both prevalent in the UK. Differentiation between these two species in particular would mean that the test could be a useful diagnostic tool for the detection of canine echinococcosis associated with *E.equinus* in the UK. The optimised assay was shown to have a detection sensitivity of up to 4.88pg, which is equivalent to approximately less than one *Echinococcus* egg. In comparison to the serial dilutions of tissue DNA, the primers were able to detect spiked faecal samples of 0.1, 1, 10 and 100ng/μl *E.equinus* tissue DNA representing 12.5, 125, 1,250 and 12,500 eggs respectively after the samples had been ethanol precipitated and diluted again.

E.equinus is still present over much of England and Wales. The current study is the first molecular confirmation of *E.equinus* in foxhounds. Future recommendations would be to re-extract the 7 Welsh farm dog samples that tested positive with the ‘Abbasi’ primers and test using the ‘cestode-specific’ primers to confirm the presence of *E.equinus* in Welsh farm dogs. Foxhound questionnaire data indicates the existence of bad practices such as feeding foxhound packs with raw liver and lungs from fallen stock, not regularly treating the foxhounds with a praziquantel (PZQ) based drug. These factors are likely to increase the risk of *Echinococcus* transmission.

A recent case has been reported whereby an ex- foxhound worker was diagnosed with *E.granulosus* (G1 genotype sheep strain) (Craig *et al.*, 2012). There were several risk

factors associated with this occupation; out of 16 hunts in England and Wales 81% reported that they fed uncooked livestock offal to fox-hounds and 56% did not use a praziquantel-based de-wormer to treat hounds (Craig *et al.*, 2012). The 2007 Council of Hunting Associations Code of Practice for the Welfare of Hounds in Hunt Kennels does not make any implications that humans may be at risk of human echinococcosis. When the Director of the Masters of Fox Hounds Association who is also the Director of the Council of Hunting Associations was contacted to ask for his approval for the current study he requested that the following questions be removed from the survey questionnaire; ‘Do you know what echinococcosis/hydatid disease is?’ and ‘If yes, how are humans infected? From dogs, from sheep or from other source?’ It was felt that these questions were ‘unnecessary’ when asked what the reasons why he wanted them to be removed. The author suggests that recommendations should be made to the Masters of Fox Hounds Association for clear guidelines to foxhound packs regarding dosing at least 4 times per year with PZQ and not allowing hounds to eat raw livestock/horse offal.

The closure of abattoirs may lead to many horses left to starve or fend for themselves in the countryside. This could also lead to a possible increased risk to increasing transmission cycles as more animals may be collected for the hunts. In addition, there could be many more animal carcasses in the field that would provide access to dogs to scavenge.

E.equinus appears to use equidae only as intermediate hosts (Jenkins *et al.*, 2005). In the current study all of the hydatid cysts originating from infected horses were identified as *E.equinus* and no other species or subspecies. The current study is the first experimental infection of dogs with *E.equinus*. The purpose of the experimental infections of dogs with *E.equinus* was to investigate whether *E.equinus* from British horse origin could be maintained in experimental dogs. In experimental infections two small infertile cysts developed in the lungs of two sheep that were fed *E.equinus* eggs suggesting that sheep is a poor host for *E.equinus* (Williams and Sweatman, 1963). Other experiments whereby sheep were injected with horse protoscoleces also failed to develop (Hatch and Smyth, 1975) and conversely cysts failed to develop in two horses when eggs of *E.granulosus* were fed (Williams and Sweatman, 1963).

The suggestion by Cook (1989) that the pre-patent period of *E.equinus* takes longer than the *E.granulosus* sheep strain is compared with the experimental infections that seem to suggest that it may be shorter than the suggested 70 days (W. Lett and S. Lahmar, unpublished observations).

E.granulosus is still present in Welsh farm dogs up to 10% coproantigen prevalence levels and indication of increase since previous control program late 1980s. Reinfection data on dogs has been recorded for first time in Wales, UK. The results showed that 4 dosing rounds can substantially reduce coproantigen levels from 10.8% to 0.7% however the prevalence rate was seen to spring back in the second year after cessation of treatment (4.2%).

REFERENCES

- Allan, J.C. (1990). Coproantigens in intestinal cestode infections. Ph.D. Thesis. University of Liverpool.
- Allan, J.C. and Craig, P.S. (1989). Coproantigens in gut tapeworm infections: *Hymenolepis diminuta* in rats. *Parasitology Research*, 76: pp. 68-73.
- Allan, J.C. and Craig, P.S. (2006). Coproantigens in taeniasis and echinococcosis. *Parasitology International*, 55: pp. S75-S80.
- Allan, J.C., Craig, P.S., Garcia-Noval, J., Mencos, F., Liu, D., Wang, Y., Wen, H., Zhou, P., Stringer, R., Rogan, M. and Zeyhle, E. (1992). Coproantigen detection for immunodiagnosis of echinococcosis and taeniasis in dogs and humans. *Parasitology*, 104: pp. 347-355.
- Allen, J.A. (1974). Baily's Hunting Directory. London
- Abbasi, I., Branzburg A., Campos-Ponce, M., Abdel Hafez, S.K., Raoul, F., Craig, P.S., and Hamburger, J. (2003). Copro-diagnosis of *Echinococcus granulosus* infection in dogs by amplification of a newly identified repeated DNA sequence. *American Journal of Tropical Medicine and Hygiene*, 69(3): pp. 324-330.
- Anon. (1960). Tenth Report Animal Health Trust, 1957-1960. Animal Health Trust, London
- Anon. (2008). Eradicating hydatid disease in Wales. *Veterinary Record*, 162: pp. 834.
- Babos, S. (1962). Untersuchungen über die Serodiagnostik der Echinokokkose. *Angewandte Parasitologie*, 3: pp. 2-4.
- Babos, S. and Nemeth, I. (1962). On the problems of the serodiagnosis of Echinococcosis. *Magyar Allatvosok Lapja*, (Budapest) 17: pp. 58-60.

- Balbinotti, H., Santosa, G.B., Badaraco, J., Arenda, A.C., Graichena, D.A.S., Haagd, K.L. and Zaha, A. (2012). *Echinococcus orteppi* (G5) and *Echinococcus granulosus* sensu stricto (G1) loads in cattle from Southern Brazil. *Veterinary Parasitology*, 188: pp. 255-260.
- Barabasi, S.S., Deplazes P., Cozma V., Pop S., Tivadar C., Bogolin I., Popescu R. (2010). *Echinococcus multilocularis* confirmed in Romania. *Scientific Parasitology*, 11(2): pp. 89-96.
- Batsch, A.J.G.C. (1786). Naturgeschichte der Bandwurm-gattung überhaupt und ihre Arten insbesondere, nach den neuen Beobachtungen in einem systematischen Auszuge. Halle.
- Baxter, J.T., Harper, W.O. and Bell, D. (1956). Hydatid disease. *Veterinary Record*, 68: pp. 104.
- Bily, S., Sterba, J. and Dykova, I., (1978). Results of an artificial feeding of eggs of *Taenia saginata* Goeze, 1782 to various beetle species. *Folia Parasitologica*, 25: pp. 257-260.
- Blutke, A., Hamel, D., Huttner, M., Gehlen, H., Romig, T., Pfister, K. and Hermanns, W. (2010). Cystic echinococcosis due to *Echinococcus equinus* in a horse from southern Germany. *Journal of Veterinary Diagnostic Investigation*, 22: pp. 458-462
- Boufana, B. (2008). Evaluation of Three PCR Assays for the Identification of the Sheep Strain (Genotype 1) of *Echinococcus granulosus* in Canid Feces and Parasite Tissues. *American Journal of Tropical Medicine and Hygiene*, 78(5): pp. 777-783.

- Boufana, B., Stidworthy, M.F., Bell, S., Chantrey, J., Masters, N., Unwin, S., Wood, R., Lawrence, R.P., Potter, A., McGarry, J., Redrobe, S., Killick, R., Foster, A.P., Mitchell, S., Greenwood, A.G., Sako, Y., Nakao, M., Ito, A., Wyatt, K., Lord, B. and Craig P.S. (2012). *Echinococcus* and *Taenia* spp. from captive mammals in the United Kingdom. *Veterinary Parasitology*, 190: pp. 95-103.
- Bowles, J. and McManus, D.P. (1993). Molecular variation in *Echinococcus*. *Acta Tropica*, 53: pp. 291-305.
- Bowles, J., Blair, D., and McManus, D.P. (1992a). Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. *Molecular and Biochemical Parasitology*, 54: pp. 165-174.
- Bowles, J., van Knapen, F. and McManus, D.P. (1992b). Cattle strain of *Echinococcus granulosus* and human infection. *Lancet*, 339: pp. 1358.
- Bretagne, S., Guillou, J.-P., Morand, M. and Houin, R. (1993). Detection of *Echinococcus multilocularis* DNA in fox faeces using DNA amplification. *Parasitology*, 106: pp. 193-199.
- Brown, C. (2004). Emerging zoonoses and pathogens of public health significance – an overview. *Revue Scientifique et Technique de l'Office International des Epizooties*, 23: pp. 435-442.
- Brouwer, A. and Willson, C. (2009). The history of hydatid control in Wales. *Government Veterinary Journal*, 20: pp. 32-35.
- Brunetti, E., Kern, P. and Vuitton, D.A. (2010). Expert consensus for the diagnosis and treatment of cystic and alveolar echinococcosis in humans. *Acta Tropica* 114: pp. 1-16.
- Budke, C.M. (2006) Global socioeconomic impact of cystic echinococcosis. *Emerging Infectious Diseases*, 12: pp. 296-303.

- Buishi, I., Walters, T., Guildea, Z., Craig, P.S. and Palmer, S. (2005a). Reemergence of canine *Echinococcus granulosus* infection, Wales. *Emerging Infectious Diseases*, 11(4): pp. 568-571.
- Buishi, I.E., Njoroge, E.M., Bouamra, O. and Craig, P.S. (2005b) Canine echinococcosis in northwest Libya: assessment of coproantigen ELISA, and a survey of infection with analysis of risk-factors. *Veterinary Parasitology*, 130: pp. 223-32.
- Cabrera, P.A., Parietti, S., Haran, G., Benavidez, U., Lloyd, S., Perera, G., Valledor, S., Gemmell, M.A. and Botto, T. (1996). Rates of re-infection with *Echinococcus granulosus*, *Taenia hydatigena*, *Taenia ovis* and other cestodes in rural dog population in Uruguay. *International Journal of Parasitology*, 26: pp. 79-83.
- Cabrera, M., Canova, S., Rosenzvit, M. and Guarnera, E. (2002). Identification of *Echinococcus granulosus* eggs. *Diagnostic Microbiology and Infectious Disease*, 44: pp. 29-34.
- Carmona, C., Perdomo, R., Carbo, A., Alvarez, C., Monti, J., Grauert, R., Stern, D., Perera, G., Lloyd, S., Bazini, R., Gemmell, M.A. and Yarzabal, L. (1998). Risk factors associated with human cystic echinococcosis in Florida, Uruguay: Results of a mass screening study using ultrasound and serology. *American Journal of Tropical Medicine and Hygiene*, 58: pp. 599-605.
- Chautan, M. Pontier, D. and Artois, M. (2000). Role of rabies in recent demographic changes in red fox (*Vulpes vulpes*) populations in Europe. *Mammalia*, 64: pp. 391-410.
- Chisholm, I.L., MacVicar, M.J. and Williams, H. (1983). Hydatid disease in the Western Isles. *Journal of Hygiene (London)*, 90(1): pp. 19-25.
- Chiou, M.T., Wang, F.I., Chang, P.H., Liu, C.H., Chian, R., Jeng, C.R., Cheng, C.H., Jou, J. and Pang, V.F. (2001). Hydatidosis in a Chapman's zebra (*Equus burchelli antiquorum*). *Journal of Veterinary Diagnostic Investigation*, 13: pp. 534-537

- Clarkson, M.J. and Walters, T.M. (1991). The growth and development of *Echinococcus granulosus* of sheep origin in dogs and foxes in Britain. *Annals of Tropical Medicine and Parasitology*, 85(1): pp. 53-61.
- Cook, B.R. (1964). The epidemiology of Echinococcus infection in Great Britain. II. The incidence of *Echinococcus granulosus* and some other cestodes in farm dogs in mid-Wales. *Annals of Tropical Medicine and Parasitology*, 58: pp. 147-152.
- Cook, B.R. (1989). The epidemiology of *Echinococcus granulosus* in Great Britain. V. The status of subspecies of *Echinococcus granulosus* in Great Britain. *Annals of Tropical Medicine and Parasitology*, 83(1): pp. 51-61.
- Craig, P.S. (1983). Immunodifferentiation between eggs of *Taenia hydatigena* and *T.pisiformis*. *Annals of Tropical Medicine and Parasitology*, 77: pp. 537-538.
- Craig, P.S. (1997). Immunodiagnosis of *Echinococcus granulosus* and a comparison of techniques for diagnosis of canine echinococcosis In: Compendium on Cystic Echinococcosis in Africa and in Middle Eastern Countries with special reference to Morocco, eds. Andersen, F.L., Ouhelli, H. and Kachani, M. Brigham Young University, Provo, Utah, 84602, pp. 85-118.
- Craig, P.S., Budke, C.M., Schantz P.M., Li, T., Qiu, J., Yang, Y., Zeyhle, E., Rogan, M.T. and Ito, A. (2007a). Human Echinococcosis: A Neglected Disease? *Tropical Medicine and Health*, 35(4): pp. 283-292.
- Craig, P.S., Gasser, R.B., Parada L., Cabera P., Parietti, S., Borgues, C., Acuttis, A., Aguilla, J., Snowden, K., and Paolillo, E. (1995). Diagnosis of canine echinococcosis: Comparison of coproantigen and serum antibody tests with arecoline purgation in Uruguay. *Veterinary Parasitology*, 56: pp. 293-301.

- Craig, P.S., Giraudoux, P., Shi, D., Bartholomot, B., Barnish, G., Delattre P., Quere J.P., Harraga, S. Bao, G. Wang, Y. Lu, F. Ito, A. and Vuitton, D.A. (2000). An epidemiological and ecological study of human alveolar echinococcosis transmission in south Gansu, China. *Acta Tropica*, 77, pp. 167-177.
- Craig, P.S., Macpherson, C.N. and Nelson, G.S. (1986). The identification of eggs of *Echinococcus* by immunofluorescence using a specific anti-oncospheral monoclonal antibody. *American Journal of Tropical Medicine and Hygiene*, 35(1): pp. 152-8.
- Craig, P.S., Macpherson, C.N.L., Watson-Jones, D.L. and Nelson, G.S. (1988). Immunodetection of *Echinococcus* eggs from naturally infected dogs and from environmental contamination sites in settlements in Turkana, Kenya. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 82 (2): pp. 268-274.
- Craig, P. S., McManus, D. P., Lightowers, M. W., Chabalgoity, J. A., Garcia, H. H., Gavidia, C. M., Gilman, R. H., Gonzalez, A. E., Lorca, M., Naquira, C., Nieto, A., Schantz, P. M. (2007b). Prevention and control of cystic echinococcosis. *The Lancet Infectious Diseases*, 7(6): pp. 385-394.
- Craig, P.S., Rogan M.T. and Allan, J.C. (1996). Detection, screening and community epidemiology of taeniid cestode zoonoses: cystic echinococcosis, alveolar echinococcosis and neurocysticercosis. *Advances in Parasitology*, 83: pp. 169-250.
- Craig, P.S., Rogan, M.T. and Campos-Ponce, M. (2003). Echinococcosis: disease, detection and transmission. In: *Echinococcosis: Transmission: Biology and Epidemiology*, eds Craig, P.S. and McManus, D.P. Cambridge University Press, *Parasitology (supplement)*, 127: 5-20.
- Craig, P.S. Woods, M.L., Boufana, B., O'Loughlin, B., Gimpel, J., Lett, W. and McManus D.P. (2012). Cystic echinococcosis in a fox-hound hunt worker, UK. *Pathogens and Global Health*, 106 (6): pp. 373-375.

- Cui, X., Li, H., Goradia, T.M., Langet, K., Kazazian, Jr., H.H., Galas, D. and Arnheim, N. (1989). Genetics Single-sperm typing: Determination of genetic distance between the γ -globin and parathyroid hormone loci by using the polymerase chain reaction and allele-specific oligomers (genetic recombination/linkage) *Proceedings of the National Academy of Sciences of the United States of America*, 86: pp. 9389-9393.
- Dailey, M.D. and Sweatman, G.K. (1965). The taxonomy of *Echinococcus granulosus* in the donkey and dromedary in Lebanon and Syria. *Annals of Tropical Medicine and Parasitology*, 59: 463-477.
- D'Alessandro, A., (1996). Polycystic echinococcosis in tropical America: *Echinococcus vogeli* and *Echinococcus oligarthrus*. *Acta Tropica*, 67: pp. 43-65.
- de la Rue, M.L., Takano, K., Brochado, J.F., Costa, C.V., Soares, A.G., Yamano, K., Yagi, K., Katoh, Y. and Takahashi, K. (2011). Infection of humans and animals with *Echinococcus granulosus* (G1 and G3 strains) and *E. ortleppi* in Southern Brazil. *Veterinary Parasitology*, 177: pp. 97-103.
- Deplazes, P., Alther, P., Tanner, I., Thompson, R.C. and Eckert, J. (1999). *Echinococcus multilocularis* coproantigen detection by enzyme-linked immunosorbent assay in fox, dog, and cat population. *Journal of Parasitology*, 85: pp. 115-121.
- Deplazes, P., Gottstein, B., Eckert, J., Jenkins, D. J., Ewald, D. and Jimenez-Palacios, S. (1992). Detection of *Echinococcus* coproantigens by enzyme-Linked immunosorbent assay in dogs, dingoes and foxes. *Parasitology Research*, 78: pp. 303-308.
- Deplazes, P., Gloor, S., Stieger, C. and Hegglin, D. (2002). Urban transmission of *Echinococcus multilocularis* In: Cestode zoonoses and cysticercosis-An Emergent and Global problem. Craig, P., Pawlowski, Z., eds. Amsterdam: IOS Press, pp. 287-97.

- Deplazes, P., Gottstein, B., Stingelin, Y. and Eckert, J. (1990). Detection of *Taenia hydatigena* coproantigens by ELISA in dogs. *Veterinary Parasitology*, 36: pp. 91-103.
- Dinkel, A., Von Nickisch-Rosenegk, M., Bilger, B., Merli, M., Lucius, R., and Romig, T. (1998). Detection of *Echinococcus multilocularis* in the definitive host: coprodiagnosis by PCR as an alternative to necropsy. *Journal of Clinical Microbiology*. 36(7): pp. 1871-1876.
- Dinkel, A., Njoroge, E.M., Zimmermann, A., Walz, M., Zeyhle, E., Elmahdic, I.E., Mackenstedt, U. and Romig T. (2004). A PCR system for detection of species and genotypes of the *Echinococcus granulosus*-complex, with reference to the epidemiological situation in eastern Africa. *International Journal for Parasitology*, 34: pp. 645-653.
- Dixon, J.B., Baker-Smith, J.K. and Greston, J.C. (1973). The incidence of hydatid cysts in horses in Great Britain. *The Veterinary Record*, 93(9): pp. 255.
- Eckert, J., (1997). Epidemiology of *Echinococcus multilocularis* and *E. granulosus* in central Europe. *Parassitologia*, 39: pp. 337-344.
- Eckert, J., Schantz, P., Gasser, R., Torgerson, P.R., Bessonov, A.S., Movsessian, S.O., Thakur, A., Grimm, F. and Nikogossian, M.A. (2001). Geographic distribution and prevalence. In: WHO/OIE manual on echinococcosis in humans and animals: a public health problem of global concern. eds. Eckert, J., Gemmell, M.A., Meslin, F-X., Pawlowski, Z.S. Paris: World Organisation for Animal Health, pp. 100-41.
- Economides, P. Christofi, G. and Gemmell, M.A. (1998). Control of *Echinococcus granulosus* in Cyprus and comparison with other island models, *Veterinary Parasitology*, 79(2): pp. 151-163.

- Eduardo, A., Guarnera, M.D., Elisa, M., Zantzottera, M.V.D, Hector Pereyra, M.D, Anibal, J. and Franco, M.V.D. (2001). Ultrasonographic diagnosis of ovine cystic echinococcosis. *Veterinary Radiology & Ultrasound*, 42: pp. 352-334.
- Edwards, -. (1962). Unpublished. Quoted by Forbes, L.S. (1964).
- Edwards, G.T., Walters, T.M. and Glossop, C. (2005). Re-emerging risk of hydatid disease in Wales. *Veterinary Record*, 157(24): pp. 782.
- Elayoubi, F.A. and Craig P.S. (2004). *Echinococcus granulosus* coproantigens: chromatographic fractionation and characterization. *Parasitology*, 128(04): pp. 455-465.
- Elayoubi, F.A., Fraser, A., Jenkins, D.J. and Craig, P.S. (2003). Partial characterisation of carbohydrate-rich *Echinococcus granulosus* coproantigens. *International Journal for Parasitology*, 33: pp. 1553-1559.
- Ellens, D.J. and Leeuw, P.W. (1977). Enzyme-Linked Immunosorbent Assay for the diagnosis of Rotavirus infections in calves. *Journal of Clinical Microbiology*, 6: pp. 530-532.
- European Commision (EC) February 2008 on agricultural insurance schemes, annex 13, fact sheet Ireland.
- Fastier, L.B. (1949). The effects of physical agents on hydatid scolex viability. *Parasitology*, 39: pp. 157-163.
- Filice, C., Strosselli, M., Brunetti, E., Colombo, P., Emmi, E., and D'Andrea F. (1991). P.A.I.R. (Puncture, Aspiration, Introduction, Reaspiration) with alcohol under US guidance of hydatid liver cysts. *Archivos de Ia Hidatidosis* 50: pp. 811-817.
- Forbes, L.S. and Cook, B.R. (1963). Hydatid disease in the United Kingdom. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 57: pp. 5.

- Foster, W. D. (1965). A history of parasitology. Livingstone, Edinburgh, United Kingdom.
- Fraser, A., Elayobi, F., and Craig, P.S, (2002). Detection of Cestode infection in Definitive Hosts. In: Cestode Zoonoses Echinococcosis and Cysticercosis. An Emergent and Global Problem. eds Craig P.S and Pawlowski Z.S. IOS press. pp. 157-172.
- Gasser, R.B., Lightowlers, M.W., Obendorf, D.L., Jenkins, D.J. and Rickard, M.D. (1988). Evaluation of a serological test system for the diagnosis of natural *Echinococcus granulosus* infection in dogs using *E.granulosus* protoscolex and oncosphere antigens. *Australian Veterinary Journal*, 65: pp. 369-373.
- Gemmell, M.A. (1977). Taeniidae: modification to the life span of the egg and regulation of tapeworm infections. *Experimental Parasitology*, 41: pp. 314-328.
- Gemmell, M.A. (1990). Australasian contributions to an understanding of the epidemiology and control of hydatid disease caused by *Echinococcus granulosus* - past, present and future. *International Journal for Parasitology*, 20(4): pp. 431-456.
- Gemmell, M.A., Lawson, J.R. and Roberts, M.G. (1987). Population dynamics in echinococcosis and cysticercosis: evaluation of the biological parameters of *Taenia hydatigena* and *T.ovis* and comparison with those of *Echinococcus granulosus* in dogs and sheep. *Parasitology*, 94: pp. 161-180.
- Gemmell, M.A., Roberts, M.G., Beard, T.C., and Lawson, J.R. (2001). Epidemiology: Quantitative epidemiology and transmission dynamics with special reference to *Echinococcus granulosus*. eds. *WHO/OIE Manual on Echinococcosis in Humans and Animals: A Public Health Problem of Global Concern*. Paris: Office Internationales Epizooties. pp. 1-19.

- Gemmell, M.A. and Schantz, P.M. (1997), Formulating Policies for Control of *Echinococcus granulosus*: An Overview of Planning, Implementation, and Evaluation. In: Andersen, F.L., Ouhelli, H. and Kachani M. (eds) Compendium of Cystic Echinococcosis, Brigham Young University Print Services, UT, USA, pp. 329-343.
- Giraudoux, P., Pleydell, D., Raoul, F., Quere, J., Wang, Q., Yang, Y., Vuitton, D.A., Qiu, J.M., Yang, W. and Craig, P.S. (2006). Transmission Ecology of *Echinococcus multilocularis*: What are the Ranges of Parasite Stability among Various Host Communities in China? *Parasitology International*, 55, S237-S246.
- Gloor, S. Bontadina, F., Hegglin, D., Deplazes, P., and Breitenmoser, U. (2001). The rise of urban fox populations in Switzerland. *Mammalian Biology*, 66, pp. 155-164.
- Goodfellow, M., Shaw S., and Morgan, E. (2006). Imported disease of dogs and cats exotic to Ireland: *Echinococcus multilocularis*. *Irish Veterinary Journal*, 59 (4): pp. 214-216.
- Goldine, A.J., Apt, W., Aguilera, X., Zulantay, I., Warhurst, D.C. and Miles, M.A. (1990). Efficient diagnosis of giardiasis among nursery and primary school children in Santiago, Chile by capture ELISA for detection of faecal Giardia antigens. *American Journal of Tropical Medicine and Hygiene*, 42: pp. 538-454.
- Gracey, (1962). Personal communication to Forbes, L.S. (1964).
- Grove, D. I. (1990). A history of human helminthology. CAB International, Wallingford, United Kingdom.
- Grundy, M.S. (1982). Preliminary observations using a multi-layer ELISA method for the detection of *Entamoeba histolytica* trophozoite antigens in stool samples. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 76: pp. 396-400.

- Guezala, MC., Rodriguez, S., Zamora, H., Garcia, H.H., Gonzalez, A.E., Tembo, A., Allan, J.C. and Craig, P.S. (2009). Development of a species-specific coproantigen ELISA for human *Taenia solium* taeniasis. *American Journal of Tropical Medicine and Hygiene*, 81(3): pp. 433-437.
- Guarnera, E.A., Parra, A., Kamenetzky, L., Garcia, G. and Gutierrez, A. (2004). Cystic echinococcosis in Argentina: evolution of metacestode and clinical expression in various *Echinococcus granulosus* strains. *Acta Tropica*, 92(2): pp. 153-9.
- Haller, M., Deplazes, P., Guscetti, F., Sardinas, J.C., Reichler, I., and Eckert, J. (1998). Surgical and chemotherapeutic treatment of alveolar echinococcosis in a dog. *Journal of the American Animal Hospital Association*, 34(4): pp. 309-314.
- Hanvanich M., Viscidi R., Laughon B.E., Bartlett J.D. and Yolken R.H. (1985). Stool desorbing activity: a possible cause of false-positive reactions in competitive enzyme immunoassays. *Journal of Clinical Microbiology*, 21: pp. 184-188.
- Hatch, C. (1967). Aspects of hydatid infestation in the horse in Ireland. Ph.D. Thesis. University of Dublin.
- Hatch, C. (1970). *Echinococcus granulosus equinus* in Irish dogs. *The Veterinary Record*, pp. 632-3.
- Hatch, C. (1972). Hydatidosis in Irish horses. *Irish Veterinary Journal*, 26: pp. 74-77.
- Hatch, C. (1975). Observations on the epidemiology of equine hydatidosis in Ireland. *Irish Veterinary Journal*, 29(10): pp. 155-157.
- Hatch, C. and Smyth, J.D. (1975). Attempted infection of sheep with *Echinococcus granulosus equinus*. *Research in Veterinary Science*, 19: pp. 340.
- Hay, -. (1962). Unpublished. Quoted by Forbes, L.S. (1964).

- Heinz, H.J. and Brauns, W. (1955). The ability of flies to transmit ova of *Echinococcus granulosus* to human foods. *South African Journal of Medical Science*, 20: pp. 131-132.
- Higginson, A.H. (1948). Foxhunting – theory and practice. London : Collins, 256.
- Holcman, B. and Heath, D.D. (1997). The early stages of *Echinococcus granulosus* development. *Acta Tropica*, 64: pp. 5-17.
- Howell, N. (1940). Hydatid disease in Wales. *Veterinary Record*, 52: pp. 493-495.
- Howells, C.H. and Taylor, R.J. (1980). Hydatid disease in Mid-Wales. *Journal of Clinical Pathology*, 33: pp. 701.
- Howkins, A.B., Gemmell, M.A. and Smyth, J.D. (1965). Experimental transmission of *Echinococcus* from horses to foxes. *Annals of Tropical Medicine and Parasitology*, 59: pp. 457-462.
- HPA, 2013. [Epidemiological data] Available at:
<http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/HydatidDisease/EpidemiologicalData/> [Accessed 21 July 2013].
- Huang, Y., Yang, W., Qiu, J., Chen, X., Yang, Y., Qiu, D., Xiao, N., Xiao, Y., and Heath, D. (2007). A modified coproantigen test used for surveillance of *Echinococcus* spp. in Tibetan dogs. *Veterinary Parasitology*, 149: pp. 229-238.
- Hunting Act 2004 (c.37) London: HMSO
- Huttner, M., Nakao, M., Wassermann, T., Siefert, L., Boomker, J.D., Dinkel, A., Sako, Y., Mackenstedt, U., Romig, T. and Ito, A. (2008). Genetic characterization and phylogenetic position of *Echinococcus felidis* (Cestoda: Taeniidae) from the African lion. *International Journal of Parasitology*, 38: pp. 861-868.

- Huttner, M., Siefert, L., Mackenstedt, U. and Romig, T. (2009). A survey of *Echinococcus* species in wild carnivores and livestock in East Africa. *International Journal for Parasitology*, 39: pp.1269-1276.
- Isaacs, R., Beeching, N. and Ellis-Pegler, R. (1985) Notification of human hydatid disease in New Zealand. *New Zealand Medical Journal*, 98: pp. 967.
- Ito, A. (2002). Review article Serologic and molecular diagnosis of zoonotic larval cestode infections. *Parasitology International*, 51: pp. 221-235.
- IWA, 2011. [press release] Available at: <http://www.clickonwales.org/wp-content/uploads/16_Factfile_Agriculture&food.pdf> [Accessed 21 Jan 2011].
- Jackson, S.G., Yip Chuck, D.A. and Brodsky, M.H. (1985). A double antibody sandwich enzyme immunoassay for *Clostridium perfringens* Type A enterotoxine detection in stool specimens. *Journal of Immunological Methods*, 83: pp. 141-150.
- Jenkins, D. J., Fraser, A., Bradshaw, H. and Craig, P.S. (2000). Detection of *Echinococcus granulosus* coproantigens in Australian canids with natural or experimental infection. *Journal of Parasitology*, 86(1): pp. 140-145.
- Jenkins, D.J. and Morris, B. (1991). Unusually heavy infections of *Echinococcus granulosus* in wild dogs in south-eastern Australia. *Australian Veterinary Journal*. 68(1): pp. 36-7.
- Jenkins, D.J., Romig, T. and Thompson, R.C.A. (2005). Emergence/re-emergence of *Echinococcus* spp. - a global update. *International Journal for Parasitology*, 35(11-12): pp. 1205-1219.
- Kamenetzky, L., Gutierrez, A.M., Canova, S.G., Haag, K.L., Guarnera, E.A., Parra, A., Garcia, G.E. and Rosenzvit, M.C. (2002). Several strains of *Echinococcus granulosus* infect livestock and humans in Argentina. *Infection, Genetics and Evolution*, 2: pp. 129-136.

- Kumaratilake, L.M. and Thompson, R.C.A. (1982). A Review of the taxonomy and speciation of the genus *Echinococcus* Rudolphi 1801. *Zeitschrift für Parasitenkunde Parasitology Research*, 68: pp. 121-146.
- Kumaratilake, L.M., Thompson, R.C.A. and Eckert, J. (1986). *Echinococcus granulosus* of equine origin from different countries possess uniform morphological characteristics. *International Journal for Parasitology*, 16(5): pp. 529-540.
- Lahmar, S., Lahmar, S., Boufana, B., Bradshaw, H., Craig, P.S. Screening for *Echinococcus granulosus* in dogs: Comparison between arecoline purgation, coproELISA and coproPCR with necropsy in pre-patent infections. (2007). *Veterinary Parasitology*, 144(3-4): pp. 287-292.
- Lapage, G. (1956). In: Monnig H. O. *Veterinary Helminthology and Entomology*. London: Tindall and Cox. pp. xv + 511.
- Larrieu, E., Mercapide, C. and Del Carpio, M. (1999). Evaluation of the losses produced by hydatidosis and cost-benefit analysis of different strategic interventions of control in the province of Rio Negro, Argentina. *Parasitology*, 33, pp. 122-128.
- Lavikainen, A., Lehtinen, M.J., Laaksonen, S., Agren, E., Oksanen, A. and Meri, S. (2006). Molecular characterization of *Echinococcus* isolates of cervid origin from Finland and Sweden. *Parasitology*, 133, 565-570.
- Lavikainen, A., Lehtinen, M.J., Meri, T., Hirvela-Koski, V. and Meri, S. (2003). Molecular genetic characterization of the Fennoscandian cervid strain, a new genotype group (G10) of *Echinococcus granulosus*. *Parasitology*, (127): pp. 207-215.
- Lawson, J.R. and Gemmell, M.A. (1990). Transmission of taeniid tapeworm egg via blowflies to intermediate hosts. *Parasitology*, 100: pp. 143-146.

- Le, T.H., Pearson, M.S., Blair, D., Dai, N., Zhang, L.H. and Mcmanus, D.P. (2002). Complete mitochondrial genomes confirm the distinctiveness of the horse-dog and sheep-dog strains of *Echinococcus granulosus*. *Parasitology*, 124(1): pp. 97-112.
- Leiby, P.D. and Kritsky, D.C. (1972). *Echinococcus multilocularis*: a possible domestic life cycle in central North America and its public health implications. *The Journal of Parasitology*, 58(6): pp. 1213-5.
- Lequin, R.M. (2005). Enzyme Immunoassay (EIA)/Enzyme-Linked Immunosorbent Assay (ELISA). *Clinical Chemistry*, 51: pp. 2415-2418.
- Li, T., Ito, A., Nakaya, K., Qiu, J., Nakao, M., Zhen, R., Xiao, N., Chen, X., Giraudoux, P., Craig, P.S. (2008). Species identification of human echinococcosis using histopathology and genotyping in northwestern China. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 102(6): pp.585-590.
- Lloyd, S., Martin, S.C., Walters, T.M.H. and Soulsby, E.J.L. (1991). Use of sentinel lambs for early monitoring of the South Powys Hydatidosis Control Scheme: Prevalence of *Echinococcus granulosus* and some other helminths. *Veterinary Record*, 129: pp. 73-76.
- Lloyd, S., Walters, T.M. and P.S. (1998). Use of sentinel lambs to survey the effect of an education programme on control of transmission of *Echinococcus granulosus* in south Powys, Wales. *Bulletin of The World Health Organisation*, 76(5): pp. 469-473.
- Logan, J.S. (1971). The low incidence of hydatidosis in man in Ulster and a record of a case. *Ulster Medical Journal*. 41(1): pp. 33-38.
- Maass, M., Delgado, E. and Knobloch, J. (1991). Detection of *Taenia solium* antigens in merthiolate-formaline preserved stool samples. *Tropical Medicine and Parasitology*, 42: pp. 112-114.

- MacDonald, D.W. (1984). The Encyclopaedia of Mammals: 1. George Allen & Unwin, London, pp. 84-85.
- Machnicka, B., Krawczuk, S. (1988). *Hymenolepis diminuta* antigen: detection in faeces of rats. *Bulletin of the Polish Academy Of Sciences-Biological Sciences*, 36: pp. 103-6.
- Macpherson, C.N.L., Bartholomot, B. and Frider, B. (2003). Application of ultrasound in diagnosis, treatment, epidemiology, public health and control of *Echinococcus granulosus* and *E. multilocularis*. *Parasitology*, (supplement) 127, (S1): S21-S35.
- Macpherson, C.N.L. and Craig, P.S. (2000). Dogs and Cestode Zoonoses. In: Dogs, Zoonoses and Public Health, eds. Macpherson, C.N.L., Meslin, F.X. and Wandeler, A.I. Wallingford, Oxon, UK, CABI Publishing. pp. 177-201.
- Macpherson, C.N.L., French, C.M., Stevenson, P., Karstad, L. and Karstad, J.H. (1985). Hydatid disease in the Turkana District of Kenya. IV. The prevalence of *Echinococcus granulosus* infections in dogs and observations on the role of the dog in the lifestyle of the Turkana. *Annals of Tropical Medicine Parasitology*, (79): pp. 57-61.
- Macpherson, C.N.L., Karstad, L., Stevenson, P., and Arundel, J.H. (1983). Hydatid disease in the Turkana District of Kenya. III. The Significance of Wild Animals in the Transmission of *Echinococcus granulosus*, with particular reference to Turkana and Masailand in Kenya. *Annals of Tropical Medicine and Parasitology*, 77 (1): pp. 66-73.
- Macpherson, C.N.L. and Wachira, T.M. (1997). Cystic Echinococcosis in Africa South of the Sahara, In: Compendium on cystic Echinococcosis in Africa and in Middle Eastern Countries with special reference to Morocco, eds Andersen, F.L. Ouhelli H. and Kachani, M. Brigham Young University, Provo, Utah 84602, pp. 245-277.

- Malgor, R., Nonaka, N., Basmadjian, I., Sakai, H., Cartimbula, B. Oku, Y. Carmona, C. and Kamiya, M. (1997). Coproantigen detection in dogs experimentally and naturally infected with *Echinococcus granulosus* by a monoclonal antibody-based enzyme-linked immunosorbent assay. *International Journal for Parasitology*, 27(12): pp. 1605-1612.
- Maravilla, P., Thompson, R.C.A., Palacios-Ruiz, J.A., Estcourt, A., Ramirez-Solis, E., Mondragon-de-la-Pena, C., Moreno-Moller, M., Cardenas- Mejia, A., Mata Miranda, P., Aguirre-Alcantara, M.T., Bonilla- Rodriguez, C. and Flisser, A. (2004). *Echinococcus granulosus* cattle strain identification in an autochthonous case of cystic echinococcosis in central Mexico. *Acta Tropica*, 92: pp. 231-236.
- Mastin, A., Brouwer, A., Fox, M., Craig, P.S., Guitián, J., Li, W. and Stevens K. (2011). Spatial and temporal investigation of *Echinococcus granulosus* coproantigen prevalence in farm dogs in South Powys, Wales. *Veterinary Parasitology* 178: pp. 100-107.
- Mathis A. and Deplazes P. (2006). Copro-DNA tests for diagnosis of animal taeniid cestodes. *Parasitology International*, 55(1): pp. S87-S90.
- Mathis, A., Deplazes, P. and Eckert, J. (1996). An improved test system for PCR-based specific detection of *Echinococcus multilocularis* eggs. *Journal of Helminthology*, 70: pp. 219-22.
- Matsuo, K., Shimizu, M., Nonaka, N., Oku, Y. and Kamiya, M. (2000). Development and sexual maturation of *Echinococcus vogeli* in an alternative definitive host, Mongolian gerbil (*Meriones unguiculatus*) *Acta Tropica*, 75: pp. 323-330.
- Matoff, K. and Kolev, G. (1964). The role of hair, muzzle and paws of echinococcic dogs in the epidemiology of echinococcosis. *Zeitschrift for Tropenmedicine Parasitologie*, 15: pp. 452-460.

- Maxson, A.D., Wachira, T.M., Zeyhile, E.E., Fine, A., Mwangi, T.W. and Smith, G. (1996). The use of ultrasound to study the prevalence of hydatid cysts in the right lung and liver of sheep and goat in Turkana, Kenya. *International Journal for Parasitology*, 26: pp. 1335-1338.
- McCurnin, M. D. (1998). Anticestodal Drugs. In: Clinical Text book for Veterinary Technicians (4th edition), eds. W.B .Sanders. Philadelphia, Pennsylvania. pp. 639.
- McManus, D.P. (2002). The molecular epidemiology of *Echinococcus granulosus* and cystic hydatid disease. *Transaction of the Royal Society of Tropical Medicine and Hygiene*, 96: pp. 151-157.
- McManus, D.P. and Thompson, R.C.A., (2003). Molecular epidemiology of cystic echinococcosis. *Parasitology* 127: pp. 37-51.
- McManus, D.P., Thompson, R.C.A. and Lymbery, A.J. (1989). Comment on the status of *Echinococcus granulosus* in the UK. *Parasitology Today*, 5(11): pp. 365-367.
- McManus, D.P., Zhang, W., Li, J. and Bartley, P.B. (2003). Echinococcosis. *Lancet*, 362: pp. 1295-305.
- Menghebat, L., Jiang, L. and Chai, J. (1993). A retrospective survey for surgical cases of cystic echinococcosis in the Xinjiang Uygur Autonomous Region, PRC (1951-90). In: *Compendium on cystic echinococcosis with special reference to the Xinjiang Uygur Autonomous Region, People's Republic of China*. Andersen, F., Chai, J., Liu, F., eds. Provo: Brigham Young University Print Services, pp. 135-45.
- Miller, W.C. and Poynter, D. (1956). Hydatid cysts in a thoroughbred mare. *Veterinary Record*, 68: pp. 51-53.

- Ming, R., Dennis Tolley, H., Andersen, F.L., Chai, J. and Sultan, Y. (1992) Frequency distribution of *Echinococcus granulosus* hydatid cysts in sheep populations in the Xinjiang Uygur Autonomous Region, China. *Veterinary Parasitology*, 44: pp. 67-75.
- Moks, E., Jogisalu, I., Saarma, U., Talvik, H., Jarvis, T. and Valdmann, H. (2006). Helminthologic survey of the wolf (*Canis lupus*) in Estonia, with an emphasis on *Echinococcus granulosus*. *Journal of Wildlife Diseases*, 42: 359-365.
- Moks, E., Jogisalu, I., Valdmann, H. and Saarma, U. (2008). First report of *Echinococcus granulosus* G8 in Eurasia and a reappraisal of the phylogenetic relationships of 'genotypes' G5-G10. *Parasitology*, 135(05): pp. 647-654.
- Moro, P.L. Bonifacio, N., Gilman, R.H., Lopera, L., Silva, B., Takumoto, R., Verastegui, M. and Cabrera, L., (1999). Field diagnosis of *Echinococcus granulosus* infection among intermediate and definitive hosts in an endemic focus of human cystic echinococcosis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 93(6) pp. 611-615.
- Moro, P. and Schantz, P.M., (2009). Echinococcosis: a review. *International Journal of Infectious Diseases*, 13: pp. 125-133.
- Mukbel, R.M. Torgerson, P.R. and Abo-Shehada, M.N. (2000). Prevalence of hydatidosis among donkeys in northern Jordan. *Veterinary Parasitology*, 88: pp. 35-42.
- Mullis K. (1990). The unusual origin of the polymerase chain reaction. *Scientific American*. pp. 56-65.
- Mwambete, D.K., Gordo, P.F and Bandera, C.C. (2004). Genetic identification and host range of the Spanish strains of *Echinococcus granulosus*. *Acta Tropica*, 91(2): pp. 87-93.

- Nageswaran, C., Devaney, E. and Craig, P.S. (1992). Coproantigen detection in rats experimentally infected with *Strongyloides ratti*. *Parasitology*, 108: pp. 335-342.
- Nakao, M., McManus, D.P., Schantz, P.M., Craig, P.S. and Ito, A. (2007). A molecular phylogeny of the genus *Echinococcus* inferred from complete mitochondrial genomes. *Parasitology*, 134: pp. 713-722.
- Naunyn, B. (1863). Ueber die zu *Echinococcus hominis* gehörige täen. Arch. Anat. Physiol. Wiss. Med. 4:412-416.
- Nelson, G.S. (1972). Human behaviour in the transmission of parasitic disease. In: *Behavioural Aspects of Parasite Transmission*, eds Canning, E.U. & Wright, C.A., London: *Zoological Journal of the Linnean Society*, pp. 109-122.
- Njoroge, E.M., Mbithi, P.M.F., Gathuma, J.M., Wachira, T.M., Magambo, J.K. and Zeyhle, E.E. (2000). Application of ultrasography in prevalence studies of hydatid cysts in goats in northwestern Turkana, Kenya and Toposaland, southern Sudan. *Onderstepoort Journal of Veterinary Research*, 67: pp. 251-255.
- Nonaka, N., Iida, M., Yagi, K., Ito, T., Ooi, H.K., Oku, Y. and Kamiya, M. (1996). Time course of coproantigen excretion in *Echinococcus multilocularis* infections in foxes and an alternative definitive host, golden hamsters. *International Journal for Parasitology*, 26(11): pp. 1271-1278.
- Ortlepp, R.J. (1937). South African Helminths, Part I. *Onderstepoort Journal of Veterinary Science and Animal Industry*, 9: pp. 311-336.
- Pallas, P.S. (1766). *Miscellanea zoologica: Quibus novae imprimus atque obscurae animalium species. Describuntur et observationibus iconibusque illustrantur.* Petrum van Cleff, The Hague, The Netherlands.
- Palmer, S.R and Biffin, A.H. (1987). The changing incidence of human hydatid Disease in England and Wales. *Epidemiology and Infection*, 99: pp. 693-700.

- Palmer, S.R, Biffin, A.H, Craig, P.S., and Walters, T.M. (1996). Control of hydatid disease in Wales. *British Medical Journal*, 312: pp. 674-675.
- Pawlowski, Z.S., Eckert, J., Vuitton, D.A., Ammann, R., Kern, P., Craig, P.S., Dar, K.F., DeRosa, F., Filice, C., Gottstein, B., Grimm, F., Macpherson, C.N.L., Sato, N., Todorov, T., Uchino, J., Von Sinner, W. and Wen, H., (2001). Echinococcosis in humans: clinical aspects, diagnosis, and treatment. Eckert, J., Gemmell, M.A., Meslin, F-X., Pawlowski, Z.S., eds. WHO/OIE Manual on Echinococcosis in Humans and Animals. Paris: OIE, pp. 48-50.
- Pearson, M., Le, T.H., Blair, D., Dai, T.H.N. and McManus, D.P. (2002). Molecular taxonomy and strain analysis in *Echinococcus*. In: *Cestode Zoonoses: Echinococcosis and Cysticercosis: An Emergent and Global Problem*, eds Craig, P.S. and Pawloski, J., Amsterdam: ISO Press, pp. 205-219.
- Petavy, A.F., Deblock, S. and Walbaum, S. (1991). Life cycles of *Echinococcus multilocularis* in relation to human infection. *Journal of Parasitology*, 77, pp. 133-137.
- Rausch, R.L. (1953). The taxonomic value and variability of certain structures in the cestode genus *Echinococcus* (Rudolphi, 1801) and a review of recognized species. *Thapar Commemoration Volume*. pp. 233-246.
- Rausch, R.L. (1967). A consideration of infraspecific categories in the genus *Echinococcus* Rudolphi, 1801 (Cestoda: Taeniidae). *The Journal of Parasitology*, 53(3): pp. 484-491.
- Rausch, R.L. (1968). Taxonomic characters in the genus *Echinococcus* (Cestoda: Taeniidae). *Bulletin. World Health Organisation*, 39: pp. 1-4.
- Rausch, R.L. (1986). Life-cycle patterns and geographical distribution of *Echinococcus* species. In: *The Biology of Echinococcus and Hydatid Disease.*, eds Thompson R.C.A. George Allan and Unwin, London, pp. 44-80.

- Rausch, R.L. (1995). Life-cycle patterns and geographical distribution of echinococcus species, eds. Thompson, R.C.A., Lymbery, A.J. *Echinococcus* and hydatid disease. CAB International, Wallingford, UK, pp. 89-134.
- Rausch, R.L. and Bernstein, J.J. (1972). *Echinococcus vogeli* sp. n. (Cestoda: Taeniidae) from the Bush Dog, *Speothos venaticus* (Lund). *Tropenmedizen and Parasitologie*, 23: pp. 25-34.
- Rausch, R.L. and D'Alessandro, A. (2002). The epidemiology of echinococcosis caused by *Echinococcus oligarthrus* and *E. vogeli* in the neotropics. In: *Cestode Zoonoses Echinococcosis and Cystercercosis. An Emergent and Global Problem*, eds. Craig, P.S. and Pawloski, P.Z. Amsterdam: ISO Press. pp. 107-130.
- Rausch, R.L. and Nelson, G.S. (1963). A review of the genus *Echinococcus* Rudolphi 1801. *Annals of Tropical Medicine and Parasitology*, 57(2): pp. 127-135.
- Rausch, R.L. and Schiller, E.L. (1956). Studies on the Helminth Fauna of Alaska. XXV. The Ecology and Public Health Significance of *Echinococcus sibiricensis* Rausch & Schiller, 1954, on St. Lawrence Island. *Faculty Publications from the Harold W. Manter Laboratory of Parasitology*, Paper 531.
- Rausch, R.L., Rausch, V.R., D'Alessandro, A. (1978). Discrimination of the larval stages of *Echinococcus oligarthrus* (Diesing, 1863) and *E. vogeli* Rausch and Bernstein, 1972 (Cestoda: Taeniidae). *American Journal of Tropical Medicine and Hygiene*, 27: pp. 1195-1202.
- Rausch, R.L., Wilson, J.F. and Schantz, P.M. (1990). A programme to reduce the risk of infection by *Echinococcus multilocularis*: the use of praziquantel to control the cestode in a village in the hyperendemic region of Alaska. *Annals of Tropical Medicine and Parasitology*, 84: pp. 239-250.
- Redi, F. (1684). Osservazioni intorno agli animali viventi che si trovano negli animali viventi. Pietro Martini, Florence, Italy.

- Rezabek, G.B., Giles, R.C. and Lyons, E.T. (1993) *Echinococcus granulosus* hydatid cysts in the livers of two horses. *Journal of Veterinary Diagnostic Investigation*, 5: pp. 122-125.
- Rishi, A.K. and McManus, D.P. (1987). Genomic cloning of human *Echinococcus granulosus* DNA: isolation of recombinant plasmids and their use as genetic markers in strain characterization. *Parasitology*, 94: pp. 369-383.
- Rogan, M.T. and Craig, P.S. (2002). Immunological approaches for transmission and Epidemiological Studies in Cestode Zoonoses- the role of serology in human infection. In: Cestode Zoonoses Echinococcosis and Cysticercosis: An Emergent and Global Problem, eds. Craig, P. and Pawloski, Z. ISO Press. pp. 135-145.
- Rogan, M.T., Craig, P.S., Zeghle, E., Romig, T., Lubano, G.M., and Deshan, L. (1991). Evaluation of a rapid dot-ELISA as a field test for the diagnosis of cystic hydatid disease. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 85: pp. 773-777.
- Rogan, M.T., Hai W.Y., Richardson, R., Zeyhle, E. and Craig, P.S. (2006). Hydatid cysts: does every picture tell a story? *Trends in Parasitology*, Volume 22(9): pp. 431–438.
- Rogan, M.T., Morris D.L., Pritchard, D.I. and Perkins, A.C. (1990). *Echinococcus granulosus*: the potential use of specific radiolabelled antibodies in diagnosis by immunoscintigraphy. *Clinical and Experimental Immunology*, 80: pp. 225 - 231.
- Ross, J.G. and Fairley, J.S. (1969). Studies of disease in the red fox (*Vulpes vulpes*) in Northern Ireland. *Journal of Zoology*, 157: pp. 375-381.
- Sakai H., Malgor R., Basmadjian I., Gallardo R., Carmona C., Sato H., Oku Y. and Kamiya M. (1995). An enzyme linked immunosorbent assay (ELISA) for the detection of *Echinococcus granulosus* coproantigen in dogs. *Japanese Journal of Parasitology*, 44: pp. 453-461.

- Sacket, D.L., Haynes, R.B. and Tugwell, P. (1985). *Clinical Epidemiology: A basic Science for Clinical Medicine*. Little, Brown & Co. Boston. USA.
- Schantz, P.M. (1982). Echinococcosis. In: *CRC Handbook Series in Zoonoses. Section C. Parasitic Zoonoses*, Volume I, eds. Jacobs, L. and Arambulo, P., CRC press, pp. 231-277.
- Schantz, P.M., Chai, J., Craig, P.S., Eckert, J., Jenkins, D.J., Macpherson, C.N.L. and Thakur, A. (1995). Epidemiology and control of hydatid. In: *Echinococcus and Hydatid disease*, eds. Thompson, R.C.A. and Lymbery, A.L. CAB International, Wallingford, Oxon, pp. 233-331.
- Schantz, P.M., Wang, H., Qiu, J., Liu, F.J., Saito, E., Emshoff, A., Ito, A., Roberts, J.M. and Delker, C. (2003). Echinococcosis on the Tibetan Plateau: prevalence and risk factors for cystic and alveolar echinococcosis in Tibetan populations in Qinghai Province, China. *Parasitology*, 127 (S1): pp. S109-S120.
- Schantz, P.M. and Schwabe, C.W. (1969). Worldwide status of hydatid disease control. *Journal of the American Veterinary Medical Association*, (155): pp. 2104-2121.
- Scherer, U., Weinzierl, M., Sturm, R., Schildberg, F.W., Zrenner, M. and Lissner, J. (1978). Computed tomography in hydatid disease of the liver: a report on 13 cases. *Journal of Computer Assisted Tomography*, 2(5): pp. 612-617.
- Silverman, P.H. and Griffiths, R.B. (1955). A review of methods of sewage disposal in Great Britain, with special reference to the epizootiology of *Cystercercus bovis*. *Annals of Tropical Medicine and Parasitology*, 49: pp. 436-450.
- Sinclair, K.B. (1956). *Echinococcus* infection in the fox. *Veterinary Record*, 68: pp. 104.

- Sirisinha, S., Chawengkirtikul, R., Sermswan, R., Monkolsuk, S. and Panyim, S. (1991). Detection of *Opisthorchis viverrini* by monoclonal antibody based ELISA and DNA hybridization. *American Journal of Tropical Medicine and Hygiene*, 44: pp. 140-145.
- Smyth, J.D. (1976). Strain differences in *Echinococcus granulosus*, with special reference to the status of equine hydatidosis in the United Kingdom. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 71: pp. 93-100.
- Smyth J.D. (1982). Speciation in *Echinococcus*: biological and biochemical criteria. *Revista Iberica de Parasitologica*, pp. 25-34.
- Smyth, J.D. and Davies, Z. (1974). Occurrence of physiological strains of *Echinococcus granulosus* demonstrated by in vitro culture of protoscoleces from sheep and horse hydatid cysts. *International Journal for Parasitology*, 4: pp. 443-5.
- Smego, Jr., R.A., Bhatti, S., Khaliq, A.A. and Beg, M.A. (2003). Percutaneous aspiration-injection-reaspiration drainage plus albendazole or mebendazole for hepatic cystic echinococcosis: a meta-analysis. *Clinical Infectious Diseases*, 37: pp. 1073-1083.
- Soulsby, E.J.L (1968) In: *Helminths, Arthropods and Protozoa of Domesticated Animals*. Veterinary Helminthology and Entomology (6th edition), Bailliere Tindall, Cassel Ltd. London. pp. 126.
- Soulsby, E.J.L. (1982). In: *Helminths, Arthropods and Protozoa of Domesticated Animals*. Veterinary Helminthology and Entomology (6th edition), Bailliere Tindall, Cassell Ltd. London, pp.126.
- Sousa, O.E. and Thatcher, V. E. (1969). Observations of the life-cycle of *Echinococcus oligarthrus* (Diesing, 1863) in the Republic of Panama. *Annals of Tropical Medicine and Parasitology*, 63: pp. 165-175.

- Southwell, T. (1927). Experimental infection of the cat and the fox with the adult *Echinococcus*. *Annals of Tropical Medicine and Parasitology*, 21: pp. 155-163.
- Sreter, T., Szell, Z., Sreter-Lancz, Z., Varga, I. (2004). *Echinococcus multilocularis* in Northern Hungary. *Emerging Infectious Diseases*, 107: pp. 1344-1346.
- Stallbaumer, M.F., Clarkson, M.J., Bailey, J.W. and Pritchard, J.E. (1986). The epidemiology of hydatid disease in England and Wales. *Journal of Hygiene (Cambridge)*, 96, 121-127.
- Stefanic, S., Shaikenov, B.S., Deplazes, P., Dinkel, A., Torgerson, P.R., Mathis, A. (2004). Polymerase chain reaction for detection of patent infections of *Echinococcus granulosus* ("sheep strain") in naturally infected dogs. *Parasitology Research*, 92: pp. 347-351.
- Swann, W.G. (1957). Annual report of the Medical Officer of Health for the City of Belfast.
- Tappe, D., Kern, P., Frosch, M. and Kern, P. (2010). A hundred years of controversy about the taxonomic status of *Echinococcus* species. *Acta Tropica*, 115: pp. 167-174.
- Tappe, D., Stich, A. and Frosch, M., (2008). Emergence of Polycystic Neotropical Echinococcosis. *Emerging Infectious Diseases*, 14(2): pp. 292-297.
- Thakur, A.S. (1999). Eradication of hydatid disease in South America. XIX *International Congress of Hydatidology*, 20-24 September 1999. *Archivos Internacionales De La Hydatidosis*, Vol. XXXIII: pp. 55-61.
- Thakur, A.S., Prezioso, U. and Marchevsky, N. (1978). Efficacy of Droncit against *Echinococcus granulosus* in dogs. *American Journal for Veterinary Research*, 39: pp. 859-860.

- Thevenet, P. S., Jensen, O., Drut, R., Cerrone, G.E., Grenovero, M.S., Alvarez, H.M., Targovnik, H.M., Basualdo, J.A. (2005). Viability and infectiousness of eggs of *Echinococcus granulosus* aged under natural conditions of inferior arid climate. *Veterinary Parasitology* 133: pp. 71-77.
- Thompson, R.C.A. (1974). Biological aspects of equine echinococcosis in Great Britain. *Proc. Third International Congress of Parasitology, Factam Munich*, 1: pp. 537-538.
- Thompson, R.C.A. (1975). Studies on equine hydatidosis in Great Britain. Ph.D. Thesis. The University of London.
- Thompson, R.C.A. (1995). Biology and systematics of *Echinococcus*. In: *Echinococcus and Hydatid Disease*. ed. Thompson, R. C. A. & Lymbery, A. J. Wallingford, Oxon, UK, CAB International. pp. 1-50.
- Thompson, R.C.A. (2008). The taxonomy, phylogeny and transmission of *Echinococcus*. *Experimental Parasitology*. 119: pp. 439-446.
- Thompson, R.C.A., Kumaratilake, L.M.N and Eckert, D.J. (1984). Observations on *Echinococcus granulosus* of cattle origin in Switzerland. *International Journal for Parasitology*, 14 (3): pp. 283-291.
- Thompson, R.C.A. and Lymbery, A.J. (1988). The nature, extent and significance of variation within the genus *Echinococcus*. *Advances in Parasitology*. 27: pp. 210-248.
- Thompson, R.C.A. and Lymbery, A.J. (1990). *Echinococcus*: Biology and strain variation. *International Journal for Parasitology*, (4): pp. 457-470.
- Thompson, R.C.A., Lymbery, A.J. and Constantine, C.C. (1995). Variation in *Echinococcus*: Towards a taxonomic revision of the genus. *Advances in Parasitology*. 35: pp. 145-175.

- Thompson, R.C.A. and McManus, D.P. (2001). Aetiology: parasites and life cycles. In: Eckert, J., Gemmell, M. A., Meslin, F. X. and Pawlowski, Z. S. (eds.), *Manual on Echinococcus in Humans and Animals a Public Health Problem of Global Concern. WHO/OIE*. pp. 1-19.
- Thompson, R.C.A., and McManus, D.P. (2002). Towards a taxonomic revision of the genus *Echinococcus*. *Trends in Parasitology*, 18(10): pp. 452-457.
- Thompson, R.C.A. and Smyth, J.D. (1974). Potential danger of hydatid disease of Horse/dog origin. *British Medical Journal*, pp. 807.
- Thompson, R.C.A. and Smyth, J.D. (1975). Equine hydatidosis: a review of the current status in Great Britain and the results of an epidemiological survey. *Veterinary Parasitology*, 1: pp. 107-127.
- Thompson, R.C.A. and Smyth, J.D. (1976). Attempted infection of the rhesus monkey (*Macaca mulatta*) with the British horse strain of *Echinococcus granulosus*. *Journal of Helminthology*, 50: pp. 175-177.
- Torgerson, P.R. and Budke, C.M. (2003). Echinococcosis – an international public health challenge. *Research in Veterinary Science*, 74: pp. 191-202.
- Torgerson, P.R. and Heath, D.D. (2003). Transmission dynamics and control options for *Echinococcus granulosus*. In: *Echinococcosis: Transmission: Biology and epidemiology*, eds Craig, P.S. and McManus, D.P. Cambridge University Press. *Parasitology* (supplement), 127: pp. 143-158.
- Torgerson, P.R., Pilkington, J. Gulland, F.M.D. and Gemmell, M.A. (1995). Further evidence for the long distance dispersal of taeniid eggs. *International Journal for Parasitology*, 25 (2): pp. 265-267.

- Torgerson, P.R., Shaikenov, B.S., Rysmukhambetova, A.T., Abdybekova, A.M., Usenbayev, A.E., Baitursinov, K.K., (2003). Modelling the transmission dynamics of *Echinococcus granulosus* in rural Kazakhstan. *Parasitology*, 126: pp. 417-424.
- Tuzun, M., Altinors, N., Ardac, I.S. and Hekimoglu, B., (2002), Cerebral Hydatid Disease CT and MR Findings. *Journal of Clinical Imaging*, 26: pp. 353-357.
- Utrilla, J., Eyre, F., Muguerza, R., Alami, H., Bueno, J. (1991). Hidatidosis en la infancia. *Arch Hidatid*, 30: pp. 721-31.
- van der Giessen, J.W., Rombout, Y.B., Franchimont, J.H., Limper, L.P. and Homan, W.L. (1999). Detection of *Echinococcus multilocularis* in foxes in The Netherlands. *Veterinary Parasitology*, 82: pp. 49-57.
- Varcasia, A., Garippa, G., Pipia, A.P., Scala, A., Brianti, E., Giannetto, S., Battelli, G., Poglayen, G. and Micagni, G. (2008). Cystic echinococcosis in equids in Italy. *Parasitology Research*, 102: pp. 815-818.
- Verster, A.J.M. (1965). Review of *Echinococcus* species in South Africa. *Onderstepoort Journal of Veterinary Research*. 32(1): pp. 7-118.
- Virchow, R. (1856). Die multiloculare, ulcerirende Echinokokkengeschwulst der Leber Verhandlungen. Sitzungsberichte der physikalisch-medicenschen. *Gesellschaft zu Wurzburg*, 6: pp. 84-95.
- Vogel, H. (1957). Uber den *Echinococcus multilocularis* Suddeneautchlands. I. Das Bandwurmstadium von Stammen menschlicher und tierischer Herkunft. *Zeitschrift Fur Tropenmedizin und Parasitologie*, 8: pp. 404-454.

- Von Nickisch-Rosenegk, M., Silva-Gonzalez, R., Lucius, R. (1999). Modification of universal 12S rDNA primers for specific amplification of contaminated *Taenia* spp. (Cestoda) gDNA enabling phylogenetic studies. *Parasitology Research*, 85: pp. 819-825.
- Von Siebold, C.T. (1853). Ueber die Verwandlung der *Echinococcus*-brut in Taenien. *Z. Wissen. Zool.* 4:409-425.
- Vuitton, D.A., Zhou, H., Bresson-Hadni, S., Wang, Q., Piarroux, M., Raoul F. and Giraudoux, P. (2003). Epidemiology of alveolar echinococcosis with particular reference to China and Europe. *Parasitology*, 127: pp. 87-107.
- Wachira, T.M., Macpherson, C.N.L. and Gathuma, J.M. (1991). Release and survival of *Echinococcus* eggs in different environments in Turkana, and their possible impact on the incidence of hydatidosis in man and livestock. *Journal of Helminthology*. (65): pp. 55-61.
- Walters, T.M.H. (1977). Hydatid disease in Wales. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 71: pp. 105-108.
- Walters, T.M.H. (1978). Hydatid disease in Wales: 1. *Epidemiology Veterinary Record*, 102: pp. 257-259.
- Walters, T.M.H. (1984). Hydatid control scheme in South Powys, Wales, U.K. *Annals of Tropical Medicine and Parasitology*, 78: pp. 138-187.
- Walters, T.M.H. (1986) Echinococcosis/ Hydatid and South Powys control scheme. *Journal of Small Animal Practice*, 27: pp. 693-703.
- Walters, T.M.H., and Clarkson, M.J. (1980). The prevalence of *Echinococcus granulosus* in farm dogs in mid-Wales. *Veterinary Parasitology*, 7: pp. 185-190.

- Walters, T.M. and Craig, P.S. (1992). Diagnosis of *Echinococcus granulosus* infection in dogs. *Veterinary Record*, 131: pp. 39-40.
- Welsh Government, 2012.
<http://wales.gov.uk/docs/statistics/2012/121121sdr2002012en.pdf> [Accessed 7 February 2013].
- Williams, B.M. (1976a). The epidemiology of adult and larval (tissue) cestodes in Dyfed (U.K.) 1: The cestodes of farm dogs. *Veterinary Parasitology*, 1: pp. 271-276.
- Williams, B.M. (1976b). The intestinal parasites of the red fox in south west Wales. *British Veterinary Journal*, 132: pp. 309-312.
- Williams, R.J. and Sweatman, G.K. (1963). On the transmission, biology and morphology of *Echinococcus granulosus equinus*, a new subspecies of hydatid tapeworm in horses in Great Britain. *Parasitology*, 53: pp. 391-407.
- Xiao, N., Qiu, J., Nakao, M., Li, T., Yang, W., Chen, X., Schantz, P.M., Craig, P.S. and Ito, A. (2005). *Echinococcus shiquicus* n. sp., a taeniid cestode from Tibetan fox and plateau pika in China. *International Journal for Parasitology*, 35: pp. 693-701.
- Yolkens, R.H., Kim, H.W., Clem, T., Wyatt, R. G., Chanock A.R. and Kapikian A.Z. (1977). Enzyme-linked immunosorbent assay (ELISA) for detection of human reovirus-like agent of infantile gastroenteritis. *The Lancet*, 11: pp. 263-266.
- Youssef, F.G., Mansour, N.S. and Aziz, A.G. (1991). Early diagnosis of human fascioliasis by detection of coproantigen, using counterimmunoelectrophoresis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 85: pp. 383-384.
- Zhang, W., Li, J. and McManus, D.P. (2003). Concepts in Immunology and Diagnosis of Hydatid Disease. *Clinical Microbiological Review*, 16 (1): pp. 18-36.

Appendices

Appendix 1

Letter to Director of the Masters of Fox Hounds Association

Miss Wai San Li
(0161) 295 4069
W.S.Li@edu.salford.ac.uk

The Hunting Office
Overley Barn,
Daglingworth
Cirencester,
Gloucestershire
GL7 7HX

Dear [REDACTED],

First of all, I would like to thank-you for speaking with me the other day about my study. I am developing diagnostic tests for dog tapeworms based on analysis of faecal samples. I am particularly interested in improving diagnostic tests for the hydatid tapeworm (*Echinococcus* species). My supervisor is Professor Philip Craig and we would like to survey as many foxhound packs as possible, at least once during 2010-2011 by collecting faecal samples from around the pen area of the hounds.

I would also like to gather information on foxhound feeding practices by issuing a short questionnaire, I have attached a copy for your reference. I have also attached a copy of the research paper by Buishi *et al.* (2005) describing the re-emergence of this parasite in Wales, which is what the current pilot control programme by the Welsh assembly was based on.

Together with the research carried out on Welsh farm dogs this would greatly help with our research into the common worm infections and recommendations for optimal treatments. Your help would be much appreciated by myself, Professor Craig and The University of Salford. All details will be kept in the strictest of confidence and we absolutely agree to be mindful of the sensitivity in the nature of this project.

I look forward to a positive response and thank-you for your time and consideration. Please do not hesitate to contact me for further information.

Yours sincerely,

Miss Wai-San Li.

Professor Philip Craig.

Appendix 2

Letter to foxhound kennelmen

Miss Wai San Li
(0161) 295 4069
W.S.Li@edu.salford.ac.uk

Dear Sir/Madam,

My name is Wai-San Li and I am a second year PhD student at The University of Salford. I am developing diagnostic tests for dog tapeworms based on analysis of faecal samples. I am particularly interested in improving diagnostic tests for the hydatid tapeworm (*Echinococcus* species). Foxhound packs are a very interesting group of dogs because they are frequently fed or have access to livestock meat/offal/carcasses and are more likely to have worms than other groups of dogs. We would like to survey foxhounds at least once during 2009-2011.

My supervisor is Professor Philip Craig and we would like you to kindly consider whether we could take faecal samples from individual dogs or from around the pen area of the dogs. Optionally, we can collect faecal samples painlessly by rectal loop, hopefully assisted by the kennel huntsman. The best time to collect faecal samples would be just prior to worming – indeed liaison at the time of worming would be ideal.

This would greatly help with our research into the common worm infections and recommendations for optimal treatments. Your help would be much appreciated by me, Professor Craig and The University of Salford. I enclose a pre-paid envelope for your convenience should you wish to respond by post.

I would be grateful also if you would kindly complete the enclosed short questionnaire and send it back in the pre-paid envelope.

I look forward to a positive response and thank-you for your time and consideration. Please do not hesitate to contact me for further information.

Yours sincerely,

Miss Wai-San Li.

Enc.

Appendix 3

Foxhunt kennel questionnaire

Questionnaire on Hunt Kennels

1. Hunt name _____
2. Hunt postcode _____
3. Name of Huntsman/contact person _____
Email _____
Tel. no. _____
4. Area covered by the hunt (district, parish, county (s)) _____
5. How many hounds do you have at the kennels? _____
6. Number of dogs: Male _____ Female _____
7. How many dogs are aged:
0-6months _____
7-12months _____
1-2years _____
3-4years _____
5-10years _____
>10years _____
8. How many times a day do you feed your dogs? _____
9. Do you feed your dogs on commercial dog food, please tick: Yes _____
No _____
10. If no, please indicate which of the following diets you normally feed your hounds, please tick:
Solely raw meat _____
Solely cooked meat _____
Solely cooked meat and cereal _____
Raw and cooked meat alternately _____
Raw and cooked meat mixed _____
Normally raw meat but cooked meat/other when raw is not plentiful _____
Livestock/carcass (whole carcass or portions) _____
Other (please state) _____
11. Which of the following animals does the meat that you feed your hounds come from? Please tick the appropriate boxes.

	Exclusively	Frequently	Occasionally	Seldom	Never
Sheep					
Lamb					
Cattle					
Calf					
Pig					
Horse					
Goat					
Donkey					

12. Other meat source, please specify and include frequency _____

13. Do you feed liver and/or lungs from any of the above animals? _____

14. If so please indicate from which animals you feed the liver and/or lungs and whether the offal is fed raw or cooked.

	Liver		Lungs	
	Raw	Cooked	Raw	Cooked
Sheep				
Lamb				
Cattle				
Calf				
Pig				
Horse				
Goat				
Donkey				

15. Have the dogs ever been fed on uncooked offal in the past and if so approximately how long ago were they last fed on this material?

16. Are there any other dogs at your kennels e.g. terriers that are fed on the same diet as the hounds? If so, please indicate what type of dog, how many there are

17. Are the hounds dewormed annually? (Y/N) _____

18. When was the last time the hounds were wormed? _____

19. If yes, which dewormer is used? _____

20. How frequently are they dewormed? 1/year ☐
2/year ☐
3/year ☐
4/year ☐
> 4/year ☐

21. Who is responsible for actual deworming? Kennel staff ☐
Huntsman ☐
Vet ☐

22. Do you know what echinococcosis/hydatid disease is? (Y/N) _____

23. If yes, how are humans infected? From dogs ☐
From sheep ☐
From other ☐

Appendix 4

Equipment list for foxhound faecal sampling

Equipment List

Hunt Name: _____

Kennel huntsman: _____

Address: _____

Tel. no: _____

Date: _____

Number of dogs: _____

- Questionnaire complete/incomplete ☐
- Faecal loops ☐
- Gloves ☐
- Autoclave bags ☐
- 30ml universals with spoons/containers ☐
- Marker pens/paper/clipboard ☐
- Bleach ☐
- Bucket & cooler box ☐
- Wooden spatulas ☐
- Sieve & plastic tweezers ☐
- 7ml Bijous ☐
- Polystyrene racks ☐
- Waterproofs/wellies ☐
- White overalls ☐

Notes:

Appendix 5

Welsh Hydatid Study farm dog questionnaire

HYDATID ERADICATION CAMPAIGN ADMINISTERED BY LVI LOCUMS LTD ON BEHALF OF WELSH ASSEMBLY GOVERNMENT. Visit Number 20__/20__

CPH of main holding ____/____/____ and linked premises (if dogs are resident/work elsewhere) ____/____/____

Owner's name and address (where dogs are resident):

Post Code				Map reference			
Name/identity of ALL DOGS on the holding	Age (Yrs)	Farm dog (F) Retired F dog (RF) Pet (Pet) Hound (H)	Faecal sample? Yes (Y) No (N) Not required (NR)	Last wormer date, product name & dosage? Excl. Campaign Tx	Weight Kg measured (M) or estimated (E)	Number of Droncit tablets given & BATCH NUMBER	Please assess if the dog has regular (R), occasional (O) or limited (L) opportunity for unsupervised roaming

I confirm that to the best of my knowledge and belief these are **ALL** the dogs on the premises and that they have been wormed as described above.

Name: _____ Date and time: _____

Signature: _____

Name of LVI Locums Limited operative: _____ Mileage shown on odometer on arrival: _____

I have observed worming and carried out sample collection of the dogs listed above as specified.

Signature of operative: _____ Date: _____

Appendix 6

Constituents of reagents used in 'Huang/Heath' test

0.15 Phosphate Buffered Saline (PBS)

8g Sodium chloride (NaCl)

1.15g di-sodium hydrogen orthophosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$)

0.2g potassium dihydrogen orthophosphate (KH_2PO_4)

0.2g potassium chloride (KCl)

in 1 litre of molecular grade water

0.3% PBS Tween 20

3ml of Tween-20 (polyoxyethylene- sorbitan monolaurate) in one litre of PBS

0.1% PBS Tween 20

1ml of Tween 20 in 1 litre of PBS

0.05M Carbonate Bicarbonate Buffer (BCB) (pH9.6)

1.59g sodium carbonate (Na_2CO_3)

2.9g sodium bicarbonate (NaHCO_3)

in 1 litre of molecular grade water

Appendix 7

Constituents of reagents used in 'Allan' test

0.15 Phosphate Buffered Saline (PBS)

8g Sodium chloride (NaCl)

1.15g di-sodium hydrogen orthophosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$)

0.2g potassium dihydrogen orthophosphate (KH_2PO_4)

0.2g potassium chloride (KCl)

in 1 litre of molecular grade water

0.3% PBS Tween 20

3ml of Tween-20 (polyoxyethylene- sorbitan monolaurate) in one litre of PBS

0.1% PBS Tween 20

1ml of Tween 20 in 1 litre of PBS

0.05M Carbonate Bicarbonate Buffer (BCB) (pH9.6)

1.59g sodium carbonate (Na_2CO_3)

2.9g sodium bicarbonate (NaHCO_3)

in 1 litre of molecular grade water

Appendix 8

Constituents of reagents for Mastermix for PCR protocols

Abassi *et al.* (2003) PCR protocol for *E.granulosus* detection (50µl reaction)

Water - 19.7µl

X2 Buffer 9 - 25µl

1000µM dNTP - 0.5µl

1µM Primer 1 - 0.5µl

1µM Primer 2 - 0.5µl

2% Formamide - 1µl

2.5U Taq - 0.8µl

DNA - 5µl

Dinkel *et al.* (1998) PCR protocol for cestode detection (100µl reaction)

Water – 40.8µl

X2 Buffer - 50µl

1000µM dNTP - 1µl

1µM Primer 1 - 0.2µl

1µM Primer 2 - 0.2µl

2.5U Taq – 2.0µl

DNA - 5µl