Epidemiology of Zoonotic and Neglected Tropical Diseases in the Lao People’s Democratic Republic

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For Finn and Alice
“If the misery of the poor be caused not by the laws of nature, but by our institutions, great is our sin”.

Charles Darwin

"As is a tale, so is life: not how long it is, but how good it is, is what matters".

Lucius Annaeus Seneca
Declaration

I declare that this thesis is my own account of my research, with due acknowledgement made to the contribution of others, and contains as its main content work that has not previously been submitted for a degree at any other tertiary educational institution.

.................................

James V Conlan

December 2013
Abstract

Laos is one of the poorest and least developed countries in Southeast Asia and living conditions, livestock production and cultural practices place large proportions of the population at risk of exposure to a range of parasitic and viral zoonoses. Surveys of humans, pigs and dogs were conducted to determine the prevalence of and risk factors associated with the transmission of *Taenia solium* and related *Taenia* species, *Trichinella* spp., soil-transmitted helminths (STH), and viral zoonoses including Japanese encephalitis virus (JEV), hepatitis E virus (HEV), swine influenza virus (SIV) and Nipah virus (NiV). Surveys were conducted in villages and slaughterhouses in four ethnically diverse provinces of northern Laos.

The human, pig and dog populations studied had a very high prevalence of parasite infection and zoonotic transmission between humans and animals was apparent for multiple species including taeniasis/cysticercosis, trichinellosis and hookworms. Cysticercosis in the human population was relatively rare with a prevalence of less than 2%, although a focal distribution and concentration of cases in a small number of villages was evident. *Taenia saginata* was the dominant *Taenia* species infecting people and *T. hydatigena* was the dominant species infecting pigs. *Trichinella spiralis* was the only species detected in pigs and we found serological evidence that human exposure to *Trichinella* larvae was common. STH infections were very common and the poorest members of the survey population and people of the Mon-Khmer ethnic group were at greatest risk of having an STH infection.

JEV was identified as being hyper-epizootic in northern Laos and remains an unmanaged threat to human health. The hemagglutination inhibition seroprevalence of JEV in the pig population was 74.7% and IgM seroprevalence of 2.3 % peaked in the monsoonal wet season months. Seroprevalence of HEV was 21.1% and the molecular characterisation of HEV isolates from village pigs demonstrated genetic homogeneity with human HEV isolates from China.

This thesis presents new data on a wide range of neglected tropical diseases, ranging from parasitic infections associated with poverty and poor sanitation through to non-discriminating zoonotic viruses. The zoonotic and neglected tropical diseases circulating in Laos are, undoubtedly, a major burden on public health and wellbeing and initiatives to prevent transmission are urgently required.
Acknowledgements

I wish to express sincere thanks and gratitude to my supervisors, Professor Andrew Thompson, Dr Stuart Blacksell and Professor Stan Fenwick for their guidance, support and most especially patience during the undertaking of this degree. I would also like to express my thanks to the staff at the Department of Livestock and Fisheries and the Department of Hygiene and Prevention in Vientiane for without their contributions this research would not have been possible. In particular, I wish to thank Dr Khamphuth Vongxay and Dr Boualam Khamlome, Mr Lapinh Phithacthep, Ms Vilaywan Soukvilay, Ms Vilayphet Viravong, Ms Manivanh Phouaravanh (National Animal Health Centre); and Mr Virasack Som, Mr Thongchan Sisouk, and Ms Khouanta Douangmala (National Centre for Laboratory and Epidemiology). My thanks and gratitude are also extended to the myriad of provincial and district staff from the Lao Ministry of Agriculture and Forestry and Ministry of Health who all made a critical contribution to the field surveys undertaken. Although not directly involved in the body of work presented in this thesis, I would like to thank Dr Syseng Khounsy for his technical and logistical support, friendship and reasoned council, which played no small part in the successful completion of this thesis.

There are a range of institutions and individuals who made contributions to the research presented in this thesis and provided me personally with technical support, and for this I offer my thanks. I would like to thank Professor's Nicholas White and Nick Day at the Mahidol-Oxford Tropical Medicine Research Unit (MORU) in Bangkok for allowing me to utilise the Units resources; also at MORU thanks to Dr Sue Lee for biostatistics advice and Stata support and other support staff for procuring laboratory equipment and reagents. I would like to thank Dr’s Paul Newton, Rattanaphone Phetsouvanh and Catrin Moore from the Wellcome Trust-Mahosot Hospital-Oxford University Tropical Medicine Research Collaboration in Vientiane for laboratory and technical support.

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Union Reference Laboratory for Parasites, Rome, Italy; and Dr Ross Lunt from the CSIRO Australian Animal Health Laboratory, Geelong, Australia.

Perhaps my greatest debt of gratitude is owed to the Lao people in the villages of northern Laos where this research was undertaken. For their incredibly generous and kind welcome and for allowing me, and the research team, to interrupt their lives for a short time to collect samples and ask questions. My one true hope is that the body of work presented in this thesis will, in some small way, make a difference to their quality of life.

I wish to finally acknowledge the support of family and friends, in particular my wife, Iwona, for her love, patience, encouragement and interest in science of all denominations.
List of publications, and work in progress, included in this thesis

Chapter 2


Chapter 3


Chapter 4


Chapter 5


Chapter 6

List of other publications related to this thesis

Review articles:


Original research articles:


Conference abstracts:


Statement of human and animal ethics approval

The protocol for surveys involving human subjects was reviewed and approved by the Murdoch University Human Ethics Committee (Project number: 2008/266) and the Lao Ministry of Health National Ethics Committee for Health Research (Number: 239/NECHR) prior to commencing field activities.

For the studies involving dogs and pigs, the protocols were reviewed and approved by the Murdoch University Animal Ethics Committee (project number: R2108/07).
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<th>Full Form</th>
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<tbody>
<tr>
<td>AAHL</td>
<td>Australian Animal Health Laboratory</td>
</tr>
<tr>
<td>ACIAR</td>
<td>Australian Centre for International Agricultural Research</td>
</tr>
<tr>
<td>AOR</td>
<td>Adjusted odds ratio</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>DALY</td>
<td>Disability adjusted life years</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>ES</td>
<td>Excretory secretory</td>
</tr>
<tr>
<td>FECT</td>
<td>Formalin-ether-concentration technique</td>
</tr>
<tr>
<td>HEV</td>
<td>Hepatitis E virus</td>
</tr>
<tr>
<td>HN</td>
<td>Haemagglutinin – neuraminidase (influenza virus membrane glycoproteins)</td>
</tr>
<tr>
<td>JEV</td>
<td>Japanese encephalitis virus</td>
</tr>
<tr>
<td>LFNC</td>
<td>Lao Front for National Construction</td>
</tr>
<tr>
<td>LECS</td>
<td>Lao economic and consumption survey</td>
</tr>
<tr>
<td>MDA</td>
<td>Mass drug administration</td>
</tr>
<tr>
<td>NiV</td>
<td>Nipah virus</td>
</tr>
<tr>
<td>NTD</td>
<td>Neglected tropical disease</td>
</tr>
<tr>
<td>OIE</td>
<td>World Organisation for Animal Health</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SIV</td>
<td>Swine influenza virus</td>
</tr>
<tr>
<td>STH</td>
<td>Soil transmitted helminth</td>
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<td>WHO</td>
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Introduction to thesis

Thesis structure

This thesis is presented as a compilation of inter-related published papers. At the time of submission, all but one of the papers have been published in high quality peer reviewed journals. The published papers (Chapters 2, 3, 5 and 6) are reproduced in this thesis as re-formatted copies of the corrected proofs. The style and format is consistent throughout the thesis with all references, figures, tables, funding sources, acknowledgements and author declarations reproduced as published after the main body of text in each chapter. As such, there is some duplication of material, particularly in the materials and methods section of each chapter and references.

Historical context

The research and results described and discussed in this thesis were assembled during the course of a project funded by the Australian Centre for International Agricultural Research (ACIAR), Project Number AH2006/161. The Australian organisation commissioned to manage the project was the School of Veterinary and Life Sciences, Murdoch University, Perth, Australia. The project entitled “Management of pig associated zoonoses in the Lao People’s Democratic Republic” commenced in January 2008 and was successfully completed in December 2010. As might be ascertained from the project title, the central objective of the project was to establish the evidence base for a range of pig associated zoonoses in Laos and identify public health interventions to prevent pathogen transmission between pigs and humans. Very early in the project, the carriage of *Taenia hydatigena* in village dogs was identified as a crucial factor in the ecology of *Taenia* cysticercosis in pigs in northern Laos. As such, surveys were also conducted in the village dog populations selected for the human and pig surveys. During the analysis of human and dog faecal samples, a very high prevalence of soil-transmitted helminths was recognised. The importance of these pathogens in the overall host-pathogen ecology could not be ignored and the research took a direction toward the broader zoonotic and neglected tropical diseases, rather than focusing solely on pig-associated zoonoses.

Thesis contents

Chapter 1 introduces the reader to Laos and the varied neglected tropical diseases and zoonoses examined in this thesis. Thereby setting the context for the epidemiological risk factors explored in this thesis, including geography, demographics, education, economic indicators and poverty,
ethnic diversity, general health and sanitation. The final section of Chapter 1 outlines the research objectives and specific questions addressed in this thesis.

Chapters 2 to 4 report the findings of the parasitic zoonoses and neglected tropical diseases surveys in multiple hosts. The epidemiology of *Taenia* spp. in humans, pigs and dogs is presented in Chapter 2. The epidemiology of soil transmitted helminths in humans and dogs, including hookworm, *Ascaris lumbricoides*, *Trichuris trichiura* and *Strongyloides stercoralis* is presented in Chapter 3. The epidemiology of trichinellosis in pigs and humans is presented in Chapter 4.

Chapters 5 and 6 report the findings of the viral zoonoses surveys in pigs. The sero-epidemiology of Japanese encephalitis virus, hepatitis E virus, swine influenza virus and Nipah virus are presented in Chapter 5. The molecular characterisation of hepatitis E virus in village pigs is presented in Chapter 6.

Chapter 7 brings together the results and discussion of the preceding chapters to present an overall synopsis of the zoonotic and neglected tropical diseases in Laos, including a discussion of the limitations of the research presented, an outline of future research needs and proposals for the sustainable control of zoonotic and neglected tropical diseases in northern Laos.

Appendix 1 details the consent form provided to participants prior to being asked to take part in the survey.

Appendix 2 is the questionnaire used in the village surveys together with a brief description of the field trial process.
Chapter 1

General Introduction
Significant proportions of this chapter are derived from two published reviews with modifications made to remove reference to data presented in this thesis. Modifications have also been made to present a consistent style and update new literature. The material presented in this chapter is the sole work of the author.


1 General introduction and literature review

1.1 Introduction

Lao People’s Democratic Republic (referred to throughout this thesis as Laos) is one of the poorest countries in Southeast Asia with an ethnically diverse population. With this poverty comes the potential for large proportions of the population to be at risk of contracting a zoonotic or neglected tropical disease via food, water, mosquito vectors, direct animal contact or from the environment. The following introductory chapter introduces the reader to Laos: its geography, its people and characteristics that influence health and wellbeing such as wealth disparities, health infrastructure, education and sanitation.

The zoonotic and neglected tropical diseases examined in this thesis were identified in close consultation with the Lao Ministry of Agriculture and Forestry and the Ministry of Health in Laos, with wider consultation encompassing international human and animal health professionals from a range of organisations. The public health significance and the epidemiology of each infectious disease in Southeast Asia is described to provide the reader with an understanding of the lack of detailed data available from Laos and to give a sense of the need for the research presented in this thesis.

1.2 The Lao People’s Democratic Republic

1.2.1 Geography and climate

Laos is the only landlocked country in Southeast Asia and shares a border with China and Myanmar in the north, Thailand in the west and northwest, Vietnam in the east and northeast and Cambodia in the south. In 2008, the country was divided into 16 provincial and one municipal administrative zone and further subdivided into 140 districts (Messerli et al., 2008). It is common for the country to be described in terms of three administrative regions: the northern region, which includes the provinces of Phongsaly, Luangnamtha, Oudomxay, Bokeo, Luangprabang, Huaphan and Xayabury; the central region, which covers Vientiane Capital and the provinces of Vientiane, Xiengkhuang, Borikhamxay and Khammuane; and the southern region, which is made up of Saravane, Sekong, Champasack and Attapeu provinces (Epprecht et al., 2008).
In 2002, the total land area of Laos was 236,800 km$^2$ of which only 9.6% was considered agricultural land, including 5% permanent agricultural land, 2.2% swidden agricultural fields and 2.4% classified as grassland (Messerli et al., 2008).

Laos has a tropical monsoonal climate with a distinct wet season from May to October, a cool dry season from November to February and a hot dry season from March to April, but variation from year to year does exist.

1.2.2 Demographics and ethnic diversity

The estimated population of Laos at the last census in 2005 was 5.6 million people and a population density of 26.7 people per square kilometre. Human density greater than 150 people per square kilometre has been observed in urban centres (Anonymous, 2006; Messerli et al., 2008), but an estimated 66% of the population are classified as rural (World Bank, 2013) where density is much lower. Laos had a relatively young population in 2005 with half of the population less than 20 years old (Messerli et al., 2008). On average, households have 5.9 inhabitants, but this can be as high as 13 or more in some areas and for some ethnic groups (Messerli et al., 2008).

Forty-nine distinct ethnic groups are recognised in Laos consisting of 160 subgroups (Messerli et al., 2008). There is some argument in scholarly circles regarding ethnic classification of Lao peoples but two methods of classification are generally used. The first method is broadly based on language and geographical location and describes three colloquial ethnic classifications, Lao loum (lowland Lao people), Lao theung (upper Lao people) and Lao soung (high Lao people), that were officially recognised until the ratification of the Lao constitution in 1991 (Messerli et al., 2008). The second and officially sanctioned ethnic group classification takes into account the intricacies of language and culture and describes groups according to ethno-linguistic characteristics (Messerli et al., 2008). The Lao Front for National Construction (LFNC) describes four ethno-linguistic families and ten ethno-linguistic categories (Messerli et al., 2008). Table 1.1 lists the ethno-linguistic classifications recognised by the LNFC and their corresponding grouping according to the colloquial classifications Lao loum, Lao theung and Lao soung.
Table 1.1 General ethnic categorisation of the Lao population

<table>
<thead>
<tr>
<th>Ethno-linguistic family †</th>
<th>Ethno-linguistic category †</th>
<th>Estimated Proportion of population (%) ‡</th>
<th>Colloquial classifications †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lao-Taï (Tai-Kadai)</td>
<td>Lao Tai-Thay</td>
<td>67</td>
<td>Lao loum</td>
</tr>
<tr>
<td>Mon-Khmer (Austroasiatic)</td>
<td>Khmuic</td>
<td>24</td>
<td>Lao theung</td>
</tr>
<tr>
<td></td>
<td>Palaungic</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Katui</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bahnaric-Khmer</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vietic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sino-Tibetan</td>
<td>Tibeto-Burman</td>
<td>1</td>
<td>Lao soung</td>
</tr>
<tr>
<td>Hmong-Mien</td>
<td>Hmong</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mien</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

References: † Messerli et al., 2008; ‡ Anonymous, 2006.

There are distinct spatial differences in the population structures of the country by ethno-linguistic family (Anonymous, 2006; Messerli et al., 2008). In broad terms, the majority of the Lao-Taï ethno-linguistic family are concentrated on the Mekong flood plain of the central and southern regions but make up a substantial proportion of the population in other areas. The Mon-Khmer predominantly occupy the mountainous areas of the northern and southern regions; the Hmong-Mien occupy the mountainous areas of the northern and central regions and the Sino-Tibetan occupy the mountainous areas of Phonsaly and Luangnamtha provinces in the far north of the country (Anonymous, 2006; Messerli et al., 2008).

1.2.3 Economic indicators and poverty

Laos is classified as a least-developed country (UNCTAD, 2007) and in 2011 had a per capita gross national income of US$1130 (World Bank, 2013). Laos was once predominantly an agricultural economy, but the economy has evolved significantly over the past two decades. In 2011, agricultural production made up 30.8% of gross domestic product, down from 58.2% in 1991 (World Bank, 2013). Though the agricultural economy has shrunk, the sector remains a very important source of employment and livelihood for a majority of the population. The 2005 population census indicated that approximately 79% of the population was engaged mainly in agricultural work and that 67% of households operate agricultural land (Messerli et al., 2008). Up to 40% of urban dwellings also operate agricultural land, indicating that a substantial amount of agricultural production occurs in or on the fringes of urban centres (Messerli et al., 2008).

The summary statistics on average income presented by World Bank (2013) do not tell the story of wealth disparity and poverty experienced across Laos. Poverty and economic opportunity vary greatly across the country. The highest rates of poverty were recorded in the southern region.
close to the border with Vietnam where, in 2002, an estimated 60-75% of households were classified as living below the poverty line (Epprecht et al., 2008). In the mountainous areas of the northern region an estimated 42-46% of households were living below the poverty line in 2002. The Mon-Khmer (51%) and Hmong-Mien (44%) people experience the highest rates of poverty compared Lao-Tai (26%) people (Epprecht et al., 2008) and rates of poverty are highest in rural areas. Geographical features also have an impact on poverty and households living in rugged mountainous terrain with access to poor quality soils experience the greatest rates of poverty (Epprecht et al., 2008). In absolute terms, areas with the highest rates of poverty are sparsely populated and the greatest numbers of impoverished people are found in the more densely populated cities and Mekong corridor (Epprecht et al., 2008).

1.2.4 Health indicators

Communicable diseases account for the highest proportion of lives lost in Laos. In 2008, an estimated 58% of lives were lost due to communicable diseases compared to 28% due to non-communicable diseases and 13% due to injuries (WHO, 2013b). The average life-expectancy at birth in 2011 was 68 years and the under 5 mortality rate was 42 per 1,000 live births; health indicators that are well below the average for the WHO Western Pacific Region (WHO, 2013b). The density of physicians per population in Laos is one of the lowest in Southeast Asia (WHO, 2013a). In 2011, there were an estimated 2.4 physicians and 7.5 nurses per 10,000 population with the Lao Ministry of Health and the WHO warning of a critical shortage of health care providers (WHO, 2012). There is a concentration of health care services in urban centres compared to rural communities and the delivery of health services is highly reliant on pharmacies, village health volunteers and practitioners of traditional medicine (LECS, 2010; WHO, 2012).

1.2.5 Sanitation

An estimated 63% of the Lao population had access to improved sanitation facilities in 2010 and 28% reported open defecation, the remainder shared a facility or had access to an unimproved latrine (UNICEF and WHO, 2012). Approximately 33% of the Lao population were classified as an urban resident in 2010 and 89% of these people had access to improved sanitation facilities, compared to 50% of rural residents. Only 3% of urban residents reported open defecation compared to 41% of rural residents (UNICEF and WHO, 2012).
1.2.6 Education indicators

In 2002, 79% of all villages in Laos had a primary school and 8% had a high school (LECS, 2004). Data from the 2007-2008 Lao Expenditure and Consumption Survey (LECS) indicates that 29% of women and 13% of men had never attended school (LECS, 2010). For those attending school in 2002, on average, children in primary and secondary school received 3 hours of schooling per day and went to school for an estimated 6 years (LECS, 2004). The school life-expectancy in 2007 increased to an estimated 9 and 7 years for men and women over 15 years old, respectively (LECS, 2010). According to the latest Population Census (Anonymous, 2006), literacy was defined for people ≥15 years old and able to read and write the official Lao language (Messerli et al., 2008). Eighty-five percent of men and 75% of women were classified as literate in 2007 and 64% of men and 56% of women reported the ability to read and write without difficulty (LECS, 2010). In rural areas, the proportion of the population with access to education and the ability to read and write was lower than urban areas. Only 45% of adults in rural areas with poor road access were able to read and write in 2007 compared to 84% in urban areas and 60% in rural areas with road access (LECS, 2010).

Lao people of non-Lao-Tai ethnicity are at a significant disadvantage. Enrolment rates in school tend to be lower for non-Lao-Tai ethnicities and non-Lao Tai children who are enrolled in school face language problems since the curriculum is taught exclusively in the Lao language (King and van de Walle, 2007). In addition to language and enrolment constraints, primary school children from economically disadvantaged households in rural areas have the highest labour demands placed on their school time (King and van de Walle, 2007), likely resulting in limited attendance and absenteeism for these children who are enrolled and captured in the official data.

1.2.7 Veterinary services

The competent veterinary authority in Laos, the Department of Livestock and Fisheries, is the principal supplier of veterinary services in Laos and is currently critically under-resourced in terms of staff numbers and quality and the provision of inadequate funding to deliver services (OIE, 2011). The delivery of services is highly reliant on para-veterinarians and village volunteers with limited training and financial support. As a consequence, the technical capacity to undertake disease surveillance and control activities is greatly constrained and limits the ability to respond effectively to disease outbreaks or other threats to animal and human health (OIE, 2011). Importantly with respect to zoonotic diseases, the capacity to interact and communicate with
stakeholders such as the Ministry of Health was deemed to of a low standard by the World Organisation for Animal Health (OIE, 2011).

1.2.8 Summary

There are clear differences in ethnicity, wealth, health care delivery and education across Laos and each of the selected indicators described above has the potential to have a substantial impact on health outcome. Communicable diseases account for the greatest loss of life in Laos and to date, the community level factors that influence the risk of having contracted a zoonotic or neglected tropical disease (NTD) are poorly understood.

1.3 Taenia solium taeniasis and cysticercosis

1.3.1 Introduction and public health significance

*Taenia solium* is a zoonotic tapeworm that has a life cycle involving humans as the definitive adult-stage host (taeniasis) and pigs as the intermediate larval-stage host (cysticercosis). In humans, who can also be inadvertently infected with larval-stage cysticerci after ingesting eggs, the most severe clinical manifestation of infection is neurocysticercosis when cysticerci establish in the central nervous system, causing serious neurological sequelae such as epilepsy and in severe cases, death. In 2010, the global burden of cysticercosis was estimated to be 503 thousand disability adjusted life years (DALYs) or 7 per 100,000 population, down from the 1990 estimate of 514 thousand DALYs or 10 per 100,000 population (Murray et al., 2012). This figure is substantially lower than other NTDs such as the soil-transmitted helminths, rabies and food-borne trematodes (Murray et al., 2012), however cysticercosis tends to be geographically clustered where conditions suit transmission between pigs and humans. In endemic countries the burden can be far greater. In the Cameroon for instance where pig cysticercosis has been estimated to be 11% (Pouedet et al., 2002), the corresponding burden of disease in the human population has been estimated to be 900 DALYs per 100,000 population (Praet et al., 2009), some 130 times greater than the global estimate.

1.3.2 Life-cycle and transmission

The *T. solium* taeniasis and cysticercosis infection complex involves two distinct disease transmission processes and requires both humans and pigs to maintain the lifecycle. Humans are the definitive host, acquiring the adult tapeworm (taeniasis) following ingestion of viable larvae (cysticerci) in contaminated pork. Eggs are shed into the environment by the adult worm via
faeces; pigs become infected following ingestion of contaminated feed or water or through direct coprophagia, thus completing the lifecycle (Figure 1.1). Human cysticercosis cases are not involved in perpetuating the lifecycle but are clinically important since cysticerci may form in the brain causing neurocysticercosis, leading to seizures, epilepsy, neurological sequelae or death.

Table 1.2 Biological parameters influencing the transmission of *Taenia solium*, *T. asiatica*, *T. hydatigena* and *T. saginata* (Source: Conlan et al., 2009)

<table>
<thead>
<tr>
<th></th>
<th><em>T. solium</em> ‡</th>
<th><em>T. asiatica</em> *</th>
<th><em>T. hydatigena</em> #</th>
<th><em>T. saginata</em> †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prepatent period in definitive host (months)</td>
<td>2</td>
<td>2-6</td>
<td>7-11</td>
<td>3</td>
</tr>
<tr>
<td>Number of eggs per proglottid</td>
<td>30-60,000</td>
<td>-</td>
<td>30-50,000</td>
<td>50-100,000</td>
</tr>
<tr>
<td>Number of proglottids shed per day</td>
<td>4-5</td>
<td>4-5</td>
<td>1-5</td>
<td>3-10</td>
</tr>
<tr>
<td>Usual number of worms per infected definitive host</td>
<td>1</td>
<td>1</td>
<td>1-4</td>
<td>1</td>
</tr>
<tr>
<td>Duration of infection in the definitive host (years)</td>
<td>1-5††</td>
<td>Not known</td>
<td>0.5-1</td>
<td>3-5</td>
</tr>
<tr>
<td>Time taken for cysts to become viable (months)</td>
<td>2-3</td>
<td>4.5</td>
<td>3½</td>
<td>2.3</td>
</tr>
</tbody>
</table>

References cited:
‡ (Allan et al., 1996; Pawlowski, 2002; Garcia et al., 2003; Craig and Ito, 2007; Kyvsgaard et al., 2007);
* (Chao et al., 1988; Eom and Rim, 1992; Chang et al., 2005; Anantaphruti et al., 2007);
# (Gemmell, 1987; Gemmell et al., 1987; Gemmell and Lawson, 1989);
† (Anantaphruti et al., 2007; Craig and Ito, 2007)
†† Unknown (Garcia et al., 2003), but estimated to be approximately 12 months (cited by (Kyvsgaard et al., 2007)).
§ Intermediate host in this case refers to sheep (Gemmell, 1987), not pigs.
Figure 1.1 Lifecycle of *Taenia* tapeworms infecting humans, dogs, pigs and bovines in the Southeast Asian region. Humans become infected with *T. solium* or *T. asiatica* adult tapeworms after ingesting metacestodes (cysticerci) in uncooked pork or pig visceral organs, respectively, and become infected with *T. saginata* adult worms after ingesting metacestodes in uncooked beef. Dogs become infected with *T. hydatigena* adult worms after ingesting metacestodes in uncooked pig visceral organs. *T. saginata* and *T. solium* are musculotropic whereas *T. asiatica* and *T. hydatigena* are viscerotropic. The solid black line within grey shading indicates death of the adult tapeworm; with no lasting immunity the host becomes susceptible. Pigs are coprophagic and become infected with cysticerci of *T. hydatigena* after ingesting eggs shed in the faeces of infected dogs. Pigs become infected with cysticerci of *T. asiatica* and *T. solium* after ingesting eggs shed in the faeces of infected humans. Cattle and buffalo are not coprophagic and become infected with cysticerci of *T. saginata* after ingesting eggs on pasture or feed contaminated with the faeces of infected humans. The dashed line indicates metacestode death and a waning of host immunity to susceptible animal status.

*Humans can become infected with cysticerci of *T. solium* after ingesting eggs shed in the faeces of tapeworm carriers. However, humans infected with *T. solium* cysticerci are dead-end hosts and play no role in transmission or maintenance of the lifecycle (Source: Conlan et al., 2009).
1.3.3 Taeniid ecology in Southeast Asia

Taeniasis and cysticercosis caused by *T. solium* has been the subject of a number of recently published reviews with an Asian focus (Ito et al., 2003; Rajshekhar et al., 2003; Willingham et al., 2003; Dorny et al., 2004; Wandra et al., 2007; Conlan et al., 2008; Conlan et al., 2009; Willingham et al., 2010). Perhaps the most consistent underlying element of these reviews is the distinct lack of high quality data from community level studies describing the epidemiology and distribution of *T. solium* in Southeast Asia. In addition, the epidemiology of *T. solium* in Southeast Asia is complicated by the co-endemicity of other *Taenia* species, where three species cause taeniasis in humans (*T. solium, T. saginata* and *T. asiatica*) and three species cause cysticercosis in pigs (*T. solium, T. asiatica* and *T. hydatigena*) (Willingham et al., 2003; Anantaphruti et al., 2007; Somers et al., 2007; Conlan et al., 2009; Willingham et al., 2010).

The distribution and epidemiology of *T. solium* in Thailand, Vietnam, Laos and Cambodia are described in detail by Willingham et al. (2010), and the distribution of *T. asiatica* in Southeast Asia has recently been described by Eom et al. (2009). *T. solium* is widely distributed in the region (Ito et al., 2003; Rajshekhar et al., 2003; Ito et al., 2004; Willingham et al., 2010) but few systematic and well-structured epidemiological surveys have been conducted (Willingham et al., 2010). The quality of the data available is quite variable and not without limitations. Intestinal helminth studies in Laos rarely describe the species of *Taenia* detected in stool (Conlan et al., 2008), however *Taenia* tapeworms were partially identified to the species level in three recent studies. In a study on *Opisthorchis viverrini* infection (Sayasone et al., 2009) in southern Laos, 23 tapeworms were recovered following praziquantel treatment and 18 were identified as *T. saginata* and the remaining five were not identified to the species level. The methods used by Sayasone et al., (2009) to characterise the tapeworms were not reported. A similar study in Khammuane province in central Laos recovered 15 worms from 12 patients and all were morphologically identified as *T. saginata* (Chai et al., 2009). *T. asiatica* can be misclassified as *T. saginata* in the absence of molecular confirmation (Anantaphruti et al., 2007) and caution should be exercised in interpreting data from the studies of Sayasone et al., (2009) and Chai et al., (2009) without the exclusion of *T. asiatica* from the differential diagnosis. The third study, conducted nationally, reported an overall taeniasis prevalence of 1.1% (408/37,090) from which 120 tapeworms were genetically and morphologically typed; three *T. solium* cases were identified from Luangprabang province in northern Laos (Eom et al., 2009). To date, *T. solium* has only been reported in the north of the country whereas *T. saginata* has a national distribution; *T. asiatica* taeniasis has not yet been detected in Laos.
*T. solium* taeniasis and cysticercosis have been confirmed in two regions of the Indonesian archipelago (see Willingham et al., 2010). In Bali, *T. solium* is endemic but transmission and prevalence seems to be on the decline (Wandra et al., 2006; Wandra et al., 2007; Sudewi et al., 2008). In Papua, *T. solium* appears to be hyperendemic in at least two districts where seroprevalence levels in the human population are some of the highest in the world and endemic in a further two districts (Salim et al., 2009). No other human *Taenia* species are known to be co-endemic in Papua (Wandra et al., 2007). North Sumatra, Flores, Sulawesi and other regions are often reported in the literature to be endemic for *T. solium*, but there are no verifiable contemporary published data to support this assertion. *T. solium* taeniasis in humans or cysticercosis in pigs has not been reported from North Sumatra; rather, evidence indicates the area is endemic for *T. asiatica* (Wandra et al., 2006; Wandra et al., 2007) although this seems to be on the decline (Ito et al., 2003). In Flores, published literature (Simanjuntak et al., 1997) does not provide evidence of *T. solium* endemicity. The most reliable and conclusive data come from studies conducted in Bali and Papua (Wandra et al., 2007; Sudewi et al., 2008; Salim et al., 2009).

There is evidence, albeit limited, that *T. solium* is present in Timor-Leste and Indonesian West Timor based on reports of suspected cases by district health officials (see Willingham et al., 2010) and a case report of several *T. solium* worms being extracted from a patient presenting with a perforated intestine after blunt trauma to the abdomen (Abu-Salem and Hassan, 2003). Further studies of the human and pig populations are required to understand better the epidemiology of *Taenia* spp. on Timor Island.

Limited data are available for taeniasis and cysticercosis in countries such as Malaysia and the Philippines although evidence presented by Willingham et al. (2010) indicates endemicity. Sporadic human neurocysticercosis cases are infrequently observed in Malaysia (Arasu et al., 2005; Nor Zainura et al., 2005) and typically detected in migrant workers (Arasu et al., 2005). However, a recent survey of 135 people from a single rural village in Ranau district, Sabah, East Malaysia found 2.2% seroprevalence for antibodies against cysticercosis (Noor Azian et al., 2006). These authors used a cut-off calculated as the mean of the 135 serum samples tested plus three standard deviations rather then a more robust use of a panel of negative control sera. It is not clear why this ‘arbitrary’ cut-off was used and as such Noor Azian et al. (2006) may have substantially underestimated the seroprevalence in the Ranau community. It is difficult to draw conclusions from this study, but *T. solium* cysticercosis in non-Muslim indigenous communities of Malaysia may be an unrecognised problem. To date, no surveys of swine cysticercosis or human taeniasis have been reported in the scientific literature in Malaysia. In the Philippines, *T. solium* cysticercosis has been detected in swine (see Martinez-Hernandez et al., 2009) and a single
seroprevalence survey for human cysticercosis found that 24.6% of the Macanip community in Eastern Visayas had antibodies. As with other regions in Southeast Asia, human cysticercosis may cluster in poor, remote communities of Malaysia and the Philippines.

In addition to *T. solium*, two other taeniid species cause human taeniasis in Southeast Asia. *T. saginata* and *T. asiatica*, which are associated with bovines and pigs as intermediate hosts, respectively (Figure 1.1), are also prevalent in the region with variable distribution (see Ito et al., 2003; Eom et al., 2009). Neither *T. saginata* or *T. asiatica* are associated with human cysticercosis, but they could potentially influence the transmission dynamics of *T. solium* through competitive mechanisms associated with crowding in the human gut (Conlan et al., 2009). In addition to *T. solium* and *T. asiatica*, pigs are also the intermediate host for the dog tapeworm *T. hydatigena* and through immune-mediated processes in the intermediate host this canine taeniid may limit the reproductive potential of related species, including *T. solium* (Conlan et al., 2009). The biological parameters influencing the transmission of the respective *Taenia* species are described in Table 1.2.

Kanchanaburi province in western Thailand appears to be the only locality where the sympatric occurrence of all three human *Taenia* species has been definitively established in a single geographically restricted area (Anantaphruti et al., 2007; Anantaphruti et al., 2010). All three human *Taenia* species are endemic in the vast Indonesian archipelago (Wandra et al., 2007) but there appears to be geographic partitioning of the three tapeworms. *T. asiatica* has been reported from Bali (Simanjuntak et al., 1997), but there are no contemporary data to verify this assertion and recent reviews indicate that only *T. saginata* and *T. solium* are endemic (Wandra et al., 2006; Wandra et al., 2007). A hospital based study in Vietnam detected all three species (Somers et al., 2007), but it is not clear if this constituted sympatric occurrence or if the patients were from geographically distinct areas. Likewise, in the Philippines all three human *Taenia* worms have been detected (Eom et al., 2009; Martinez-Hernandez et al., 2009) but sympatric distribution cannot be determined from the limited data.

The co-distribution of canine *Taenia* is difficult to determine since there is scarce literature on *T. hydatigena* infecting pigs or dogs in Southeast Asia. From the limited literature it appears that *T. hydatigena* has only been reported in pigs in Vietnam (Willingham et al., 2003) and that four *Taenia* species of humans, dogs, pigs and bovines are co-endemic and likely to occur sympatrically. In west Thailand where three human *Taenia* species are co-endemic (Anantaphruti et al., 2007; Anantaphruti et al., 2010), there are no published data on *T. solium*, *T. asiatica* or *T. hydatigena* prevalence in the pig population. From the human data though, it seems *T. solium* and *T. asiatica* co-exist in the pig population in Kanchanaburi province.
Cross-immunity between *Taenia* species has been well documented and characterised for the ovine cysticerci of *T. ovis* and *T. hydatigena* and is capable of significantly modifying the transmission dynamics of competing species (Gemmell et al., 1987). No such studies have been undertaken in Southeast Asia to examine the immune-mediated interactions between *T. solium*, *T. hydatigena* and *T. asiatica* in pigs (Conlan et al., 2009). The potential for *T. hydatigena* to moderate the transmission dynamics of *T. solium* has important implications for *Taeniiid* ecology and epidemiology in Southeast Asia (Conlan et al., 2009) and needs to be considered when formulating parasite control programs for dogs and pigs so as not to afford a competitive advantage to *T. solium* (Thompson and Conlan, 2011).

There are significant knowledge gaps regarding taeniasis and cysticercosis in Southeast Asia generally and Laos more specifically. These gaps are primarily related to the prevalence and risk of taeniasis and cysticercosis in humans, including a detailed understanding of meat consumption habits; the species of *Taenia* causing taeniasis in humans in northern Laos; the prevalence of cysticercosis in pigs and the *Taeniiid* species involved and the prevalence of *T. hydatigena* taeniasis in dogs.

1.4 *Trichinella* spp.

1.4.1 *Introduction and public health significance*

The majority of data on trichinellosis in Asia comes from China and Thailand even though *Trichinella* spp. are endemic throughout Southeast Asia (Odermatt et al., 2010). Little data is available in Southeast Asia regarding the public health significance and burden of disease and is most commonly associated with point source outbreaks and sporadic case reports. The lack of data does not necessarily indicate a low disease burden, but rather the true burden of disease in the region is most likely underestimated (Odermatt et al., 2010). In Laos, the specific risk factors associated with disease, other than consumption of undercooked meat, are largely unresolved, however in Laos the outbreaks reported in the international literature identify Lao-Tai people as being the ethnic group predominantly affected (Sayasone et al., 2006; Barennes et al., 2008).

1.4.2 *Life-cycle and transmission*

Trichinellosis is a direct zoonosis caused by infection with nematodes of the genus *Trichinella* and is one of the most widely distributed parasitic zoonoses worldwide (Dupouy-Camet, 2000; Pozio and Murrell, 2006). Infection occurs via the consumption of encysted larvae in the muscle of infected animals and involves an enteral phase associated with excystment, sexual maturation,
reproduction and larval penetration of the intestinal wall and a parenteral phase associated with
the migration of larvae, via lymphatic and blood vessels, to striated muscles where they encyst in
a nurse cell complex. Clinical symptoms in humans are related to the number of viable larvae
consumed and are typically associated with the parenteral phase (Dupouy-Camet et al., 2002).
Humans are a dead-end host and not involved in perpetuating the lifecycle.

1.4.3 Epidemiology of Trichinella spp. in Southeast Asia

Three species of *Trichinella* have been documented in the Southeast Asian region, the
encapsulated *T. spiralis* and the non-encapsulated *T. pseudospiralis* and *T. papuae*, and all have been
associated with human disease (Pozio et al., 2009). *T. spiralis* has a regional distribution (Pozio,
2001) with the majority of outbreaks recorded in the ethnically diverse regions of central and
northern Laos, northern Thailand and northwest Vietnam where consumption of uncooked pork
is common (Sayasone et al., 2006; Barennes et al., 2008; Kaewpitoon et al., 2008; Taylor et al.,
2009; Van De et al., 2012). Recent outbreaks of *T. papuae* originating from wild pigs in Thailand
(Khumjui et al., 2008; Kusolsuk et al., 2010) together with cases from Papua New Guinea
(PNG)(Pozio et al., 1999; Pozio et al., 2004) and Malaysia (Intapan et al., 2011) suggests the
geographic range of this sylvatic species encompasses continental Southeast Asia and all the main
islands to PNG (Kusolsuk et al., 2010) and the Torres Strait Islands, off the north coast of
Australia (Cuttell et al., 2012). *T. pseudospiralis* was detected in southern Thailand where villagers
were infected after consuming wild pig meat in 1994/1995 (Jongwutiwes et al., 1998).

Data on trichinellosis of wildlife and domestic animals in Southeast Asia are scarce. Surveys of
pigs in Southeast Asia, specifically addressing trichinellosis prevalence and burden of infection,
are limited. In Son La province in northwest Vietnam, an outbreak of human trichinellosis
occurred in 2008 (Taylor et al., 2009) leading to an investigation of swine trichinellosis from
December 2008 to April 2009 (Vu Thi et al., 2010). Vu Thi et al. (2010) found almost one fifth of
pigs in the survey area had serological evidence of *Trichinella* infection as determined by the
excretory-secretory (ES)-ELISA and 15% of these serologically reactive animals had evidence of
muscle larvae. *T. spiralis* was the only species detected and the muscle burden ranged from 0.04 to
0.38 larvae per gram (lpg) of muscle (Vu Thi et al., 2010), indicating a relatively low burden of
infection but still posing a risk for human disease. The disproportionate serological and muscle
digestion results in this study were interpreted as being due to low sensitivity of muscle digestion
or lack of ES-ELISA specificity (Vu Thi et al., 2010). Since 50 grams of muscle per animal was
digested, it seems reasonable to assume that poor test specificity was the strongest controlling
factor in a study environment where polyparasitism in the pig population is common. A recent
survey over a wider geographic range in two northern provinces of Vietnam observed a seroprevalence of 5.6% in pigs, 4% in dogs and 0% in cats (Vu Thi et al., 2013). In Oudomxay province in northern Laos, *T. spiralis* was isolated from one of eleven pigs sampled during an outbreak investigation in 2005; 380 lpg of muscle tissue was detected (Barennes et al., 2008). A slaughterhouse survey in Cambodia in 2005 found a very low seroprevalence of swine trichinellosis (1.13%, 5/440) and there was no difference between intensively produced and free-range pigs (Sovyra, T., 2005 unpublished thesis, Chiang Mai University, Thailand and Frei University, Berlin, Germany).

The majority of reports of trichinellosis arise from outbreaks in human populations (Pozio, 2007) and for the most part these have been discussed in detail elsewhere (Pozio, 2001, 2007; Kaewpitoon et al., 2008; Odermatt et al., 2010). Community level surveys of trichinellosis in Southeast Asia specifically addressing prevalence and risk factors of exposure to this food-borne nematode are scarce. In part this is a consequence of the difficulty of interpreting serological data based on the ES-ELISA and the excessive cost of western blot analysis.

Even with an apparent decline in the number of outbreaks in northern Thailand (Kaewpitoon et al., 2008) and an apparent increase in northwest Vietnam (Taylor et al., 2009; Van De et al., 2012), there is insufficient evidence to suggest that trichinellosis is emerging or re-emerging in the Southeast Asian region. The minimum number of larvae required to cause clinical disease has been estimated to be between 70 and 150 larvae (Dupouy-Camet et al., 2002). The prevalence of *T. spiralis* larvae in backyard and free-range pigs is relatively low and the majority harbour a low worm burden (<1 lpg) (Vu Thi et al., 2010) suggesting that in communities where uncooked pork is consumed, many infections will be subclinical. Severe clinical cases predominantly occur as sporadic point source outbreaks or sporadic isolated cases (Odermatt et al., 2010). The epidemiology of *Trichinella* spp. infection in pigs and the risk factors associated with trichinellosis in humans in Laos and Southeast Asia more generally remains to be determined.

### 1.5 Zoonotic hookworm

#### 1.5.1 Introduction and public health significance

Human hookworm infections continue to be a major public health problem in Southeast Asia with approximately one quarter of the 563 million inhabitants infected (Hotez and Ehrenberg, 2010). Worldwide, enteric human hookworm infections are predominantly associated with two species, *Ancylostoma duodenale* and *Necator americanus* (Brooker et al., 2004), and neither is considered zoonotic. However, pigs have been implicated as transport hosts of *N. americanus*...
(Steenhard et al., 2000) and may have an important role in the natural history of human disease. Of the zoonotic hookworm species that cause human disease, *A. ceylanicum* is the only species capable of establishing a patent enteric infection in humans, canines and felines (Anten and Zuidema, 1964; Wijers and Smit, 1966; Yoshida et al., 1968; Carroll and Grove, 1984, 1986). Historically, *A. ceylanicum* has received little attention despite it being known to cause human disease for at least the past 40-50 years (Anten and Zuidema, 1964; Wijers and Smit, 1966; Carroll and Grove, 1986; Traub et al., 2008).

Zoonotic hookworm disease resulting in anaemia was first described in 1964 in Dutch marines returning from service in West New Guinea (now Indonesian West Papua) (Anten and Zuidema, 1964). Nine of eleven (82%) returning marines were found to have a patent enteric infection with *A. braziliense* (Anten and Zuidema, 1964) which was later referred to as *A. ceylanicum* (Wijers and Smit, 1966; Chowdhury and Schad, 1972). Three marines were infected with more than 100 adult worms and two of these otherwise healthy well-fed marines were anaemic (Anten and Zuidema, 1964) which is in stark contrast to most reports where only a few adult *A. ceylanicum* worms have been recovered (Kian Joe and Kok Siang, 1959; Yoshida et al., 1968; Chowdhury and Schad, 1972). A follow-up study using *A. ceylanicum* worms originating in West New Guinea and passaged through dogs showed that infection in healthy volunteers produced severe clinical symptoms within 15-20 days after cutaneous exposure to L3 larvae, including severe abdominal pain and epigastric spasms (Wijers and Smit, 1966). All volunteers were exposed to relatively few larvae and patent infections with low egg counts were detected in all cases. Of particular note was the finding that a small worm burden resulted in cognitive impairment and difficulty concentrating (Wijers and Smit, 1966). A second experimental infection study involving two well-fed healthy volunteers in Australia (Carroll and Grove, 1986) reported similar severe abdominal pain 5 weeks after infection with associated diarrhoea in one case; Carroll and Grove (1986) were also able to demonstrate recurrent bouts of abdominal disturbance over several months.

*A. ceylanicum* is the most neglected of all human hookworm species, typically considered to be an unimportant pathogen (Chowdhury and Schad, 1972; Brooker et al., 2004; Hotez et al., 2004) due to an absence of demonstrated heavy infections and subsequent anaemia (Brooker et al., 2004). *A. ceylanicum* is described as a poorly adapted human hookworm (Chowdhury and Schad, 1972) and ill-suited to the human gastrointestinal tract, resulting in patent infections with low fecundity. The evidence for clinical insignificance however comes from experimental studies involving healthy well-fed adults (Wijers and Smit, 1966; Carroll and Grove, 1986) and urban inhabitants (Kian Joe and Kok Siang, 1959; Chowdhury and Schad, 1972). The clinically significant findings from West New Guinea (Anten and Zuidema, 1964), with vastly different environmental
exposures, has been largely overlooked for 45 years. In addition, the non-blood loss symptoms associated with *A. ceylanicum* infection, including cognitive impairment from light infections (Wijers and Smit, 1966), rarely receive a mention. Furthermore, there is a distinct similarity between acute clinical presentation caused by *A. ceylanicum*, including severe abdominal pain (Wijers and Smit, 1966; Carroll and Grove, 1986; Traub et al., 2008) and recurrent abdominal disturbance (Carroll and Grove, 1986), and eosinophilic enteritis caused by *A. caninum* that is indicative of intestinal hypersensitivity (Prociv and Croese, 1996).

1.5.2 **Life-cycle and transmission**

Hookworms have a direct life cycle involving a soil developmental stage (geo-helminth stage) whereby eggs excreted in the faeces of a host embryonate and hatch in warm moist conditions. Free-living rhabditiform larvae are released and feed on organic debris and bacteria before moulting into non-feeding infective filariform larvae (L3). The infective larvae remain viable for several weeks under warm moist conditions and gain entry to the definitive host via skin penetration (Para-site, 2010). *N. americanus* larvae must penetrate the skin to infect humans whereas *Ancylostoma* species can infect hosts by penetrating the skin or oral mucosa, being passed to an infant in breast milk or transplacental transmission to the foetus (Para-site, 2010). *Ancylostoma* species may also survive in the muscle of paratenic hosts and lead to infection in a competent definitive host after ingesting uncooked meat of an infected paratenic host (Bowman et al., 2010; Para-site, 2010).

1.5.3 **Epidemiology of hookworm in southeast Asia**

Several community surveys in Southeast Asia over the past 5 years report hookworm to the species level and *A. ceylanicum* is prevalent, to varying degrees in all studies (Traub et al., 2008; Sato et al., 2010; Mahdy et al., 2012; Ngui et al., 2012a; Ngui et al., 2012b). *Ancylostoma duodenale* may be the predominant *Ancylostoma* species in people in southern Laos (Sato et al., 2010), but this study was only conducted in a single village and no dog data were reported. A survey of humans and dogs in Bangkok recorded *A. ceylanicum* as the predominant hookworm species in dogs and almost a third of human hookworm carriers in the study population (2/7) harboured *A. ceylanicum* (Traub et al., 2008). Notably, only the *A. ceylanicum* cases suffered chronic abdominal disturbance (Traub et al., 2008). These recent surveys from Thailand and Laos indicate that dogs have an important role in the natural history of human infection. Unfortunately, no detailed clinical or worm burden data were reported in these studies but the high prevalence of *A. ceylanicum* in humans and dogs warrants further investigation.
Zoonotic infections caused by dog and cat hookworm species, *A. caninum*, *A. braziliense* and *A. tubaeforme* can also occur and the pathogenic nature of the infection is dependent on the migration of larvae to ectopic sites in the paratenic human host (see Bowman et al., 2010). Cutaneous larva migrans (CLM) is the most common disease described (Bowman et al., 2010), other clinical manifestations include eosinophilic enteritis (Croese, 1988; Prociv and Croese, 1990; Croese et al., 1994; Prociv and Croese, 1996), eosinophilic pneumonia (Löffler's syndrome), myositis, folliculitis, erythema multiforme or ophthalmological manifestations (see Bowman et al., 2010). Cutaneous larva migrans is predominantly associated with *A. braziliense* (Bowman et al., 2010) and published reports of CLM from Southeast Asia tend to be limited to tourists returning home (Jelinek et al., 1994; Malvy et al., 2006). Since *A. braziliense* is rarely reported in Southeast Asia, with just a few reports from Malaysia and Indonesia (Yoshida et al., 1973; Margono et al., 1979), it is not clear what hookworm species were the cause of these CLM cases, possibly *A. ceylanicum* or *A. caninum*. In light of the advances in *Ancylostoma* molecular diagnostics (Traub et al., 2004; Palmer et al., 2007; Traub et al., 2007; Traub et al., 2008), the geographic range and prevalence of *A. braziliense* in Southeast Asia should be reappraised. *A. ceylanicum* on the other hand is endemic in Southeast Asia with a wide geographic range, encompassing Indonesia, Borneo, Malaysia, Philippines and Thailand (Kian Joe and Kok Siang, 1959; Anten and Zuidema, 1964; Velasquez and Cabrera, 1968; Yoshida et al., 1968; Yoshida et al., 1973; Setasuban et al., 1976; Margono et al., 1979; Choo et al., 2000; Scholz et al., 2003; Traub et al., 2008; Sato et al., 2010; Mahdy et al., 2012; Ngui et al., 2012a; Ngui et al., 2012b) and can cause CLM, presenting as a maculopapular ‘ground itch’ (Haydon and Bearup, 1963; Wijers and Smit, 1966).

Eosinophilic enteritis has been well described for *A. caninum* infections in northeastern Australia (Croese, 1988; Prociv and Croese, 1990; Croese et al., 1994; Prociv and Croese, 1996) with sporadic case reports from the United States of America (Khoshoo et al., 1994; Khoshoo et al., 1995) and Egypt (Bahgat et al., 1999). It is notoriously difficult to make a definitive diagnosis of *A. caninum* eosinophilic enteritis due to the vagaries of clinical symptoms, the variability of serological results and the difficulties of recovering worms (Prociv and Croese, 1996). While the symptoms are non-specific, abdominal pain is almost invariably observed and can range from severe acute pain mimicking appendicitis to more mild discomfort; pain can become chronic or recurrent and in rare cases bowel obstruction or bleeding can occur (Prociv and Croese, 1996). Other symptoms may include anorexia, nausea and diarrhoea (Prociv and Croese, 1996). *Ancylostoma caninum* eosinophilic enteritis has not been documented in Southeast Asia even though this hookworm is prevalent with a wide geographic range (Setasuban et al., 1976; Margono et al., 1979; Traub et al., 2008; Mahdy et al., 2012). In part, this may be due to the
difficulty of establishing hookworm as a cause of obscure and/or recurrent abdominal pain or eosinophilic enteritis.

Mass drug administration (MDA) using a single dose of mebendazole or albendazole for the control of soil-transmitted helminths is widespread in Southeast Asia with greater than 90% of school age children in Laos and Cambodia and greater than 70% in Vietnam treated (Montresor et al., 2008; WHO, 2009). Mebendazole is commonly administered in Southeast Asia due to safety and low cost (Flohr et al., 2007; Phommasack et al., 2008). However, hookworm disease continues to be an important public health problem throughout the region and there is little evidence that MDA is effectively reducing the burden of hookworm disease. There are multiple reasons for this issue; foremost among them is the low efficacy of a single dose of mebendazole in reducing egg output (Flohr et al., 2007; Keiser and Utzinger, 2008), but there is no data describing the efficacy of mebendazole in clearing or reducing egg counts for *A. ceylanicum*.

An investigation of the role that dogs play in the natural history of human hookworm disease in northern Laos is warranted owing to the potential for adverse health outcomes and the close interactions of dogs and humans in Lao villages.

### 1.6 Zoonotic pig associated viruses

#### 1.6.1 Introduction

It has been estimated that around 75% of emerging or novel viral diseases are zoonotic and their emergence and spread is primarily a consequence of human activities. Such activities may include land clearing or deforestation, newly developed or expanded irrigated agriculture, construction of dams for hydropower generation, increased urbanisation or other human-made alterations to the ecosystem (Mackenzie, 2005). Just such activities have been occurring in Laos at an accelerating rate over the past decade. Despite these ongoing changes, there has been relatively little attention paid to viral zoonoses, especially in pigs which have been demonstrated to be an important source of human infections (Mackenzie, 2005).

#### 1.6.2 Japanese encephalitis virus

With the exception of Japanese encephalitis virus (JEV), there remains little information on the impact or burden of viral zoonoses in Laos and Southeast Asia. JEV is a major cause of death and disability in Asia and is the major mosquito-borne flavivirus in East, Southeast and South Asia (Mackenzie, 2005). In 2004, the burden of disease was estimated to be 681 thousand DALYs and 11,000 deaths in the Southeast Asia and Western Pacific regions (WHO, 2008).
Children and young adults are at greatest risk of developing clinical disease associated with Japanese encephalitis (Solomon et al., 2000; Campbell et al., 2011). The majority of JEV infections are subclinical (Vaughn and Hoke, 1992) and by the time children reach adulthood they have developed immunity through previous exposures (WHO, 2010). For those that develop clinical signs, cases typically develop non-specific febrile illness, with or without diarrhoea and rigors. In patients that develop more serious encephalitis symptoms, the initial non-specific febrile symptoms are followed by headache, vomiting, convulsion and a reduced level of consciousness. The disease is typically severe with a quarter of encephalitis cases being fatal and a third resulting in permanent neurological sequelae (WHO, 2010).

JEV is transmitted by paddy breeding *Culex* mosquitoes, primarily *Culex tritaeniorhynchus*, in a zoonotic cycle involving ardeid wading birds (herons and egrets), pigs and humans (van den Hurk et al., 2009). During times of peak transmission, amplification of JEV in naïve pigs within the vicinity of human habitation precedes epidemic transmission to people (van den Hurk et al., 2009). Pig production industries are also affected by JEV infection and economic losses occur through decreased productivity associated with reproductive failure (Joo and Platt, 2006). In Laos, a significant proportion of the human population live within the vicinity of rice paddy (Messerli et al., 2008) and pig production is practiced in rural, peri-urban and urban environs (Blacksell, 2000; Millar and Photakoun, 2008) ensuring suitable conditions for JEV transmission from pigs to humans.

The incidence of Japanese encephalitis has reportedly been increasing in Laos in recent years (Erlanger et al., 2009), there are however no reliable estimates of incidence presently available. The incidence of Japanese encephalitis in high-incidence countries without vaccination, including Laos, has been estimated to be 3.7 cases per 100,000 population for all ages; increasing to 10.6 cases per 100,000 population for children under 15 years old and decreasing to 0.7 cases per 100,000 population for those 15 years and older (Campbell et al., 2011). The seroprevalence of flavivirus exposure, including dengue virus and JEV, increases with age in Laos (Vongxay, 1995; Vallee et al., 2009). In a survey conducted in Vientiane Municipality in 2006, the seroprevalence of flavivirus exposure was 84.6% for people 35 years and older and 9.4% for children less than 6 years old (Vallee et al., 2009). In neighbouring countries with historically high incidence but with expanding vaccination programs, including Thailand and Vietnam, the incidence of Japanese encephalitis has been estimated to be 1.5 cases per 100,000 population; 4.5 and 0.3 cases per 100,000 population for children under 15 years old and 15 years and older, respectively (Campbell et al., 2011).
There is currently no data available on the prevalence or incidence of JEV infection in pigs in Laos, including the age and season of exposure, which greatly impacts on the knowledge base for designing and implementing vaccination programs.

1.6.3 Hepatitis E virus

Hepatitis E virus (HEV) is comprised of four distinct genotypes (Emerson and Purcell, 2003; Lu et al., 2006). Genotypes 1 and 2 are primarily associated with humans and account for the majority of HEV infections worldwide, including epidemics (Aggarwal and Naik, 2009). Genotype 3 and 4 HEV have a zoonotic origin (Aggarwal and Naik, 2009; Meng, 2009) with a diverse animal host range (Lu et al., 2006; Okamoto, 2007). However, pigs are an especially important reservoir of genotype 3 and 4 HEV and a common source of human HEV infection (Lu et al., 2006).

Hepatitis E virus (HEV) typically causes a self-limiting acute viral hepatitis followed by recovery and on occasion a fulminant hepatitis develops (Aggarwal and Naik, 2009). It is the causative agent of large-scale and sporadic acute hepatitis outbreaks worldwide with endemicity primarily centred in regions with poor sanitation and hygiene, encompassing large areas of Asia, Africa and Central and South America (Emerson and Purcell, 2003; Aggarwal and Naik, 2009). Faecal-oral transmission is the predominant route of HEV infection and contaminated drinking water is the most common cause of epidemic human disease (Emerson and Purcell, 2003; Aggarwal and Naik, 2009; Meng, 2010). Consumption of raw shellfish and uncooked meat and viscera from HEV infected animals are also important sources of human disease and result in sporadic cases (Meng, 2009, 2010). Consumption of contaminated meat and occupational exposure are also recognised as important modes of zoonotic transmission (Meng, 2009, 2010).

The majority of human HEV infection in Asia can be attributed to genotype 1 virus (Wei et al., 2006; Aggarwal and Naik, 2009) with sporadic acute disease associated with genotypes 3 and 4 (Aggarwal and Naik, 2009; Fu et al., 2010). However, recent evidence from China indicates that genotype 4 HEV is emerging as the predominant cause of human disease (Li et al., 2006; Zhang et al., 2010). All pig HEV infections in Asia have been attributed to genotype 3 or 4 (Meng, 2009) with a single genotype 1-like virus isolated from a pig in Cambodia (Caron et al., 2006), but this finding has not yet been verified (Meng, 2010). Genotype 3 HEV is prevalent in pig populations of Cambodia and Thailand (Cooper et al., 2005; Caron et al., 2006; Hinjoy et al., 2013) and Genotype 4 is prevalent in pigs of southern China (Ji et al., 2008). Genotype 4 HEV has also been associated with shellfish and human disease in northern Vietnam (Koizumi et al., 2004).
In Laos, prevalence estimates of anti-HEV antibodies range from 16-18% of the general human population (Corwin et al., 1999; Syhavong et al., 2010), and 2-4% of hospital admitted hepatitis cases have been attributed to HEV infection (Corwin et al., 1999; Syhavong et al., 2010). In a sero-epidemiological study of Lao pigs, anti-HEV antibodies were detected in 51% of slaughter-pigs and 15% of village-surveyed pigs, with a peak sero-prevalence in 7-9 month-old animals (Blacksell et al., 2007). Neither pig nor human HEV isolates have been genetically typed in Laos and the link between pig and human infections have not been examined.

1.6.4 Swine influenza virus

The prevalence of swine influenza virus (SIV) in pig populations around the world can vary greatly and a number of HN subtypes may circulate in any particular country. Pigs have a role in the emergence of novel pathogenic strains through the mixing and re-assortment of human, avian and swine adapted influenza viruses (Shinde et al., 2009). The antigenicity of SIV strains circulating in Southeast Asia are, however, poorly characterised. In Vietnam and Thailand, three subtypes H1N1, H1N2, and H3N2 (Chutinimitkul et al., 2008; Takemae et al., 2013) have been reported in their respective pig populations, whereas in China, five subtypes H1, H3, H5, H7 and H9 (Liu et al., 2009) have been described.

The public health importance of SIV in Southeast Asia is not well defined in the international literature. However, the significance of SIV in human disease lies in virus re-assortment in pigs; genes from seasonal human influenza have been detected in SIV strains isolated from pigs in Vietnam (Ngo et al., 2011) and a gene fragment detected in a pig virus isolate (subtype H3N2) was closely related to an avian subtype H5N1 virus strain in China, indicating that the subtype H5N1 viruses may be able to transfer pathogenic genes in pigs (Bi et al., 2010). With the emergence of highly pathogenic H5N1 avian influenza virus in Laos (Boltz et al., 2006) and the emergence of the pandemic H1N1 influenza virus in 2009, the characterisation of SIV strains circulating in the Lao pig population has become a priority and remains unknown.

1.6.5 Nipah virus

Nipah virus (NiV) is an emerging encephalitic virus that has been associated with significant outbreaks of human disease in Malaysia, Singapore, India and Bangladesh (Chua et al., 1999; Paton et al., 1999; Harcourt et al., 2005; Harit et al., 2006). The natural reservoir hosts of NiV are Pteropid fruit bats (flying foxes) which can serve as the source of infection for a range of susceptible mammals, including humans and pigs (Mackenzie, 2005). Pigs can be a critical intermediary host acting as a bridge in transmission between infected wild fruit bats and people.
This was demonstrated in the Malaysian and Singaporean outbreaks where human NiV disease was associated with exposure to infected pigs or pig carcasses (Chua et al., 2000). However, food-borne and human-to-human transmission has also been demonstrated in South Asia (Luby et al., 2006; Gurley et al., 2007).

The known geographical range of the virus has extended over recent years with serological and molecular evidence demonstrating NiV to be present in fruit bat species in Cambodia, Indonesia and Thailand (Olson et al., 2002; Reynes et al., 2005; Wacharapluesadee et al., 2005; Sendow et al., 2009). Pteropid bats are resident in Laos, but are currently threatened by habitat loss and hunting (Duckworth et al., 1999). The emergence of NiV in Laos is, therefore, possible and to date no studies have investigated the occurrence of NiV in pigs in Laos or other Southeast Asian countries outside of Malaysia.

1.7 Objectives of this study

The conditions necessary for the efficient transmission of a range of parasitic and viral zoonoses are prevalent in Laos. Coupled with a low standard of medical and veterinary infrastructure and service delivery, zoonotic and neglected tropical diseases have the potential to entrench affected people in poverty and have a detrimental impact on quality of life. The country remains a challenging place to conduct research owing to significant resource and logistical constraints. As a consequence, there is relatively little data describing the epidemiology and ecology of zoonotic and neglected tropical diseases in a host assemblage that includes humans, pigs and dogs. No studies have attempted to conduct concurrent surveys in these three host populations in Laos to draw together the results and infer interactions that would otherwise not be possible. The principle objectives of this research thesis were to:

1. Determine the prevalence and risk of zoonotic and neglected infectious diseases in the human population of northern Laos;
2. Determine the prevalence and risk of zoonotic and neglected infectious diseases in the pig and dog populations of northern Laos;
3. Characterise the ecology of host-pathogen interactions in a complex host assemblage that includes humans, pigs and dogs.
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Chapter 2

A Cross-Sectional Study of *Taenia solium* in a Multiple Taeniid-Endemic Region Reveals Competition May be Protective
This chapter is a published paper:


Author contributions:

*Conceived and designed the study: JVC, KV, BK, SDB, SF, RCAT*

*Conducted the surveys: JVC, KV, BK*

*Performed the laboratory testing: BS (human enteric parasitology), JVC (molecular identification of expelled *Taenia* worms), AE (dog enteric parasitology)*

*Provided reagents for antigen capture ELISA: PD*

*Analysed the data: JVC*

*Wrote the manuscript: JVC*

*Proofed and critically appraised the manuscript: KV, BK, PD, BS, AE, SDB, SF, RCAT*
2 A cross-sectional study of *Taenia solium* in a multiple Taeniid-endemic region reveals competition may be protective

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2.1 Abstract

We conducted cross-sectional surveys for taeniasis and cysticercosis in humans, pigs and dogs in four northern provinces of Laos. Human cysticercosis and taeniasis prevalence was 2.2% (95% confidence interval (CI): 1.4-3.0%) and 8.4% (95% CI: 6.9-9.9%), respectively. Eating uncooked beef, being male, province of residence, age and ethnicity were all significant risk factors for taeniasis and only province of residence was a significant risk factor for cysticercosis. Thirty-five human tapeworms were recovered during the survey and 33 (94.3%) and 2 (5.7%) were identified as *Taenia saginata* and *T. solium*, respectively. Maximum likelihood adjusted prevalence of *T. solium* and *T. hydatigena* in pigs was 4.2% (95% CI: 0.5-7.9%) and 55.9% (95% CI: 47.5-64.3%), respectively, and *T. hydatigena* taeniasis in dogs was 4.8% (95% CI: 0.0-11.3%). *T. hydatigena* and *T. saginata* were the most prevalent taeniids in the respective pig and human populations and together may suppress *T. solium* transmission.
2.2 Introduction

*Taenia solium* is a zoonotic tapeworm that has a life cycle involving humans as the definitive adult-stage host (taeniasis) and pigs as the intermediate larval-stage host (cysticercosis). In humans, who can also be inadvertently infected with larval-stage cysticerci after ingesting eggs, the most severe clinical manifestation of infection is neurocysticercosis when cysticerci establish in the central nervous system, causing serious neurological sequelae such as epilepsy and in severe cases, death. In Southeast Asia, the epidemiology of *T. solium* is complicated by the co-endemicity of other *Taenia* species, where three species cause taeniasis in humans (*T. solium*, *T. saginata* and *T. asiatica*) and three species cause cysticercosis in pigs (*T. solium*, *T. asiatica* and *T. hydatigena*) (Willingham et al., 2003; Anantaphruti et al., 2007; Somers et al., 2007; Conlan et al., 2009; Willingham et al., 2010).

*Taenia solium* infection disproportionately affects the poorest communities worldwide where conditions are suitable for the completion of the tapeworm life cycle, including free-roaming pig production, inadequate sanitation, poor hygiene and low levels of education. Such conditions exist in many rural communities in the Lao People’s Democratic Republic (Laos), yet to date, no studies have been undertaken to investigate *T. solium* in a multi-species context. In Laos, evidence of human cysticercosis is limited to a small study that found 4.8% seroprevalence of antibody against *T. solium* cysticercosis (Tran et al., 2007) and an ill-defined case of neurocysticercosis in northern Laos (Eom et al., 2009). The only data from pigs is based on carcass inspection indicating a prevalence of 1-2% (Conlan et al., 2008). Human taeniasis is also poorly understood. Many studies have reported taeniasis prevalence without determining or reporting the species causing infection (Giboda et al., 1991a; Giboda et al., 1991b; Pholsena et al., 1991; Chai and Hongvanthong, 1998; Vannachone et al., 1998; Rim et al., 2003; Sithithaworn et al., 2006; Sayasone et al., 2007; Tran et al., 2007; Erlanger et al., 2008) and prevalence estimates range from 0-14%, with a high degree of spatial variation (Conlan et al., 2008). Only *T. saginata* has been reported in southern Laos (Chai et al., 2009; Sayasone et al., 2009) and both *T. solium* and *T. saginata* have been reported in northern Laos (Eom et al., 2009).

The principal objective of the present study was to investigate *Taenia* spp. infection in humans, pigs and dogs in four provinces of northern Laos by, (i) conducting studies to estimate the prevalence and risk of taeniasis and cysticercosis in humans, (ii) identifying the *Taenia* species causing taeniasis in humans, (iii) estimating the prevalence of cysticercosis in pigs and *T. hydatigena*
taeniasis in dogs and (iv) combining the different studies to draw conclusions on the ecological factors controlling human and pig infections.

2.3 Materials and methods

2.3.1 Ethics statement

Informed consent was obtained from all human adult participants and from the parents or legal guardians of minors (children <15 years old). The study protocol was reviewed and approved by the Murdoch University Human Ethics Committee (Project number: 2008/266) and the Lao Ministry of Health National Ethics Committee for Health Research (Number: 239/NECHR) prior to commencing this study.

For the studies involving dogs and pigs, the protocols were reviewed and approved by the Murdoch University Animal Ethics Committee (project number: R2108/07), which adheres to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The Lao Department of Livestock and Fisheries does not, at this time, have a committee to review and approve scientific research protocols involving animals.

2.3.2 Human survey

Study site. The Laos is an ethnically diverse country with 49 distinct ethnic groups classified into four ethno-linguistic families, Lao-Tai, Mon-Khmer, Hmong-Mien and Sino-Tibetan, making up 67, 24, 8 and 1% of the population, respectively (Anonymous, 2006). The study was conducted in four provinces in northern Laos, Oudomxay, Luangprabang, Huaphan and Xiengkhuang (Figure 2.1), where all four ethno-linguistic families are represented. Provinces were selected in consultation with the Lao government and the guiding principles of selection were accessibility from Vientiane and priority areas for poverty alleviation, rural development and improving pig production.

Survey design. In each province, one district was randomly selected for inclusion in this study (Figure 2.1). The survey was conducted in six randomly selected villages in the dry season from January to March 2009 to maximise study participation and minimise negative impacts on seasonal labour demands. The number of villages selected was constrained by the human resources available at the three levels of government administration, national, provincial and district. Villages were selected from official listings provided by provincial government offices and villages were excluded if a four-wheel drive vehicle could not access them. For the sample
size calculation, we conservatively estimated that 4% of households would have at least one
cysticercosis or taeniasis case based on prevalence data from northern Vietnam (Somers et al.,
2006) and prevalence estimates for Laos (Pawlowski, 2002). At a precision of 10% and 95% confidence level (eq. 1) and correcting for a finite population of 150 households per village based on averaged data supplied by district agriculture and forestry offices (eq. 2), 14 households were randomly selected from each village using a random number table.

\[ N_0 = \frac{Z^2 \times P(1-P)}{\alpha^2} \]  
\[ N_1 = \frac{nN_0}{n + (N_0 - 1)} \]  

Where \( N_0 \) was the sample size required for simple random sampling, \( Z \) was the z-score for the required confidence level (1.96), \( P \) was the estimated proportion of affected households and \( \alpha \) was the required precision expressed as a proportion (0.1). \( N_1 \) was the corrected sample size and \( n \) the finite population size.

All household members \( \geq 6 \) years old were asked to participate. Village chiefs were given advance notice of the survey timing and meetings to select households were conducted in villages the day prior to sampling. In cases where a household refused to participate, the village chief selected a household with similar characteristics. A household questionnaire was administered to the head of each household with his/her family present to assess the house characteristics, assets owned, ownership of animals, ethnicity, education levels and literacy of the male and female heads of household, the person with greatest responsibility for preparing food and person with the greatest responsibility for primary care. An individual questionnaire was administered to all study participants, with younger participants (<15 years) interviewed in the presence of a parent or guardian who may have provided assistance in answering questions. Data on frequency of raw meat consumption, latrine use, tapeworm segments seen in stool and a history of taeniasis were collected. For those who consumed raw meat, we asked them to estimate the frequency of raw meat consumption; weekly, monthly, every few months and infrequent (once or twice per year or less often). Questionnaires were administered in the Lao language and were pre-tested with people who did not otherwise participate in the survey. In the circumstances where a person could not understand the Lao language, a household member, relative or village chief provided verbal translation of questions to study participants with their consent. A venous blood sample of 2-3 ml was collected and the serum fraction stored at minus 20°C. Labelled plastic bags for a single stool sample were handed out. Stool samples were collected from the participants the
following day and stored in two preservation solutions, 10% formalin and 80% ethanol, for microscopy and molecular analysis, respectively.

Individuals who were *Taenia* egg positive or self-reported seeing stool segments were treated with niclosamide and a purgative (bisacodyl) according to manufacturers instructions (Vechaphant Baesaj, Visonic, Thailand) from November to December 2009. All participants were provided with detailed information on the risks associated with *T. solium* taeniasis and the need to safely dispose of all stools and adhere to strict hand hygiene measures. Buckets and soap were provided to all participants. Adults were treated with 2 g niclosamide and 15 mg bisacodyl two hours post-treatment, children >34 kg were treated with 1.5 g niclosamide and 10 mg bisacodyl and children 11-34 kg were treated with 1 g niclosamide and 5 mg bisacodyl. All stool samples were examined for scolecites and proglottids for two days after treatment. Stool samples were preserved in 80% ethanol and transported back to Vientiane Capital at ambient temperature where they were stored at 4°C until testing.

### 2.3.3 Animal surveys

Opportunistic pig surveys were conducted at three slaughter-points in Xiengkhuang and Oudomxay provinces from May to September 2008 and at two collection points in Huaphan and Luangprabang provinces from October 2008 to January 2009. The survey team consisted of trained district and provincial agricultural and forestry government staff who visited the slaughter-points approximately every two weeks. All pigs brought for slaughter on the nights the survey team visited were examined post-mortem and a blood sample was collected. The tongue and diaphragm pillar muscles were excised and examined for cysts. Pork traders prevented muscle slicing; as such, the heart, liver, mesentery and omentum and other viscera were examined for the presence of *Taenia* cysts, as were all exposed muscle surfaces. Presence of cysts and data on location, age, breed, sex and production system at last point of sale were recorded on a data collection sheet and sent to Vientiane Capital with the blood sample and any cysts found.

Dog faecal samples were collected in the same villages as the human survey described above from January to March 2009 using a semi-structured approach. Dogs were selected if they belonged to the same household as those randomly selected for the human survey, if no dogs were present in a household, then dogs were opportunistically identified in the village. The permission of owner’s was granted before sampling was undertaken. Faecal samples were collected by manual digital extraction and preserved in two preservation solutions, 10% formalin and 80% ethanol. Demographic data and the age and sex were recorded with the sample. The
samples were sent to Vientiane Capital at ambient temperature and subsequently stored at 4 °C prior to processing.

2.3.4 Laboratory analysis

Formalin preserved human faeces were transported at room temperature to Khon Kaen University, Thailand, where the samples were analysed by the formalin-ether-concentration technique (FECT) followed by microscopy (Elkins et al., 1990). Formalin preserved dog faeces were transported to Murdoch University, Perth, Australia, at ambient temperature and examined for the presence of taeniid eggs by the saturated sodium nitrate flotation technique followed by microscopy (O’Grady and Slocombe, 1980).

The preserved human and pig serum samples were tested by enzyme linked immunosorbent assay (ELISA) for the presence of *Taenia* metacestode circulating antigens (Brandt et al., 1992; Dorny et al., 2000; Dorny et al., 2002) using modifications introduced by Dorny *et al.* (Dorny et al., 2004b). The optical density (OD) of the human samples were read at 490nm with a reference at 650nm, the OD of the pig samples were read at 490nm. The cut-off was calculated as described by Dorny *et al.* (Dorny et al., 2000) using a panel of eight negative sera from the Lao human and pig populations. A ratio for each test was calculated by dividing the optical density of the test sample by the cut-off value, a ratio >1 was considered positive. Samples were retested if the coefficient of variation was more than 50% or if the optical density of the test sample was close to the cut-off.

DNA was isolated from proglottids expelled post-niclosamide treatment using DNeasy Blood and Tissue extraction kit (QIAGEN, Hilden, Germany). Multiplex PCR for cytochrome c oxidase subunit 1 gene (cox1) was performed for species identification of *T. saginata*, *T. solium* and *T. asiatica*. Primers and PCR protocols were as described earlier (Yamasaki et al., 2004) with modifications. A PCR cocktail contained 0.4 μM of each primer (Sigma-Aldrich,St. Louis, USA), 1.25 U of GoTaq DNA polymerase in GoTaq reaction buffer supplemented with 2 mM MgCl₂ (Promega, Madison, USA) and 0.2 mM of each dNTP (Promega, Madison, USA) in a final 50 μl reaction volume. Amplification protocol consisted of 3 min at 94 °C followed by 35 cycles of 30 s at 94°C, 30 s at 56°C and 90 s at 72°C, plus one cycle of 5 min at 72 °C. Subsequently, PCR-amplified products were electrophoresed on 1.5% agarose gels with a DNA ladder (Hyperladder II; Bioline, London, United Kingdom). Positive control DNA for the three *Taenia* species were extracted from proglottids kindly supplied by the Institute of Tropical Medicine (Antwerp, Belgium).
2.3.5 Data analysis

The questionnaire and laboratory test result data were entered into a spreadsheet (Excel®; Microsoft Corporation, Redmond, USA) and subsequent analysis was carried out in STATA/IC version 10 (StataCorp LP, College Station, USA). The socio-economic status of each household was calculated by use of principal component analysis of household assets (Raso et al., 2005; Steinmann et al., 2007) after replacement of missing values with the mean of the respective asset for that ethnic group. All assets were dichotomous. The households were ranked into wealth quintiles according to their cumulative standardised asset scores.

Prevalence of cysticercosis seropositivity in the human and pig populations were calculated as the proportion of positive AgELISA results in the sampled population. Taeniasis prevalence in humans and dogs was calculated as the proportion of faecal samples with taeniid eggs in the sampled population. In addition, human taeniasis prevalence was calculated for those individuals who had eggs detected and/or self-reported tapeworm segments in their stool. In the pig study, the Pearson’s $\chi^2$-test and Fisher’s exact test were used to explore associations between infection status (carcass inspection and AgELISA seropositivity) and age, breed, sex and production system at last point of sale.

For risk factor analysis in the human study, taeniasis was defined as taeniid egg positive and/or self-reporting segments. Univariate logistic regression without adjustment was used to test associations between infection status (cysticercosis or taeniasis) and gender, location, ethno-linguistic family, age, wealth status, defecation site, taeniasis, history of taeniasis, uncooked meat consumption habits and literacy of selected household members. Risk factors significant or borderline significant (cut-off $P \leq 0.10$) in the univariate analyses were included in a multivariate random effects logistic regression model adjusting for the effect of household clustering. The results are reported as adjusted odds ratio (AOR) and 95% confidence intervals (CI). The final analysis only considered individuals with complete parasitological and serological data. Missing data on literacy of household members due to death, divorce or other factors were replaced with the mean for that village and rounded to zero or one, illiterate or literate.

A Maximum Likelihood Estimator (MLE) (eq. 3) with 95% CI (eq. 4) (Rahme, 1998) was used to calculate adjusted prevalence for dog *T. hydatigena* taeniasis and pig cysticercosis detected by inspection at slaughter, for *T. solium, T. hydatigena* and *T. asiatica*.

$$\text{MLE} = \frac{p - (1 - sp)}{se + sp - 1}$$  \hspace{1cm} (eq. 3)
\[ 95\% \text{ CI} = \frac{p + sp - 1 - 1.96\sqrt{\frac{p(1 - p)}{N}}}{se + sp - 1}, \quad \frac{p + sp - 1 + 1.96\sqrt{\frac{p(1 - p)}{N}}}{se + sp - 1} \]  
(eq. 4)

Where \( p \) was the observed prevalence, \( sp \) the test specificity, \( se \) the test sensitivity and \( N \) was the sample size. Calculations were made through 10% increments of test sensitivity assuming specificity was 100%. For dog \( T. \ hydatigena \) taeniasis, 100% specificity was assumed since \( Echinococcus \ spp. \) are not endemic in Southeast Asia. Cystic echinococcosis has been rarely reported from mainland Southeast Asia and nothing is known of its epidemiology (McManus, 2010). For pig cysticercosis, carcass inspection specificity has been estimated to be at or very close to 100% (Dorny et al., 2004b).

2.4 Results

2.4.1 Human study

A total of 1582 people in 332 households were eligible to participate in this survey, of these, 1306 (82.7%) individuals from 321 households aged 6-91 years provided blood and faecal samples, a completed questionnaire and had valid laboratory test results. Overall, the Mon-Khmer and Lao-Tai ethnic families had the highest compliance, 88.9 and 88.6% of eligible persons, respectively. The Hmong-Mien ethnic family had the lowest compliance with only 62.5% of eligible persons providing a stool and blood sample and a completed questionnaire. Non-compliance due to mental illness was negligible with two persons, one each from the Lao-Tai and Hmong-Mien ethnic groups, protected by their respective families. The final survey population consisted of 553 Lao-Tai (42.3%), 523 Mon-Khmer (40.1%) and 230 (17.6%) Hmong-Mien. No Sino-Tibetan people were recruited into this study. Table 2.1 describes differences between the compliant and non-compliant persons who were eligible to participate in the study stratified by ethnic family.

Table 2.2 summarises the survey population structures stratified by ethnicity. Significant differences were observed for all characteristics with the exception of gender. The majority of Lao-Tai people were from Huaphan province (49.0%), no Mon-Khmer people were selected in Huaphan and Xiengkhuang province and the majority of Hmong-Mien people were from Xiengkhuang province (70.4%). The highest proportion of impoverished participants were from the Mon-Khmer ethnic family and the highest proportion of least and less poor participants were from the Lao-Tai ethnic family. The Mon-Khmer and Hmong-Mien ethnic families had the highest proportion of participants defecating in the open and the highest proportion of people living in a household with an illiterate female head of household (Table 2.2).
The prevalence of cysticercosis and taeniasis stratified by ethnicity for population and individual variables are summarised in Table 2.3. The prevalence of cysticercosis AgELISA positivity was 2.2% (95% Confidence interval (CI): 1.4-3.0%), ranging at the village level from 0.0-11.3%; 14 villages had no detectable cysticercosis cases and 10 villages had at least one seropositive case. Greater than half the cases (15/29) were detected in three villages in Oudomxay province. Univariate analysis showed that province was the only variable significantly associated with cysticercosis AgELISA positivity. After controlling for clustering at the household level by random effects logistic regression, only Luangprabang province (Adjusted OR=0.26, 95% CI=0.07, 0.98) was significantly associated with reduced risk of cysticercosis AgELISA positivity.

The prevalence of Taenia egg positivity was 2.9% (95% CI: 2.0-3.8%) and the estimated taeniasis prevalence, egg positive plus self-reported, was 8.4% (95% CI: 6.9-9.9%), ranging at the village level from 0.0-6.9% and 0.0-17.0%, respectively. The proportion of people reporting a history of taeniasis was 27.0% (95% CI: 24.5-29.4%). For individuals with current taeniasis, 90.0% (95% CI: 84.3-95.7%) reported having a history of taeniasis compared to 21.2% (95% CI: 18.8-23.5%) for uninfected individuals. Only egg positive and/or self-reporting cases were considered in the risk factor analysis. On univariate analysis, a history of taeniasis was very strongly associated with increased risk of having a current taeniasis infection. Other factors significantly associated with taeniasis were gender, province, age, ethnicity and consumption of raw meat. Two multivariate analyses of risk factors associated with taeniasis were carried out, including and excluding the variable “history of taeniasis” (Table 2.4). History of taeniasis was strongly correlated with increasing age ($\chi^2=121.9$, $P<0.001$) and inclusion in the model gave the perception that increasing age was protective (Table 2.4). The exclusion of “history of taeniasis” from the analysis resulted in gender, province of origin, age, ethnicity, and infrequent consumption of uncooked beef being significantly associated with a current taeniasis infection (Table 2.4). The consumption of uncooked pork and uncooked fermented pork were not significantly associated with taeniasis after controlling for other risk factors.

The proportion of people reporting consumption of any uncooked meat, uncooked pork, fermented pork sausage and uncooked beef was 50.2% (95% CI: 47.5-52.9%), 13.9% (12.1-15.8%), 28.8% (26.3-31.2%) and 36.7% (34.1-39.4%), respectively. The prevalence of eating any uncooked meat increased significantly with age ($\chi^2=145.8$, $P<0.001$), similar results were observed for eating uncooked beef ($\chi^2=214.2$, $P<0.001$), uncooked pork ($\chi^2=48.7$, $P<0.001$) and fermented pork sausage ($\chi^2=41.3$, $P<0.001$) (Figure 2.2). Uncooked beef consumption had the highest peak prevalence of 56.9% (95% CI: 50.4-63.4%) in the 40-54 year age group; the peak
prevalence of eating uncooked pork and uncooked fermented pork sausage was 20.9% (95% CI: 15.5-26.2%) and 37.8% (95% CI: 31.3-44.2%), respectively, also in the 40-54 year age group (Figure 2.2).

Of the 110 taeniasis positive individuals who were treated with niclosamide, proglottids were expelled from 35 people and PCR revealed 33 tapeworms were *T. saginata* and two were *T. solium*. The *T. solium* worms were recovered from a 7-year-old male from Oudomxay province who was AgELISA negative and from a 34-year-old male from Xiengkhuang province who was AgELISA positive. Both cases reported not eating uncooked pork or fermented pork sausage. The *T. saginata* worms were recovered from 27 males and six females (age range: 19-78) from all provinces. Thirty-two of the *T. saginata* cases were AgELISA negative and 32 reported eating uncooked beef. Both *T. solium* cases were egg positive by FECT and one was self-reported. Sixteen of the 33 (48.5%) *T. saginata* cases were egg positive by FECT and 29/33 (87.9%) were self-reported.

### 2.4.2 Pig study

The inspection data results from Oudomxay province were dropped from the analysis due to the submission of incorrectly completed forms. A total of 590 pig carcasses, with a matching serum sample, were inspected in three provinces, 209 in Luangprabang, 190 in Huaphan and 191 in Xiengkhuang. Data on the variables ‘breed’, ‘age’, ‘sex’ and ‘production system at last point of sale’ were collected for 538, 528, 540 and 518 pigs, respectively (Table 2.5). Carcass inspection detected five pigs (0.8%) with cysts consistent with the morphology of *T. solium*, 1.0-1.5 cm fluid filled muscle cysts with a single white scolex. All infected pigs were heavily infected (without counting) and had viable and degenerated cysts and increasing age was significantly associated with *T. solium* detection (Table 2.5). One hundred and thirty two (22.4%) carcasses were detected with cysts consistent with the morphology of *T. hydatigena*; large, visceral, fluid filled cysts with a single white scolex. The prevalence of *T. hydatigena* detection was significantly greater in free-range pigs (Table 2.5). Two pigs, one each from Luangprabang and Huaphan, had a dual infection with *T. solium* and *T. hydatigena*. One pig in Huaphan province was detected with cysts consistent with *T. asiatica*, very small fluid filled cysts present in the liver, spleen and lung. This pig was a 15-month-old female purchased from a penned production system. Non-specific liver lesions consistent with parasitemia were detected in 16 (2.7%) pigs; these were considered inspection negative in the absence of histopathology to definitively detect *Taenia*.

The sera of 404 (68.5%) pigs were reactive in the AgELISA and no significant association was observed for province, breed, age, sex and production system (Table 2.5). All five *T. solium* and
one *T. asiatica* inspection positive pigs had serum samples reactive in the AgELISA. Of the 132 *T. hydatigena* inspection positive pigs, 129 were reactive and three were non-reactive. Fourteen of the pigs with non-specific liver lesions were serum reactive in the AgELISA and two were non-reactive.

Table 2.6 describes the estimates of true prevalence of *T. solium*, *T. hydatigena*, *T. asiatica* cysticercosis in pigs and *T. hydatigena* taeniasis in dogs using the Maximum Likelihood Estimator (MLE) for carcass inspection with sensitivity ranging from 0.1 to 1.0 and assuming specificity was equal to 1.0.

### 2.4.3 Dog study

Faecal samples were collected from 105 dogs from 21 villages, 32 (30.5%), 30 (28.6%), 11 (10.5%) and 32 (30.5%) from Oudomxay, Luangprabang, Huaphan and Xiengkhuang provinces, respectively. All dogs were raised in an unrestrained manner, the median age was 12 months (range: 2-108) and 63 (60%) were female and 42 (40%) were male. Two dogs (1.9% (95% CI: 0.0-4.6)) were *Taenia* egg positive; one a 12-month-old male dog from village 5 in Xiengkhuang province and the other a 3-year-old male dog from village 15 in Luangprabang province. Estimates of true prevalence in the plausible range of diagnostic sensitivity ranged from 4.8-6.3% (Table 2.6).

### 2.5 Discussion

This study was the first of its kind to investigate *T. solium* in the context of multiple *Taenia* species interacting in a host assemblage that includes humans, pigs, dogs and bovines. We have documented the sympatric occurrence of four *Taenia* species in northern Laos where conditions suit *T. solium* transmission and hyperendmicity. We observed a substantial proportion of the population practicing open defecation, uncooked pork consumption was relatively common and pig production systems were rudimentary. However we observed a low prevalence of human cysticercosis in the survey population. The following discussion draws on the human and animal studies to hypothesise that *T. hydatigena* hyperendemicity in pigs and a high prevalence of Lao people eating uncooked beef were the strongest factors controlling *Taenia* ecology in the Laos.

This study had a number of important limitations and most notable was the relatively small sample size, this was evident in a lack of statistical power to detect significant risk factors associated with cysticercosis even though the majority of cases occurred in three Mon-Khmer villages in Oudomxay province. Secondly, we sought to recruit all eligible household members ≥6
years old and compliance varied for the different ethnic groups. The majority of the low compliance in the Hmong-Mien ethnic family was evident in Huaphan province and could be explained by an aversion to venipuncture and embarrassment in giving a faecal sample, both closely linked to cultural beliefs and customs. This could be corrected in future studies by using a finger-prick and blood spot sampling method, the use of trained Hmong-Mien women to administer the surveys and strengthening of the consultation process. This study limitation possibly lead to an under estimation of cysticercosis prevalence in this ethnic group as the poor household members were most likely to have refused participation and the three cysticercosis cases were identified in the three poorest quintiles. Taeniasis however was more common in the wealthier quintiles of the Hmong-Mien ethnic group and our data may have represented an over estimate. Families also tended to exclude household members who were mentally ill or frail, possibly having a limited effect on the estimate of taeniasis and cysticercosis prevalence within the survey population. Since the number of refusals due to frailty or mental illness was small, this effect was assumed to be negligible.

We required an accurate estimate of current human cysticercosis infection and used a circulating antigen ELISA rather than an antibody ELISA which tends to overestimate prevalence in endemic areas (Garcia et al., 2001; Dorny et al., 2004a; Somers et al., 2006). One preliminary study in Vietnam, using CT-scan and cutaneous nodule biopsy as gold standard, found a high sensitivity (94.4%) and specificity (100%) of detecting active human cysticercosis with the AgELISA (Erhart et al., 2002), indicating the results in our study were reliable. Human cysticercosis was relatively rare in northern Laos at the community level (2.2%) but there was strong evidence of a focal distribution since just over half of the seropositive cases came from three villages in Oudomxay province. In Asia, a focal distribution of human cysticercosis has also been observed in northern Vietnam (Somers et al., 2006), Indonesian Papua (Salim et al., 2009) and China (Chen et al., 2004), so our results support evidence that cases tend to cluster in geographically restricted localities. The relatively high prevalence of cysticercosis in young children was unexpected and corresponded with a relatively high prevalence of taeniasis in the same age group, even though the 7-year old boy with T. solium taeniasis was cysticercosis AgELISA negative. The specific exposures leading to increased prevalence in this age group warrants further investigation.

We observed high taeniasis prevalence (8.4%) with spatial variation based on self-reporting and detection of Taenia eggs in a single stool and these results were comparable with other studies in southern and central Laos (Giboda et al., 1991a; Giboda et al., 1991b; Pholsena et al., 1991; Chai and Hongvanthong, 1998; Vannachone et al., 1998; Rim et al., 2003; Sithithaworn et al., 2006;
Sayasone et al., 2007; Tran et al., 2007; Conlan et al., 2008; Erlanger et al., 2008; Sayasone et al., 2009). Somers et al. (2006) estimated that self-reporting in Vietnam grossly over-estimated true prevalence, but in our study almost half (17/35) of the treated cases who expelled proglottids were initially detected by self-reporting alone. This was consistent with Flisser et al. (2005) who reported improved detection of tapeworm carriers using self-reporting methods. Somers et al. (2006) also found Taenia egg prevalence almost identical to copro-antigen prevalence, which was inconsistent with studies conducted elsewhere (Allan et al., 1996; Garcia-Noval et al., 1996). Our results show conclusively that Taenia egg detection was a gross underestimate of true prevalence and we obtained 18 additional tapeworm specimens after treating egg negative individuals who self-reported infection. Unfortunately, no scoleces were recovered following treatment and proglottids were not recovered from more than half of the egg positive cases (20/38). These results are comparable with other studies using niclosamide and magnesium sulphate (Sanchez et al., 1997) but poor in comparison to the use of pre- and post-niclosamide purging with electrolyte-polyethyleneglycol salt (Jeri et al., 2004). The later was not suitable for use in the field in Laos. Thirty-three (94.3%) of the tapeworm specimens were identified by PCR as T. saginata and since the efficacy of niclosamide in treating T. solium and T. saginata are assumed to be similar (Flisser, 2006), the data indicates that T. saginata was the dominant species infecting Lao people.

The strongest risk factor for having a current taeniasis infection was having a history of taeniasis. Ninety percent of taeniasis cases reported having a history of taeniasis, although this could refer to a single infection with sporadic shedding of segments. Despite this potential bias, the results indicated a high prevalence of taeniasis re-infection, which was not surprising considering the very high prevalence of eating uncooked meat; particularly beef. Infrequent consumption of beef, which is typically prepared as “laap” (a traditional meat salad prepared with lime juice, mint, chilli and pounded rice), was strongly associated with taeniasis in both multivariate models and indicates a possible link with raw meat consumption at festivals. More detailed investigations will be required to understand specific cultural practices and food preparation. We did not undertake studies of cysticercosis in cattle and buffalo in this study, however a recent slaughterhouse based study in five northern provinces detected cysticercosis antigen in 52% of cattle and 21% of buffalo (Vongxay et al., 2012). The evidence therefore suggests that taeniasis re-infection was predominantly due to eating uncooked beef and infection with T. saginata. The two T. solium cases were not associated with knowingly eating uncooked pork and may have arisen simply from inadvertent undercooking.

The animal studies provide important insights into the ecology of Taenia in the Laos and mainland Southeast Asia in general. The evidence suggests that T. hydatigena was the dominant
species infecting pigs in the Laos, with a strong infection pressure being exerted by a relatively large, unmanaged dog population (*Canis lupus familiaris*). No other definitive hosts for *T. hydatigena* are found in the Laos. Under a strong infection pressure, immunity in the intermediate host can be acquired within two weeks of exposure to eggs and embryos entering muscles after one week will not survive (Gemmell, 1987; Gemmell et al., 1987), meaning there exists a short window of opportunity for infection throughout the life of the intermediate host. Immune-mediated *Taenia* competition in the intermediate host has been well documented for the ovine tapeworms, *T. ovis* and *T. hydatigena*, where *T. ovis* cyst development can be inhibited by pre-exposure to *T. hydatigena* eggs (cited by Ref. (Gemmell et al., 1987)). There are no plausible reasons why these same immune-mediated competitive interactions do not occur in pigs, meaning that *T. solium* cyst development would be inhibited by ingestion of *T. hydatigena* eggs. The evidence presented here indicates that pigs may be exposed to *T. hydatigena* eggs at a young age through coprophagia, feed, water and/or soil contamination and provide pigs with protective immunity due to genus conserved immunogenic antigens. However, controlled studies will be required to elucidate the immune-mediated interactions in pigs, if they exist, and determine the impact on transmission dynamics.

The pig and dog studies were limited by the diagnostic protocols employed. We used egg detection for *T. hydatigena* taeniasis in dogs instead of copro-antigen ELISA as we were unable to source suitable diagnostic reagents during the course of this study. No published data provide a reliable estimate of the diagnostic sensitivity and specificity of *T. hydatigena* egg detection in dog faeces. Since proglottids are predominantly shed without defecation (cited by Ref. (Deplazes et al., 1990)), we believe sensitivity would be much lower than the estimated 62.5% observed for *T. solium* taeniasis (Allan et al., 1996; Allan et al., 2003). Our prevalence estimates of *T. hydatigena* were in the order of 5-6% of village dogs and this corresponded to prevalence in pigs of 50-60%.

In the pig study, the AgELISA was not able to differentiate the three *Taenia* species (Dorny et al., 2004a; Deckers and Dorny, 2010) and the inspection data could only account for one third of the serologically positive animals. The sensitivity of detecting *T. solium* cysts at slaughter can be variable and estimates range from 20-60% (Sciutto et al., 1998; Dorny et al., 2004b; OIE, 2010), but specificity has been estimated to be 100% (Dorny et al., 2004b). Bayesian approaches have been applied to model true prevalence of *T. solium* cysticercosis in the face of imperfect tests (Dorny et al., 2004b), however, in this present study, *T. hydatigena* and *T. asiatica* co-endemcity meant the exclusion of AgELISA results and the Bayesian model could not be applied. Instead of a Bayesian approach, we used a standard maximum likelihood estimator to calculate true prevalence, which was only valid if both specificity and sensitivity were known (Rahme, 1998).
We made the assumption that inspection was 100% specific and calculated true prevalence through a range of sensitivities of detecting cysts at slaughter; meaning prevalence was estimated from one degree of freedom in the observed data at each increment of sensitivity. Since inspection was constrained by traders restricting muscle slicing, we assumed the sensitivity of detecting *T. solium* cysts was at the lower end of the range. No data was available that allowed us to estimate the sensitivity of detecting *T. hydatigena* in pigs. Since *T. hydatigena* metacestodes mature in the fat of the mesentery and omentum and the size can vary from 1-7 cm (OIE, 2010), these cysts could be easily missed, particularly in Lao indigenous breed pigs that typically have a very high fat content (Phengsavanh et al., 2010). The adjusted inspection prevalence data for *T. solium*, *T. hydatigena* and *T. asiatica* accounted for the majority of the serologically positive animals.

It is evident that future *Taenia* research in Southeast Asia will require the use of more robust diagnostic protocols for the animal and human studies. The ‘gold standard’ for cysticercosis in pigs, dissection taking 0.5 cm slices through half a carcass (Boa et al., 2002), is both expensive and time consuming and not conducive to large a geographically diverse studies. There is a genuine need for a robust, cheap, sensitive, specific, validated and readily available test that can differentiate *Taenia* species in pigs (Sciutto et al., 1998; Conlan et al., 2009). Similarly, improved tests for the sensitive and specific detection of human *T. solium* taeniasis cases are required, and these tests are currently being developed and validated (Handali et al., 2010), but validation in multiple populations around the world should be a priority.

We have documented the occurrence of four *Taenia* species in the Laos. The study indicated a low prevalence of *T. solium* cysticercosis and taeniasis in the human population with a focal distribution in northern Laos. The evidence also suggests that natural parasite competition and local food customs have an influence on *T. solium* transmission, presenting real opportunities for control and possible elimination. With a concerted effort to identify, treat and follow-to-cure *T. solium* tapeworm carriers, thereby reducing the infection pressure on pigs, continued exposure of pigs to *T. hydatigena* eggs may assist in further reducing *T. solium* transmission. With time, and continued improvements to sanitation and pig husbandry in Laos, we might expect significant reductions in human cysticercosis prevalence.

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2.8 References


http://www.oie.int/eng/normes/mmanual/2008/pdf/2.09.05_CYSTICERCOSIS.pdf.


Figure 2.1 Study sites in northern Laos. 1, Xay district, Oudomxay province; 2, Xiengngeun district, Luangprabang province; 3, Pek district, Xiengkuang province; 4, Viengxay district, Huaphan province.
Figure 2.2 Proportion of study population consuming uncooked meat by age category. Solid black line, any uncooked meat; grey line with circles, uncooked pork; grey line with triangles, uncooked fermented pork sausage; grey line with diamonds, uncooked beef.
Table 2.1 Demographic differences between survey participants compliant (C) and non-compliant (NC), stratified by ethnicity, Laos

<table>
<thead>
<tr>
<th></th>
<th>Lao-Tai</th>
<th>NC, no. (%)</th>
<th>P</th>
<th>Mon-Khmer</th>
<th>NC, no. (%)</th>
<th>P</th>
<th>Hmong-Mien</th>
<th>NC, no. (%)</th>
<th>P</th>
</tr>
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<tr>
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<td>277 (89.1)</td>
<td>34 (10.9)</td>
<td>0.168†</td>
<td>270 (91.5)</td>
<td>25 (8.5)</td>
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<td>109 (67.3)</td>
<td>53 (32.7)</td>
<td>0.267†</td>
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<td>276 (92.3)</td>
<td>23 (7.7)</td>
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<td>25 (9.0%)</td>
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<td>121 (72.9)</td>
<td>45 (27.1)</td>
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</tr>
<tr>
<td>Province</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Oudomxay</td>
<td>58 (96.7)</td>
<td>2 (3.3)</td>
<td>0.029‡</td>
<td>297 (90.8)</td>
<td>30 (9.2)</td>
<td>0.779‡</td>
<td>28 (96.6)</td>
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<tr>
<td>Luangprabang</td>
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<td>0</td>
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<td>12 (6.9)</td>
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C=compliant; NC=non-compliant; Missing data for non-compliant: Lao-Tai=14, Mon-Khmer=16, Hmong-Mien=41.
† For chi-square test for difference between C and NC.
‡ For Fisher’s exact test for difference between C and NC.
Table 2.2 Survey population characteristics stratified by ethnicity, Laos

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<th>Population characteristics</th>
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<th>Hmong-Mien</th>
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<td>31.4</td>
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HH=household
† Calculated across all groups and ethnicities.
Table 2.3 Prevalence of cysticercosis (antigen capture ELISA) and taeniasis (egg detection plus self-reported) by population level and individual level characteristics, stratified by ethnicity, Laos

<table>
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<tr>
<th>Population characteristics</th>
<th>Proportion Cysticercosis antigen ELISA positive (95% CI)</th>
<th>Proportion egg positive or self-reported taeniasis (95% CI)</th>
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<td>Total LT MK HM</td>
<td>Total LT MK HM</td>
</tr>
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<td>Total</td>
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<td>8.4 (6.9, 9.9) 7.8 (5.5, 10.0) 11.7 (8.9, 14.4) 2.6 (0.5, 4.7)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>2.1 (1.0, 3.2) 1.1 (0.0, 2.3) 3.7 (1.4, 6.0) 0.9 (0.0, 2.7)</td>
<td>4.7 (3.1, 6.4) 3.9 (1.7, 6.3) 7.0 (4.0, 10.1) 0.9 (0.0, 2.7)</td>
</tr>
<tr>
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<td>2.3 (1.2, 3.5) 2.2 (0.4, 3.9) 2.7 (0.7, 4.8) 1.7 (0.0, 4.0)</td>
<td>12.2 (9.6, 14.7) 11.6 (7.8, 15.4) 16.6 (12.0, 21.2) 4.1 (0.5, 7.7)</td>
</tr>
<tr>
<td>Province</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oudomxay</td>
<td>3.9 (2.0, 5.9) 0.0</td>
<td>5.1 (2.5, 7.6) 0.0</td>
</tr>
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<td>1.1 (0.0, 2.3) 1.1 (0.0, 2.4)</td>
<td>0.0</td>
</tr>
<tr>
<td>Xiengkhuang</td>
<td>2.4 (0.6, 4.1) 3.0 (0.0, 5.9)</td>
<td>1.9 (0.0, 4.0)</td>
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<tr>
<td>Wealth status</td>
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<tr>
<td>Most poor</td>
<td>3.1 (0.8, 5.4) 0.0</td>
<td>3.7 (0.8, 6.6) 2.0 (0.0, 6.1)</td>
</tr>
<tr>
<td>Very poor</td>
<td>4.1 (1.6, 6.6) 0.0</td>
<td>5.9 (2.1, 9.7) 2.9 (0.0, 8.7)</td>
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<td>7.3 (4.3, 10.3) 8.4 (4.3, 12.5) 7.4 (0.2, 14.6) 3.6 (0.0, 8.6)</td>
</tr>
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<td>7.6 (4.4, 10.8) 6.3 (2.0, 10.7)</td>
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<td>Age (years)</td>
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<td>7.3 (3.9, 10.9) 2.9 (0.0, 7.0)</td>
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<td>1.4 (0.0, 4.3) 2.6 (0.0, 8.0)</td>
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<td>4.3 (1.8, 6.7) 2.9 (0.0, 6.1) 6.9 (1.9, 11.9) 2.0 (0.0, 5.9)</td>
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<td>13.5 (9.5, 17.5) 13.3 (7.5, 19.1) 14.7 (7.9, 21.4) 10.5 (0.3, 20.7)</td>
</tr>
<tr>
<td>40-54</td>
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<td>11.6 (7.3, 15.8) 12.9 (6.0, 19.8) 13.3 (6.4, 20.1) 2.9 (0.0, 8.9)</td>
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<td>8.8 (6.9, 10.7) 7.6 (5.2, 10.0) 13.5 (9.4, 17.5) 2.7 (0.0, 5.8)</td>
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<td>8.2 (6.5, 9.8) 8.0 (5.6, 10.3)</td>
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<td>Female head of HH</td>
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<tr>
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<td>8.2 (6.3, 10.1)</td>
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</tbody>
</table>
ELISA=enzyme linked immunosorbent assay, CI=confidence interval, LT=Lao-Tai ethnicity, MK=Mon-Khmer ethnicity, HM=Hmong-Mien ethnicity, HH=household.
Table 2.4 Risk factors significantly (P<0.050) associated with taeniasis, as determined by multiple logistic regression modelling controlling for household clustering, Laos

<table>
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<th>Model†</th>
<th>Population characteristic</th>
<th>Risk factor</th>
<th>Adjusted OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1</td>
<td>Province</td>
<td>Oudomxay</td>
<td>Ref.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Luangprabang</td>
<td>0.71</td>
<td>0.40, 1.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Huaphan province</td>
<td>0.32</td>
<td>0.12, 0.84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xiengkhuang province</td>
<td>0.38</td>
<td>0.15, 0.93</td>
</tr>
<tr>
<td>Age (years)</td>
<td>6-10 years old</td>
<td>Ref.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11-14 years old</td>
<td>0.17</td>
<td>0.05, 0.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15-24 years old</td>
<td>0.24</td>
<td>0.09, 0.67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25-39 years old</td>
<td>0.42</td>
<td>0.17, 1.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40-54 years old</td>
<td>0.29</td>
<td>0.11, 0.75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥55 years old</td>
<td>0.22</td>
<td>0.08, 0.60</td>
<td></td>
</tr>
<tr>
<td>Ethnicity</td>
<td>Lao-Tai ethnicity</td>
<td>Ref.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mon-Khmer ethnicity</td>
<td>0.90</td>
<td>0.45, 1.83</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hmong ethnicity</td>
<td>0.34</td>
<td>0.12, 0.96</td>
<td></td>
</tr>
<tr>
<td>Previous taeniasis</td>
<td>No previous taeniasis</td>
<td>Ref.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>History of taeniasis</td>
<td>32.98</td>
<td>15.63, 69.56</td>
<td></td>
</tr>
<tr>
<td>Raw beef consumption</td>
<td>Doesn’t eat</td>
<td>Ref.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Weekly</td>
<td>0.81</td>
<td>0.27, 2.37</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Monthly</td>
<td>1.48</td>
<td>0.71, 3.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Every few months</td>
<td>1.07</td>
<td>0.49, 2.35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Infrequent</td>
<td>4.13</td>
<td>1.50, 11.36</td>
<td></td>
</tr>
<tr>
<td>Model 2</td>
<td>Gender</td>
<td>Female</td>
<td>Ref.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>2.20</td>
<td>1.34, 3.63</td>
<td></td>
</tr>
<tr>
<td>Province</td>
<td>Oudomxay province</td>
<td>Ref.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Luangprabang province</td>
<td>0.65</td>
<td>0.38, 1.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Huaphan province</td>
<td>0.26</td>
<td>0.11, 0.63</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Xiengkhuang province</td>
<td>0.36</td>
<td>0.15, 0.86</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>6-10 years old</td>
<td>Ref.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11-14 years old</td>
<td>0.24</td>
<td>0.07, 0.75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15-24 years old</td>
<td>0.42</td>
<td>0.17, 1.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25-39 years old</td>
<td>1.11</td>
<td>0.53, 2.34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40-54 years old</td>
<td>0.86</td>
<td>0.39, 1.90</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥55 years old</td>
<td>0.82</td>
<td>0.34, 1.94</td>
<td></td>
</tr>
<tr>
<td>Ethnicity</td>
<td>Lao-Tai ethnicity</td>
<td>Ref.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mon-Khmer ethnicity</td>
<td>0.86</td>
<td>0.45, 1.65</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hmong ethnicity</td>
<td>0.26</td>
<td>0.10, 0.70</td>
<td></td>
</tr>
<tr>
<td>Raw beef consumption</td>
<td>Doesn’t eat</td>
<td>Ref.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Weekly</td>
<td>1.68</td>
<td>0.61, 4.65</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Monthly</td>
<td>2.43</td>
<td>1.27, 4.65</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Every few months</td>
<td>1.88</td>
<td>0.91, 3.87</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Infrequent</td>
<td>5.99</td>
<td>2.51, 14.25</td>
<td></td>
</tr>
</tbody>
</table>

OR=odds ratio; CI=confidence interval; Ref.=referent; Infrequent=consumes once or twice per year or less often.

†Model 1=taeniasis OR adjusted for gender, province, age, history of taeniasis, ethnicity and frequency of raw fermented pork sausage, raw pork and raw beef consumption; Model 2=taeniasis OR adjusted for gender, province, age, ethnicity, raw fermented pork sausage consumption, raw pork consumption, raw beef consumption
Table 2.5 Prevalence of pig cysticercosis (*Taenia solium*, *T. hydatigena* and *T. asiatica*) by carcass inspection and seroprevalence of pig cysticercosis (antigen capture ELISA) by location, sex, breed, age and production system at last point of sale, Laos

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>N (%)</th>
<th>Carcass inspection prevalence of pig cysticercosis (95% CI)</th>
<th></th>
<th>Cysticercosis AgELISA positivity (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>T. solium</em></td>
<td><em>T. hydatigena</em></td>
<td>Prevalence</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(95% CI)</td>
<td>(95% CI)</td>
<td>(95% CI)</td>
</tr>
<tr>
<td>Total</td>
<td>590</td>
<td>0.8 (0.1-1.6)</td>
<td>22.4 (19.0-25.7)</td>
<td>68.5 (64.7-72.2)</td>
</tr>
<tr>
<td>Province (N=590)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luangprabang</td>
<td>209 (35.4)</td>
<td>1.4 (0.0-3.1)</td>
<td>22.5 (16.8-28.2)</td>
<td>0.173‡</td>
</tr>
<tr>
<td>Huaphan</td>
<td>190 (32.2)</td>
<td>1.1 (0.0-2.5)</td>
<td>26.3 (20.0-32.6)</td>
<td></td>
</tr>
<tr>
<td>Xiengkhuang</td>
<td>191 (32.4)</td>
<td>0.0 (0.0-0.0)</td>
<td>18.3 (12.7-23.8)</td>
<td></td>
</tr>
<tr>
<td>Age (months) (N=528)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤6</td>
<td>70 (13.3)</td>
<td>0</td>
<td>27.1 (16.6-37.7)</td>
<td>0.629‡</td>
</tr>
<tr>
<td>7-12</td>
<td>250 (47.4)</td>
<td>0</td>
<td>22.8 (17.6-28.0)</td>
<td></td>
</tr>
<tr>
<td>13-18</td>
<td>128 (24.2)</td>
<td>1.6 (0.0-3.7)</td>
<td>19.5 (12.6-26.4)</td>
<td></td>
</tr>
<tr>
<td>≥18</td>
<td>80 (15.2)</td>
<td>3.8 (0.0-8.0)</td>
<td>25.0 (15.4-34.6)</td>
<td></td>
</tr>
<tr>
<td>Breed (N=538)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lao indigenous</td>
<td>473 (87.9)</td>
<td>1.1 (0.1-2.0)</td>
<td>24.7 (20.8-28.6)</td>
<td>0.077†</td>
</tr>
<tr>
<td>Exotic</td>
<td>37 (6.9)</td>
<td>0</td>
<td>10.8 (6.9-21.0)</td>
<td></td>
</tr>
<tr>
<td>Cross-breed</td>
<td>28 (5.2)</td>
<td>0</td>
<td>14.3 (1.1-27.5)</td>
<td></td>
</tr>
<tr>
<td>Sex (N=540)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>275 (50.9)</td>
<td>0.7 (0.0-1.7)</td>
<td>22.5 (17.6-27.5)</td>
<td>0.423‡</td>
</tr>
<tr>
<td>Male</td>
<td>130 (24.1)</td>
<td>2.3 (0.0-4.9)</td>
<td>20.0 (13.1-26.9)</td>
<td></td>
</tr>
<tr>
<td>Castrated male</td>
<td>135 (25.0)</td>
<td>0</td>
<td>26.7 (19.2-34.2)</td>
<td></td>
</tr>
<tr>
<td>Production system at last point of sale (N=518)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penned/Corralled</td>
<td>345 (66.6)</td>
<td>1.2 (0.0-2.3)</td>
<td>19.4 (15.2-23.6)</td>
<td>0.024‡</td>
</tr>
<tr>
<td>Free-roaming</td>
<td>167 (32.2)</td>
<td>0.6 (0.0-1.8)</td>
<td>29.3 (22.4-36.3)</td>
<td></td>
</tr>
<tr>
<td>Mixed</td>
<td>6 (1.2)</td>
<td>0</td>
<td>33.3 (0.0-74.7)</td>
<td></td>
</tr>
</tbody>
</table>

ELISA=enzyme-linked immunosorbent assay; CI=confidence interval; Mixed=sometimes penned and sometimes free-roaming.
†By Fisher’s exact test.
‡By Pearson’s chi-square test.
Table 2.6 Estimated true prevalence of pig cysticercosis (*Taenia solium, T. hydatigena* and *T. asiatica*) and dog taeniasis (*T. hydatigena*) adjusted by the Maximum-Likelihood-Estimation model for increments of test sensitivity, assuming specificity of 100%, Laos

<table>
<thead>
<tr>
<th>Test sensitivity (%)</th>
<th>Estimated true prevalence of pig cysticercosis, % (95% CI)</th>
<th>Estimated true prevalence of <em>T. hydatigena</em> taeniasis in village dogs (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>T. solium</em></td>
<td><em>T. hydatigena</em></td>
</tr>
<tr>
<td>100</td>
<td>0.8 (0.1 – 1.6)</td>
<td>22.4 (19.0 – 25.7)</td>
</tr>
<tr>
<td>90</td>
<td>0.9 (0.1 – 1.8)</td>
<td>24.9 (21.1 – 28.6)</td>
</tr>
<tr>
<td>80</td>
<td>1.1 (0.1 – 2.0)</td>
<td>28.0 (23.8 – 32.2)</td>
</tr>
<tr>
<td>70</td>
<td>1.2 (0.2 – 2.3)</td>
<td>32.0 (27.2 – 36.8)</td>
</tr>
<tr>
<td>60</td>
<td>1.4 (0.2 – 2.6)</td>
<td>37.3 (31.7 – 43.0)</td>
</tr>
<tr>
<td>50</td>
<td>1.7 (0.2 – 3.2)</td>
<td>44.7 (38.0 – 51.5)</td>
</tr>
<tr>
<td>40</td>
<td>2.1 (0.3 – 4.0)</td>
<td>55.9 (47.5 – 64.3)</td>
</tr>
<tr>
<td>30</td>
<td>2.8 (0.4 – 5.3)**</td>
<td>74.6 (63.4 – 85.8)</td>
</tr>
<tr>
<td>20</td>
<td>4.2 (0.5 – 7.9)**</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>8.5 (1.1 – 15.9)**</td>
<td>-</td>
</tr>
</tbody>
</table>

CI=confidence interval; = >100% prevalence calculated.

†Biologically plausible estimates of the true prevalence of respective *Taenia* species.
Chapter 3

Soil-Transmitted Helminthiasis in Laos: A Community-Wide Cross-Sectional Study of Humans and Dogs in a Mass Drug Administration Environment
This chapter is a published paper:


Author contributions:

Conceived and designed the study: JVC, BK, KV, SDB, SF, RCAT

Conducted the surveys: JVC, BK, KV

Performed the laboratory testing: AE (dog enteric parasitology), LP (dog enteric parasitology and hookworm molecular identification), BS (human enteric parasitology)

Analysed the data: JVC

Wrote the manuscript: JVC

Proofed and critically appraised the manuscript: BK, KV, AE, LP, BS, SDB, SF, RCST
3 Soil-transmitted helminthiasis in Laos: a community-wide cross-sectional study of humans and dogs in a mass drug administration environment

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\textsuperscript{6} Centre for Tropical Medicine, Nuffield Department of Clinical Medicine, John Radcliffe Hospital, Oxford, United Kingdom.
3.1 Abstract

We conducted a community cross-sectional survey of soil-transmitted helminthiasis in humans and dogs in four provinces in northern Lao People’s Democratic Republic. We collected and tested human and dog faecal samples and analysed results against socio-demographic data. The prevalence of *Ascaris lumbricoides*, *Trichuris trichiura*, hookworm and *Strongyloides stercoralis* was 26.1 (95% confidence interval: 23.7-28.4%), 41.5 (38.8-44.1), 46.3 (43.3-49.0) and 8.9 (7.4-10.4), respectively. We observed strong heterogeneity for helminthiasis by ethnicity, province and wealth status, coinciding with a risk profile demonstrating that Mon-Khmer people and the poorest households are highly vulnerable. *Necator americanus* was the dominant hookworm species infecting people and *Ancylostoma ceylanicum* was the only *Ancylostoma* species detected. Hookworm prevalence in village dogs was 94% and the dominant species was *A. ceylanicum*. *N. americanus* was also detected in dogs. It appears that dogs have a role in human hookworm transmission and warrants further investigation.
3.2 Introduction

Southeast Asia accounts for almost one third of all cases of human ascariasis, trichuriasis and hookworm infections worldwide (de Silva et al., 2003; Hotez and Ehrenberg, 2010). Pre-school and school-age children and pregnant women are considered high risk due to the negative impacts of infection in these groups, including anaemia, malabsorption and decreased linear growth, adverse birth outcomes and cognitive impairment (Bethony et al., 2006). Health outcomes are generally density dependent, whereby high intensity infections lead to more severe clinical outcomes (Bethony et al., 2006; Pullan and Brooker, 2008). Infection with multiple soil-transmitted helminths (STH) is common in poor tropical countries where these helminths are endemic (Pullan and Brooker, 2008) and synergistic polyparasitic interactions may lead to higher worm burdens, thereby exacerbating the problem (Ezeamama et al., 2008; Pullan and Brooker, 2008). In Southeast Asia, those infected with a STH commonly harbour at least one other concurrent infection (Steinmann et al., 2010).

Lao People’s Democratic Republic (Laos) has one of the highest prevalences of STH in the Southeast Asian region and polyparasitism is common (Rim et al., 2003). Rim et al. (2003) provided the evidence base for the introduction of a mass drug administration (MDA) program that achieved nationwide coverage in 2006 (WHO, 2009). Eight provinces have biannual treatment of 6-11 year old children with a single dose of 500 mg mebendazole and the remaining nine provinces have an annual treatment (Phommasack et al., 2008; WHO, 2009). Coverage in the highly endemic provinces was estimated to be greater than 98% in 2008 and greater than 93% for coverage across the whole country (WHO, 2009). Activity of mebendazole against *Ascaris lumbricoides* remains high and historic cure rates are consistently greater than 90% (Keiser and Utzinger, 2008). Recent studies have, however, raised doubts about the efficacy of mebendazole in clearing or reducing the intensity of hookworm infection and to a lesser degree *Trichuris trichiura* (Flohr et al., 2007; Keiser and Utzinger, 2008, 2010; Knopp et al., 2010a). In addition, evidence exists in China and Southeast Asia that peak hookworm prevalence and intensity occurs during adulthood (Humphries et al., 1997; Gandhi et al., 2001; Bethony et al., 2002) indicating that targeting school age children alone may have limited impact in reducing environmental contamination with hookworm eggs.

*Necator americanus* is the predominant hookworm species infecting people in the Mekong countries (Flohr et al., 2007; Sato et al., 2010; Jiraanankul et al., 2011); however, historical evidence suggests that the zoonotic hookworm species, *Ancylostoma ceylanicum*, is an important human pathogen in the Southeast Asian region. This particular hookworm species has been
associated with clinical disease (Anten and Zuidema, 1964) but has been overlooked as a human pathogen for the past 50 years. Recent surveys conducted in Thailand and Laos indicate the potential significance of zoonotic transmission of this species (Traub et al., 2008; Sato et al., 2010; Jiraanankul et al., 2011).

We report the results of a cross-sectional epidemiological survey of humans and dogs to investigate the patterns and risks of soil transmitted helminths (STH) in communities of four provinces of northern Laos. Furthermore, we report the results of a micro-study of a randomly selected subset of hookworm positive stool samples to determine the hookworm species infecting humans and dogs.

3.3 Materials and Methods

3.3.1 Ethics statement

Informed written consent was obtained from all human adult participants and from the parents or legal guardians of minors. All participants were free to withdraw from the study at any time during the consultation with no further obligation. The study protocol was reviewed and approved by the Murdoch University Human Ethics Committee (Project number: 2008/266) and the Lao Ministry of Health National Ethics Committee for Health Research (Number: 239/NECHR) prior to commencing this study.

For the studies involving dogs and pigs, the protocols were reviewed and approved by the Murdoch University Animal Ethics Committee (project number: R2108/07), which adheres to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The Lao Department of Livestock and Fisheries does not, at this time, have a committee to review and approve scientific research protocols involving animals.

3.3.2 Study sites

Laos is an ethnically diverse country with 49 distinct ethnic groups classified into four ethno-linguistic families, Lao-Tai, Mon-Khmer, Hmong-Mien and Sino-Tibetan, making up 67, 24, 8 and 1% of the population, respectively (Anonymous, 2006). The study was conducted in four provinces in northern Laos, Oudomxay, Luangprabang, Huaphan and Xiengkhuang (Figure 3.1), where all four ethno-linguistic families are represented. One district in each province, Xay, Xiengngeun, Viengxay and Pek district, respectively, was randomly selected for inclusion in this study. All provinces selected are categorised as highly endemic for STH and 6-11 year old children receive biannual mebendazole treatment (WHO, 2008, 2009). Mass drug administration
was delivered in November and December 2008 to all four provinces included in our study (Dr Padmasari; personal communication), approximately 1-3 months before we commenced. The coverage was estimated to be 98% (WHO, 2009). No detailed data on the timing of treatment of children in individual schools was available (Dr Padmasari; personal communication).

### 3.3.3 Survey design

The human survey was conducted in the dry season from January to March 2009 to maximise study participation and minimise negative impacts on seasonal labour demands. Six accessible villages in each district were randomly selected from official listings provided by provincial government offices and villages were excluded if a four-wheel drive vehicle could not access them. Sample size calculation was by simple random sampling based on estimated prevalence data for cysticercosis and taeniasis (Chapter 2); 14 households were randomly selected in each village from the official list of households provided by the village chief using a random number table. All household members ≥6 years old were asked to participate. Village chiefs were given advance notice of the survey timing and meetings were conducted in villages the day before to select households. In cases where a household refused to participate, the village chief selected another household with similar characteristics. A household questionnaire was administered to the head of each household with his/her family present to assess the house characteristics, assets owned, ownership of animals, age of each household member, ethnicity and education levels and literacy of the male and female heads of household. Individuals were also asked about their defecation practices.

### 3.3.4 Field procedures and sample collection

Questionnaires were pre-tested with people who did not otherwise participate in the survey. Labelled plastic bags for a single stool sample were distributed to household members to minimise risk of sample mix-up and stool samples were collected from the participants the following day. Approximately 1g of faecal material was transferred to separate tubes containing 8 ml 10% formalin and 80% ethanol, for microscopy and molecular analysis, respectively.

Dog faecal samples were collected with the permission of owners in the same villages as the human survey described above from January to March 2009. Samples were collected digitally and collected into two preservation solutions, 10% formalin and 80% ethanol, for microscopy and molecular analysis, respectively. Demographic data and the age and sex were recorded with the sample. The samples were sent to Vientiane Capital at ambient temperature and subsequently stored at 4 °C prior to processing.
### 3.3.5 Laboratory analysis

Formalin preserved human faeces were transported at room temperature to Khon Kaen University, Thailand, where the samples were analysed by the standard formalin-ether-concentration technique followed by microscopy. Formalin preserved dog faeces were transported to Murdoch University, Perth, Australia, at ambient temperature and examined for the presence of helminth eggs by the saturated sodium nitrate flotation technique followed by microscopy.

A subset of ethanol preserved human and dog samples that were hookworm egg positive were randomly selected from the total pool of positive samples stratified by province and sent to Murdoch University, Perth, Australia, at ambient temperature. Total DNA was extracted directly from faeces using the automated Maxwell® 16 Tissue DNA Purification Kit (Promega, Madison, USA) as per manufacturer’s instructions. A fragment encompassing the ITS1, 5.8S and ITS2 regions of *N. americanus* (485bp) and *Ancylostoma* spp. (380bp), were amplified as described previously (Traub et al., 2008) with some modifications. In brief, a reaction volume of 25 µl contained 1 unit of Tth Plus DNA polymerase in Tth Plus reaction buffer supplemented with 2.5 mM MgCl₂ (Fisher Biotech, Perth, Australia), 0.5 µM of each primer-RTHW1F and RTHW1R, 0.2 mM of each dNTP and 1 µl of template DNA. Initial PCR reactions were carried out using undiluted template; those that failed to amplify were retested using template diluted 1:4 in DNA grade water. Samples were heated to 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 64 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 7 min. All amplified bands of the correct size were subject to DNA sequencing. PCR products were purified using Agencourt AMPure XP DNA Purification and Cleanup Kit (Beckman Coulter Australia, Gladesville, Australia) according to manufacturer’s instructions. Purified products were sequenced in both directions using the Big Dye Terminator system, Version 3.1 (Applied Biosystems, Foster City, USA) on an ABI 373 x1 capillary sequencer. Sequence chromatograms were edited using FinchTV Version 1.4.0 (Geospiza Inc., Seattle, USA). Hookworm sequences were aligned and compared to previously published sequences of *A. duodenale* (Genbank accession numbers: EU344797, AJ001679, AJ001594), *N. americanus* (AF217891, AJ001680), *A. ceylanicum* (DQ780009, DQ831517), *A. caninum* (AM850106, DQ438079), *A. braziliense* (DQ438054) and the sequences generated from positive controls of *A. duodenale* and *A. ceylanicum using CLUSTALX version 2.0 (Larkin et al., 2007).
3.3.6 Data management and statistical analysis

The questionnaire and laboratory test result data were entered into a spreadsheet (Excel®, Microsoft Corporation, Redmond, USA) and subsequent analysis was carried out in STATA/IC version 10 (StataCorp LP, College Station, USA). The socio-economic status of each household was calculated by use of principal component analysis of household assets (Raso et al., 2005; Steinmann et al., 2007) after missing values were replaced by the mean of the respective asset for that ethnic group. All assets were dichotomous. The households were ranked into wealth quintiles according to their cumulative standardised asset scores. Missing data on literacy of household members due to death, divorce or other factors were replaced with the mean for that village and rounded to zero or one, illiterate or literate. The final analysis only considered individuals with complete parasitological data.

Age was stratified into five groups to reflect the mass drug administration target group (6-11 years), adolescents and young adults (12-19 years), adults (20-34 years), older adults (35-49 years) and elderly (≥50 years). Prevalence of A. lumbricoides, T. trichiura and hookworm was calculated as the proportion of egg positive results in the sampled population, prevalence of S. stercoralis was calculated as the proportion of rhabditiform larvae detected in the sampled population. In the human study, the Pearson’s \( \chi^2 \)-test was used to explore associations between infection status and gender, location, ethnicity, age group, wealth status, defecation site, and literacy of the male and female heads-of-household. Multiple logistic regression analysis was used to calculate odds ratio (OR) and 95% confidence intervals to determine the risk associations between helminth infections and each population characteristic and the risk of co-infection with another STH. Explanatory variables that were significant (\( P<0.05 \)) or borderline significant (cut-off \( P\leq0.10 \)) in univariate analyses were included in a multivariate logistic regression model allowing for clustering in households by applying a random effects estimator. Risk factors were calculated for the entire survey population and a sub-population analysis was performed for school age children (6-11 years old) and women of childbearing age (defined as women 15-49 years old).

For the dog study, the Kruskal-Wallis rank test was used to test for an association between dog age and hookworm infection and Pearson’s \( \chi^2 \)-test was used to explore associations between hookworm infection status and gender and location.
3.4 Results

3.4.1 Human study cohort, participation rates and population structures

In total, 1,579 people in 333 households from the 24 selected villages were eligible to participate in this survey. Of these 1,358 (86.0%) individuals from 330 households were included in the final analysis. Of these households, the village chief in a single Hmong-Mien village in Xiengkhuang province selected eight participating households, rather than 14 randomly selected villages. The Lao-Tai and Mon-Khmer ethno-linguistic families had the highest participation rates with 95.9 and 89.5% of eligible persons, respectively, fully compliant. The Hmong-Mien ethno-linguistic family had the lowest participation rate with 72.8% of eligible persons compliant. The final survey population consisted of 564 (41.5%) Lao-Tai, 526 (38.7%) Mon-Khmer and 268 (19.7%) Hmong-Mien. The median age was 24.0 years, range 6-91. By ethnicity, the median age was 28.0, 22.5 and 19.0 for Lao-Tai, Mon-Khmer and Hmong-Mien, respectively (Kruskal-Wallis rank test, $\chi^2=21.6$, $P<0.001$).

Characteristics of the survey population, stratified by province are presented in Table 3.1. Significant differences were observed for all characteristics with the exception of gender and age category. The Mon-Khmer people were the predominant ethno-linguistic family in Oudomxay and Luangprabang provinces, Lao-Tai were the majority in Huaphan and Hmong-Mien were the majority in Xiengkhuang province (Table 3.1). The survey population in Oudomxay province was comprised predominantly of most or very poor people (30.2 and 37.5%), Luangprabang province consisted predominantly of least poor people (42.6%), Huaphan province was comprised predominantly of poor people (46.8%) and Xiengkhuang province was comprised predominantly of less poor people (36.6%). The highest rates of open defecation were observed in Oudomxay and Luangprabang provinces, as were the highest rates of illiteracy of male and female heads of households (Table 3.1). Mon-Khmer people were significantly more likely to defecate in the open ($\chi^2=182.9$, $P<0.001$), have an illiterate male ($\chi^2=138.3$, $P<0.001$) or female ($\chi^2=249.5$, $P<0.001$) head of household and belong to a most poor or very poor household ($\chi^2=298.6$, $P<0.001$).

3.4.2 Prevalence and risks of STH infections

The overall prevalence of infection with any STH tested by the formalin-ether concentration technique was 70.6%; the prevalence of *A. lumbricoides*, *T. trichiura*, hookworm and *S. stercoralis* were 26.1, 41.5, 46.3 and 8.9%, respectively (Table 3.2). The species-specific prevalence of STH by population characteristic are described in detail in Table 3.2. There was no significant
difference ($P<0.05$) in prevalence between male and females for all parasite species with the exception of $S. stercoralis$, where the prevalence in males was more than double that of females. There was a significant difference in province specific prevalence for all parasite species; Xiengkhuang province had the lowest prevalence for all STH and Luangprabang province had the highest prevalence for all species except hookworm. Prevalence of $A. lumbricoides$, $T. trichiura$ and hookworm decreased significantly with increasing household wealth and $S. stercoralis$ increased significantly with increasing wealth. There was significant difference between age groups for $A. lumbricoides$ with peak prevalence observed in 20-34 year-olds, $T. trichiura$ prevalence decreased significantly with increasing age up to 49 years old with a slight rise in the oldest cohort, whereas $S. stercoralis$ increased significantly with increasing age up to 49 years old. The prevalence of helminthiasis was significantly greatest in the Mon-Khmer ethno-linguistic family for all STH and $A. lumbricoides$ and $T. trichiura$ infection were significantly greater in the cohort who defecated in the open. Infection with $S. stercoralis$ was not associated with the literacy of the male or female head-of-household, whereas $A. lumbricoides$ and $T. trichiura$ infections were both significantly greater in households with an illiterate male or female head-of-household. Hookworm infection was significantly greater in households with an illiterate female head-of-household but not the male head-of-household.

The significant risk factors associated with STH infection after controlling for household clustering at the community level and in high-risk sub-populations are summarised in Table 3.3. At the community level, there was strong evidence of spatial, ethnicity and wealth variation in having a STH infection. Study participants living in Luangprabang and Huaphan provinces had an increased risk of ascariasis, trichuriasis or having any STH infection as compared to Oudomxay province residents. Study participants from Xiengkhouang province had a reduced risk of ascariasis or strongyloidiasis and people from Huaphan province had an increased risk of hookworm infection. People in the wealthier quintiles had a reduced risk of having an STH infection with the exception of strongyloidiasis. People of Mon-Khmer ethnicity had increased risk of ascariasis, trichuriasis or any STH infection compared to Lao-Tai people, whereas people of Hmong-Mien ethnicity had a moderately increased risk of hookworm infection but a marked reduction in risk of trichuriasis or strongyloidiasis. At the community level, open defecation was a significant risk for trichuriasis and overall, men had a slightly increased risk of having any STH infection.

For the 6-11 year old children sub-group analysis, a significant increase in risk of ascariasis, trichuriasis, strongyloidiasis or any STH infection was observed in Luangprabang province. Children in Huaphan province were also more likely to have trichuriasis compared to Oudomxay
province and children from least poor households had a reduction in risk of ascariasis, trichuriasis, hookworm or any STH infection. Literacy of the heads of household were also significant, whereby children from households with a literate male head had a marked reduction in risk of ascariasis and children from a household with a literate female head were less likely to have a hookworm infection. Boys had an increased risk of hookworm infection compared to girls.

Women of childbearing age from Luangprabang and Huaphan provinces were at increased risk of ascariasis and trichuriasis, furthermore women from Huaphan and Luangprabang province were also at increased risk of hookworm and strongyloidiasis, respectively. Women of Mon-Khmer ethnicity had an increased risk of ascariasis, trichuriasis or any STH infection, conversely women from the least poor households had a reduced risk of ascariasis, trichuriasis, hookworm or any STH infection. Women who reported open defecation had an increased risk of trichuriasis.

3.4.3 Patterns of STH polyparasitism

The overall prevalence of co-infection with *A. lumbricoides* and *T. trichiura* was 16.6% (95% CI: 14.6-18.5), *A. lumbricoides* and hookworm was 14.7% (95% CI: 12.8-16.6), *T. trichiura* and hookworm was 23.0% (95% CI: 20.7-25.2) and prevalence of infection with all three STH, *A. lumbricoides*, *T. trichiura* and hookworm, was 9.6% (95% CI: 8.1-11.2). The patterns of polyparasitism by population characteristics are graphically summarised in Figure 3.2. The prevalence of co-infection was significantly lower in Xiengkhuang province compared to the other three provinces and the prevalence of co-infection with *A. lumbricoides* and *T. trichiura* was 30.1% in Luangprabang province and this was significantly higher than that in the other provinces. The prevalence of polyparasitism was significantly greater in Mon-Khmer participants for all co-infections and prevalence of polyparasitism uniformly declined with increasing household wealth status. No significant differences were observed for the prevalence of polyparasitism across age groups (Figure 3.2).

The patterns of polyparasitism in children was similar to the community as a whole; children in Luangprabang province had a significantly greater prevalence of dual infections with *A. lumbricoides* and *T. trichiura* and triple infections with *A. lumbricoides*, *T. trichiura* and hookworm (Figure 3.2a). Mon-Khmer children had significantly higher prevalence of all co-infections with the exception of dual infection with *A. lumbricoides* and hookworm (Figure 3.2b). Furthermore, prevalence of polyparasitism in Mon-Khmer children and Luangprabang children was markedly greater than the community level prevalence. Children from the most poor households had a
higher prevalence of dual infections with *A. lumbricoides* and *T. trichiura* than the less and least poor and there was a uniform but non-significant decrease in prevalence of triple infections with *A. lumbricoides*, *T. trichiura* and hookworm from most poor to least poor (Figure 3.2c).

The associations between the different helminth co-infections are described in Table 3.4. The strongest associations were between *A. lumbricoides* with *T. trichiura* and hookworm with *S. stercoralis*, however significant associations were also observed between *A. lumbricoides* with hookworm, *T. trichiura* with hookworm and *S. stercoralis* with *T. trichiura*.

### 3.4.4 Human hookworm characterisation

Forty-six faecal samples were randomly selected from the 629 human hookworm microscopy positive samples and subjected to PCR and sequencing analysis to identify the species. PCR amplification and sequencing was successful for 17 (37.0%) samples and a further 15 samples had a PCR product that failed to sequence or sequenced a non-specific product. Of the 17 characterised samples, all were identified as a single infection with either *N. americanus* (14/17; 82.4%) or *A. ceylanicum* (3/17; 17.6%). All three *A. ceylanicum* positive samples originated in Oudomxay province from Mon-Khmer people and the 14 *N. americanus* positive samples originated in Oudomxay (2), Luangprabang (6) and Huaphan provinces (6). None of the samples from Xiengkhuang province returned a conclusive PCR result.

### 3.4.5 Dog hookworm characterisation

Thirty-two, 30, 11 and 32 dog samples were collected from Oudomxay, Luanprabang, Huaphan and Xiengkhuang provinces, respectively, and analysed by saturated sodium nitrate flotation and microscopy. Four villages in Huaphan province had no dogs. All dogs were free to roam, scavenge and defecate without hindrance in their respective villages and 60% were female and 40% were male. In total, 94 of 105 dogs (89.5%) had hookworm eggs detected by microscopy. Prevalence in each of the four provinces was 93.5, 90.0, 90.9 and 84.4%, respectively, and no significant difference was detected ($\chi^2=1.5, P=0.672$). Dog age was not associated with hookworm egg detection (Kruskal-Wallis $\chi^2=2.0, P=0.657$), however prevalence in male dogs (97.6%) was significantly greater than in female dogs (84.1%)($\chi^2=4.9, P=0.027$).

Twenty-three of 94 (24.5%) faecal samples positive for hookworm were analysed by PCR and sequencing to identify the species. The PCR-sequencing protocol was able to successfully amplify and characterise 18 (78.3%) of these samples. Single species amplification of DNA from *A. ceylanicum*, *A. caninum*, *A. braziliense* and *N. americanus* were detected in 7 (38.9%), 2 (11.1%), 1
(5.6%) and 1 (5.6%) dogs, respectively. Dual species amplification of DNA from *A. ceylanicum* and *A. caninum* were detected in 4 (22.2%) dogs and dual species amplification of DNA from *A. ceylanicum* and *N. americanus* were detected in 3 (16.7%) dogs. Overall, *A. ceylanicum* was the most prevalent hookworm species detected in village dogs, 14/18 (77.8%). Dogs infected with *A. ceylanicum* or *A. caninum* were detected in all four provinces, *A. braziliense* was detected only in Luangprabang province and *N. americanus* was detected in two villages in Oudomxay province and two villages in Luangprabang province. In Oudomxay province where *N. americanus* was detected in dogs, all residents of one village reported open defecation and all residents of the other village reported latrine use. The residents of both villages in Luangprabang province where *N. americanus* was detected in dogs predominantly defecated in the open (65 and 68%, respectively, reported open defecation).

### 3.5 Discussion

Despite the MDA program providing a high coverage of mebendazole treatment in school age children since 2006 (WHO, 2009), we found a high prevalence of STH in school children and in the general community. Unfortunately there is a dearth of good quality data with which we can compare our results. However, we have been able to provide a detailed analysis of the patterns of infection and we are the first to report specific risk factors for STH infections from four highly prevalent provinces in northern Laos.

This study has a number of important limitations. Firstly, we collected a single stool sample and tested using a single diagnostic protocol without egg count data. We used the formalin-ether concentration method and this test used alone has poor sensitivity, 35.5, 51.1 and 43.8%, for hookworm, *T. trichiura* and *A. lumbricoides*, respectively (Glinz et al., 2010) indicating that our results are an underestimation of true prevalence. However, our preservation protocol involved a 1:8 w/v ratio of stool to formalised buffer compared to a 1:5 w/v ratio for the Glinz et al. (2010) study, making it difficult to compare diagnostic performance, particularly for the detection of hookworm eggs. Variable intensities of STH infections also affect the interpretation of diagnostic performance data between regions, whereby high intensity infections are more sensitively detected. More accurate prevalence results would have been obtained if we used multiple diagnostic protocols in a single study. Secondly, the sample size was relatively small and this was evident in the large confidence intervals in the risk factor analysis. The WHO guidelines for evaluating STH at the community level (Montresor et al., 1998) recommend that 200 to 250 compliant individuals in an ecologically homogenous environment, including climate, humidity, soil type and temperature, is sufficient for an accurate measure of prevalence. If the assumption
of ecological homogeneity holds true at the district level then our sample size was sufficient. Even though the confidence intervals for the risk analysis were wide, we were still able to detect significant risk for STH infection for several population level explanatory variables. Thirdly, we had a high non-participation rate for the Hmong-Mien ethnic group. This was most likely a result of socio-cultural aversion to giving faecal samples and discussing personal health details with doctors from a different ethnic group. We believe this could be improved in the future with a more consultative process and the involvement of Hmong-Mien doctors in the survey.

People of Mon-Khmer ethnicity are a highly vulnerable group with respect to STH infection in northern Laos. This group of people were more likely to defecate in the open, were more likely to be poor and more likely to have an illiterate male or female head of household. These findings were reflected in the high prevalence of STH infection and the increased risk in comparison with other ethnic groups. Mon-Khmer school age children were greater than six times more likely to have an *A. lumbricoides* infection and 12 times more likely to have any STH infection compared to Lao-Tai children.

Of particular note was the high prevalence of *A. lumbricoides* in children from Mon-Khmer and poor households so soon after the MDA program. Mebendazole is recognised as having good activity against *A. lumbricoides*, recent and historical data consistently shows *A. lumbricoides* cure rates in excess of 90% using a single 500 mg dose of mebendazole (Keiser and Utzinger, 2008; Knopp et al., 2010a). The prepatent period for *A. lumbricoides* is approximately 2-3 months (Bogitsh et al., 2005) and we would therefore not expect the cases we detected to be a result of re-infection or larvae transiting through the body at the time of treatment. The very high prevalence of *A. lumbricoides* in the children in Luangprabang province and Mon-Khmer children (50.8 and 47.1%, respectively) indicates these children may be overlooked in the system, possibly as a consequence of not being enrolled in school, absenteeism or other socio-cultural beliefs affecting the uptake of therapy. School enrolment in Laos is highly variable and low enrolment rates have been observed for the poorest households and for the non-Lao Tai ethnic groups (King and van de Walle, 2007). In addition to low enrolment rates, non-Lao Tai children who are enrolled in school face language problems since the curriculum is taught exclusively in the Lao language and labour demands on primary school children are highest for economically disadvantaged households in rural areas (King and van de Walle, 2007), possibly resulting in limited attendance and absenteeism. Our findings are consistent with those of Nokes and Bundy (Nokes and Bundy, 1993) who found that children in Jamaica with the heaviest infections came from the most socioeconomically disadvantaged households and were the least likely to comply with a treatment programme based on screening. Compliance with the MDA program in Laos is
undoubtedly a complex and multifaceted issue, however, education and out-reach programs targeting non-Lao Tai ethnic groups may help to address this issue. Interestingly in our study, children with a literate male head of household had a marked decrease in risk of ascariasis.

A recent review of STH in Southeast Asia (Jex et al., 2011) singles out the MDA program in Laos as a success and cites a reduction in STH prevalence in a small pilot survey from one school in each of four northern provinces (Phommasack et al., 2008) in comparison with a large study conducted in 2000-2002 (Rim et al., 2003). The pilot study (Phommasack et al., 2008) failed to disclose the school locations, ethnicity and relative wealth status of the children tested and compares pooled data whereas Rim et al (2003) stratified prevalence data by province. The review by Jex et al (2011) and the study by Phommasack et al (2008) represents an over-interpretation of the data and specifically fails to take into account the spatial, ethnic and wealth heterogeneity of STH infection in the Lao population and the different risk profiles that we encountered in our study. We cannot readily compare the data from our study with the work of Rim et al (2003) from 2000-2002 since study sites differed, but we can use it to approximate overall trends. In 2001, Rim et al (Rim et al., 2003) found 65.2 and 62.6% of children in Luangprabang and Oudomxay, respectively, had evidence of *A. lumbricoides* infection by cellophane-thick smear compared to our finding of 50.8 and 30.5%, respectively. Similarly in 2002, Rim et al (2003) found 72.0 and 50.6% of children in Huaphan and Xiengkhuang province were infected with *A. lumbricoides* compared to our finding of 18.6 and 4.1%, respectively. The overall trends presented here and elsewhere indicate the program is having a measure of success, but robust monitoring will be required to prevent the most vulnerable from missing out.

Almost all of the Mon-Khmer children with ascariasis had concurrent infections with either *T. trichiura* or hookworm or both, as was the case for the poorest children, indicating that health and development consequences were potentially greater in these groups. Recent studies in the Philippines demonstrated that low to moderate intensity multiple infections were associated with anaemia (Ezeamama et al., 2005b; Ezeamama et al., 2008), although schistosomiasis seemed to be the dominant controlling infection, as was observed in a similar Brazilian study (Brito et al., 2006). These results have not been supported by studies conducted in Africa where schistosomiasis is also prevalent (Mupfasoni et al., 2009; Knopp et al., 2010b), although these later studies could be complicated by the administration of drugs to control helminthiasis. Less clear again is the impact of multiple infections on cognitive function. Multiple studies indicate that STH infections have a detrimental effect on cognitive performance and educational outcomes in school children (Drake and Bundy, 2001), of particular note are recent data from geographically diverse regions indicating that light and moderate polyparasitic infections have a
detrimental effect (Ezeamama et al., 2005a; Jardim-Botelho et al., 2008). In the Lao context, polyparasitic STH infections are highly prevalent in high-risk groups and may have a strong role in perpetuating the cycle of poverty.

Few studies have sought to determine the species of hookworm causing human or dog infections in Laos. The results we present indicate that *N. americanus* is the predominant species causing human hookworm disease in northern Laos and *A. ceylanicum* is the predominant species causing dog infections. Our results are in contrast to those from recently published studies from a single village in Savannakhet province in southern Laos where *Ancylostoma* species and *Trichostrongylus colubriformis* were more prevalent than *N. americanus* (Sato et al., 2010; Sato et al., 2011). We experienced a high failure rate in the PCR for human hookworm and this could be due to a variety of reasons. It could simply be the result of PCR inhibition, which has been previously noted in faecal samples, or more complex factors such as high prevalence of hookworm infection with low worm burdens. Sato et al., (2010) found a strong correlation between PCR success and eggs per gram of faeces, whereby low egg count samples, indicative of low worm numbers, were more likely to fail in a PCR assay. This could account for PCR failure in our study. A similarly strong correlation between egg count and cycle threshold (Ct) values in real-time PCR for *N. americanus* have been observed (Verweij et al., 2007). With further validation and incorporation of *A. ceylanicum* and *Trichostrongylus* spp. specific primer sets, the latter method may be a more appropriate molecular tool since real-time PCR affords greater sensitivity than conventional PCR. The presence of *T. colubriformis* in northern Laos could also account for the discrepancy between our microscopy and PCR results; *N. americanus* and *Ancylostoma* eggs cannot be differentiated from *T. colubriformis* by microscopy (Sato et al., 2010) and our PCR protocol would not detect *T. colubriformis* DNA.

The molecular diagnosis and subsequent identification of hookworm species was far more successful in the faecal samples of dogs than humans and possibly reflects a higher worm burden in the former. The three *A. ceylanicum* human cases we detected by PCR had a worm count sufficient to produce enough eggs to be detected by conventional PCR. *A. ceylanicum* is often associated with poorly established infection in the human gut leading to low worm numbers, low egg output and an absence of blood loss symptoms (Chowdhury and Schad, 1972; Carroll and Grove, 1986). Patent *A. ceylanicum* infections with high worm numbers have caused significant human disease, including blood loss (Anten and Zuidema, 1964), intestinal pain and discomfort (Wijers and Smit, 1966; Carroll and Grove, 1986) and cognitive impairment (Wijers and Smit, 1966). That we found almost a fifth of PCR positive samples were *A. ceylanicum* is significant since this represents a parasite species that will not be controlled by a MDA program targeting...
only people. Moreover, all but four of the surveyed villages had a relatively large unmanaged dog population representing a strong infection pressure on human inhabitants. Our data also shows that village dogs have a role in *N. americanus* epidemiology in northern Laos. From our data we cannot definitively say if dogs are a transport host or if they are harbouring a patent infection, but experimental data from Japan has documented patent *N. americanus* infection in young dogs exposed to 1000 larvae (Yoshida et al., 1960). If the human population is exerting a strong and continuous infection pressure on dogs then there remains the possibility of spill-over into the dog population and zoonotic transmission between host species. Since we found almost one quarter of PCR positive dogs harbouring *N. americanus* eggs, we believe further investigations are warranted to discount patent infection and zoonotic transmission.

We present the first study to describe the patterns of STH infection in the context of MDA in northern Laos. Our evidence serves to highlight an important limitation of the MDA program. Poor compliance, absenteeism or delivery failure results in poor uptake of mebendazole treatment by children of the Mon-Khmer ethno-linguistic group and children from the poorest households. The efficacy of mebendazole in controlling *T. trichiura* and hookworm infections may also need to be examined in the Lao context. This is consistent with studies on efficacy conducted elsewhere (Flohr et al., 2007; Keiser and Utzinger, 2008, 2010; Knopp et al., 2010a). At the community level, we have conclusively demonstrated that people of the Mon-Khmer ethnic group are a highly vulnerable people as are people from the poorest households. There is a need for effective outreach programs designed in conjunction with improvements to sanitation and health services and the delivery of improved therapy, possibly albendazole plus ivermectin (Knopp et al., 2010a), to reach the most vulnerable people. The emerging trend of the Lao MDA program is positive but far from conclusive and we strongly question the use of mebendazole as a lone therapy. Owing to a lack of good quality parasitological field data from northern Laos, we do not concur with other authors (Montresor et al., 2008; Phommasack et al., 2008; Jex et al., 2011) that MDA success is adequately measured by high rates of drug delivery. A thorough appraisal of the Lao MDA program is warranted and a robust monitoring program should be urgently initiated, taking into account the role of dogs in the natural history of human hookworm infection.

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WHO, 2009. First Mekong-plus programme managers workshop on lymphatic filariasis and other helminthiasis. Available at:

Figure 3.1 Study sites in northern Laos, 1, Xay district, Oudomxay province; 2, Xiengngeun district, Luangprabang province; 3, Pek district, Xiengkhuang province and 4, Viengxay district, Huaphan province
Figure 3.2 Prevalence of dual and triple infections of STH at the community level (left column) and 6-11 year-old children (right column) by (a) province, (b) ethnicity and (c) wealth status. Error bars represent 95% confidence intervals. As, A. lumbricoides; Tr, T. trichiura; Hkw, Hookworm. OUD, Oudomxay; LP, Luangprabang; HUA, Huaphan; XK, Xiengkhuang. LT, Lao-Tai; MK, Mon-Khmer; HM, Hmong-Mien.
Table 3.1 Survey population characteristics stratified by province, Laos

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<th>Huaphan</th>
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*\(\chi^2\) calculated across all groups and provinces.
Table 3.2 Unadjusted prevalence of soil-transmitted helminths by population characteristics, Laos

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<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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</tr>
<tr>
<td>(P)</td>
<td>&lt;0.001</td>
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</tr>
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<td></td>
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<td>&lt;0.001</td>
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</tr>
</tbody>
</table>

* Including all four STH infections.

HH=household; STH=soil-transmitted helminth; CI=confidence interval.
Table 3.3 Risk factors significantly (P<0.050) associated with soil-transmitted helminth infections at the community level and in vulnerable sub-populations (6-11 year-old children and women of childbearing age, 15-49 years-old), as determined by multiple logistic regression modelling controlling for household clustering

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Population</th>
<th>Risk factor</th>
<th>Adjusted OR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. lumbricoides</em> †</td>
<td>Community-wide</td>
<td>Luangprabang province</td>
<td>5.08</td>
<td>2.95, 8.72</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Huaphan province</td>
<td>5.55</td>
<td>2.62, 11.76</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Less poor</td>
<td>0.36</td>
<td>0.19, 0.69</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Least poor</td>
<td>0.27</td>
<td>0.13, 0.54</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mon-Khmer ethnicity</td>
<td>4.11</td>
<td>2.16, 7.82</td>
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</tr>
<tr>
<td></td>
<td>6-11 year-old children</td>
<td>Luangprabang province</td>
<td>7.84</td>
<td>2.29, 26.84</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Least poor</td>
<td>0.12</td>
<td>0.02, 0.57</td>
<td>0.008</td>
</tr>
<tr>
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<td></td>
<td>Mon-Khmer ethnicity</td>
<td>6.51</td>
<td>1.30, 32.62</td>
<td>0.023</td>
</tr>
<tr>
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<td>Literate male head of household</td>
<td>0.45</td>
<td>0.20, 0.99</td>
<td>0.048</td>
</tr>
<tr>
<td></td>
<td>Women of childbearing age</td>
<td>Luangprabang province</td>
<td>9.02</td>
<td>3.06, 26.64</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Huaphan province</td>
<td>24.49</td>
<td>4.92, 121.87</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Less poor</td>
<td>0.14</td>
<td>0.04, 0.54</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Least poor</td>
<td>0.23</td>
<td>0.07, 0.82</td>
<td>0.024</td>
</tr>
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<td></td>
<td></td>
<td>Mon-Khmer ethnicity</td>
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<td>1.94, 25.55</td>
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</tr>
<tr>
<td><em>T. trichiura</em> §‡</td>
<td>Community-wide</td>
<td>Luangprabang province</td>
<td>4.11</td>
<td>2.29, 7.39</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Huaphan province</td>
<td>5.46</td>
<td>2.58, 11.56</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xiengkhuang province</td>
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<td>0.04, 0.32</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Least poor</td>
<td>0.46</td>
<td>0.21, 0.99</td>
<td>0.048</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 – 34 year-olds</td>
<td>0.46</td>
<td>0.29, 0.73</td>
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<tr>
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<td>35 – 49 year-olds</td>
<td>0.42</td>
<td>0.27, 0.67</td>
<td>&lt;0.001</td>
</tr>
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<td></td>
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<td>Mon-Khmer ethnicity</td>
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<td>1.27, 4.55</td>
<td>0.007</td>
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<tr>
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<td></td>
<td>Hmong-Mien ethnicity</td>
<td>0.36</td>
<td>0.17, 0.76</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Open defecation</td>
<td>1.62</td>
<td>1.05, 2.51</td>
<td>0.030</td>
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<td>6-11 year-old children</td>
<td>Luangprabang province</td>
<td>12.13</td>
<td>3.04, 48.49</td>
<td>&lt;0.001</td>
</tr>
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<td></td>
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<td>Huaphan province</td>
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<td>1.33, 22.84</td>
<td>0.019</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Least poor</td>
<td>0.15</td>
<td>0.03, 0.83</td>
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<tr>
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<td>Women of childbearing age</td>
<td>Luangprabang province</td>
<td>2.78</td>
<td>1.28, 6.02</td>
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<tr>
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<td>Huaphan province</td>
<td>12.29</td>
<td>4.10, 36.88</td>
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<td></td>
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<td>Mon-Khmer ethnicity</td>
<td>4.13</td>
<td>1.71, 9.95</td>
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<tr>
<td></td>
<td></td>
<td>Open defecation</td>
<td>3.06</td>
<td>1.63, 5.76</td>
<td>0.001</td>
</tr>
<tr>
<td><em>Hookworm</em> ¶§</td>
<td>Community-wide</td>
<td>Huaphan province</td>
<td>1.89</td>
<td>1.01, 3.53</td>
<td>0.045</td>
</tr>
<tr>
<td>Age Group</td>
<td>Ethnicity</td>
<td>Condition</td>
<td>OR (95% CI)</td>
<td>p-value</td>
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</tr>
<tr>
<td>-----------</td>
<td>--------------------</td>
<td>-----------</td>
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<td>---------</td>
<td></td>
</tr>
<tr>
<td>6-11 year-old children</td>
<td>Least poor</td>
<td>S. stercoralis</td>
<td>0.42 (0.24, 0.76)</td>
<td>0.004</td>
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<td>Hmong-Mien ethnicity</td>
<td>Male</td>
<td>1.74 (1.04, 2.90)</td>
<td>0.033</td>
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<td></td>
<td>Least poor</td>
<td>0.13 (0.02, 0.68)</td>
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<td></td>
<td></td>
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<td>0.019</td>
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<td></td>
<td>Women of childbearing age</td>
<td>Least poor</td>
<td>0.26 (0.10, 0.67)</td>
<td>0.005</td>
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<tr>
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<td>S. stercoralis #¶</td>
<td>Community-wide</td>
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<td>2.87 (1.85, 4.45)</td>
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<td>Xiengkhuang province</td>
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<td>20 – 34 year-olds</td>
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<tr>
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<td>35 – 49 year-olds</td>
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<td></td>
<td>≥ 50 years-old</td>
<td>2.19 (1.03, 4.67)</td>
<td>0.042</td>
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<td></td>
<td>Hmong-Mien ethnicity</td>
<td>0.26 (0.09, 0.69)</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
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<td>Women of childbearing age</td>
<td>Luangprabang province</td>
<td>17.73 (2.21, 142.33)</td>
<td>0.007</td>
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<td>Huaphan province</td>
<td>17.45 (1.89, 160.51)</td>
<td>0.012</td>
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<tr>
<td></td>
<td></td>
<td>Poor</td>
<td>1.42 (1.06, 1.90)</td>
<td>0.020</td>
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<td>6-11 year-old children</td>
<td>3.07 (1.67, 5.63)</td>
<td>&lt;0.001</td>
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<tr>
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<td>Huaphan province</td>
<td>3.81 (1.80, 8.06)</td>
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<tr>
<td></td>
<td></td>
<td>Poor</td>
<td>0.36 (0.17, 0.73)</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Less poor</td>
<td>0.33 (0.16, 0.68)</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Least poor</td>
<td>0.22 (0.10, 0.49)</td>
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<td>Mon-Khmer ethnicity</td>
<td>2.90 (1.54, 5.50)</td>
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<tr>
<td></td>
<td>Women of childbearing age</td>
<td>Luangprabang province</td>
<td>17.14 (1.31, 131.91)</td>
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<td>Huaphan province</td>
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<td>0.029</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poor</td>
<td>0.09 (0.01, 0.83)</td>
<td>0.034</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6-11 year-old children</td>
<td>0.03 (0.00, 0.39)</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mon-Khmer ethnicity</td>
<td>12.04 (1.31, 110.88)</td>
<td>0.028</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Least poor</td>
<td>0.30 (0.09, 0.95)</td>
<td>0.040</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mon-Khmer ethnicity</td>
<td>3.04 (1.24, 7.44)</td>
<td>0.015</td>
<td></td>
</tr>
</tbody>
</table>

* Reference comparators: Province, Oudomxay province; Wealth status, most poor; Ethnicity, Lao-Tai; Age group, 6-11 years of age.
† Community-wide OR adjusted for province, wealth status, ethnicity, age group, defecation site and male and female head-of-household literacy
6-11 year-old children OR adjusted for province, wealth status, ethnicity, defecation site and male and female head-of-household literacy
Women of childbearing age OR adjusted for province, wealth status, ethnicity and defecation site
§‡ Community-wide OR adjusted for province, wealth status, ethnicity, age group, defecation site and male and female head-of-household literacy
6-11 year-old children OR adjusted for province, wealth status, ethnicity and male head-of-household literacy
Women of childbearing age OR adjusted for province, wealth status, ethnicity, defecation site and male and female head-of-household literacy
Community–wide OR adjusted for gender, province, wealth status, ethnicity and female head-of-household literacy
6-11 year-old children OR adjusted for gender, wealth status, ethnicity and male and female head-of-household literacy
Women of childbearing age OR adjusted for wealth status, ethnicity, defecation site and female head-of-household literacy

# Community–wide OR adjusted for gender, province, wealth status, ethnicity and age group
6-11 year-old children OR adjusted for province
Women of childbearing age OR adjusted for province

# Community–wide OR adjusted for gender, province, wealth status, ethnicity, defecation site and male and female head-of-household literacy
6-11 year-old children OR adjusted for province, wealth status, ethnicity, defecation site and male head-of-household literacy
Women of childbearing age OR adjusted for province, wealth status, ethnicity, defecation site and male and female head-of-household literacy

OR=odds ratio; CI=confidence interval; STH=soil-transmitted helminth
Table 3.4 Significant associations (P<0.050) between the different soil-transmitted helminths infecting people in northern Laos, as determined by multiple logistic regression modelling controlling for household clustering.

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Co-infection (risk factor)</th>
<th>Adjusted OR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. lumbricoides</em></td>
<td>T. trichiura</td>
<td>2.97</td>
<td>2.11, 4.18</td>
<td>&lt;0.001</td>
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<tr>
<td></td>
<td>Hookworm</td>
<td>1.86</td>
<td>1.35, 2.56</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>T. trichiura</td>
<td><em>A. lumbricoides</em></td>
<td>2.87</td>
<td>1.99, 4.13</td>
<td>&lt;0.001</td>
</tr>
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<td>Hookworm</td>
<td>2.04</td>
<td>1.48, 2.83</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hookworm †</td>
<td><em>A. lumbricoides</em></td>
<td>1.70</td>
<td>1.25, 2.30</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>T. trichiura</td>
<td>1.83</td>
<td>1.38, 2.42</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>S. stercoralis</td>
<td>2.41</td>
<td>1.53, 3.79</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>S. stercoralis ‡</td>
<td>T. trichiura</td>
<td>1.55</td>
<td>1.01, 2.40</td>
<td>0.045</td>
</tr>
<tr>
<td></td>
<td>Hookworm</td>
<td>2.28</td>
<td>1.47, 3.52</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Adjusted for province, wealth status, age group, ethnicity, defecation site and male and female head-of-household literacy.
† Adjusted for gender, province, wealth status, ethnicity and female head-of-household literacy.
‡ Adjusted for gender, province, wealth status, age group and ethnicity.

OR=Odds ratio; CI=confidence interval.
Chapter 4

Patterns and risks of *Trichinella* infection in humans and pigs in northern Laos
This chapter will be submitted for publication:


Author contributions:

*Conceived and designed the study:* JVC, KV, BK, SDB, SF, RCAT

*Conducted the surveys:* JVC, KV, BK

*Performed the laboratory testing:* JVC (4.3.4 ES ELISA & 4.3.6 artificial muscle digestion with support from technical staff acknowledged in 4.6), MAGM (4.3.4 western blot and ES ELISA), EP (4.3.4 western blot and ES ELISA)

*Provided reagents for ELISA:* EP

*Analysed the data:* JVC

*Wrote the manuscript:* JVC

*Proofed and critically appraised the manuscript:* KV, BK, MAGM, EP, SDB, SF, RCAT
Patterns and risks of *Trichinella* infection in humans and pigs in northern Laos

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⁶Centre for Tropical Medicine, Nuffield Department of Clinical Medicine, Churchill Hospital, Oxford, United Kingdom.
Abstract

Several outbreaks of trichinellosis associated with the consumption of raw pork have occurred in Laos since 2004. This study was conducted in four provinces of northern Laos and designed to investigate the seroepidemiology of trichinellosis in the human population and determine the prevalence and species of *Trichinella* infection in the domestic pig population. Serum samples and questionnaire data were obtained from 1419 individuals. Serum samples were tested for *Trichinella* antibodies by ELISA using larval excretory–secretory (ES) antigens and a subset of 68 ES ELISA positive samples were tested by western blot. The seroprevalence of *Trichinella* antibodies was 19.1% (95% confidence interval (CI)=17.1-21.1%). The risk of having antibodies detected by ES ELISA increased with age, being of Lao-Tai ethnicity, living in Oudomxay province and being male. Tongue and diaphragm muscle samples were collected from 728 pigs and tested for *Trichinella* larvae by the artificial digestion method. *Trichinella* larvae were isolated from 15 pigs (2.1%) of which 13 were identified as *T. spiralis* by molecular typing; the species of the two remaining isolates could not be determined due to DNA degradation. *Trichinella* spp. are endemic in the domestic environment of northern Laos and targeted preventative health measures should be initiated to reduce the risk of further outbreaks occurring.
4.2 Introduction

Trichinellosis is one of the most widely distributed zoonoses worldwide and is caused by infection with nematodes of the genus *Trichinella* (Pozio and Murrell, 2006). Infection occurs after consuming larvae in the muscle of infected animals with domestic and wild pigs the most common vehicles of human infections (Murrell and Pozio, 2011). The severity of human disease is dependent on multiple factors including the number of viable larvae consumed, the frequency of consuming infected meat, meat being consumed raw or rare, the *Trichinella* species involved and individual susceptibility (Dupouy-Camet et al., 2002).

*Trichinella* spp. are endemic throughout Southeast Asia, from southern China to the Indonesian archipelago (Pozio, 2007; Odermatt et al., 2010) in domestic pigs and wildlife, causing frequent outbreaks of human disease. Three species of *Trichinella* have been detected in the Southeast Asian region, the encapsulated *T. spiralis* and the non-encapsulated *T. pseudospiralis* and *T. papuae* (Pozio et al., 2009). *Trichinella spiralis* has a regional distribution (Pozio, 2001) with many of the recognised outbreaks occurring in the ethnically diverse regions of central and northern Laos, northern Thailand and northwest Vietnam where consumption of uncooked pork is common (Barennes et al., 2008; Kaewpitoon et al., 2008; Taylor et al., 2009; Conlan et al., 2011).

Outbreaks of human trichinellosis involving *T. pseudospiralis* and *T. papuae* have occurred in Thailand after consuming wild pig meat (Jongwutiwes et al., 1998; Khumjui et al., 2008; Kusolsuk et al., 2010) and cases of trichinellosis involving *T. papuae* have been detected in Papua New Guinea (Pozio et al., 1999; Pozio et al., 2004) and a Thai patient returning from Malaysia (Intapan et al., 2011).

Several outbreaks and sporadic cases of trichinellosis have occurred in Laos over the past five years (Sayasone et al., 2006; Suwansrinon et al., 2007; Barennes et al., 2008) with the majority of the reported cases being associated with consumption of raw pork. Notwithstanding the propensity for Lao people to consume uncooked meat, including pork (Conlan et al., 2012b), little is known of the population and individual level risk factors of exposure and the meat consumption habits across an ethnically diverse country. Furthermore, relatively little is known about the prevalence of *Trichinella* infection in pigs and the species circulating in the domestic pig population. We report here the results of a cross-sectional serological survey of the human population and a concurrent survey in domestic pigs using muscle digestion in four provinces of northern Laos.
4.3 Materials and methods

4.3.1 Human and animal ethics statement

Informed consent was obtained from all human adult participants and from the parents or legal guardians of minors (children < 15 years of age). The study protocol was reviewed and approved by the Murdoch University Human Ethics Committee (Project no. 2008/266) and the Lao Ministry of Health National Ethics Committee for Health Research (no. 239/NECHR) before commencing this study. For the pig study, the protocol was reviewed and approved by the Murdoch University Animal Ethics Committee (Project no. R2108/07), which adheres to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

4.3.2 Study sites

Laos is an ethnically diverse country with 49 distinct ethnic groups classified into four ethnolinguistic families (Lao-Tai, Mon-Khmer, Hmong-Mien, and Sino-Tibetan), comprising 67%, 24%, 8%, and 1% of the population, respectively (Anonymous, 2006). The study was conducted in four provinces in northern Laos (Oudomxay, Luangprabang, Huaphan, and Xiengkhuang), where all four ethnolinguistic families are represented. One district in each province (Xay, Xiengngeun, Viengxay, and Pek Districts, respectively) was randomly selected for inclusion in this study.

4.3.3 Human study design and risk factor questionnaire

The human survey was conducted in the dry season during January–March 2009 to maximize study participation and minimize negative impacts on seasonal labour demands. The survey design, sample size calculations and methodology have been described in detail elsewhere (Conlan et al., 2012a; Conlan et al., 2012b). The sample size calculations were based on estimates of taeniasis prevalence in the target populations. In brief, 14 households were randomly selected in each village and all household members ≥6 years of age were asked to participate. A venous blood sample of 2–3 mL was collected and the serum fraction was stored at −20°C. A household questionnaire was administered to the head of each household with his/her family present to assess the house characteristics, assets owned, ownership of animals, age of each household member, ethnicity and education levels, literacy of the male and female heads of household. Individual questionnaires were administered to collect data on meat consumption. For those family members who consumed raw meat, either pork, beef or fermented pork sausage, we asked...
them to estimate the frequency of raw meat consumption: weekly, monthly, every few months, and infrequently (once or twice per year or less often).

4.3.4 Serological analysis and quality control

*Trichinella* excretory secretory antigens (ESA) prepared from *T. spiralis* larvae (Gomez-Morales et al., 2012) and four positive control human serum samples were prepared by the European Union Reference Laboratory for Parasites (EURLP; Department of Infectious, Parasitic and Immunomediated Diseases, Istituto Superiore di Sanita, Rome, Italy) and supplied lyophilised and stored at 4 °C on receipt in Laos. The ESA and control sera were reconstituted in analytical grade water, aliquoted and stored at -20 °C according to the manufacturer’s instructions immediately prior to use. A panel of eight negative control serum samples were sourced from Lao people with no reported history of trichinellosis or of consuming raw pork.

The ES ELISA was performed in Laos at the National Centre for Laboratory and Epidemiology (NCLE) using a standard protocol (Gomez-Morales et al., 2012) with some minor modifications. Two positive control serum samples, 40 test serum samples and conjugate and substrate controls were added in duplicate to each plate; eight negative control serum samples were added to single wells of each plate. The optical density (OD) was measured at a wavelength of 450nm using a microtiter plate reader (HumaReader, Germany). The cut-off on each plate was calculated as the mean OD of the eight negative control reference sera plus three standard deviations; a test ratio was calculated by dividing the OD of the test sample by the plate cut-off value and a test ratio ≥1 was considered reactive in the ES ELISA.

Sixty-eight samples that had an ES ELISA test ratio ≥1 were randomly selected from the pool of positive samples and sent to the EURLP for confirmatory testing. Samples were tested by the ES ELISA and western blot according to methods described elsewhere (Gomez-Morales et al., 2012).

4.3.5 Abattoir survey design

The abattoir survey design has been described elsewhere (Conlan et al., 2012b). In brief, pig surveys were conducted at three slaughter-points in Xiengkhuang and Oudomxay Provinces from May–September 2008 and at two collection points in Huaphan and Luangprabang Provinces from October 2008–January 2009. The survey team consisted of trained district and provincial agricultural and forestry government staff who visited the slaughter points approximately every two weeks. The tongue and diaphragm pillar muscles were excised from all pigs brought for
slaughter on the nights the survey team visited. Muscle samples were collected into labelled plastic containers and stored at 4 °C before transport on ice to the National Animal Health Laboratory in Vientiane where samples were stored at 4 °C prior to artificial muscle digestion.

4.3.6 Artificial muscle digestion

Tongue and diaphragm muscle samples were artificially digested by the magnetic stirrer method in 1% pepsin (1:10,000 US National Standard Formulary) and 1% hydrochloric acid (HCl) after removal of fat and fascia (Gamble et al., 2000; OIE, 2012). Samples were tested in pools by muscle type with a maximum of 100 g per pool using 10 g of tissue per animal (if the animal was small >5 g of tissue was processed per animal). Tongue samples from positive pools were artificially digested as per the above protocol using 20 g muscle tissue (>10 g for small animals). Larvae were counted, transferred to 100% ethanol and sent to the EURLP for molecular species identification by multiplex PCR as previously described (Pozio and La Rosa, 2003).

4.3.7 Data analysis

The prevalence of human serum reactivity with Trichinella ES antigens was calculated for three diagnostic cut-offs in the ES ELISA, test ratios ≥1, ≥1.2 and ≥1.4. The level of agreement between the ES ELISA results from Laos and EURLP, and the level of agreement between the ES ELISA results from Laos and the western blot test were calculated for the three diagnostic cut-offs using the Kappa statistic. Sensitivity and specificity could not be calculated since no ES ELISA negative samples from Laos were subjected to further testing at the EURLP.

The questionnaire and laboratory test data were entered into a spreadsheet (Excel®, Microsoft Corporation, Redmond, USA) and subsequent analysis was carried out in STATA/IC version 10 (StataCorp LP, College Station, USA). The socioeconomic status of each household was calculated by use of principal component analysis of household assets (Raso et al., 2005; Steinmann et al., 2007) after replacement of missing values with the mean of the respective asset for that ethnic group. All assets were dichotomous. The households were ranked into wealth quintiles according to their cumulative standardized asset scores.

Univariate logistic regression without adjustment was used to test associations between ES ELISA reactivity and gender, location, ethnicity, age, wealth status and uncooked meat consumption habits. Risk factors significant or borderline significant ($P \leq 0.20$) in the univariate analyses were included in a multivariate random effects logistic regression model adjusting for the effect of household clustering. The results are reported as adjusted odds ratios and 95%
confidence intervals (CIs). The final analysis only considered persons with serologic and questionnaire data.

In the pig study, the Pearson’s chi-square test was used to explore associations between infection status (larvae detected by artificial digestion) and age, breed, sex and production system at last point of sale.

4.4 Results

4.4.1 Human study

A total of 1,582 persons in 332 households were eligible to participate in this survey. Of these persons, 1,419 (89.7%) individuals from 324 households aged 6–91 years provided a blood sample, a completed questionnaire, and had valid laboratory test results. The final survey population consisted of 583 Lao-Tai (41.1%), 564 Mon-Khmer (39.8%), and 272 (19.2%) Hmong-Mien. No Sino-Tibetan persons were recruited into this study. Survey population structures stratified by province are shown in Table 4.1. Significant differences in the survey population structure were observed for ethnicity and wealth status. Lao-Tai people made up the majority of the population surveyed in Huaphan province (95.6%), Mon-Khmer people made up the majority of the survey population in Oudomxay and Lauangprabang provinces (78.6% and 64.3%, respectively) and Hmong-Mien people made up the majority of the survey population in Xiengkhuang province (58.5%) (Table 4.1). Oudomxay province had the greatest proportion of participants who were very poor or most poor (68.5%) and this was reflected in the finding that Mon-Khmer people were the most impoverished ethnic group and Lao-Tai people were the least poor overall.

Sixty-eight samples with a test ratio ≥1 in the ES ELISA in Laos were tested at the EURLP by the ES ELISA method and all but one were confirmed positive, corresponding to 98.5% agreement. In comparison with the western blot test, only 35.3% (24/68) of the Lao samples had three diagnostic bands detected, a banding profile consistent with clinically confirmed trichinellosis (Gomez-Morales et al., 2008; Gomez-Morales et al., 2012). Using a diagnostic cut-off test ratio ≥1.0, ≥1.2 and ≥1.4, the level of agreement with the western blot test was 35.3%, 50.0% and 62.3%, respectively. The two-by-two tables comparing the western blot test results and ES ELISA at different diagnostic cut offs are presented in Table 4.2.

Using a diagnostic cut-off test ratio ≥1.0, ≥1.2 and ≥1.4, the prevalence of *Trichinella* antibodies detected by ES ELISA were 19.1%, 12.7% and 7.5%, respectively (Table 4.3). The prevalence of antibody detection by ES ELISA was highest in males, increased with increasing age to a peak in
35-49 year olds, increased with increasing wealth and was highest in the Lao-Tai ethnic group (Table 4.3). Prevalence was highest in Oudomxay province when a cut-off test ratio ≥1.2 and ≥1.40 were applied, and was highest in Xienghuang province when a cut-off test ratio ≥1.0 was applied (Table 4.3).

The proportion of people reporting the consumption of uncooked beef, pork and fermented sausage peaked in older age groups for all ethnic groups (Figure 4.1) with the exception of Hmong-Mien people consuming fermented pork sausage, which was comparatively low for all age groups. The prevalence of antibody detection using a cut-off test ratio ≥1.0 was highest in people reporting consumption of raw pork (26.2% versus 18.0%), raw beef (27.1% versus 14.7%) and fermented pork sausage (29.8% versus 15.0%). Similarly, the prevalence of antibody detection using a cut-off test ratio ≥1.2 was highest in people reporting consumption of raw pork (18.5% versus 11.8%), raw beef (19.3% versus 9.1%) and fermented pork sausage (21.7% versus 9.2%). Using a cut-off test ratio ≥1.4, prevalence was highest in people reporting consumption of raw pork (10.8% versus 7.0%), raw beef (12.3% versus 4.6%) and fermented pork sausage (13.3% versus 5.2%). The prevalence of antibody detection stratified by frequency of raw meat consumption are summarised in Table 4.3.

After controlling for clustering at the household level, the risk of having *Trichinella* antibodies detected in the ES ELISA was significantly greater for people residing in Oudomxay province, people of Lao-Tai ethnicity, increasing age and being male. Increasing wealth was no longer associated with increased risk of having *Trichinella* antibodies detected after controlling for other risk factors (Figure 4.2). The frequency of consuming raw pork and beef were not associated with increased risk of having antibodies detected. Only consumption of fermented pork sausage on a weekly basis was significantly associated with increased risk of having antibodies detected when the diagnostic cut-off test ratio ≥1.2 (Odds ratio=3.29 (95% CI=1.35-7.99); Figure 4.2).

To explore the influence of potential cross-reactivity with enteric parasites prevalent in Laos using data collected from the same survey population (Conlan et al., 2012a; Conlan et al., 2012b), the prevalence of ES ELISA positivity, using a diagnostic cut-off test ratio ≥1.0, was calculated for people for whom enteric parasitic data was available (N=1305). The enteric parasites detected were *Taenia* spp (2.9%), *Opisthorchis viverrini* (3.1%), *Echinostoma* spp. (2.2%), Minute intestinal fluke (0.8%), hookworm (45.8%), *Strongyloides stercoralis* (9.3%), *Ascaris lumbricoides* (26.0%), *Trichuris trichiura* (41.8%), *Giardia duodenalis* (1.4%), *Entamoeba* spp. (1.8%), *Paragonimus* spp. (0.1%), *Enterobius* spp. (0.5%) and *Hymenolepis* spp. (0.3%). Seroprevalence of trichinellosis decreased with increasing polyparasitism, with the highest prevalence detected in the cohort with no parasite eggs or oocysts detected (Figure 4.3).
4.4.2 Pig abattoir study

Tongue and diaphragm muscle samples were tested by the artificial digestion method from 728 pigs sampled from all four northern provinces included in the study. *Trichinella* larvae were isolated from 15 pigs (2.1%) of which 13 were identified as *T. spiralis* by molecular typing. Two isolates were not identified to the species level due to damaged DNA that may have occurred during tissue storage and muscle digestion. Prevalence of *Trichinella* spp. infection in pigs varied significantly \( (P<0.05) \) by province whereby the highest prevalence was recorded in Xiengkhuang (4.8%) and Oudomxay (2.8%) provinces (Table 4.4). No samples collected in Luangprabang province were infected with *Trichinella* larvae at the time of this survey. There was no significant difference in prevalence by breed, sex or the production system where the pigs were purchased immediately prior to slaughter.

Of the 15 pigs infected with *Trichinella* larvae, 0.1-0.9 larvae per gram (lpg) of tongue tissue was detected in 10 pigs, 1-10 lpg was detected in three pigs and greater than 10 lpg was detected in two pigs. The highest recorded intensity of infection was 69 lpg in a pig slaughtered in Xiengkhuang province.

4.5 Discussion

Trichinellosis is endemic in Southeast Asia with a concentration of outbreaks occurring in the ethnically diverse regions of northern Thailand, northern Vietnam and Laos (Barennes et al., 2008; Kaewpitoon et al., 2008; Taylor et al., 2009; Conlan et al., 2011). Our study confirms endemicity of *T. spiralis* in the pig population of Laos together with a spatial difference in prevalence of *T. spiralis* infection in pigs with worm burdens sufficient to cause severe human disease. One of the principle aims of the present study was to determine population and individual level risk factors associated with human exposure to *Trichinella* spp. larvae. Therefore we conducted a randomised cross-sectional survey of the human population in four northern provinces of Laos and used the ES ELISA as a serological measure of exposure. A high prevalence of *Trichinella* antibodies was detected by ES ELISA, with significant increased risk being associated with increasing age, Lao-Tai ethnicity, residing in Oudomxay province, being male and regular consumption of fermented pork sausage.

An important limitation of this study was the low participation rate of people from the Hmong-Mien ethnic group. The reasons for this low participation rate have been discussed elsewhere (Conlan et al., 2012a; Conlan et al., 2012b). Overall, the prevalence of ES ELISA reactivity across all ethnic groups increased with increasing age and prevalence was highest for males. In the
Hmong-Mien ethnic group the ratio of females to males was similar for all age groups except the youngest group, where boys represented 60% of the age group. This discrepancy indicates that older males were over-represented in the survey and our prevalence estimates are possibly higher than would otherwise have been the case if participation rates were higher. In addition, the highest non-participation rates in the Hmong-Mien group were observed in Huaphan province and this may have led to an over-estimation of prevalence of ES ELISA reactivity in this province.

No diagnostic test for trichinellosis, in any host species, has been validated for cross-sectional studies in Southeast Asia. The *Trichinella* ES ELISA lacks specificity owing to the relatively large population of antigens resulting in the detection of non-specific cross-reacting antibodies (Gomez-Morales et al., 2008; Gomez-Morales et al., 2012). The western blot test described by Gomez-Morales et al. (2008; 2012) was used as the gold-standard comparator for a small subset of ES ELISA positive samples in this study. The full spectrum of exposures, from subclinical infection, exposure to inactivated or injured larvae, old exposures through to acute and chronic clinical disease would lead to a varied serological spectrum at a population level. The ES ELISA results presented here are therefore imperfect but provide a measure of exposure at the population level. The evidence presented in Figure 4.3, showing ES ELISA positivity highest in the cohort of individuals with no enteric parasites detected, demonstrates that the high seroprevalence as determined by ES ELISA cannot be disregarded out of hand as being associated with non-specific reactions. We are, however, unable to rule out the possibility of cross-reactions with non-detected enteric parasites or other parasitic and non-parasitic infections, as was observed by Gomez-Morales et al. (2008; 2012). Notwithstanding cross-reactivity in the ES ELISA, the polyparasitism results are suggestive of a possible protective mechanism with increasing parasite load. Polyparasitism is known to stimulate different components of the host’s immune system (Thompson and Smith, 2011) and well-designed studies would be required to measure the immunological interactions, if they exist, that may lead to resistance to infection (Supali et al., 2010).

Despite these limitations, we were able to demonstrate widespread serological evidence of exposure to *Trichinella* larvae in the human population. Increasing the diagnostic cut-off in the ES ELISA resulted in improved agreement with the western blot test, likely as a consequence of improved specificity at the expense of sensitivity. For this reason, prevalence was calculated for a range of diagnostic cut-offs in the ES ELISA to test the effect on the subsequent risk factor analysis. The pattern of risk for age, province of residence, gender, ethnicity and wealth status remained essentially the same as the diagnostic cut-off increased. For raw meat consumption,
only the self-reported consumption of fermented pork sausage on a weekly basis was significantly associated with antibody detection in the ES ELISA at a cut-off test ratio $\geq 1.2$. The consumption of fermented pork has previously been linked with an outbreak of trichinellosis in Bolikhamxay province in central Laos (Sayasone et al., 2006).

The lack of association with raw meat consumption, particularly raw pork, was somewhat unexpected since previously reported outbreaks of trichinellosis in Oudomxay and Bolikhamxay provinces have been linked with consumption of raw pork at festivals (Sayasone et al., 2006; Barennes et al., 2008). This might be explained by the limitations of using single point-in-time self-reporting as opposed to asking the survey participants to keep a more detailed food diary. In general, methods of assessing dietary intake are imperfect and subject to error (Willett, 2001), especially in an ethnically diverse population with high rates of illiteracy and where Lao may have been the second language. From the data collected we were unable to estimate or correct for recall bias and the possibility that some survey participants may have misinterpreted the questionnaire cannot be ruled out. Future studies assessing risk associated with consuming uncooked pork should consider the use of a food diary to better estimate the prevalence of consuming uncooked meat.

In all risk models, the risk of having *Trichinella* antibodies detected by ES ELISA was highest in Oudomxay province compared to all other provinces. This finding may be an artefact of the large and widespread outbreak of trichinellosis that occurred in this province in 2005 (Barennes et al., 2008) and be indicative of more widespread exposure over and above the clinical cases that were reported. More research, using more statistically powered surveys, is warranted in Oudomxay province to further investigate the risk of trichinellosis in this province.

The majority of pigs in which *T. spiralis* larvae were detected had a worm burden of less than 1 lpg and the infecting dose for clinically apparent trichinellosis has been estimated to range from $\sim 70$-150 larvae (Dupouy-Camet et al., 2002). The serological results together with the meat consumption habits and the abattoir survey results suggests that subclinical exposure may be common in Laos. Barennes and others (2008) reported apparently low morbidity associated with the 2005 outbreak in Oudomxay province and hypothesised that alcohol consumption may have diminished the severity of disease. Our results indicate that population immunity may have had a protective role.

The risk of *Trichinella* antibody detection in the ES ELISA increased significantly with increasing age and Lao-Tai people were at significantly greater risk. Regionally, trichinellosis has been associated with ethnically diverse mountainous areas of northern Vietnam and Thailand.
(Kaewpitoon et al., 2008; Taylor et al., 2009), whereas in Laos we found the greatest risk associated with the majority lowland Lao-Tai population and the lowest risk was associated with people from the minority upland Mon-Khmer and Hmong-Mien ethnic groups. A high proportion of people from all ethnic groups reported consuming uncooked meat. Public health interventions, including a detailed assessment of the risks posed by ceremonial food preparation and the development of food safety education and awareness programs, could potentially reduce the transmission of *Trichinella* and other foodborne pathogens in Laos.

### 4.6 Acknowledgements

We would like to thank the study participants in northern Laos for giving us their valuable time and the national, provincial and district staff from the Ministry of Agriculture and Forestry and the Ministry of Health for their support and valued contribution to this study. In particular, we wish to acknowledge the contribution of Mr. Lapinh Phithacthep, Ms. Vilaywan Soukvilay and Ms Vilayphet Viravong from the National Animal Health Centre and Mr Virasack Som, Mr Thongchan Sisouk and Ms. Khouanta Douangmala from the National Centre for Laboratory and Epidemiology, who provided logistic, technical and laboratory support. We are also grateful to Dr. Alessandra Ludovisi, Dr. Marilena Interisano, and Mr. Marco Amati of the EURLP for their technical support.

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### 4.8 References


Figure 4.1 Proportion of the survey population reporting consumption of uncooked pork, uncooked beef and uncooked fermented pork, A-C, respectively, by age and ethnicity. Black columns, Lao-Tai ethnic group; Grey columns, Mon-Khmer ethnic group; and white columns, Hmong-Mien ethnic group.
Figure 4.2 Adjusted odds ratio (AOR) of population characteristics associated with *Trichinella* ES-ELISA reactivity, as determined by random effects multiple logistic regression modeling controlling for household clustering. All models adjusted for gender, province, wealth, ethnicity, age, frequency of raw pork consumption, raw beef consumption and fermented pork sausage consumption. Model 1; Diagnostic cut-off in *Trichinella* ES ELISA = standardised ratio ≥1.00; Model 2, Diagnostic cut-off in *Trichinella* ES ELISA = standardised ratio ≥1.20; Model 3, Diagnostic cut-off in *Trichinella* ES ELISA = standardised ratio ≥1.40. In descending order population characteristics are: Gender; female (referent), male. Province; Oudomxay (referent), Luangprabang, Huaphan, Xiengkhuang. Wealth status; most poor (referent), very poor, poor, less poor, least poor. Ethnicity; Lao-Tai (referent), Mon-Khmer, Hmong-Mien. Age category; 6-11 years (referent), 12-19 years, 20-34 years, 35-49 years, ≥50 years. Raw pork consumption; does not eat (referent), weekly, monthly, every few months, infrequent. Raw beef consumption; does not eat (referent), weekly, monthly, every few months, infrequent. Raw fermented pork consumption; does not eat (referent), weekly, monthly, every few months, infrequent.
Figure 4.3 Prevalence of *Trichinella* ES ELISA reactivity verses enteric polyparasitism for 1,305 people where faecal sample results were matched to trichinellosis serology data. Error bars indicate 95% confidence interval. The enteric parasites detected were *Taenia* spp (2.9%), *Opisthorchis viverrini* (3.1%), *Echinostoma* spp. (2.2%), Minute intestinal fluke (0.8%), hookworm (45.8%), *Strongyloides stercoralis* (9.3%), *Ascaris lumbricoides* (26.0%), *Trichuris trichiura* (41.8%), *Giardia duodenalis* (1.4%), *Entamoeba* spp. (1.8%), *Paragonimus* spp. (0.1%), *Enterobius* spp. (0.5%) and *Hymenolepis* spp. (0.3%). No significant difference for polyparasitism and age was observed ($\chi^2=12.4$, $P=0.714$).
Table 4.1 Survey population structure, stratified by province, ethnicity, wealth status, age and gender, Laos

<table>
<thead>
<tr>
<th>Population characteristic</th>
<th>Total (%)</th>
<th>Oudomxay (%)</th>
<th>Luangprabang (%)</th>
<th>Huaphan (%)</th>
<th>Xiengkhuang (%)</th>
<th>$\chi^2$</th>
<th>$P$</th>
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<td><strong>Gender</strong></td>
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<tr>
<td>Female</td>
<td>719 (50.7)</td>
<td>217 (52.7)</td>
<td>187 (50.1)</td>
<td>141 (48.0)</td>
<td>174 (51.2)</td>
<td>1.6</td>
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<td>700 (49.3)</td>
<td>195 (47.3)</td>
<td>186 (49.9)</td>
<td>153 (52.0)</td>
<td>166 (48.8)</td>
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<td><strong>Ethnicity</strong></td>
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<tr>
<td>Lao-Tai</td>
<td>583 (41.1)</td>
<td>59 (14.3)</td>
<td>102 (27.4)</td>
<td>281 (95.6)</td>
<td>141 (41.5)</td>
<td>1.6</td>
<td>0.659</td>
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<tr>
<td>Mon-Khmer</td>
<td>564 (39.8)</td>
<td>324 (78.6)</td>
<td>240 (64.3)</td>
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<td>0 (0.0)</td>
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<td>29 (7.0)</td>
<td>31 (8.3)</td>
<td>13 (4.4)</td>
<td>199 (58.5)</td>
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<tr>
<td>Most poor</td>
<td>253 (17.8)</td>
<td>123 (29.9)</td>
<td>55 (14.8)</td>
<td>17 (5.8)</td>
<td>58 (17.1)</td>
<td>1.6</td>
<td>0.659</td>
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<tr>
<td>Very poor</td>
<td>270 (19.0)</td>
<td>159 (38.6)</td>
<td>32 (8.6)</td>
<td>36 (12.2)</td>
<td>43 (12.7)</td>
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<tr>
<td>Poor</td>
<td>297 (20.9)</td>
<td>27 (6.6)</td>
<td>57 (15.3)</td>
<td>147 (50.0)</td>
<td>66 (19.4)</td>
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<td>Less poor</td>
<td>313 (22.1)</td>
<td>40 (9.7)</td>
<td>67 (18.6)</td>
<td>86 (29.3)</td>
<td>120 (35.3)</td>
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<td>63 (15.3)</td>
<td>162 (43.4)</td>
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<td>53 (15.6)</td>
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<td><strong>Age (years)</strong></td>
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<td>296 (20.9)</td>
<td>106 (25.7)</td>
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<td>66 (17.7)</td>
<td>70 (23.8)</td>
<td>75 (22.1)</td>
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<td>35-49</td>
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<td>76 (18.5)</td>
<td>83 (22.3)</td>
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<td>63 (18.5)</td>
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<td>220 (15.5)</td>
<td>51 (12.4)</td>
<td>67 (18.0)</td>
<td>53 (18.0)</td>
<td>49 (14.4)</td>
<td>20.3</td>
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Table 4.2 *Trichinella* spp. western blot positivity verses ES ELISA positivity for diagnostic cut-off equal to standardised ratios of ≥1.0, ≥1.2 and ≥1.4 for 68 human samples, Laos

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<th>Western blot</th>
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<td>ES ELISA (≥1.0)</td>
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<tr>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>44</td>
<td>24</td>
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<tr>
<td>ES ELISA (≥1.2)</td>
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<td>-</td>
<td>15</td>
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<tr>
<td>+</td>
<td>29</td>
<td>19</td>
</tr>
<tr>
<td>ES ELISA (≥1.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>31</td>
<td>12</td>
</tr>
<tr>
<td>+</td>
<td>13</td>
<td>12</td>
</tr>
</tbody>
</table>
Table 4.3 Unadjusted prevalence of *Trichinella* ES ELISA positivity (95% CI) for diagnostic cut-off equal to standardised ratios of ≥1.0, ≥1.2 and ≥1.4, Laos

<table>
<thead>
<tr>
<th>Population characteristic</th>
<th>No. (%)</th>
<th>Proportion of human serum reactive in <em>Trichinella</em> ES ELISA</th>
<th>Cut-off ratio ≥1.0</th>
<th>Cut-off ratio ≥1.2</th>
<th>Cut-off ratio ≥1.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total survey population</td>
<td>1419</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>719 (50.7)</td>
<td>15.4 (12.8, 18.1)</td>
<td>11.0 (8.7, 13.3)</td>
<td>6.7 (4.8, 8.5)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>700 (49.3)</td>
<td>22.9 (19.7, 26.0)</td>
<td>14.4 (11.8, 17.0)</td>
<td>8.4 (6.4, 10.5)</td>
<td></td>
</tr>
<tr>
<td><strong>Province</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oudomxay</td>
<td>412 (29.0)</td>
<td>22.8 (18.8, 26.9)</td>
<td>17.2 (13.6, 20.9)</td>
<td>12.6 (9.4, 15.8)</td>
<td></td>
</tr>
<tr>
<td>Luangprabang</td>
<td>373 (26.3)</td>
<td>17.7 (13.8, 21.6)</td>
<td>11.0 (7.8, 14.2)</td>
<td>6.2 (3.7, 8.6)</td>
<td></td>
</tr>
<tr>
<td>Huaphan</td>
<td>294 (20.7)</td>
<td>11.2 (7.6, 14.8)</td>
<td>7.5 (4.5, 10.5)</td>
<td>3.7 (1.6, 5.9)</td>
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<tr>
<td>Xiengkuang</td>
<td>340 (24.0)</td>
<td>22.9 (18.5, 27.4)</td>
<td>13.5 (9.9, 17.2)</td>
<td>6.2 (3.6, 8.7)</td>
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</tr>
<tr>
<td><strong>Wealth status</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Most poor</td>
<td>253 (17.8)</td>
<td>9.8 (6.2, 13.6)</td>
<td>5.5 (2.7, 8.4)</td>
<td>2.4 (0.5, 4.3)</td>
<td></td>
</tr>
<tr>
<td>Very poor</td>
<td>270 (19.0)</td>
<td>17.4 (12.9, 22.0)</td>
<td>12.2 (8.3, 16.1)</td>
<td>8.5 (5.2, 11.9)</td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>297 (20.9)</td>
<td>14.8 (10.8, 18.9)</td>
<td>11.1 (7.5, 14.7)</td>
<td>5.7 (3.1, 8.4)</td>
<td></td>
</tr>
<tr>
<td>Less poor</td>
<td>313 (22.1)</td>
<td>24.0 (19.2, 28.7)</td>
<td>16.0 (11.9, 20.0)</td>
<td>9.6 (6.3, 12.9)</td>
<td></td>
</tr>
<tr>
<td>Least poor</td>
<td>286 (20.2)</td>
<td>28.0 (22.7, 33.2)</td>
<td>17.5 (13.1, 21.9)</td>
<td>10.8 (7.2, 14.5)</td>
<td></td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lao-Tai</td>
<td>583 (41.1)</td>
<td>26.1 (22.5, 29.6)</td>
<td>19.2 (16.0, 22.4)</td>
<td>12.7 (10.0, 15.4)</td>
<td></td>
</tr>
<tr>
<td>Mon-Khmer</td>
<td>564 (39.8)</td>
<td>13.1 (10.3, 15.9)</td>
<td>7.6 (5.4, 9.8)</td>
<td>3.9 (2.3, 5.5)</td>
<td></td>
</tr>
<tr>
<td>Hmong-Mien</td>
<td>272 (19.2)</td>
<td>16.5 (12.1, 21.0)</td>
<td>9.2 (5.7, 12.6)</td>
<td>4.0 (1.7, 6.4)</td>
<td></td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>6-11</td>
<td>296 (20.9)</td>
<td>6.8 (3.9, 9.6)</td>
<td>4.1 (1.8, 6.3)</td>
<td>2.7 (0.9, 4.6)</td>
<td></td>
</tr>
<tr>
<td>12-19</td>
<td>329 (23.2)</td>
<td>12.1 (8.6, 15.7)</td>
<td>7.6 (4.7, 10.5)</td>
<td>3.3 (1.4, 5.3)</td>
<td></td>
</tr>
<tr>
<td>20-34</td>
<td>297 (20.9)</td>
<td>22.6 (17.8, 27.3)</td>
<td>14.8 (10.8, 18.9)</td>
<td>8.4 (5.3, 11.6)</td>
<td></td>
</tr>
<tr>
<td>35-49</td>
<td>277 (19.5)</td>
<td>30.7 (25.2, 36.2)</td>
<td>20.6 (15.8, 25.4)</td>
<td>15.2 (10.9, 19.4)</td>
<td></td>
</tr>
<tr>
<td>≥50</td>
<td>220 (15.5)</td>
<td>26.8 (20.9, 32.7)</td>
<td>19.1 (13.9, 24.3)</td>
<td>9.5 (5.7, 13.4)</td>
<td></td>
</tr>
<tr>
<td><strong>Raw pork consumption</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Does not eat</td>
<td>1224 (86.3)</td>
<td>18.0 (15.8, 20.1)</td>
<td>11.8 (6.0, 13.6)</td>
<td>7.0 (5.6, 8.5)</td>
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</tr>
<tr>
<td>Weekly</td>
<td>20 (1.4)</td>
<td>15.0 (0.0, 31.1)</td>
<td>10.0 (0.0, 23.5)</td>
<td>5.0 (0.0, 14.8)</td>
<td></td>
</tr>
<tr>
<td>Monthly</td>
<td>66 (4.7)</td>
<td>18.2 (8.8, 27.6)</td>
<td>13.6 (5.2, 22.0)</td>
<td>7.6 (1.1, 14.0)</td>
<td></td>
</tr>
<tr>
<td>Every few months</td>
<td>71 (5.0)</td>
<td>31.0 (20.1, 41.8)</td>
<td>22.5 (12.7, 32.3)</td>
<td>15.5 (7.0, 24.0)</td>
<td></td>
</tr>
<tr>
<td>Infrequent</td>
<td>38 (2.7)</td>
<td>36.8 (21.3, 52.4)</td>
<td>23.7 (10.0, 37.4)</td>
<td>10.5 (0.6, 20.4)</td>
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</tr>
<tr>
<td><strong>Raw beef consumption</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Does not eat</td>
<td>917 (64.6)</td>
<td>14.7 (12.4, 17.0)</td>
<td>9.1 (7.2, 10.9)</td>
<td>4.6 (3.2, 5.9)</td>
<td></td>
</tr>
<tr>
<td>Weekly</td>
<td>52 (3.7)</td>
<td>30.8 (18.1, 43.4)</td>
<td>21.2 (9.9, 32.4)</td>
<td>13.5 (4.1, 22.8)</td>
<td></td>
</tr>
<tr>
<td>Monthly</td>
<td>205 (14.5)</td>
<td>29.8 (23.5, 36.0)</td>
<td>22.0 (16.3, 27.6)</td>
<td>17.2 (11.9, 22.2)</td>
<td></td>
</tr>
<tr>
<td>Every few months</td>
<td>168 (11.8)</td>
<td>24.4 (17.9, 30.9)</td>
<td>17.9 (12.0, 23.7)</td>
<td>9.5 (5.1, 14.0)</td>
<td></td>
</tr>
<tr>
<td>Infrequent</td>
<td>77 (5.4)</td>
<td>23.4 (13.8, 32.9)</td>
<td>14.3 (6.4, 22.1)</td>
<td>9.1 (2.6, 15.6)</td>
<td></td>
</tr>
<tr>
<td><strong>Raw fermented pork consumption</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Does not eat</td>
<td>1023 (72.1)</td>
<td>15.0 (12.8, 17.1)</td>
<td>9.2 (7.4, 11.0)</td>
<td>5.3 (3.9, 6.7)</td>
<td></td>
</tr>
<tr>
<td>Weekly</td>
<td>96 (6.8)</td>
<td>25.0 (16.3, 33.7)</td>
<td>24.0 (15.4, 32.5)</td>
<td>13.5 (6.7, 20.4)</td>
<td></td>
</tr>
<tr>
<td>Monthly</td>
<td>149 (10.5)</td>
<td>33.6 (25.9, 41.2)</td>
<td>20.8 (14.3, 27.4)</td>
<td>14.1 (8.5, 19.7)</td>
<td></td>
</tr>
<tr>
<td>Every few months</td>
<td>104 (7.3)</td>
<td>33.7 (24.5, 42.8)</td>
<td>25.0 (16.6, 33.4)</td>
<td>16.3 (9.2, 23.5)</td>
<td></td>
</tr>
<tr>
<td>Infrequently</td>
<td>47 (3.3)</td>
<td>19.1 (7.8, 30.5)</td>
<td>12.8 (3.1, 22.4)</td>
<td>4.3 (0.0, 10.1)</td>
<td></td>
</tr>
</tbody>
</table>

CI=confidence interval
Table 4.4 Prevalence of *Trichinella* spp. larvae isolated by artificial digestion from the tongue and diaphragm of pigs slaughtered at official slaughter points in four provinces of northern Laos

<table>
<thead>
<tr>
<th>Animal production characteristic</th>
<th>No.</th>
<th>Prevalence of <em>Trichinella</em> spp. larvae detection</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total survey population</td>
<td>728</td>
<td>2.1 (1.0, 3.1)</td>
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<tr>
<td>Province slaughtered</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Oudomxay</td>
<td>144</td>
<td>2.8 (0.1, 5.5)</td>
<td>0.005</td>
</tr>
<tr>
<td>Luangprabang</td>
<td>209</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Huaphan</td>
<td>189</td>
<td>1.1 (0.0, 2.5)</td>
<td></td>
</tr>
<tr>
<td>Xiengkhuan</td>
<td>186</td>
<td>4.8 (1.7, 7.9)</td>
<td></td>
</tr>
<tr>
<td>Age (months)</td>
<td>656</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-6</td>
<td>97</td>
<td>1.0 (0.0, 3.1)</td>
<td>0.282</td>
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<td>7-12</td>
<td>3343</td>
<td>2.6 (0.9, 4.3)</td>
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<tr>
<td>&gt;12</td>
<td>216</td>
<td>0.9 (0.0, 2.2)</td>
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</tr>
<tr>
<td>Breed</td>
<td>666</td>
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<tr>
<td>Indigenous</td>
<td>559</td>
<td>2.0 (0.8, 3.1)</td>
<td>0.691</td>
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<tr>
<td>Exotic</td>
<td>78</td>
<td>1.3 (0.0, 3.8)</td>
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<tr>
<td>Cross-breed</td>
<td>29</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>668</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>356</td>
<td>2.0 (0.5, 3.4)</td>
<td>0.532</td>
</tr>
<tr>
<td>Male</td>
<td>140</td>
<td>0.7 (0.0, 2.1)</td>
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<tr>
<td>Castrated male</td>
<td>172</td>
<td>2.3 (0.0, 4.6)</td>
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<tr>
<td>Production system at last point of sale</td>
<td>641</td>
<td></td>
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<tr>
<td>Penned/corralled</td>
<td>464</td>
<td>2.4 (1.0, 3.8)</td>
<td>0.118</td>
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<tr>
<td>Free roaming</td>
<td>171</td>
<td>0.0</td>
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</tr>
<tr>
<td>Mixed</td>
<td>6</td>
<td>0.0</td>
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</tr>
</tbody>
</table>

CI=confidence interval
Chapter 5

SeroLogic Study of Pig-Associated Viral Zoonoses in Laos
This chapter is a published paper:


Author contributions:

*Conceived and designed the study:* JVC, KV, SF, RCAT, SDB

*Conducted the surveys:* JVC, KV

*Performed the laboratory testing:* RGJ, RVG, RAL

*Analysed the data:* JVC, RAL (interpretation of SIV results)

*Wrote the manuscript:* JVC, RGJ (5.3.3), RAL (5.3.3 & interpretation of SIV results)

*Proofed and critically appraised the manuscript:* KV, RGJ, RVG, RAL, SF, RCAT, SDB
5 Serologic study of pig-associated viral zoonoses in Laos

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³ Department of Virology, United States Army Medical Component, Armed Forces Research Institute of Medical Sciences, Rajvithi Road, Bangkok, Thailand
⁴ CSIRO Livestock Industries, Australian Animal Health Laboratory, Portarlington Rd, Geelong, Victoria, Australia
⁵ Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand
⁶ Centre for Clinical Vaccinology and Tropical Medicine, Nuffield Department of Clinical Medicine, Oxford University, Oxford, United Kingdom
5.1 Abstract

We conducted a serological survey of four high priority pig associated viral zoonoses, Japanese encephalitis virus (JEV), hepatitis E virus (HEV), Nipah virus (NiV) and swine influenza virus (SIV) in Lao People’s Democratic Republic. We collected blood from pigs at slaughter from May 2008 to January 2009 in four northern provinces. JEV haemagglutination inhibition (HI) seroprevalence was 74.7% (95% CI: 71.5-77.9%), JEV IgM seroprevalence was 2.3% (95% CI: 1.2-3.2%) and HEV seroprevalence was 21.1% (95% CI: 18.1-24.0%). Antibodies to SIV were detected in 1.8% (95% CI: 0.8-2.8%) of pigs by screening ELISA and only subtype H3N2 was detected by HI in two animals with an inconclusive ELISA test result. No NiV antibody positive pigs were detected. Our evidence indicates that peak JEV and HEV transmission coincides with the start of the monsoonal wet season and poses the greatest risk for human infection.
5.2 Introduction

Pig associated viral zoonoses pose a significant threat to human populations in Southeast Asia. Of particular importance are the encephalitic Japanese encephalitis (JEV) and Nipah viruses (NiV), the pathogenic strains of swine influenza virus (SIV) and zoonotic genotypes of hepatitis E virus (HEV). All have been reported in Southeast Asia and pigs have been directly linked to human disease (Kitikoon et al., 2000; Chua et al., 2000; Wibawa et al., 2007; Erlanger et al., 2009).

Japanese encephalitis virus is a major cause of death and disability in Asia and is transmitted by paddy breeding Culex mosquitoes, primarily Culex tritaeniorhynchus, in a zoonotic cycle involving ardeid wading birds (herons and egrets), pigs and humans (van den Hurk et al., 2009). During times of peak transmission, amplification of JEV in naïve pigs within the vicinity of human habitation precedes epidemic transmission to people (van den Hurk et al., 2009). Pig production industries are also affected by JEV infection and economic losses occur through decreased productivity associated with reproductive failure (Joo and Platt, 2006). In Laos, a significant proportion of the human population live within the vicinity of rice paddy (Messerli et al., 2008) and pig production is practiced in rural, peri-urban and urban environs (Blacksell and (editor), 2000; Millar and Photakoun, 2008) ensuring suitable conditions for JEV transmission from pigs to humans.

Hepatitis E virus is primarily a water-borne virus generally causing a self-limiting acute hepatitis in humans, but noted for fulminant hepatitis and a high case-fatality rate during pregnancy in certain environments (Meng, 2009, 2010). Consumption of contaminated meat and occupational exposure are also recognised as important modes of zoonotic transmission (Meng, 2009, 2010). Four distinct genotypes of HEV have been characterised but only genotypes 3 and 4 are considered zoonotic and pigs are recognised as an especially important source of human infection (Lu et al., 2006). Previous studies in Laos indicate that 16-18% of the human population (Corwin et al., 1999; Syhavong et al., 2010) and up to 50% of the pig population (Blacksell et al., 2007) have serological evidence of past exposure to HEV. Zoonotic HEV is emerging as the dominant form of human HEV disease in eastern and southern China (Li et al., 2006; Zhang et al., 2010) and clearly demonstrates the risks posed to the human population of Laos.

Nipah virus is an emerging encephalitic virus that has been associated with significant outbreaks of human disease in Malaysia, Singapore, India and Bangladesh (Chua et al., 1999; Paton et al., 1999; Harcourt et al., 2005; Harit et al., 2006). The natural reservoir hosts of NiV are Pteropid
fruit bats (flying foxes) which can serve as the source of infection for a range of susceptible mammals, including humans and pigs (Mackenzie, 2005). The known geographical range of the virus has extended over recent years with serological and molecular evidence demonstrating NiV to be present in fruit bat species in Cambodia, Indonesia and Thailand (Olson et al., 2002; Reynes et al., 2005; Wacharapluesadee et al., 2005; Sendow et al., 2009). Pigs can be a critical intermediary host acting as a bridge in transmission between infected wild fruit bats and people. This was demonstrated in the Malaysian and Singaporean outbreaks where human NiV disease was associated with exposure to infected pigs or pig carcasses (Chua et al., 2000). However, food-borne and human-to-human transmission has also been demonstrated in South Asia (Luby et al., 2006; Gurley et al., 2007). Pteropid bats are prevalent in Laos, but are currently threatened by habitat loss and hunting (Duckworth et al., 1999). This incursion into the natural fruit bat habitat possibly exposes human and pig populations to NiV infection and an investigation of infection in pigs is required.

The prevalence of SIV in pig populations around the world can vary greatly and a number of HN subtypes may circulate in any particular country. In China, five subtypes H1, H3, H5, H7 and H9 (Liu et al., 2009) and in Thailand three subtypes H1N1, H1N2, and H3N2 (Chutinimitkul et al., 2008) have been reported in their respective pig populations. Pigs have a role in the emergence of novel pathogenic strains through the mixing and re-assortment of human, avian and swine adapted influenza viruses (Shinde et al., 2009). In light of the highly pathogenic avian influenza virus epidemic in Laos (Boltz et al., 2006) and the emergence of the pandemic H1N1 influenza virus in 2009, SIV warrants investigation in Laos.

There remains a scarcity of good quality data relating to the role of pigs as a reservoir of pathogens causing human disease in Laos. The present study was conducted within the scope of a broader pig zoonoses project and aimed to determine the serological prevalence of these four important viral zoonoses and to assess if age, breed, temporal and spatial factors were associated with serological evidence of infection.

5.3 Materials and methods

5.3.1 Ethics statement

The research protocols were reviewed and approved by the Murdoch University Animal Ethics Committee (project number: R2108/07), which adheres to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The Lao Department of Livestock and
Fisheries does not, at this time, have a committee to review and approve scientific research protocols involving animals.

5.3.2 Study sites and survey design

The surveys were conducted at three slaughter-points each in Xiengkhuang and Oudomxay provinces from May to September 2008 and at two collection points each in Huaphan and Luangprabang provinces from October 2008 to January 2009 (Figure 5.1). The survey team consisted of trained district and provincial agricultural and forestry government staff who visited the slaughter-points approximately every two weeks. All pigs brought for slaughter on the nights the survey team visited had a blood sample collected. Blood samples were centrifuged and the serum fraction removed and stored at -20°C prior to testing. An aliquot of each serum sample was sent to the Armed Forces Research Institute for Medical Sciences (AFRIMS), Bangkok, Thailand, for JEV and HEV testing and to the CSIRO Australian Animal Health Laboratory (AAHL), Geelong, Australia, for SIV and NiV testing.

5.3.3 Laboratory techniques

**JEV Hemagglutination inhibition (HI) serology.** Serum samples were pre-treated with acetone and the ability of test serum antibodies to inhibit JEV sucrose acetone extracted mouse-brain antigens agglutinating goose red blood cells was assayed using a microtiter adaptation of the method of Clarke and Casals (Clarke and Casals, 1958) with starting dilution of 1:10. Sera were serially diluted to 1:80 and HI titres > 10 were considered positive for JEV specific antibody (Burke et al., 1985c).

**JEV IgM ELISA protocol.** The AFRIMS in-house JEV IgM ELISA for pig serum (Burke et al., 1985c), adapted from human JEV IgM immunoassays (Burke and Nisalak, 1982; Burke et al., 1982; Burke et al., 1985b), was used to test pigs for evidence of an active or recent infection at the time the survey was conducted. In brief, IgM in the test sample was captured out of solution onto the solid phase of polystyrene plate wells previously coated with goat anti-swine IgM. A 1:100 dilution of test sera were added to the wells. Sucrose- and acetone-extracted suckling mouse brain JEV antigen was added to each well followed by peroxidase-conjugated human anti-flavivirus-hyperimmune IgG was added to label the bound antigen, and the colour developed by the addition of o-phenylenediamine (OPD) substrate, the colour development was stopped by 4M H2SO4. Absorbance at 492nm (A492) was read spectrophotometrically on a microplate reader (Spectra Max 340 PC; Molecular Devices, Inc., Sunnyvale, USA). Negative (NS, A492 < 0.1) and positive standards (WPC, A492 0.4 + 0.15) were added to each test plate. Assay results were
expressed as units which were calculated by: units = 100 x (A492 test sample - A492 NS) / (A492 WPC - A492 NS) and 40 units of antibody defines the lower limit of positivity (Burke et al., 1985c).

**HEV serology.** Detection of HEV IgG was modified from an ELISA procedure previously described by Innis et al (Innis et al., 2002) and Wang et al (Wang et al., 2001). Briefly, 96 well plates were coated with 100 μl of recombinant capsid protein antigen of HEV ORF2/3 (GenWay Biotech, Inc., San Diego, USA) diluted 1:5000 in 0.05 M carbonate-bicarbonate buffer (pH 9.6) (Sigma-Aldrich, St. Louis, USA). The plate was incubated at 37°C for 4 hr and then overnight at 4°C. The coating buffer was discarded and the plate was machine washed (SkanWasher; Skatron, Sterling, USA) with wash buffer (PBST) (0.5% Tween 20 in PBS) (Sigma-Aldrich, St. Louis, USA). Three hundred microliters of blocking buffer (0.5% Casein, 0.5% BSA in PBS, pH 7.4) was added to each well and incubated for 1 hour at 37°C and then machine-washed. One hundred microliters of two negative control, three positive control and each pig serum diluted 1:500 in blocking buffer was added in duplicate wells and incubated at 37°C for 1 hour. Horseradish peroxidase conjugated goat anti-swine IgG (Kirkegaard and Perry Laboratories, Gaithersburg, USA) diluted 1:4000 in blocking buffer containing 0.2% Tween 20 was added to each well. The plate was incubated at 37°C for 30 minutes and then machine-washed. Samples were visualized with the addition of SureBlue (3,3',5,5'-Tetramethylbenzidine) substrate (Kirkegaard and Perry Laboratories, Gaithersburg, USA) and after incubation at room temperature for 10 minutes the reaction was stopped with 100 μl of diluted sulfuric acid (0.18 N H₂SO₄). Within 10 minutes the absorbance 450/650 nm was read on a microplate reader (Spectra Max 340 PC; Molecular Devices, Inc., Sunnyvale, USA). The positivity cut-off was determined by calculating the mean OD±3 SD of 30 Thai HEV-negative pig serum.

**NiV serology.** Sera were initially screened for antibodies to NiV by an indirect ELISA using irradiation-inactivated virus, extracted as a soluble lysate from infected cells by treatment with a non-ionic detergent (Daniels et al., 2001; OIE, 2010b). Sera that tested positive or inconclusive were tested by a microtiter virus neutralisation test (Middleton et al., 2002; OIE, 2010b) using a Malaysian isolate of NiV. Any neutralisation at or beyond the initial 1 in 2 serum dilution was regarded as positive.

**SIV serology.** Sera were tested by a competition ELISA (Sergeant et al., 2009) previously used to detect antibody to influenza nucleoprotein (NP) in avian and equine sera. In brief, plates were coated with influenza NP derived from yeast cells transfected to express a recombinant long form of the influenza A NP. Coated plates were first exposed to test sera diluted 1/10 and then
anti-NP monoclonal antibody (H16-l10-4R5) reacted in the mixture. Washed plates were exposed to anti-mouse HRPO conjugate, and binding of conjugate ultimately assessed by TMB substrate conversion. Sera causing 40% or greater inhibition of the monoclonal antibody were then tested for HI antibody to swine influenza subtypes A/California/07/2009 (pandemic H1N1), A/swine/Ratchaburi/2000 H1N1 (H1N1) and A/swine/Nakhon Pathom/2002 (H3N2). Sera were tested in accordance with OIE protocols (OIE, 2010a). In brief, sera were treated with RDE and heated for 30 min at 56°C to remove non-specific inhibitors adsorbed with packed chicken red blood cells to remove non-specific agglutinins, and assessed in the HI using 4 HA units. Positive sera were recognised as sera with a HI titre of 40 or greater.

5.3.4 Data analysis

Seroprevalence was calculated as the proportion of serum samples with a positive test result in the sampled population. Pearson’s $\chi^2$-test and Fisher’s exact test were used to explore associations between infection status, as measured by JEV HI and HEV ELISA, and the location of the slaughterhouse, age, breed, production system at last point of sale and the month of slaughter. Fisher’s exact test was used to explore associations for JEV IgM positivity due to small numbers in the respective contingency tables.

5.4 Results

5.4.1 Characteristics of the survey pig population

Seven hundred and twenty nine and 724 serum samples were tested at AFRIMS for antibodies to JEV and HEV, respectively; two samples were mislabelled and excluded from the analysis. Seven hundred and twenty six serum samples were tested at CSIRO AAHL for SIV and NiV; seven samples were mislabelled and excluded from the analysis. Complete data was collected for location of slaughterhouse and the collection date and only partial data was available for pig age, breed and production system at last point of sale (Table 5.1 and 5.2). The median age was 12 months (25-75 percentile range: 8-16 months) for the 656 pigs for which age data was available. The majority of slaughtered animals were indigenous breed swayback black pigs (83.9%) and the majority were purchased by slaughter-traders from a penned production system (72.4%). The last point of sale provides no indication of the production systems encountered during the life of the animals.
5.4.2 JEV serology

Antibodies to JEV were detected by HI in 543 of 727 pigs (74.7%; 95% CI: 71.5-77.9%), of which, inhibition titres of 20, 40 and ≥ 80 were observed in 26 (3.6%), 55 (7.6%) and 462 (63.6%) pigs, respectively. In Oudomxay and Xiengkhuang provinces, where samples were collected in the wet season months, there was a significantly lower seroprevalence of anti-JEV antibodies detected by HI in pigs in Xiengkhuang province and pigs in the 4-6 month old age range. In contrast, 4-6 month old pigs had the highest prevalence of anti-JEV IgM antibodies (Table 5.1). In Luangprabang and Huaphan provinces, where samples were collected in the dry season months, there was a significantly lower seroprevalence of anti-JEV antibodies detected by HI in pigs in Luangprabang province and pigs raised in a free range production system (Table 5.2). Anti-JEV IgM antibodies were not detected in samples collected from Luangprabang and Huaphan provinces.

IgM antibody was detected in 17 of 329 pigs (5.2%; 95% CI: 2.7-7.6%) in Oudomxay and Xiengkhuang provinces, and comprised 11 pigs sampled from Oudomxay province and six from Xiengkhouang province. Fifteen of the 17 IgM positive sera had HI titres ≥ 80 and the remaining two samples had HI titres of 40. Age data was available for 265/329 pigs and peak seroprevalence (11.6%) was observed in the youngest age class of animal (Table 5.1) and was 20.7% (6/29) and 5.0% (2/40) of 4-6 month-old pigs in Oudomxay and Xiengkhuang provinces, respectively.

5.4.3 HEV serology

Seven hundred and twenty-two serum samples were analysed for the presence of anti-HEV antibodies by ELISA and 152 (21.1%) had an optical density (OD) ≥ 0.500 and were considered positive. One hundred and sixty-three (22.6%) samples had an inconclusive borderline test result, OD > 0.260 and < 0.500; the remaining 407 (56.4%) samples were negative for antibodies to HEV with an OD < 0.260. In Oudomxay and Xiengkhuang provinces, where samples were collected in the wet season months, there was no observed spatial difference in seroprevalence. Seroprevalence was significantly higher in the early stages of the wet season, May and June, and significantly higher in indigenous and cross-breeds compared to exotic breed pigs (Table 5.1). In Luangprabang and Huaphan provinces, where samples were collected in the dry season, there was no observed spatial difference in seroprevalence and no observed difference for pig breeds. There was, however, a significant decrease in seroprevalence for December 2008 (Table 5.2). In the wet season collection sites, seroprevalence peaked in 4-6 month old pigs (41.2%; Table 5.1),
however, the reverse was observed for the dry season collection sites where 4-6 month old pigs had the lowest seroprevalence (3.6%; Table 5.2).

5.4.4 **NiV serology**

Seven hundred and nineteen serum samples were tested by the NiV comparative ELISA of which 716 (99.6%) were negative and three (0.4%) returned an inconclusive test result. All three ELISA inconclusive sera were tested by the NiV neutralisation assay of which two samples were negative and the third was toxic to the cell line.

5.4.5 **SIV serology**

Twenty-three out of 719 (3.2%) pig serum samples were reactive in the ELISA, of which 13 (1.8%) had a percent inhibition (PI) > 60 and were considered positive, the remaining 10 samples had a PI of 40-60 and were considered inconclusive. Twenty ELISA reactive sera were tested by HI for H3N2 (Nakorn Pathom), two samples were HI positive with titres 160 and 640 the remaining 18 sera were negative by HI for H3N2. Twenty-one and 23 sera were tested by HI for H1N1 (Ratchaburi) and H1N1 (2009-Pandemic), respectively, and all were negative. Furthermore, 14 ELISA negative sera were tested by HI for H3N2 (Nakorn Pathom) and two samples were HI positive. No ELISA negative samples were tested by HI for H1N1 due to an insufficient volume of sera.

5.5 **Discussion**

This is the first study to report the seroprevalence of four viral zoonoses in the pig population of Laos. Previous published studies on JEV in Laos have focused on human populations (Vongxay, 1995; Vallee et al., 2009) and our study represents the first assessment of the role pigs might play in transmission to Lao people. Previous swine HEV studies have been reported from Laos (Blacksell et al., 2007; Conlan et al., 2011) and our survey supports and adds weight to the argument that pigs have a potential role in the natural history of human HEV disease. No published studies have reported seroprevalence of NiV and SIV in the Lao pig population and we provide strong evidence that pigs posed little risk for human NiV and SIV disease, at least at the time the survey was conducted. Anti-NiV antibodies were not detected and only a limited number of animals had serological evidence of a previous SIV infection with a non-pathogenic subtype.
This study demonstrates unequivocally that JEV was widespread in the pig population of northern Laos and a seroprevalence of 74.7% was indicative of a hyper-epizootic state. There is no JEV vaccination of pigs in northern Laos and these results represent natural transmission. Furthermore, maternal antibodies wane after 2 months (Scherer et al., 1959) and the youngest pigs in our survey population were 4 months old, indicating the detected antibodies were raised against active JEV infections rather than via passive immunity.

Antibodies to JEV were detected in all provinces and significant differences in prevalence were observed between provinces for both temporal sampling frames. Since HI can detect anti-JEV antibodies in pigs for up to three years post-infection (Geevarghese et al., 1994) and the median age at slaughter was 12 months, it was unlikely that the observed seroprevalence between the two temporal sampling frames was influenced by the timing of sample collection. The differences we encountered were possibly due to factors such as pig density, rice paddy production and *Culex* mosquito abundance. This was further supported by the finding that pigs purchased for slaughter from free-range production systems had lower seroprevalence compared to penned pigs, with the former production systems being encountered predominantly in upland rice growing areas with limited paddy. However, the observed prevalence in all four provinces was very high.

Prevalence of IgM antibodies against JEV peaked in June and July, corresponding with the start of the wet season and water filling of rice paddies providing suitable breeding conditions for *Culex* mosquitoes. In pigs, IgM antibodies are detected within 2-3 days post-infection and can be detected in serum for up to 3 weeks (Burke et al., 1985c), giving us confidence that the IgM positive pigs we detected were recently infected and that peak transmission and greatest risk for human infection corresponds with the first half of the wet season. This peak in Lao pigs was consistent with a peak transmission to Thai people in June and July 1983 (Burke et al., 1985a). Since we do not present a single sampling frame over a complete year, caution should be exercised in interpreting seasonal transmission patterns. We would not expect, however, highly active transmission in the dry season months due to a lack of mosquito breeding sites but the impact of irrigated rice production on *Culex* mosquito abundance in the dry season of northern Laos remains to be determined.

The IgM ELISA results for pigs provide limited evidence that JEV is not maintained in the pig population throughout the year, being consistent with an epizootic pattern of transmission. This could be due to a combination of relatively low animal densities (FAO, 2005), a short duration of viremia, ranging from 1-3 days (Williams et al., 2001), and a decrease in mosquito vector abundance in the dry season winter months. The migration patterns of ardeid birds could therefore have a strong influence on JEV transmission patterns and several ardeid bird species
breed in Laos during the wet season months and other species over-winter during the dry season months (Duckworth et al., 1999; Kushlan and Hancock, 2005). The role of these migratory birds in maintaining JEV in an epizootic state in Laos warrants further investigation.

In pigs, the most clinically significant manifestation of a JEV infection is reproductive failure in sows due to abortion and abnormal farrowing (Joo and Platt, 2006). The very high seroprevalence of JEV in young pigs ≤ 6 months indicates that JEV would have little or no impact on the reproductive potential of local indigenous breed sows. Southeast Asian indigenous breed sows sexually mature from 6 - 8 months (Dang-Nguyen et al., 2010) and the majority of sows in Laos would have protective immunity by the age of first oestrus. The impact on the reproductive potential of indigenous breed boars may however be more significant. Southeast Asian indigenous breed boars can reach sexual maturity from 2-3 months of age (Dang-Nguyen et al., 2010) and infection of sexually mature boars can cause infertility (Joo and Platt, 2006).

Since the smallholder pig sector in Laos has low productivity (Conlan et al., 2008; Phengsavanh et al., 2010), we believe the effect of JEV on this pig producing sector warrants greater scrutiny, with particular reference to boar infertility.

Two recent swine HEV studies in Laos (Blacksell et al., 2007; Conlan et al., 2011) together with this present study demonstrate the relative importance of pigs as a reservoir of human HEV disease. Blacksell et al (Blacksell et al., 2007) observed a high seroprevalence of HEV in pigs sampled at provincial slaughterhouses in northern Laos; 85.7%, 47.1%, 60.0% and 72.1% for Huaphan, Luangprabang, Oudomxay and Xiengkhuang provinces, respectively. These data are substantially higher than those observed during this current study, 29-30% was observed in Xiengkhuang and Oudomxay provinces sampled in the wet season and 12-15% in Huaphan and Luangprabang sampled in the dry season. The difference may have been due to seasonal variation since Blacksell et al (Blacksell et al., 2007) observed very high seroprevalence in Huaphan and Luangprabang when sampling was conducted in the wet season months. Further work will be required to confirm seasonal peaks of transmission, however the data from Oudomxay and Xiengkhuang provides evidence that the peak seroprevalence was at the start of the wet season.

Age-related seroprevalence in the current study peaked in 4-6 month old pigs sampled in the wet season and 7-12 month old pigs sampled in the dry season. The combined temporal and age prevalence data indicates that young animals are an important reservoir of HEV at the beginning of the wet season. We can speculate that management of young animals differs from older animals and predisposes them to infection during the wet season, but further research will be required to understand the specific production practices associated with increased risk of HEV transmission. The importance of young animals in the epidemiology of HEV in Laos was further
demonstrated by Conlan et al. (Conlan et al., 2011) who observed 11.6% of pigs ≤ 6 months were shedding virus during the dry season months January to March. Only genotype 4 HEV has been recognised in northern Laos (Conlan et al., 2011), and this same genotype has been identified as the most common cause of human HEV disease in southern and eastern China (Li et al., 2006; Zhang et al., 2010). The seroprevalence of human HEV in Laos has been estimated at 16-18% and 2-4% of acute hepatitis hospital admissions were caused by HEV (Corwin et al., 1999; Syhavong et al., 2010). To date, no data exists describing the genotypes causing human HEV disease in Laos and work should now be undertaken to establish the source of human infections.

Nipah virus has been detected in Pteropid fruit bats from Malaysia, Cambodia and Thailand by serology, virus isolation or RNA amplification (Yob et al., 2001; Reynes et al., 2005; Wacharapluesadee et al., 2005) and NiV antibodies have also been detected in fruit bats in Indonesia (Sendow et al., 2009), providing evidence that NiV is endemic in Southeast Asia. The most human cases and deaths of any outbreak to date occurred during the Malaysian and Singaporean outbreak of 1999 and pigs were the source of human infections, demonstrating the importance of pigs as an intermediary amplification host. We found no serological evidence of NiV infection of pigs in Laos, but by no means does this confirm absence of NiV in this country. Pteropid fruit bats are present in Laos and to better understand the epidemiology and risks of NiV, surveys of these competent reservoir host species will be required.

Serological data for SIV indicated low levels of virus circulation in the pig survey population for the period May 2008 to January 2009. We found no evidence of H1N1 (Ratchaburi or 2009 pandemic strain) infection and only H3N2 subtype was detected. We could only confirm HI positivity for 2/23 ELISA reactive sera, both of which were inconclusive in the ELISA, indicating that the ELISA has not been fully optimised for pig sera or that different subtypes are circulating in Lao pigs. The influenza ELISA is well defined for avian and equine sera but less well characterised for testing pig sera, this was further evident in our finding that two ELISA negative samples were positive for H3N2 by HI. The influenza HI is affected by the degree of homology between the assay virus and the virus to which animals have been exposed. It was possible that many of the 14 sera that were ELISA reactive, but not HI positive, could represent animals with exposure to a sufficiently different but homologous subtype, or to an untested subtype such as H9 or H5 subtype. In Southern China, near the border region with northern Laos, H9 subtype has been shown to be prevalent at 10.5% (Liu et al., 2009) and could explain some of the discrepancies we observed between the ELISA and HI results. However, the 2/14 ELISA-negative HI (H3N2)-positive sera suggests that additional work is required to define ELISA sensitivity with pig sera in Southeast Asia. The low frequency of antibody to SIV may also
derive from a low-density pig population acting as a natural barrier to maintenance of virus endemicity and therefore to the timing of the test cohort relative to the opportunity for periodic spread of the virus.

We provide confirmation that pigs are an important reservoir of the viral zoonoses JEV and HEV in Laos and the limited evidence suggests the early months of the monsoonal wet season, May to July, coincide with peak transmission in pigs and greatest risk for human disease. Our study supports the need for continued surveillance of pig associated viral zoonoses and the integration of human and veterinary public health authorities to control these important diseases.

5.6 Acknowledgements

We wish to acknowledge the contributions made by district, provincial and national government staff from the Lao Department of Livestock and Fisheries, Ministry of Agriculture and Forestry, whose contribution was critical to the conduct of this study. In particular, we acknowledge Mr. Lapinh Phithacthep, Ms. Vilaywan Soukvilay, Ms Manivanh Phouarawan and Ms Vilayphet Viravong from the National Animal Health Centre.

5.7 Funding

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5.8 Disclaimer

The opinions or assertions contained herein are the private views of the authors, and not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense (RGJ, RVG).

5.9 References


Figure 5.1 Study sites in northern Laos. 1, Xay, district, Oudomxay province; 2, Luangprabang district, Luangprabang province; 3, Xiengngeun district, Luangprabang province; 4, Pek district, Xiengkhuang province; 5, Xamneua district, Huaphan province; 6, Viengxay, Huaphan province. Black dots, abattoirs or slaughterhouses (> 20 pigs per night); black circles, slaughter-points (< 5 pigs per night). Black dot in Luangprabang district (2) represents an amalgamation of home slaughter-points due to re-construction of abattoir at time of survey.
Table 5.1 Seroprevalence of antibodies against Japanese encephalitis virus (JEV) and Hepatitis E virus (HEV) during May-September 2008 (wet season months) in Oudomxay and Xiengkhuang provinces, Laos

<table>
<thead>
<tr>
<th>Population characteristic</th>
<th>Japanese encephalitis virus (JEV) serology</th>
<th>Hepatitis E virus (HEV) serology</th>
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<tr>
<td></td>
<td>No. tested</td>
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* Fishers Exact Test (all other P-values calculated using χ²-test)  
HI=hemagglutination inhibition; ELISA=enzyme-linked immunosorbent assay; CI=confidence interval.
<table>
<thead>
<tr>
<th>Population characteristic</th>
<th>Japanese encephalitis virus (JEV) serology *</th>
<th>Hepatitis E virus (HEV) serology</th>
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* All serum samples collected in Luangprabang and Huaphan provinces were not reactive in the JEV IgM ELISA.

HI=hemagglutination inhibition; ELISA=enzyme-linked immunosorbent assay; CI=confidence interval.
Chapter 6

Hepatitis E virus is Prevalent in the Pig Population of Lao People’s Democratic Republic and Evidence Exists for Homogeneity with Chinese Genotype 4 Human Isolates
This chapter is a published paper:


Author contributions:

*Conceived and designed the study*: JVC, KV, SF, RCAT, SDB

*Conducted the surveys*: JVC, KV

*Performed the laboratory testing*: RGJ, PC, MCM

*Analysed the data*: JVC, MCM (phylogenetic tree analysis)

*Wrote the manuscript*: JVC, RG (6.3.5), PC (6.3.5), MCM (6.3.5 & phylogenetic tree interpretation)

*Proofed and critically appraised the manuscript*: RGJ, KV, PC, MCM, SF, RCAT, SDB
6 Hepatitis E virus is prevalent in the pig population of Laos and evidence exists for homogeneity with Chinese Genotype 4 human isolates

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6.1 Abstract

The objective of this study was to determine the prevalence and genotypic range of Hepatitis E virus (HEV) in the pig population of northern Lao People’s Democratic Republic (PDR). We collected 181 faecal samples from indigenous-breed pigs ≤6 months of age and the faeces was stored in RNA stabilisation buffer due to cold-chain and transport limitations. Twenty-one (11.6%) pigs had detectable HEV RNA and 43.5% of village pig herds were infected. Based on a 240 base pair-nucleotide sequence flanking the junction of open reading frames 1, 2 and 3 (ORF1, ORF2 and ORF3) the isolates were phylogenetically classified within genotype 4. Phylogenetic analyses revealed distinct genetic groupings of the Lao HEV isolates and two groups clustered with human and pig HEV isolates from China. This was the first study to demonstrate genotype 4 HEV in Laos and indicates pigs are a potential reservoir for human HEV infection.
6.2 Introduction

Hepatitis E virus (HEV) typically causes a self-limiting acute viral hepatitis followed by recovery and on occasion a fulminant hepatitis develops (Aggarwal and Naik, 2009). It is the causative agent of large-scale and sporadic acute hepatitis outbreaks worldwide with endemicity primarily centred in regions with poor sanitation and hygiene; encompassing large areas of Asia, Africa and central and south America (Aggarwal and Naik, 2009; Emerson and Purcell, 2003). Faecal-oral transmission is the predominant route of HEV infection and contaminated drinking water is the most common cause of epidemic human disease (Aggarwal and Naik, 2009; Emerson and Purcell, 2003; Meng, 2009b). Consumption of raw shellfish and uncooked meat and viscera from HEV infected animals are also important sources of human disease and result in sporadic cases (Meng, 2009a, b).

Hepatitis E virus is a non-enveloped, single-stranded positive sense RNA virus of the family Hepeviridae, genus Hepevirus and is comprised of four distinct genotypes (Emerson and Purcell, 2003; Lu et al., 2006). Genotypes 1 and 2 are primarily associated with humans and account for the majority of HEV infections worldwide, including epidemics (Aggarwal and Naik, 2009). Genotype 3 and 4 HEV have a zoonotic origin (Aggarwal and Naik, 2009; Meng, 2009a) with a diverse animal host range (Lu et al., 2006; Okamoto, 2007). However, pigs are an especially important reservoir of genotype 3 and 4 HEV and a common source of human HEV infection (Lu et al., 2006).

The majority of human HEV infection in Asia can be attributed to genotype 1 virus (Aggarwal and Naik, 2009; Wei et al., 2006) with sporadic acute disease associated with genotypes 3 and 4 (Aggarwal and Naik, 2009; Fu et al., 2010). However, recent evidence from China indicates that genotype 4 HEV is emerging as the predominant cause of human disease (Li et al., 2006; Zhang et al., 2010). All pig HEV infections in Asia have been attributed to genotype 3 or 4 (Meng, 2009a) with a single genotype 1-like virus isolated from a pig in Cambodia (Caron et al., 2006), but this finding has not yet been verified (Meng, 2009b). Genotype 3 HEV is prevalent in pig populations of Cambodia and Thailand (Caron et al., 2006; Cooper et al., 2005) and Genotype 4 is prevalent in pigs of southern China (Ji et al., 2008). Furthermore, genotype 4 HEV has been associated with shellfish and human disease in northern Vietnam (Koizumi et al., 2004).

In the Laos, anti-HEV antibodies are prevalent in 16-18% of the general human population (Corwin et al., 1999; Syhavong et al., 2010) and 2-4% of hospital admitted hepatitis cases have been attributed to HEV infection (Corwin et al., 1999; Syhavong et al., 2010). In a sero-
epidemiological study of the Lao pig population, anti-HEV antibodies were detected in 51% of slaughter-pigs and 15% of village-surveyed pigs, with a peak sero-prevalence in 7-9 month-old animals (Blacksell et al., 2007). Neither pig nor human HEV isolates have been genetically typed in the Laos and the link between pig and human infections have not been examined. This study describes the detection and molecular epidemiology of HEV in pigs in northern Laos.

6.3 Materials and methods

6.3.1 Ethics statement

The research protocols were reviewed and approved by the Murdoch University Animal Ethics Committee (project number: R2108/07) prior to commencing the study. The Lao Department of Livestock and Fisheries does not, at this time, have an ethics committee to review and approve scientific research protocols involving animal subjects.

6.3.2 Study site

The study was conducted in four provinces in northern Laos, Luangprabang, Oudomxay, Xiengkhuang and Huaphanh, where >90% of pigs are produced in the smallholder sector using traditional low-input production practices. One district in each province was selected for inclusion in the study (Figure 6.1).

6.3.3 Faecal samples and storage

Faecal samples were collected from 181 pigs ≤6 months of age in the dry season from January to March 2009 and stored in RINAlater® (Ambion, Carlsbad, USA). Approximately 1 g of faecal material was added to 5 ml of RINAlater® and mixed thoroughly with a clean wooden stick. Data on age, breed, sex and village origin were collected for each animal. Samples were stored at 4 °C in the field and at -85 °C upon return to the central laboratory in Vientiane Capital. Samples were shipped on dry ice to the Armed Forces Research Institute for Medical Sciences (AFRIMS), Bangkok, Thailand, for molecular analysis.

6.3.4 Statistical analysis

Data were entered into a spreadsheet (Excel®, Microsoft Corporation, Redmond, USA) and analysis was performed using Stata/IC version 10 (StataCorp LP, College Station, USA). HEV prevalence was calculated as the proportion of animals that had detectable HEV RNA in the sample population. The Fisher's exact test was used to explore associations between infection
status and sex, province and age category. The effect of sample storage time at 4 °C on HEV RNA detection was tested by Kruskal-Wallis test for non-parametric difference of means. Associations were considered significant if \( P \leq 0.05 \).

### 6.3.5 Polymerase chain reaction, sequencing and phylogenetic analysis

Virus RNA was extracted from stool suspension using QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instruction. The RNA was tested by nested RT-PCR using primers flanking the junction of open reading frame (ORF) 1, 2 and 3 with external primers F2782 (5’-GGDCTBGTTCAAACTGAT-3’) and R2783 (5’-GGTTGTTGGATGAATAGG-3’) and internal primers F2781 (5’-GGTTCAACCTGATWGGYATGCT-3’) and R2784 (5’-GGATTGCGAAGGGCTGAGAATCA-3’). Briefly, genomic RNA was converted to cDNA using specific primer R2783 with the AMV-RT (Promega, Madison, USA) according to the manufacturer’s instruction. The first and second round PCR were amplified by using AmpliTaq DNA polymerase (Applied Biosystems, Foster City, USA) with the external and internal primer sets, respectively. Cycling conditions included 35 amplification cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C. After electrophoresis in a 1.5% agarose gel stained with ethidium bromide, the expected 310 bp bands of the second round PCR product were visualized on a UV transiluminator. The PCR amplified DNA fragments were purified using QIAquick PCR purification kit and the QIAquick gel extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions.

Sequencing reactions were performed using the DYEnamic ET dye terminator sequencing kit (GE Healthcare, Little Chalfont, UK) according to the manufacturer’s instruction, with sequencing primers. The sequencing products were cleaned by standard precipitation before sequencing on a MegaBACE 500 automated DNA sequencer (GE Healthcare, Little Chalfont, UK). Overlapping nucleic acid sequences were combined for analysis and edited with the aid of Sequencher software (Gene Code Corporation, Ann Arbor, USA). The 21 Lao isolates were combined with 20 known HEV swine and human sequences obtained from GenBank (Figure 6.3); including 10 human HEV strains (Genotype and GenBank Accession numbers in parenthesis) from Burma (genotype 1; M73218), Mexico (genotype 2; M74506), China (genotype 4; AB108537, AJ272108, AB197673, AB369688), Japan (genotype 4; AB253420, AB097812, AB291966), South Korea (genotype 4; FJ763142), and 10 pig HEV strains, including Thailand (genotype 3; EU375463), China (genotype 4; EU676172, GU119960, EF570133, AY594199, FJ610232, GU361892, GU206559), Japan (genotype 4; AB097811) and India (genotype 4;
AY723745). Sequences were aligned using MUSCLE v2.1 (Edgar, 2004) and a phylogenetic tree was constructed using PhyML v3.0 (Guindon and Gascuel, 2003) under the model GTR+G+I determined using jModelTest (Podsada, 2008) and with aLRT, SH-like support (indicated at tree nodes). Pairwise analysis was performed in CLC Main Workbench (CLC bio, Denmark) and Fisher’s exact test was used to detect significant evidence of heterogeneity with respect to host.

6.4 Results

6.4.1 Detection and epidemiology of HEV in pigs

One hundred and eighty-one faecal samples were collected from pigs ≤6 months old (median: 3 months old), stored in RNAlater® and kept at 4 °C in the field before storage at -85 °C in the laboratory. The median storage time at 4 °C was 11 days (range: 1 – 44). Twenty-one samples (11.6%) had detectable HEV RNA in the faeces and no significant effect of storage time on HEV RNA PCR-amplification was observed ($P=0.420$). Samples were collected from pigs from 95 households in 23 villages (Table 6.1). There was a significant difference in observed prevalence between the 4 provinces ($P=0.012$) as no pigs sampled in Huaphanh province had detectable HEV RNA (Table 6.1). Age-stratified prevalence peaked at 15.8% (3/19) in 1-2 month old pigs (Figure 6.2) but no significant difference ($P=0.514$) in HEV infection was observed for piglet age due to the small sample size. There was no significant association between HEV infection status and the sex of pigs ($P=0.831$).

6.4.2 Genetic analysis of HEV isolates from viremic pigs

Sequence analysis of the 240-bp fragment indicated that all 21 Lao HEV isolates belonged to genotype 4 (Figure 6.3) and were 89.6-100% identical to each other. When Lao sequences were compared to known human and pig HEV isolates obtained from GenBank, they had 88.3-97.1% sequence homology with genotype 4 strains, 80.0-83.3% homology with Thai genotype 3 HEV (EU375463), 80.8-84.2% homology with Burmese genotype 1 HEV (M73218) and 79.6-83.3% homology with Mexican genotype 2 HEV (M74506). Genotype 4 sequences from both human and swine HEV isolates appeared to cluster indiscriminately within the phylogenetic tree (Figure 6.3) and no significant clustering for host was evident ($P=0.392$).

Genetic variability was observed within the 21 Lao HEV isolates and we tentatively assigned them into four apparent groups and a lone genetically distinct isolate (Figure 6.3). Group 1 was comprised of four isolates from a single litter in Xiengkuang province (HQ541429-HQ541432) and a piglet in Oudomxay province (HQ541425) and had 100% sequence homology. Group 1
could not be classified to the subtype level as there was 96.3% sequence homology with subtype 4b swine HEV isolated in Guangxi province in southern China (GenBank accession number: EU676172) (Zhang et al., 2010), 95.4% homology with subtype 4g human HEV isolated in Jilin province in northeast China (AB108537) (Lu et al., 2006) and 95.0% homology with subtype 4d swine HEV isolated in Jiangxi province in southeast China (AY594199) (Lu et al., 2006). A single isolate from Oudomxay province (HQ541423) had 95.0% sequence homology with subtype 4b swine HEV (EU676172) and 94.2% homology with subtype 4c swine HEV isolated in Hokkaido, Japan (AB097811) (Lu et al., 2006). Group 2 was comprised of two isolates, one each from Luangprabang (HQ541412) and Oudomxay (HQ541427) provinces and had 99.6% sequence homology. Group 2 isolates had 96.7-97.1% sequence homology with subtype 4a human HEV isolated from a Japanese patient who travelled to Shaanxi province in central China (AB197673) and subtype 4a swine HEV isolated in Xinjiang province in far northwest China (GU119960), giving us confidence that these isolates can be classified as subtype 4a. Group 3 was comprised of isolates from two litters in a village in Luangprabang province (HQ541414-HQ541417; HQ541418-HQ541419), from a piglet in another village in Luangprabang province (HQ541413) and a piglet from Xiengkhuang province (HQ541428) and had 98.3-100% sequence homology. Group 4 was comprised of five isolates from five different litters and three villages in Oudomxay province (HQ541420-HQ541422, HQ541424, HQ541426) and had 94.2-98.3% sequence homology. Group 3 and 4 Lao isolates did not cluster with other Asian HEV strains (88.3-93.8% homology) and were not classified at the subtype level.

The sequence data for the Lao HEV isolates were deposited on GenBank, accession numbers: HQ541412 – HQ541432.

6.5 Discussion

We report for the first time genotype 4 HEV in the pig population of northern Laos and that almost half of the village pig herds were infected. This finding has epidemiological significance since the growing body of evidence indicates possible geographical partitioning of genotypes 3 and 4 in the Mekong sub-region of Southeast Asia. To our knowledge, only genotype 3 has been isolated from pigs in Cambodia and Thailand (Caron et al., 2006; Cooper et al., 2005) and genotype 4 from humans in Vietnam (Hijikata et al., 2002; Koizumi et al., 2004) and pigs in Laos. The Lao and Vietnamese HEV isolates originate from northern regions and to date no data on the genotypes of HEV circulating in the southern regions of these countries are available. Wide reaching studies are required to confirm geographical partitioning, however, classical swine fever
virus, which is endemic throughout the region, provides a precedent for genotypic partitioning in the Mekong region (Blacksell et al., 2005; Blacksell et al., 2004).

The success of this study was dependent on the ability to store and transport faecal samples in a manner that prevented HEV RNA degradation. We used the proprietary RNA stabilisation buffer RNAlater® to preserve HEV RNA for subsequent molecular analysis. RNA stabilisation buffers have been used to preserve RNA in faecal samples in studies involving rotavirus (Whittier et al., 2004) and avian influenza virus (Forster et al., 2008), but to our knowledge this is the first report using RNAlater® to preserve HEV in faeces. Our study clearly demonstrates proof-of-principle and further studies to validate the protocol and determine analytical and diagnostic performance characteristics are warranted. Hepatitis E virus could be detected in faeces stored at 4 °C for 41 days, indicating the usefulness of this method in conducting an epidemiological survey in a hot, tropical country with poor quality infrastructure and cold chains. The most significant drawback of RNAlater® is the incompatibility with virus isolation (Forster et al., 2008) and dual sampling will be needed if live virus manipulations are required. Since our study was primarily a genotyping study, RNAlater® preservation served our purpose.

Previous studies in Laos indicate that tropical monsoon weather patterns may influence the epidemiology of swine HEV (Blacksell et al., 2007), such that peak seroprevalence corresponds with the wet season months May to September (Conlan et al 2010, In prep). The timing of this present survey coincided with the dry season and the observed prevalence of 11.6% may be indicative of the baseline from which epidemic transmission can occur during the wet season. However, longitudinal studies are needed to determine seasonal influence on virus transmission in Laos; a study in Eastern China demonstrated little seasonal influence on transmission (Lu et al., 2009), but rainfall patterns and pig production practices are vastly different.

The age-stratified HEV RNA positivity data suggested that pigs were infected at a young age with peak prevalence observed in 1-2 month old piglets. This finding is consistent with experimental studies showing piglets start to shed virus from 1 month of age (Kanai et al., 2010) after the waning of IgA maternal antibodies by 3 weeks of age (de Deus et al., 2008). Piglets can shed virus for up to 4 months post infection (Kanai et al., 2010) providing a continued source of environmental contamination and enabling HEV to remain endemic over the dry season months, November to March.

It is important to note the limitations of interpreting sequence data from a 240-bp fragment; nevertheless, genetic variability was evident amongst the Lao HEV isolates. Interestingly, four genetically distinct isolates were detected in Oudomxay province and all but those belonging to
Group 4 clustered with other Asian isolates, and could reflect the movement of people and animals in a region at the junction of five countries (Figure 6.1). Groups 1, 2 and 3 were comprised of isolates from different provinces and provide evidence for HEV dispersal in Laos through pig trade and or human movements. All but three of the Lao isolates fell externally in the phylogenetic tree when compared to the other Asian genotype 4 isolates included in the analysis. The data provides evidence that Groups 3 and 4 HEV isolates from Laos were unique and possibly geographically partitioned, whereas the remaining isolates were related to Chinese genotype 4 HEV isolates from humans and pigs.

Host related factors are generally thought to be closely associated with the severity of HEV infection (Okamoto, 2007), however, recent studies indicate that genotype 4 HEV was more commonly associated with severe and fulminant hepatitis compared to genotype 3 HEV (Mizuo et al., 2005; Okamoto, 2007). Severe and fulminant hepatitis was reported in 36% and 8% of cases, respectively, for genotype 4 HEV infection compared to no severe or fulminant hepatitis observed in genotype 3 HEV infections (Okamoto, 2007). This finding could have important implications for high-risk groups in northern Laos considering we exclusively found genotype 4 HEV and that genotype 4 HEV is emerging as the dominant cause of human HEV disease in southern and eastern China (Li et al., 2006; Zhang et al., 2010).

The results of the present study showed that genotype 4 HEV was prevalent in Laos and that almost half the herds were infected. The high pig and herd level prevalence in the Lao pig population provides an abundant reservoir of virus for human HEV disease in rural communities of northern Laos. Research will be required to understand the cause of human HEV disease and the use of a validated protocol using RNA stabilisation buffer could provide a suitable means of achieving this objective. Our study provides the first data set from Laos that can be used to examine the source of human HEV disease. In addition, further work will be required to determine if the dry season prevalence we observed was baseline and a source of epidemic transmission in the monsoonal wet season.

6.6 Acknowledgements

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6.7 Funding

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6.8 Disclaimer

The opinions or assertions contained herein are the private views of the authors, and not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense (RGJ, PC, MCM).

6.9 Conflict of interest statement

The authors have declared that no competing interests exist.

6.10 References


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storage methods for reverse-transcriptase PCR amplification of rotavirus RNA from gorilla 
(Gorilla g. gorilla) fecal samples. J. Virol. Methods 116, 11-17.

Figure 6.1 Map of study sites in Laos. 1, Xay district, Oudomxay province; 2, Xieng Ngeun district, Luangprabang province; 3, Paek district, Xiengkhuang province; and 4, Viengxay district, Huaphan province
Figure 6.2 Proportion of pigs HEV RNA positive by age-category
Figure 6.3 Phylogenetic tree constructed by the maximum-likelihood method (GTR+G+I, model) based on 41 sequences and 240 nucleotides flanking the junction of open reading frames 1, 2 and 3 (ORF1-3) of Hepatitis E virus (HEV). Twenty-one HEV isolates from Lao pigs are listed with the province of origin, compared to human and swine sequences obtained from GenBank. Genotypes are denoted on the tree and genetically distinct Lao groups are enclosed in brackets.
Table 6.1 Detection of HEV RNA in faeces of pigs in northern Laos

<table>
<thead>
<tr>
<th>Province</th>
<th>Number of villages</th>
<th>Number of households</th>
<th>Number of pigs</th>
<th>Median age (months)</th>
<th>Age range (months)</th>
<th>Proportion pigs HEV positive (95%CI)</th>
<th>HEV positive villages (%)</th>
<th>HEV positive households (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xiengkhuang</td>
<td>6</td>
<td>20</td>
<td>39</td>
<td>3</td>
<td>1.5 - 4.5</td>
<td>12.8 (1.8 - 23.8)</td>
<td>2 (33.3)</td>
<td>2 (10.0)</td>
</tr>
<tr>
<td>Huaphanh</td>
<td>5</td>
<td>26</td>
<td>45</td>
<td>3</td>
<td>2.0 - 5.0</td>
<td>0.0 (0.0 - 0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Oudomxay</td>
<td>6</td>
<td>30</td>
<td>46</td>
<td>4</td>
<td>1.5 - 6.0</td>
<td>17.4 (6.0 - 28.8)</td>
<td>5 (83.3)</td>
<td>8 (26.7)</td>
</tr>
<tr>
<td>Luangprabang</td>
<td>6</td>
<td>19</td>
<td>51</td>
<td>4</td>
<td>2.0 - 6.0</td>
<td>15.7 (5.4 - 26.0)</td>
<td>3 (50.0)</td>
<td>3 (15.8)</td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>95</td>
<td>181</td>
<td>3</td>
<td>1.5 - 6.0</td>
<td>11.6 (6.9 - 16.3)</td>
<td>10 (43.5)</td>
<td>13 (13.7)</td>
</tr>
</tbody>
</table>
Chapter 7

General discussion and concluding comments
7 General discussion and concluding comments

7.1 Introduction

This thesis has sought to determine and characterise the ecological and epidemiological aspects of parasitic and viral zoonoses in Laos where living conditions, livestock production and cultural practices place large proportions of the population at risk of exposure to debilitating neglected tropical diseases. In so doing, this thesis has made a substantial and important contribution to the limited, but growing, body of knowledge on the prevalence and risks of a broad range of neglected tropical diseases in northern Laos. To achieve this in a resource poor country, a consultative ‘one health’ approach was undertaken in design, implementation, analysis and communication of research findings.

The formulation and implementation of ‘one health’ research, while embraced across human and animal health disciplines, may be susceptible to discipline bias depending on funding and resource allocation. Furthermore, researchers may fail to seek input from policy makers at the outset to ensure the research outcomes will inform policy decisions. To address these potential handicaps, strong collaborations were formed with the Lao Ministry of Health with the support of the Institut Francophone pour la Médecine Tropicale, Vientiane, Laos and the Wellcome Trust-Mahosot Hospital-Oxford University Tropical Medicine Research Collaboration, Microbiology Laboratory, Mahosot Hospital, Vientiane, Laos. Lao government officials were consulted from the outset to inform the research design through informal discussions and a more structured and inclusive workshop to inform the design and implementation process. Officials from the Ministry of Health and the Ministry of Agriculture and Forestry, including national and provincial government officials were consulted on research and policy priorities and the data gaps that were unresolved. In addition to the Lao government officials, human and animal health professionals from a range of international organisations were consulted on the research priorities and design.

Chapter 1 outlined three broad objectives that would contribute to our understanding of the ecology and epidemiology of neglected zoonoses in Laos and provide insights into how they might be better controlled. These objectives were:

1. To determine the prevalence and risk of zoonotic infectious diseases in the human population of northern Laos;
2. To determine the prevalence and risk of zoonotic infectious diseases in the pig and dog populations of northern Laos;

3. To characterise host-pathogen interactions in a complex host assemblage that includes humans, pigs and dogs.

The following discussion draws on the results of the preceding chapters, bringing together the three overarching objectives to provide a linkage between the various infectious diseases studied and how they collectively impact on the health and well being of the populations studied. In so doing, a framework for disease control prioritisation will be discussed in the context of infectious disease ecology and human and animal health policy advocacy. Finally, recommendations on potential disease control options will be presented together with recommendations for future research to better inform policy decisions to reduce the risk of exposure to zoonotic infectious diseases.

7.2 Limitations of conducting research in a resource-poor developing country

A number of limitations have been outlined in the discussion sections of previous chapters, these were primarily related to the interpretation of the data and were not specifically related to the complexity and difficulty of conducting research in remote locations in a resource poor setting. These limitations will be discussed in more detail here, together with more general factors that added to the complexity of designing and conducting infectious diseases surveys in northern Laos.

7.2.1 Ethnic diversity and language

Laos is an ethnically diverse country, with a large proportion of this diversity centred in the northern provinces where this research was conducted. This ethnic diversity presented a number of important challenges. First and foremost was the issue of language. In many of the villages in which surveys were conducted people spoke Lao as a second language even though Lao is the official language and is taught in schools. Research design and questionnaire development was initially conducted in English and survey questionnaires were then translated into Lao, in many instances in non-Lao Tai villages, questions were then verbally translated into a third language, either Hmong or Khmu. In all circumstances no professional translation services were used, rather Lao counterparts from the Department of Livestock and Fisheries and the Ministry of Health administered the questionnaire and an inhabitant of the village, usually the village chief,
provided translation. As a consequence, the questionnaires were non-technical in nature and the scope of the questions that could be asked was limited. Even with the non-technical nature of questioning, there remains the possibility that miscommunication had a detrimental influence on responses and the reproducibility of some surveys remains uncertain.

Secondly, there was significant mistrust of the survey team from many Hmong people who were asked to participate in the survey. This has been discussed in some length in previous chapters and may have had a negative impact on the representativeness of the survey population.

7.2.2 Village access

Northern Laos is a mountainous region with poor road infrastructure linking villages with district and provincial capitals, the problem is further compounded in the wet season months from June to September when access worsens due to roads being cut-off or made impassable due to the wet conditions. The issue of village access was a limiting factor in survey design; only villages that could be accessed by four-wheel drive vehicle in the dry season were included in this study. The impact that these exclusions had on the survey results cannot be estimated, other than to say that remoteness and poor access to district and provincial capitals tends to be associated with increased poverty. By extension, we could expect that pathogens that were highly prevalent in the poorest households, such as STHs, would also be highly prevalent in villages excluded due to remoteness. The omission of inaccessible remote villages would then likely have resulted in a reduction in overall prevalence for pathogens associated with poverty, and the reverse would likely be true for pathogens, such as trichinellosis, that were more prevalent in the least poor households.

7.2.3 Diagnostic capacity, human resource constraints and outsourcing diagnostic testing

Laos is a least developed country with limited technical resources. There are a very small number of trained veterinarians and para-veterinarians with variable competencies spread across national and provincial governments in sectors including policy development, disease investigation, control and prevention, diagnostics, international relations and research (OIE, 2011) to name a few. Likewise, a critical shortage of health workers and poor standards of basic training severely constrains the health system (WHO, 2012) and there are a limited number of technically trained laboratory staff and infectious diseases researchers.
The National Animal Health Centre (NAHC) and the National Centre for Laboratory and Epidemiology (NCLE) have staff trained in ELISA methodology. With the support of the Istituto Superiore di Sanita, Rome, Italy, the ES ELISA protocol for detecting antibodies to trichinellosis in human serum samples was transferred to the NCLE. With the support of the Institute of Tropical Medicine, Antwerp, Belgium, the Ag ELISA protocol for detecting cysticercosis antigen in human and pig serum was transferred to both laboratories. There was however, insufficient capacity to undertake testing for JEV, HEV, NiV and SIV in the pig samples collected. Quality of results was a very high priority and the pig samples were tested at AFRIMS (JEV and HEV) and the AAHL CSIRO (NiV and SIV).

The artificial digestion method for the detection of *Trichinella* larvae was suitable for technology transfer to the parasitology laboratory within the NAHC. Artificial digestion does not require maintenance of sophisticated equipment and was not expensive to sustain, furthermore the test method was relatively robust and has been shown to be reliable if conducted under an appropriate quality control system (Rossi and Pozio, 2008). The artificial digestion method using a magnetic stirrer was conducted in the NAHC laboratory under a quality system (Gajadhar et al., 2009) with the exception of proficiency testing to ensure the test was performing as intended. A 5g tissue sample allows for the detection of ≥1 lpg (Gamble, 1996; Gajadhar et al., 2009) and a 10g tissue sample <1 lpg. Without method validation and proficiency testing using meat samples spiked with known numbers of larvae, we cannot be absolutely certain the test was performing as intended and that the expected detection limit was achieved. In stating this limitation however, we can have a high degree of confidence the artificial digestion results are accurate since 15 of the 16 quality assurance guidelines recommended by Gajadher et al (2009) were implemented. Future work in the NAHC laboratory using the artificial muscle digestion method should take this extra critical step in quality assurance and perform proficiency testing and method validation. The Istituto Superiore di Sanita, Rome, Italy, performed molecular testing to identify the *Trichinella* species detected by artificial muscle digestion.

The PCR protocol for differentiating the three *Taenia* species circulating in Southeast Asia was established in the NAHC laboratory with special permission from the Directors General of the Department of Livestock and Fisheries and the Department of Hygiene and Prevention since the samples originated from human subjects rather than animals. This permission was granted primarily as a result of the author’s good standing with both departments and contingent on the author performing the test. As a consequence, it is unlikely that this protocol will be used in future studies conducted at the NAHC laboratory.
As a result of the logistical complexity of coordinating sample collection and diagnostic services, particular attention was placed on sample control and labelling, using multiple identifiers including sample number, date, origin and age marked on tube labels and record sheets, to ensure with a high degree of confidence that samples were not mislabelled and mishandled. As described in previous chapters, only a small number of samples were excluded from the final analysis due to mislabelling and poor sample quality. This small number of omissions reflects positively on the controls put in place to dispatch multiple sample types to multiple locations for multiple diagnostic tests.

7.3 The neglected tropical zoonoses and poverty

Laos is a least developed country with large proportions of the population living in poverty; indeed the classifications of wealth status used in this thesis - least poor to most poor - reflects this unfortunate reality. The neglected tropical diseases contribute to the cycle of poverty but they are also, in many instances, the direct result of poverty (Hotez et al., 2007). The high prevalence of zoonotic and neglected tropical diseases identified in humans, pigs and dogs in northern Laos are indicative of poverty and the environmental and social conditions suitable for the transmission of pathogens between and within host populations.

This thesis provides sufficient evidence to suggest that Lao people, in general, are exposed to a wide range of NTDs. Although there were spatial differences in prevalence and risk observed for the diseases investigated, the human and animal populations in Oudomxay province experienced the highest prevalence of all the diseases investigated. The Mon-Khmer people of Oudomxay province experienced the highest prevalence of cysticercosis and evidence indicates that three of the six villages in Oudomxay province may be ‘hot-spots’ of *T. solium* transmission (Chapter 2). The Mon-Khmer people of Oudomxay province were also more likely to be infected with an STH and eggs of *A. ceylanicum* and *N. americanus* were recovered from people and dogs (Chapter 3). Further still, the highest prevalence of *Trichinella* seropositivity was observed in Oudomxay province, however this was predominantly restricted to the less poor Lao-Tai people (Chapter 4). This greater prevalence in the human population of Oudomxay province could, however, simply be due to a large and sustained outbreak in the province in 2005 (Barennes et al., 2008).

The highest prevalence of JEV HI and IgM seropositivity was observed in the pig population of Oudomxay province (Chapter 5), as was the highest prevalence of HEV isolation from the faeces of young pigs less than 6 months old. Furthermore, the greatest genetic diversity of HEV isolates was observed in Oudomxay province (Chapter 6).
What could be driving this apparent concentration of NTD transmission in Oudomxay province; poverty, cultural practices, education? The answer is likely to include a great many factors, but as was outlined in the results section of Chapter 3, poverty, open defecation and illiteracy were all concentrated in Oudomxay province, and predominantly in the Mon-Khmer population. The make up of the survey population of Luangprabang province also included a large proportion of Mon-Khmer people, the major difference being 23% of the survey population in Luangprabang province were classified as very poor or most poor, compared to almost 70% of the survey population in Oudomxay province falling into these two wealth quintiles. Likewise, the proportion of female heads of household who were illiterate in Luangprabang province was 39% compared with 63% in Oudomxay province (Chapter 3). Women in Lao predominantly are tasked with the roles of primary care giver and food preparation, so it seems that poverty and poor education standards, especially in women, could be contributing to a greater risk of acquiring an NTD.

The conditions under which people live and interact with domestic animals and livestock in Oudomxay province places them at great risk of acquiring a zoonotic disease in their lifetime. The results also indicate that substantial risk of infection exists in other population groups, however, the most poor and uneducated members of the population are highly vulnerable. It is these people, disproportionately made up of Mon-Khmer people, who are in greatest need of interventions to control NTDs, and we, as a public health community, need to be innovative about how we go about addressing this challenge.

In an ethnically diverse and resource poor country such as Laos, where the most vulnerable populations are disenfranchised from the political and policy setting process, the immediate challenge will be framing health policy to benefit these people. Surely the first priority must be to clear STH infections in affected villages so that general health and wellbeing can begin to be addressed. Or at least reduce parasite burdens below a threshold that causes ill health. Chronic ill health caused by the suite of STHs observed in northern Laos has detrimental impacts on cognitive function, productivity and educational performance. The chronic disease state may not be recognised by the individual afflicted or by the health profession more generally and in such a situation affected individuals will be continuously living and functioning in a sub-optimal state. To affect positive change for other infectious diseases and bring people out of poverty, the underlying burden of STH infections must be addressed as a priority.

A combination of good policy, making available the necessary resources, having the right tools or biomedical solutions and understanding the social determinants of health and education will all
be important to address NTD control in Laos. Future research needs and potential disease control options are discussed below.

7.4 Future research to inform decision making processes

7.4.1 Controlled in vivo studies of the competitive immunological interactions between T. hydatigena and T. solium metacestodes in the pig intermediate host

The results presented in Chapter 2 on T. solium ecology identified a potential novel interaction between two related Taenia species in the pig intermediate host. The survey design and the diagnostic tests used were not suitable for characterising the immunological interactions between T. solium and T. hydatigena in pigs and the study could not definitively demonstrate that a strong infection pressure of T. hydatigena from dogs suppresses T. solium transmission in Laos. In this study, the antigen capture ELISA could not discriminate between the three different Taenia species infecting pigs in Southeast Asia and at present no antibody ELISA for pigs has been fully validated in Southeast Asia where three Taenia species are circulating in the pig population. Meaning that, using the antigen capture ELISA, we could not discern if pig’s infected with T. hydatigena were co-infected with T. solium in the absence of visual detection, and we had no tools to determine if pigs had been exposed to T. solium if they had a current T. hydatigena infestation, or vice versa. In this context, well-designed and controlled pen trials to measure the competitive interactions in the pig intermediate host, serologically and by cyst enumeration, would be very valuable.

Evidence from controlled pen trials of T. solium infection in 1, 3 and 5 month old pigs suggests that pigs develop innate resistance to the establishment of viable cysticerci (Deckers et al., 2008) and this may have an impact on the investigation of competitive interference and could lead to a false interpretation of results if older animals (>1 month old) were to be used. The value of understanding the competitive interactions, if they exist, lies in the knowledge that maintaining the infection pressure from dogs would in effect provide a natural vaccination against T. solium in pigs. The infection pressure exerted by dogs could then potentially be manipulated to greater effect. Perhaps most importantly however, T. hydatigena infection from dogs would need to be taken into account when controlling other zoonotic helminths, such as A. ceylanicum, so as not to inadvertently reduce the T. hydatigena infection pressure on pigs (Thompson and Conlan, 2011).
7.4.2 Develop a model to identify high risk areas/regions/villages for the transmission and maintenance of T. solium and use structured surveillance employing the best available discriminatory diagnostics to validate the model

Like other countries in the Southeast Asian region, T. solium taeniasis and cysticercosis tends to have a focal distribution with ‘hot-spots’ of transmission between humans and pigs. These ‘hot-spots’ exist where the suite of factors needed for transmission to occur; open defecation, free-ranging pig production and associated coprophagia, consumption of undercooked or raw pork, poor sanitation and hygiene and generally low levels of education. At the population level, a low prevalence of human cysticercosis was found but the majority of cases were detected in a small number of villages. At the population level the burden of disease may not be high enough to garner political support for comprehensive disease control initiatives, both nationally and internationally, but in affected villages, the burden of disease is unquestionably high. A critical component of a future T. solium control program will be the ability to reach, with a high degree of certainty, these high-risk groups or ‘hot-spots’ for targeted treatment of carriers and pig management interventions.

A well-developed and validated model using existing data, where available, to identify villages or regions that are likely to harbour active transmission would be a valuable tool. Existing data would include such things as ethnicity, pig husbandry, poverty maps and sanitation maps. A second level of data used to refine and further pin point hot-spots would involve rapid surveys, using minimal resources, asking about meat consumption habits, unexplained epilepsy cases, pig cysticercosis and general hygiene measures. To validate the model, intensive and more invasive surveys would be needed to collect blood and faecal samples to test for human cysticercosis and T. solium taeniasis, the latter using diagnostic tests capable of discriminating between the three sympatric Taenia species circulating in the human population in Southeast Asia. The value of such a model would lie in maximising the efforts of a limited human resource and ensuring the initial identification of target areas is cost effective. Large-scale surveys of villages across geographically diverse areas are very expensive and would not receive support from the national government or international donors.

7.4.3 Controlled clinical trials of niclosamide and praziquantel for the treatment-to-cure of taeniasis

Praziquantel and niclosamide are cost effective, safe and efficacious for the treatment of taeniasis. Both drugs are listed on the WHO list of essential drugs (WHO, 2011), however the efficacy of
both drugs in treating taeniasis in Laos and the Southeast Asian region in general has not been tested. Treatment failures have been demonstrated elsewhere (Vermund et al., 1986; Koul et al., 1999; Lateef et al., 2008) and failure to clear taeniasis infections using niclosamide and magnesium sulphate have been reported (Sanchez et al., 1997). The use of pre- and post-treatment purging with electrolyte-polyethyleneglycol salt improves the rate of cure (Jeri et al., 2004) and studies are warranted in Laos to determine the most appropriate treatment regime to clear infections. The value of clinical trials to determine efficacy lies in maximising the public health benefit of investing resources to detect and treat Taenia carriers.

7.4.4 Determine the role of dogs in the ecology of N. americanus in northern Laos and determine if, under a high infection pressure, they act as a competent reservoir host

Necator americanus was detected in 22.2% of the dog faecal samples (4/18) from which valid PCR results were obtained and the respective hookworm species were identified. The four dogs originated in two villages in Luangprabang province and two villages in Oudomxay province. In three of these villages open defecation amongst the human population was extensive. The results presented in this thesis were unable to determine if the eggs were present in dog faecal samples due to coprophagia and passage through the dogs gastrointestinal system or if a patent infection established after L3 stage larval penetration of skin surfaces and eggs were shed from mature reproductive adults. Limited data exists supporting the potential for patent N. americanus infection in dogs, however experimental studies have indicated that patent infection can establish in young dogs (<3 months old) exposed to 1000 encysted larvae (Yoshida et al., 1960). Under a strong infection pressure from a human population where N. americanus is highly prevalent, there remains the potential for patent infection in dogs, and dogs acting as an unrecognised reservoir of human infection. Very simple studies could be designed to detect N. americanus egg positive dogs first by microscopy in affected villages and then follow up with treatment of dogs to expel adult worms. Morphological characteristics of any expelled adult worms together with PCR confirmation and larval development of eggs would demonstrate fecundity or lack thereof. In the absence of patent N. americanus infections in dogs, the role that they play in distributing eggs to a wider geographical range in a village should also be investigated. If dogs play a role in the ecology of N. americanus in Laos, then they will need to be taken into consideration when controlling human infection through mass drug administration and improving access to latrines.
7.4.5 Social research assessing barriers to the uptake of drugs to treat soil transmitted helminths to complement clinical trials of drug efficacy and treatment regimes

One of the most important findings of this research thesis was the very high prevalence of STH infections in the Mon-Khmer populations of northern Laos, particularly in children who had supposedly just been exposed to mebendazole treatment through a mass drug administration program. These research findings are consistent with those observed in Jamaica (Nokes and Bundy, 1993) where the most socioeconomically disadvantaged children were least likely to comply with treatment. There are undoubtedly complex socio-cultural factors that influence access to health services and compliance with treatment in northern Laos; it will be critically important for these to be fully understood to ensure that any control measures are effective and appropriate. The value of understanding the socio-cultural barriers to uptake of health interventions will be in improving the chances of achieving the stated objectives of a disease control program.

7.4.6 Molecular characterisation of human HEV isolates to determine the source of infection

The evidence presented in Chapter 6 demonstrates that HEV genotype 4 was circulating in the pig population of northern Laos and several isolates were genetically related to human and pig genotype 4 viruses isolated in China. The supporting evidence presented in Chapter 5 indicates that pigs are an important reservoir of HEV with the potential for transmission to humans through contaminated water, handling of animals or through the consumption of food. However, this evidence only tells part of the story. Future research will be needed to determine the genotypes circulating in the human population of northern Laos, the severity and burden of disease and the source of infection, particularly for highly vulnerable populations such as pregnant women. To achieve the latter, the HEV sequence data presented in Chapter 6 may be used to determine if pigs are the source of human infections.

7.4.7 Investigation of JEV incidence in young pigs and in the dry season

The serological survey of viral zoonoses in the pig slaughter population of northern Laos provided important results to inform the epidemiology of these viruses, however, the scope of the survey was limited in its geographical and temporal range and the age of the animals surveyed. Irrigation of rice paddy fields may enable transmission of JEV in the dry season when breeding grounds for mosquitoes would otherwise be dry (WHO, 2006). Unfortunately, as a
consequence of the study limitations, the results were unable to provide insights into the impact of irrigated rice cultivation on JEV transmission in the dry season and the age group of pigs most likely to actively transmit virus. Further studies should address this shortcoming and design surveys to include younger animals and expand the geographical range to southern Laos and conduct sample collections across the wet and dry seasons.

7.5 Options for the sustainable control of neglected tropical zoonoses

7.5.1 Taeniasis and cysticercosis

The control of taeniasis and cysticercosis will require a two-pronged approach that targets the definitive host to reduce and eventually eliminate environmental contamination, implemented concurrently with improvements in pig management and sanitation to prevent transmission to the intermediate host. At present there is little or no incentive for meat traders to apply rigid controls on pork and beef supply since many traders are heavily reliant on the sale of all edible portions of the carcass to make a profit and the government’s official meat inspection program lacks sufficient resources, expertise and funding to implement. But perhaps most importantly, meat inspection prior to sale lacks sensitivity even with the best-equipped and trained inspectors. Post-slaughter treatment of carcasses, such as freezing, also lacks applicability in Laos since meat supply is almost exclusively slaughter-to-wet market and there are no facilities for holding and treating suspect or contaminated carcasses. Control efforts must be concentrated in villages where human taeniasis cases are known or likely to reside and pigs are raised in such a way that exposes them to human excrement. Vaccination of pigs could potentially be a part of the mix, however there should not be an expectation that pig farmers would voluntarily vaccinate when vaccination rates are extremely low for epidemic high mortality diseases such as classical swine fever virus. Identification of target villages could be achieved through the development of a validated risk-profiling model based on data described in section 7.4.2 above.

7.5.2 Trichinellosis

The best way to prevent human trichinellosis is to cook meat to a safe temperature. An internal temperature of 71°C maintained for at least one minute is required to inactivate infective larvae (Gottstein et al., 2009). Other methods of inactivating infective larvae, including irradiation and freezing, are not appropriate to the Lao situation. Only 16% of individuals surveyed indicated they had a refrigerator in their home and there is no capacity to irradiate food prior to sale. In an environment such as Laos where people have a preference for consuming uncooked or partially
cooked meat from domestic or wild pigs, the control and prevention of trichinellosis will be problematic. An education program raising awareness of transmission mode and impacts of disease may have a moderate impact, but without a major cultural shift away from consuming raw meat, there will continue to be sporadic cases and outbreaks of trichinellosis in Laos.

The principal reason for this pessimistic outlook is the nature of pig production in Laos. Pig production is firmly entrenched in the smallholder sector (Stür et al., 2002; Conlan et al., 2011), confinement of pigs in pens from birth to sale is rare, there are limited controls on feeding inputs and pigs are raised in such a way that they have access to rodents and wildlife. It is this category of pig that presents the highest risk of *Trichinella* infection (Gottstein et al., 2009). Until such a time that pig production is predominantly restricted to well-managed facilities or farms with systems in place to restrict rodent access and feed inputs are controlled, pigs will continue to act a major source for human trichinellosis (Conlan et al., 2011).

### 7.5.3 The soil transmitted helminths

There has been important research conducted recently to assess anthelmintic drug efficacy aimed at achieving the best clinical outcome for the soil-transmitted helminths (Ndyomugenyi et al., 2008; Steinmann et al., 2008; Kirwan et al., 2009; Knopp et al., 2010; Steinmann et al., 2011) and improvements in sanitation have a clear impact on reducing the risk of transmission to humans (Ziegelbauer et al., 2012). There is, however, an under-representation of health sociology or other social science disciplines in the field of NTDs research with the focus primarily on biomedical interventions (Allotey et al., 2010; Pokhrel et al., 2011; Reidpath et al., 2011). Disease control programs must have a broad focus taking into account the local social and cultural factors that influence the uptake of biomedical solutions and shape the way marginalised communities interface with the broader health system (Parker et al., 2008). Having good treatment options delivered concurrently with improvements to sanitation and access to clean water will not necessarily be sufficient to achieve disease control if target populations remain marginalised and they do not have ownership of actions taken to reduce their disease burden. This latter point will be critical to the success or failure of any control program; since the soil-transmitted helminths typically cause chronic disease that may not be clinically apparent, recognition of a disease state at an individual or community level may be difficult to communicate. An integrated education program to raise awareness of parasite transmission, health impacts and treatment strategies will need to be a part of a comprehensive control program.
7.5.4 Viral zoonoses

The very high prevalence of JEV in the pig population of northern Laos was unquestionably the most significant finding of the serological survey of viral zoonoses (Chapter 5) and the control of the virus in pigs will be extremely difficult, if not impossible, to achieve. Vector control is not practiced in Laos and the close proximity of water birds, pigs, human habitation and rice cultivation means the only reasonable approach will be vaccination of the human population. The WHO recommends vaccination as the single most effective control measure (WHO, 2006) and this approach in conjunction with continued surveillance to monitor the evolving epidemiology of JEV and identify areas of peak incidence will reduce the disease burden of Lao people. The case for interventions to control HEV in the human and pig populations of Laos is yet to be made, however options for control include improvements in sanitation and access to clean drinking water free from human and livestock faecal contamination. An integrated program to control STHs through improvements to sanitation and drinking water quality would, in all likelihood, have beneficial impacts on reducing the burden of enteric viruses such as HEV. For high-risk populations, such as pregnant women where fulminant hepatitis may manifest, safe and effective vaccines are now available and may be an essential intervention to prevent maternal deaths in high-risk areas (Labrique et al., 2012).

7.6 Concluding comments

This body of work presents new data on a wide range of neglected tropical diseases, ranging from parasitic infections associated with poverty and poor sanitation through to zoonotic viruses. The zoonotic and neglected tropical diseases circulating in Laos are a major burden on public health and wellbeing. The magnitude and scope of this burden varies for each of the diseases investigated. With the new data presented in this thesis, policy makers and professionals working in the areas of public health, tropical infectious diseases and veterinary medicine are better informed to take a measured approach to formulating and implementing disease control initiatives. Ideally, these measures will be primarily aimed at improving the livelihoods of highly vulnerable populations in northern Laos, which will have flow-on effects to curbing disease transmission to those less vulnerable.

7.7 References


WHO, 2012. Health Service Delivery Profile - Lao PDR. [http://www.wpro.who.int/health_services/service_delivery_profile_laopdr.pdf](http://www.wpro.who.int/health_services/service_delivery_profile_laopdr.pdf) [accessed 3 April 2013].


Appendix 1: Consent form

Detailed information was provided in the Lao language and was read by or read to participants prior to consenting to survey participation. The parents or legal guardians were asked to provide consent on behalf of minors (children <15 years old). The consent form is provided in this appendix.
### Consent Form

1. By signing this form, I agree voluntarily to take part in this study.
2. I have read or heard someone read the Information Sheet provided and I have been given a full explanation of the purpose of this study, of the procedures involved and of what is expected of me. The researcher has answered all my questions and has explained the possible problems that may arise as a result of my participation in this study.
3. I understand we are free to withdraw from the study at any time without needing to give any reason.
4. I understand we will not be identified in any publication arising out of this study.
5. I understand that our names and identities will be stored separately from the data, and these are accessible only to the investigators. All data will be analysed anonymously using code numbers.
6. I understand that all information provided by me and my family is treated as confidential and will not be released by the researcher to a third party unless required to do so by law.
7. I give my consent to have photographs taken of me and my family and I understand that these images will not be identified by name or location and will not be shared with a third party.

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Signature of Investigator (ลายมือชื่อ): __________________________ Date (วันที่): __________

Print name (ชื่อ): ___________________________________
Appendix 2: Risk factor questionnaire for human survey

The survey questionnaire was pre-tested in two villages in Phon Hong district in Vientiane province prior to commencing the survey to amend any questions that were not clear or translated incorrectly. The pre-trial also served as an opportunity to provide the survey team with experience in asking questions and recording data.
**Household Information:**

**Household Identification (Unique household ID #):**

**Village (Village name):**

**Province (Province):**

**Date (Date):**

1. **Household GPS coordinates of household (Front door):**
   - R1:
   - R2:
   - R3:
   - R4:
   - R5: Average reading

2. **If pigs are penned, GPS coordinates of pig pen, household socioeconomic status (Can GPS):**
   - R1:
   - R2:
   - R3:
   - R4:
   - R5: Average reading

3. **Household socioeconomic status:**

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<tr>
<th>Item Description</th>
<th>Yes</th>
<th>No</th>
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<td>House has a refrigerator</td>
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<td>House has a television</td>
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<td>House has a radio</td>
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<td>House has at least one mobile telephone</td>
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<td>House is made of concrete or brick</td>
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<td>House has concrete or tiled flooring</td>
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<td>House has a dirt floor</td>
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<td>House is made of wood or timber</td>
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<td>House has tiled roofing</td>
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<td>House has grass roofing</td>
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<td>House has a motorbike</td>
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<td>House has at least one bicycle</td>
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<td>House owns a small tractor</td>
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<td>House has running water inside</td>
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ACIAR AH2006/161 PIG ZOONOSES PROJECT: Risk Factor & Spatial Epidemiology Questionnaire
3.2 សារបញ្ជាក់ពាណិជ្ជការ (Education)
ក្រុមប្រឹក្សាសិស្សក្រុម (Male head of household):  
អាយុ (កាលបរិច្ឆេទ):  
សារបញ្ជាក់ពាណិជ្ជការ (Level attained):  

ក្រុមប្រឹក្សាសិស្សក្រុម (Female head of household):  
អាយុ (កាលបរិច្ឆេទ):  
សារបញ្ជាក់ពាណិជ្ជការ (Level attained):  

ក្រុមប្រឹក្សាសិស្សក្រុម (Primary care giver):  
អាយុ (កាលបរិច្ឆេទ):  
សារបញ្ជាក់ពាណិជ្ជការ (Level attained):  

ក្រុមប្រឹក្សាសិស្សក្រុម (Main person responsible for food preparation):  
អាយុ (កាលបរិច្ឆេទ):  
សារបញ្ជាក់ពាណិជ្ជការ (Level attained):  

4. សារបញ្ជាក់ពាណិជ្ជការក្នុងក្រុម (Household members):

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ACTAR AH2006/161 PIG ZOONOSES PROJECT: Risk Factor & Spatial Epidemiology Questionnaire
5. tragterung und Tragmenge (Pig production & slaughter)

5.1. tragterungsgewichte 30 kg (Pigs >30 kg) ☐ (yes), brood (Brood): ☐

5.2. tragterungsgewichte 15 kg 30 kg (Pigs 15 to 30 kg) ☐ (yes), brood (Breed): ☐

5.3. tragterungsgewichte 15 kg (Pigs <15 kg) ☐ (yes), brood (Brood): ☐

5.4. tragterungsgewichte 30 kg (Pigs >30 kg)

☐ permanent (Always freerange)
☐ temporarily in enclosures (Always confined in pen)
☐ temporarily outside (Freerange sometimes)
☐ tethered (Tethered)

5.5. tragterungsgewichte 15 kg 30 kg (Pigs 15 to 30 kg)

☐ permanent (Always freerange)
☐ temporarily in enclosures (Always confined in pen)
☐ temporarily outside (Freerange sometimes)
☐ tethered (Tethered)

5.6. tragterungsgewichte 15 kg (Pigs <15 kg)

☐ permanent (Always freerange)
☐ temporarily in enclosures (Always confined in pen)
☐ temporarily outside (Freerange sometimes)
☐ tethered (Tethered)

5.7. Volume of home-slaughtered pork consumed in household?

☐ This month ☐ kg
☐ Last month ☐ kg
☐ Two months ago ☐ kg

5.8. Volume of market purchased pork consumed in household?

☐ This month ☐ kg
☐ Last month ☐ kg
☐ Two months ago ☐ kg

ACIAR AH2006/161 PIG ZOONOSSES PROJECT: Risk Factor & Spatial Epidemiology Questionnaire
6.  អារម្មណ៍ខ្លែមិនអាចរំលាយបាន៖ (Other livestock and animals associated with household):
   6.1  គ្រុស (Number of cattle): __________ (ទំនិញ)
   6.2  គ្រុសប្រៃមាន (Number of buffalo): __________ (ទំនិញ)
   6.3  គ្រុសកៃហ្វូ (Number of dogs): __________ (ទំនិញ)
   6.4  គ្រុសវែង (Number of cats): __________ (ទំនិញ)
   6.5  គ្រុសស្រុកសាច់ (Number of poultry): __________ (ទំនិញ)

7.  ការទិញទិញទូរស័ព្ទ (Water supply & use)   

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.1</td>
<td>គ្រុសរាប់ដូងដោយការទិញទូរស័ព្ទរបស់អង្គ (Where does your water supply come from)?</td>
<td></td>
</tr>
<tr>
<td>7.1a</td>
<td>Running tap water in house</td>
<td>☐ 1</td>
</tr>
<tr>
<td>7.1b</td>
<td>គ្រុសរាប់ដូងដោយការក្លាយទូរស័ព្ទរបស់អង្គ (Where does your water supply come from)?</td>
<td></td>
</tr>
<tr>
<td>7.1c</td>
<td>Piped ground water</td>
<td>☐ 1</td>
</tr>
<tr>
<td>7.1d</td>
<td>Direct from river or stream</td>
<td>☐ 1</td>
</tr>
<tr>
<td>7.1e</td>
<td>Gravity fed mountain spring water</td>
<td>☐ 1</td>
</tr>
<tr>
<td>7.2</td>
<td>គ្រុសរាប់ដូងដោយការក្លាយទូរស័ព្ទរបស់អង្គ (Where does your water supply come from)?</td>
<td></td>
</tr>
<tr>
<td>7.3</td>
<td>គ្រុសរាប់ដូងដោយការក្លាយទូរស័ព្ទរបស់អង្គ (Where does your water supply come from)?</td>
<td></td>
</tr>
</tbody>
</table>

8.  ការសម្រមៗនិងសុវត្ថិភាព (Sanitation & hygiene)   

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.1</td>
<td>ការសម្រមៗតម្រូវការបន្ថយបន្ថយទូរស័ព្ទ (Household has a flushable latrine with septic tank)?</td>
<td>☐ 1</td>
</tr>
<tr>
<td>8.2a</td>
<td>ការសម្រមៗតម្រូវការបន្ថយបន្ថយទូរស័ព្ទ (Household has a flushable latrine with no septic tank)?</td>
<td>☐ 1</td>
</tr>
<tr>
<td>8.2b</td>
<td>Household with flushable latrine</td>
<td>☐ 1</td>
</tr>
<tr>
<td>8.2c</td>
<td>Household with flushable latrine</td>
<td>☐ 1</td>
</tr>
<tr>
<td>8.2d</td>
<td>Household with flushable latrine</td>
<td>☐ 1</td>
</tr>
<tr>
<td>8.3</td>
<td>ការសម្រមៗតម្រូវការបន្ថយបន្ថយទូរស័ព្ទ (Household has a nonflushable latrine)</td>
<td>☐ 1</td>
</tr>
<tr>
<td>8.4</td>
<td>ការសម្រមៗតម្រូវការបន្ថយបន្ថយទូរស័ព្ទ (Household has no latrine)</td>
<td>☐ 1</td>
</tr>
</tbody>
</table>

ACIAR AH2006/161 PIG ZOOHOSES PROJECT: Risk Factor & Spatial Epidemiology Questionnaire
8.5 Is human excrement ever used as a fertilizer for vegetables?  

8.6 Select the method of garbage disposal that best describes the situation in your household. 

8.6a All household waste is collected and taken to a garbage dump  

8.6b All household waste is burnt  

8.6c All household waste is buried in a pit  

8.6d None of the above 

8.7 Are pigs fed food scraps and waste from the household?  

8.8 Do dogs, cats or vermin scavenge household waste?  

9. Access to veterinary and human health services: 

9.1 How often do you receive veterinary services from DAFO or PAFO? For example, vaccination of livestock, treatment of sick animals, advice on pen construction etc. 

9.2 How often do you receive veterinary services from private sector specialists? For example, vaccination of livestock, treatment of sick animals, advice on pen construction etc.
| 9.3. How often do you use traditional or local medicine for animal health? (How often do you use traditional or local medicine for animal health?) |
|---|---|
| □ Never | □ More than 6 times a year (less than once a year) |
| □ Once a year | □ 6 times a year (about once every 6 months) |
| □ Twice a year | □ 3 times a year (about once every 3 months) |
| □ More than 3 times a year | □ I don't know |

| 9.4. How often do you receive animal health services from a project? |
|---|---|
| □ Never | □ More than 6 times a year (less than once a year) |
| □ Once a year | □ 6 times a year (about once every 6 months) |
| □ Twice a year | □ 3 times a year (about once every 3 months) |
| □ More than 3 times a year | □ I don't know |

| 9.5. How often do you receive human health services from the district or provincial health department? (How often do you receive human health services from the district or provincial health department? For example, for vaccination, treatment of illness, public health advice etc.) |
|---|---|
| □ Never | □ More than 6 times a year (less than once a year) |
| □ Once a year | □ 6 times a year (about once every 6 months) |
| □ Twice a year | □ 3 times a year (about once every 3 months) |
| □ More than 3 times a year | □ I don't know |

| 9.6. How often do you receive human health services from private sector specialists (private clinic, pharmacist, doctor, hospital)? (How often do you receive human health services from private sector specialists (private clinic, pharmacist, doctor, hospital)? For example, for vaccination, treatment of illness, public health advice etc.) |
|---|---|
| □ Never | □ More than 6 times a year (less than once a year) |
| □ Once a year | □ 6 times a year (about once every 6 months) |
| □ Twice a year | □ 3 times a year (about once every 3 months) |
| □ More than 3 times a year | □ I don't know |
9.7 คุณใช้ยาแผนโบราณหรือยาแผนปัจจุบันบ้าง? (How often do you use traditional or local medicine for human health?)

☐ ถ้าไม่เคย (Never)  ☐ เรียบร้อยครั้งละ 1 ครั้งต่อปี (less than one time per year)
☐ ประมาณปีละ 1 ครั้ง (about once per year)  ☐ ประมาณปีละ 6 ครั้งต่อปี (about once every 6 months)
☐ ประมาณปีละ 3 ครั้งต่อปี (about once every 3 months)  ☐ ประมาณปีละ 1 ครั้งต่อเดือน (about once per month)
☐ อย่ารู้ (don’t know)

9.8 คุณได้รับบริการทางการแพทย์จากโครงการหรือไม่? (How often do you receive human health services from a project?)

☐ ถ้าไม่เคย (Never)  ☐ เรียบร้อยครั้งละ 1 ครั้งต่อปี (less than one time per year)
☐ ประมาณปีละ 1 ครั้ง (about once per year)  ☐ ประมาณปีละ 6 ครั้งต่อปี (about once every 6 months)
☐ ประมาณปีละ 3 ครั้งต่อปี (about once every 3 months)  ☐ ประมาณปีละ 1 ครั้งต่อเดือน (about once per month)
☐ อย่ารู้ (don’t know)

10. ความรู้และความเข้าใจ (Knowledge & understanding)

ตอบให้ตรงที่มี ตอบละเอียดในระดับที่ไร้ประโยชน์

(Answer the following question to the best of your knowledge) ______________________ Yes 1 No 2

10.1 คุณคิดว่าโรคที่มาจากสัตว์ป่วยจากหมูได้ไหม? (In your opinion, can people contract diseases from pigs)? ______________________ 1 2

10.2 คุณเคยเห็นกลุ่มต่างๆในกลูมหนูไหม? (Have you ever seen white cysts in the meat of pigs)? ______________________ 1 2

10.3 คุณเคยเห็นสัตว์ต่างๆมีกลูม และ ยังรู้ไหมแม้คุณไม่ได้เห็น?

Do you know why some pigs have cysts and others do not? ______________________ 1 2

หากคุณตี ต้องบอกย่อเกี่ยวกับความเข้าใจ (If yes to Q10.3, explain in your own words).

10.4 คุณกินเนื้อหมูที่มีกลูมไหม? (Do you eat the meat of pigs containing cysts)? ______________________ 1 2

10.5 คุณขายเนื้อหมูที่มีกลูมไหม? (Do you sell the meat of pigs containing cysts)? ______________________ 1 2

10.6 คุณรู้ว่าสัตว์บางชนิดมีกลูมและสัตว์อื่นๆไม่มี? (Do you know why some people have tapeworms and others don’t)? ______________________ 1 2

หากคุณตี ต้องบอกย่อเกี่ยวกับความเข้าใจ (If yes to Q9.4, explain in your own words).

จึงขอรับมติ I (END OF PART I)
**PART 2: QUESTIONNAIRE FOR INDIVIDUALS**

**Unique household ID #: ________________________________**

**Household member name: _______________________________________________________________**

**Number 1-12 (Refer to Part 1, Q4): ___________________________**

**Age:** ___________________________

11. **Hygiene & sanitation practices**

11.1 Do you usually defecate? (Where do you usually defecate?)
   11.1a Latrine
   11.1b Forest
   11.1c Rice field
   11.1d River or stream
   11.1e Other

11.2 Do you always defecate in a latrine? (If no, where?)
   11.2a Forest
   11.2b Rice field
   11.2c River or stream
   11.2d Other

11.3 Do you wash your hands after defecating? (If yes to Q11.3, prompt to determine washing method)
   11.3a Water only
   11.3b Water & soap

11.4 Do you wash your hands before eating? (If yes to Q11.4, prompt to determine washing method)
   11.4a Water only
   11.4b Water & soap

**ACIAR AH2006/151 PIG ZOONOSES PROJECT: Risk Factor & Spatial Epidemiology Questionnaire**
### 12. Pork meat consumption

<table>
<thead>
<tr>
<th>Question</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Do you eat fermented pork sausage? (If yes, how often?)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 time per week</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>More than 1 time per week</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you eat uncooked pig meat (dry or fresh)?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 time per week</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>More than 1 time per week</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you eat partially cooked pig meat (dry or fresh)?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 time per week</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>More than 1 time per week</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you eat uncooked beef (cattle &amp; buffalo / dry or fresh)?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 time per week</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>More than 1 time per week</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you eat partially cooked beef (cattle &amp; buffalo / dry or fresh)?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 time per week</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>More than 1 time per week</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ACIAR AH2006/161 PIG ZOOONES PROJECT: Risk Factor & Spatial Epidemiology Questionnaire
13. ประวัติการรับประทานอาหารที่มีการเสียบ (History of taeniasis)

Yes ☐  No ☐

13.1 คุณเคยเห็นส่วนของตัว))(ในอุจจาระในอดีตหรือไม่?

(Have you seen tapeworm segments in your faeces in the past?) ☐ 1 ☐ 2

13.2 คุณเห็นส่วนของตัว)) ในอุจจาระในปัจจุบันหรือไม่?

(Do you currently see tapeworm segments in your faeces?) ☐ 1 ☐ 2

13.3 ถ้าคุณตอบไปในข้อ 13.2 ว่า 'ใช่', คุณได้รับการรักษาหรือไม่?

(If you answered yes to question Q13.2, did you seek treatment?) ☐ 1 ☐ 2

หากคุณได้รับการรักษา, คุณได้รับการรักษาอย่างไร? (If yes, what treatment was taken?)

_________________________________________________________________________________________

_________________________________________________________________________________________

_________________________________________________________________________________________

13.4 ถ้าคุณตอบไปในข้อ 13.2 ว่า 'ใช่', คุณได้รับการรักษาเองหรือไม่?

(If you answered yes to Q13.2, did you undertake selftreatment?) ☐ 1 ☐ 2

หากคุณได้รับการรักษาเอง, คุณได้รับการรักษาอย่างไร? (If yes, what treatment was taken?)

_________________________________________________________________________________________

_________________________________________________________________________________________

_________________________________________________________________________________________

หากคุณได้รับการรักษาเอง, คุณได้รับการรักษาเองจากใคร? (If yes, where did you seek advice for self treatment?)

_________________________________________________________________________________________

_________________________________________________________________________________________

jìbhàpài IRONMENT OF PART 2

ACIAR AH2006/161 PIG ZOONOSES PROJECT: Risk Factor & Spatial Epidemiology Questionnaire