Canine echinococcosis in the Alay Valley, southern Kyrgyzstan

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Declaration

This thesis is the result of approximately three years of research conducted at the School of Environment and Life Sciences, University of Salford. As well as being enrolled as a PhD student, I have been simultaneously employed as a Research Assistant on a larger Wellcome Trust funded research project (grant number #094325/10/Z/10). This project aimed to study canine echinococcosis in the Alay Valley of southern Kyrgyzstan, as well as in Xinjiang and the Tibetan Plateau in China.

As this project is part of a larger collaborative study, some aspects of this research have been conducted in collaboration with other members of the project, although all research ideas were generated by the author. Specifically, in Chapters 5 and 6 the work described includes a research expedition to the Alay Valley which included Prof. P.S Craig, Prof. M. Rogan, A. Mastin (from Salford University), I. Ziadinov (from the Institute of Parasitology, University of Zurich) and B. Mytynova (from the Kyrgyz Veterinary Institute in Bishkek). All expedition members assisted in collecting dog faecal samples, and as such some of the samples described in these chapters were collected by these collaborators and all questionnaires were administered by native Kyrgyz speakers I. Ziadinov and B. Mytynova.

A. Mastin (also a Research Assistant and PhD student on the Wellcome Trust project) assisted with some spatial tools including generating shapefiles and random GPS coordinates as described in Chapters 6 and 7, and assessing dog roaming behaviour in Chapter 6. A. Mastin also assisted with statistical analyses in Chapter 6, including the Poisson-normal generalised model and 'Hub lines' analysis, and associated figures. ROC curve cut-offs were also computed by A. Mastin.

Furthermore, parts of this research made use of resources previously created by other researchers in the Cestode Zoonoses Research Group. In Chapter 3, the hyperimmune rabbit sera had been previously created by other researchers including previous PhD students (see Feng, 2012). However the antibody extractions and optimization and testing was done by the author. Similarly, many of the faecal and DNA panel samples described in Chapters 3 and 4 were provided by Dr. Belgees Boufana from samples obtained as part of previous research studies (for example Naidich *et al.*, 2006; Boufana *et al.*, 2012) and/or work conducted by Dr. Boufana for Cestode Diagnostics (www.star.salford.ac.uk/page/Cestode_Diagnostics). However later panel samples, including arecoline purge samples from Kyrgyzstan and necropsy samples from Xinjiang, were collected and tested by the author. The primers described and optimized in Chapter 4 were originally designed by Dr. Boufana, but the optimization and testing described in the chapter was done by the author.

Abstract

Echinococcosis is a serious and often fatal zoonotic disease caused by parasites in the genus *Echinococcus*. *Echinococcus* spp. cycle between intermediate and final hosts, and it is the accidental ingestion of eggs in faeces of final hosts (usually canids) that causes the disease in humans. There is evidence that echinococcosis is re-emerging in Kyrgyzstan, with increasing numbers of human cases reported from the south of the country. However, little is known about canine echinococcosis in the local domestic dog population, despite the fact that dogs are the main source of human infection. As such, this thesis focuses on canine echinococcosis in the Alay Valley, southern Kyrgyzstan.

In order to study canine echinococcosis, reliable tools for diagnosing infection in dogs are needed. Previous studies have found that coproELISAs measuring *Echinococcus* spp. antigens in faecal samples can accurately detect canine echinococcosis. As part of this study, polyclonal antibodies were extracted from hyperimmune rabbit sera and optimized in a hybrid sandwich coproELISA for the detection of *Echinococcus* spp. in faecal samples with high diagnostic sensitivity and specificity. However, coproELISAs are genus specific, and identifying species/strains of *Echinococcus* spp. requires coproPCR. Although previously published coproPCR protocols were available for detection of *E. granulosus* and *E. multilocularis*, such a protocol was not available for *E. canadensis*, which was found to occur in the Alay Valley as part of this study. As such, a new analytically specific and sensitive coproPCR protocol for the detection of *E. canadensis* was developed.

The prevalence of canine echinococcosis in four communities in the Alay Valley was estimated by sampling 333 dogs in May 2012. The coproELISA prevalence was found to be high, with an average of 26.4%. All faecal samples collected in May 2012 were DNA extracted and tested by coproPCR. CoproPCR testing of coproELISA positives found that 33.3% tested positive for *E. canadensis*, 8.2% tested positive for *E. granulosus*, and 11.0% tested positive for *E. multilocularis*. Establishing pre-intervention canine coproELISA prevalences is crucial for evaluating the impact of any future control programs. As the ecology of dogs is important when studying diseases spread by them, dog demography, dog roles, dog husbandry and dog roaming was studied in four communities in the Alay Valley, as well as environmental faecal contamination being assessed. The local dog population was large, with 1 dog/9.36 people. Most dogs were male and below five years of age. Dogs played various roles in the communities, including as sheep dogs, guard dogs, and pets. Most dogs were free-roaming and could move up to 2km away from their homes. The large population of free-roaming dogs was reflected in high levels of environmental contamination, with between 0.11 and 1.20 faecal samples/100m^2 recorded.

Following the implementation of a World Bank control scheme which aimed to dose all owned dogs with praziquantel four times a year, the effects of this programme on canine echinococcosis were evaluated. In order to do this, Lot Quality Assurance Sampling (LQAS) was applied to ten communities in the Alay Valley, with communities sampled 9 and 21 months after the start of dosing. Results suggested that after 21 months of dosing, at least 75% of dogs were being dosed in 8/10 communities, and coproELISA prevalences were reduced in 5/4 communities respectively after 9 and 21 months of dosing. As control programmes require large commitments of time and resources, it is important to be able to evaluate how well these are meeting their targets. Here, reliable tools were developed to study canine echinococcosis, the pre-intervention canine echinococcosis coproELISA prevalence was established, dog ecology and demographics were studied, and LQAS was used to assess the first two years of an echinococcosis control programme. It is hoped that these studies contribute to a better understanding of the re-emergence of echinococcosis in Kyrgyzstan and the impacts of control schemes on canine echinococcosis.

Chapter 1: Introduction

1.1 General Introduction

Echinococcosis is a zoonotic parasitic disease that has been recognized in humans for thousands of years (WHO/OIE, 2001). It is caused by cestode tapeworms in the genus *Echinococcus*, that, in humans, cause echinococcosis, which is often characterized by the formation of cysts, usually in the liver, lungs, or, less commonly in the spleen, kidneys, heart, bone, and central nervous system (Moro and Schantz, 2009). If untreated, echinococcosis is often fatal (Fujikura, 1991; Moro and Schantz, 2009). Despite the long history and seriousness of this zoonotic disease, control and eradication of echinococcosis has proved difficult, largely due to the complex life cycle of the parasites, which cycle between mammalian intermediate and final hosts (Fujikura, 1991; Moro and Schantz, 2009).

1.2 Taxonomy and species of *Echinococcus*

The taxonomy of *Echinococcus* spp. has been subject to much controversy (Tappe *et al.*, 2010), and this continues to this day, with different authors recognizing different species and/or genotypes (e.g. Badaraco *et al.*, 2008; Moro and Schantz, 2009). Although authors still disagree on the taxonomy of *Echinococcus* spp., nine species are currently considered valid by most researchers. These include *E. granulosus*, *E. canadensis*, *E. multilocularis*, *E. vogeli*, *E. oligarthrus*, *E. equinus*, *E. ortleppi* (see Thompson and McManus, 2002), *E. shiquicus* (Xiao *et al.*, 2006) and *E. felidis* (Huttner *et al.*, 2008). *Echinococcus granulosus* is still commonly divided into different strains or genotypes, indicated by numbers (G1-G10) and by the intermediate host they were first discovered in, although each genotype may infect multiple intermediate hosts (Thompson and McManus, 2002). The strains include *E. granulosus* G1 (common sheep strain), *E. granulosus* G2 (Tasmanian sheep strain), and *E.*

granulosus G3 (buffalo strain, Thompson and McManus, 2002). These three strains together are now often referred to as *E. granulosus sensu stricto* (*s.s*. Nakao *et al.*, 2013a; Alvares Rojas *et al.*, 2014). Two previously described strains of *E. granulosus* have been elevated to species status, namely the previous *E. granulosus* G4 (horse) strain, which has now been reclassified as *E. equinus* and the previous *E. granulosus* G5 (cattle) strain has now been reclassified as *E. ortleppi* (Thompson and McManus, 2002). In addition a group of strains, namely *E. granulosus* G6 (camel strain), *E. granulosus* G7 (pig strain), *E. granulosus* G8 (cervid strain, Thompson and McManus, 2002), *E. granulosus* G9 (Polish strain, Scott *et al.*, 1997) and *E. granulosus* G10 (Fennoscandian cervid strain, Lavikainen *et al.*, 2003) together have now been classified as *E. canadensis* (Tappe *et al.*, 2010).

1.2.1 *E. vogeli* and *E. oligarthrus*

E. vogeli and *E. oligarthrus* are Neotropical and cause polycystic echinococcosis in humans (Tappe, 2008). Polycystic echinococcosis is characterized by a polycystic structure and development in visceral organs, usually the liver (WHO/OIE, 2001). *E. vogeli*, like other *Echinococcus* species, cycles between herbivorous intermediate and carnivorous final hosts. Identified intermediate hosts include Neotropical rodents, mainly pacas, *Cuniculus paca*, while the bush dog, *Speothos venaticus*, has been identified as a final host (Rausch *et al.*, 1981). *E. oligarthrus* has been found in South American rodents including nutria, *Myocastor coypus*, and final hosts include felids such as pumas, *Puma concolor*, and jaguars, *Panthera onca* (Rausch *et al.*, 1981). Although potentially the zoonotic incidence of polycystic echinococcosis is increasing, it is a rare disease, with only just over a hundred human cases documented up to 2007 (Tappe, 2008). Because *E. vogeli* and *E. oligarthrus* and polycystic echinococcosis are restricted to South America, and the current study is based in Asia, these parasites and the disease they cause will not be discussed in further detail.

1.2.2 *E. equinus*

E. equinus has now been designated as a separate species, although for a long time it was considered a strain (G4, horse) of *E. granulosus* (Thompson and McManus, 2002). However, molecular studies have shown that *E. equinus* and *E. granulosus* are genetically and evolutionarily distinct (Thompson and McManus, 2002). As the name implies, *E. equinus* uses equids as its intermediate host, and domestic dogs serve as final hosts (Thompson and McManus, 2002). Furthermore, *E. equinus* has to date not been found to be infective to humans (Eckert and Thompson, 1997; Thompson and McManus, 2002). However, *E. equinus* has been described in a captive lemur, *Varecia rubra*, from the United Kingdom (Boufana *et al.*, 2012).

1.2.3 *E. ortleppi*

E. ortleppi has now been designated as a separate species, although it was previously classified as a strain (G5, cattle strain) of *E. granulosus* (Thompson and McManus, 2002). *E. ortleppi* utilises cattle as its intermediate host, and is quite distinct from *E. granulosus* and *E. equinus* in morphology and genetics (Thompson, 2008). It is infective to humans (Eckert and Thompson, 1997; Thompson and McManus, 2002), although infection rates are low and few cases are known (De la Rue *et al.*, 2011).

1.2.4 *E. felidis*

A lion strain of *Echinococcus granulosus* has been considered based on necropsy findings (Eckert and Thompson, 1997). It has now been classified as a distinct species after DNA analysis found it to be genetically different from *E. granulosus*, and was designated as *E. felidis* (Huttner *et al.*, 2008). *E. felidis* uses lions, *Panthera leo*, as its final host, and

intermediate hosts may include zebras, *Equus quagga* (Huttner *et al.*, 2008) and warthogs, *Phacochoerus africanus* (Huttner and Romig, 2009). To date, no infections have been found in humans (Eckert and Thompson, 1997; Huttner *et al.*, 2008).

1.2.5 *E. shiquicus*

E. shiquicus was first described from plateau pika, *Ochotona curzoniae* and Tibetan foxes, *Vulpes ferrilata* on the Tibetan Plateau in China (Xiao *et al.*, 2005). Although morphologically similar to *E. multilocularis* in the adult stage, *E. shiquicus* is smaller and genetically distinct (Xiao *et al.*, 2005). In contrast, the larval stage of *E. shiquicus* in pika is morphologically more similar to *E. granulosus* (Xiao *et al.*, 2005). *E. shiquicus* DNA has also been found in domestic dog faeces, although its infectivity to humans remains unknown (Boufana *et al.*, 2013a).

1.2.6 *E. multilocularis*

E. multilocularis has long been recognized as a distinct species of *Echinococcus* (Tappe *et al.*, 2010). It is the cause of alveolar echinococcosis in humans, characterized by multi-vesicular lesions, primarily in the liver, although other organs, including the lungs, spleen and brain may be affected (WHO/OIE, 2001). *E. multilocularis* uses small mammals as intermediate hosts, including, primarily, microtine voles *Arvicola terrestris*, *Microtus arvalis*, *Microtus limnophilus* (Craig *et al.*, 2000; Duscher *et al.*, 2006), Tibetan hares, *Lepus oiostolus* (Xiao *et al.*, 2004), shrews, *Sorex jacksonii*, ground squirrels *Citellus undulatus lyratus*, and harvest mice, *Peromyscus gossypinus* (Smyth and Smyth, 1964). Carnivores and usually canids serve as final hosts, and *E. multilocularis* has been found in red fox, *Vulpes vulpes* (e.g. Duscher *et al.*, 2006), grey wolves, *Canis lupus* (Martinek, 2007), coyotes, *Canis latrans* (Hildreth *et al.*, 2000) and domestic dogs, *Canis familiaris* (Budke *et al.*, 2005).

1.2.7 *E. granulosus*

E. granulosus is the most geographically widespread of the *Echinococcus* spp., occurring in Europe (e.g. Casulli *et al.*, 2012), North America (e.g. Sweatman and Williams, 1963), South America (e.g. Reyes *et al.*, 2012), Africa (e.g. Lahmar *et al.*, 2004), Asia (e.g. Bart *et al.*, 2006), and Australasia (e.g. Jenkins *et al.*, 2006). *E. granulosus* has several intermediate hosts, most commonly sheep, *Ovis aries* (WHO/OIE, 2001), but also, among others, camels, *Camelus dromedarius* (Lahmar *et al.*, 2004), moose, *Alces alces* (Sweatman and Williams, 1963), and goats, *Capra hircus* (Varcasia *et al.*, 2007). Final hosts are carnivores and usually canids including grey wolf (Sobrino *et al.*, 2006) and domestic dogs (Budke *et al.*, 2005).

There are variations between the different strains of *E. granulosus*, including in hook number and dimensions, host infectivity and specificity, biochemical composition, and genetic makeup (Thompson and McManus, 2002). The different strains also vary in relation to their infectivity to humans. The most common cause of human cystic echinococcosis is the G1 genotype (common sheep strain) (e.g. Bart *et al.*, 2006; Kia *et al.*, 2010), and the G1 strain is thought to be responsible for >88% of human cystic echinococcosis cases (Alvares Rojas *et al.*, 2014). The G2 (Tasmanian sheep) strain has also been found to be infective to humans (see Eckert and Thompson, 1997). The infectivity of the G3 (buffalo) strain to humans is unclear (see Jenkins *et al.*, 2005), although rare cases of human infection with the G3 strain have been described (De la Rue *et al.*, 2011).

1.2.8 *E. canadensis*

Although the taxonomy of *E. canadensis* is still somewhat controversial (e.g. Badaraco *et al.*, 2008; Casulli *et al.*, 2012), it is now accepted by many researchers as a separate species (e.g. Thompson, 2008; Nakao *et al.*, 2013a). *E. canadensis* includes what was previously known

as the G6-G10 strains of *E. granulosus* (Tappe *et al.*, 2010), although the status of G9 is still somewhat unclear (see Nakao *et al.*, 2013a). Recent genetic analysis has revealed that the G6-G8 and G10 strains are very closely related to each other (Nakao *et al.*, 2013a). *E. canadensis* is infective to humans, although *E. canadensis* is the cause of a minority of human cases, with strains G6 and G7 estimated to be the cause of 11.04% of human echinococcosis cases (Alvares Rojas *et al.*, 2014). The G6 (camel) strain has been found to be infective to humans, (e.g. Bart *et al.*, 2006), although the G6 strain is thought to be much less infective to humans than the G1 strain (McManus and Thompson, 2003). The G9 strain has been found in Polish echinococcosis patients (Scott *et al.*, 1997) and the G7 strain has been found in Slovakian echinococcosis patients (Turcekova *et al.*, 2003). The G8 (cervid) strain has also been found to be infective to humans (see Eckert and Thompson, 1997), as has the G10 (Fennoscandian cervid) strain (see Jenkins *et al.*, 2005).

1.3 Detection of *Echinococcus* spp.

Echinococcus spp. cycle between an intermediate and final host. The adult cestode inhabits the small intestine of a definitive host, which is a carnivore, and produces eggs containing infective oncospheres (Eckert and Deplazes, 2004). Eggs are released from the intestinal tract of the carnivore into the environment, and may then be ingested orally by an intermediate host, which is usually a herbivore such as a sheep (Craig *et al.*, 1995) or a rodent (Craig, 2006). In the intermediate host, a larval stage, the metacestode, develops in internal organs (Eckert and Deplazes, 2004). If the intermediate host is consumed by a definitive host, the cycle is complete. *Echinococcus* spp. can therefore be detected in both intermediate and final hosts.

1.3.1 Detecting *Echinococcus* spp. in intermediate hosts

Although some serodiagnostic tests have been assessed for the detection of *Echinococcus* infection in intermediate hosts (Kittelberger *et al.*, 2002), and pre-mortem diagnosis of echinococcosis can be achieved using ultrasound (Lahmar *et al.*, 2007a; Dore *et al.*, 2014), most studies on intermediate hosts involve post-mortem examination of the internal organs for parasites. In the case of *E. multilocularis*, *E. shiquicus*, *E. vogeli* and *E. oligarthrus*, which use small mammals as intermediate hosts, this usually involves trapping small rodents using live traps or break-back traps and dissecting these (see for example Craig *et al.*, 2000). Other *Echinococcus* species cycle through larger intermediate hosts, including sheep (*E. granulosus*, e.g. Ahmadi and Dalimi, 2006), horses (*E. equinus*, e.g. Williams and Sweatman, 1963), bovines (*E. ortleppi*, e.g. Casulli *et al.*, 2008), and zebras (*E. felidis*, Huttner *et al.*, 2008; for a review see Craig *et al.*, in press.). In this case, the intermediate hosts (if these are domestic species) are usually inspected at slaughterhouses and cysts are examined using morphology (e.g. Ahmadi and Dalimi, 2006) or molecular techniques (e.g. Casulli *et al.*, 2008). If the intermediate hosts include wild herbivores, specimens may be provided by hunters/cullers for inspection (e.g. Rau and Caron, 1979; Schurer *et al.*, 2013).

1.3.2 Detecting *Echinococcus* spp. in final hosts

Echinococcus spp. use carnivores as a final host, and these are often canids, although *E. felidis* use African lions as their final hosts (Huttner *et al.*, 2008) and *E. oligarthrus* uses felids such as pumas and jaguars as final hosts (Rausch *et al.*, 1981). In the final host, the adult cestode inhabits the intestine (Eckert and Deplazes, 2004), and, as with the intermediate hosts, necropsy and post mortem evaluation is the gold standard for detection of *Echinococcus* spp. infection (e.g. Abdybekova and Torgerson, 2012). However, whereas intermediate hosts for *Echinococcus* spp. are usually either small mammals that may be

easily trapped, or domesticated herbivores which can be inspected in slaughterhouses, final hosts are not as easy to necropsy and analyse. In addition, domestic dogs function as final hosts for almost all *Echinococcus* spp., including *E. granulosus*, *E. multilocularis*, *E. ortleppi*, *E. equinus* (see Thompson and McManus, 2002), *E. canadensis* (Nakao *et al.*, 2013a) and *E. shiquicus* (Boufana *et al.*, 2013a). Domestic dogs usually belong to people, or play some role in a community, which often makes larger scale necropsy studies infeasible. Fortunately, alternative diagnostic tools are available. Serodiagnosis by ELISA has been investigated to test for canine echinococcosis, though diagnostic sensitivity was generally poor with natural infections (35-40%), and lower than that achieved with coproantigen detection ELISAs (Jenkins and Rickard, 1986; Gasser *et al.*, 1988; Craig *et al.*, 1995). This, together with the fact that faecal samples (unlike serum samples) can be collected non-invasively, means that most methods of diagnosis of echinococcosis focus on copro-tests. These include arecoline purgation, coproELISA and coproPCR.

Arecoline purgation involves the administration of arecoline hydrobromide to dogs, which induces them to purge the contents of their intestine. These purges can then be examined for *Echinococcus* spp. (Craig *et al.*, 1995). Arecoline purgation has the advantage of allowing for estimation of worm burdens, but disadvantages include logistical difficulties in larger scale studies, the need for trained personnel, the biohazard posed by purges, potential distress to dogs and failure rates of between 10-20% (Craig *et al.*, 1995). CoproELISAs use polyclonal (e.g. Jenkins *et al.*, 2000) or monoclonal (Morel *et al.*, 2013) antibodies to detect *Echinococcus* spp. antigens in final host faeces. CoproELISAs therefore do not require necropsy or purgation. In addition, coproELISAs are relatively simple to carry out, allow for high sample throughput, and often achieve good diagnostic sensitivity and specificity (Craig *et al.*, 1995; Allan and Craig, 2006).

CoproPCR (polymerase chain reaction) depends on the presence of *Echinococcus* spp. DNA in faecal samples. Target DNA can be isolated from eggs after flotation concentration (Štefanić *et al.*, 2004), or from total DNA in faecal samples (Abbasi *et al.*, 2003). CoproPCR can achieve high analytic sensitivity and specificity (Lahmar *et al.*, 2007b). However coproPCR usually requires specialist equipment such as a thermocycler (e.g. Knapp *et al.*, 2008; De la Rue *et al.*, 2011), although recently more low-tech protocols have been developed using loop-mediated isothermal amplification (LAMP) for detection of *Echinococcus* spp. (Ni *et al.*, 2014).

Protocols for the detection of *E. granulosus* include those developed by Abbasi *et al.* (2003), Štefanić *et al.*, (2004), Dinkel *et al.*, (2004), and Boufana *et al.*, (2013b). The protocol developed by Štefanić *et al.*, (2004) targets the mitochondrial 12S RNA gene and aimed to identify only *E. granulosus* "sheep strain" in domestic dog faecal samples, but was found by Boufana *et al.*, (2008) to cross react with *E. equinus*, *E. ortleppi*, and strains G6, G7, G8, and G10 of *E. canadensis*, as well *E. multilocularis* and *E. shiquicus*. The protocol developed by Dinkel *et al.*, (2004) also targets the mitochondrial 12S RNA gene and aimed to identify the G1 strain of *E. granulosus*, with additional possible protocols to identify the *E. ortleppi* (G5) and *E. canadensis* (G6/G7). However, the primers were found by Boufana *et al.*, (2008) to cross react with *E. shiquicus, Taenia hydatigena, T. multiceps, T. ovis, T. pisiformis, D. caninum*, and *T. solium* although the test was found to be specific for the G1 strain of *E. granulosus* when tested against *E. equinus*, *E. ortleppi*, and strains G6, G7, G8, and G10 of *E. canadensis.* Furthermore the Dinkel *et al.*, (2004) protocol has not been specifically optimized for coproPCR. The protocol developed by Abbasi *et al.* (2003) targets a repeat sequence in the genomic DNA and aimed to detect *E. granulosus* infections in dogs, although the strains are not specified. The "Abbasi test" was found by Boufana *et al.*, (2008) to be the most species specific of the protocols tested, although the test did detect DNA from *E.*

equinus, *E. ortleppi*, and strain G7 of *E. canadensis.* Furthermore the only sequence available for these primers on the ncbi database (http://ncbi.nlm.nih.gov/) is for *E. granulosus* without a specified strain (accession number DQ157697.1). As such these primers are not suitable for use in areas where several strains may be present. The protocol developed by Boufana *et al*. (2013b) targets a fragment within the NADH dehydrogenase subunit 1 (ND1) mitochondrial gene and aims to identify only the G1 strain of *E. granulosus*. Although highly analytically specific, this protocol is less sensitive (Boufana *et al.*, 2013b). In addition, as this protocol is specific for the G1 strain, its applicability in areas where several strains occur needs be complemented with other protocols.

Protocols for the detection of *E. multilocularis* DNA include those by Bretagne *et al*., (1992), Dinkel *et al*., (2011) and Boufana *et al*., (2013b). The protocol developed by Bretagne *et al*., (1992) involves primers that target ribosomal DNA in the U1 snRNA gene. The protocol was found to have a high analytic sensitivity, but required isolation of eggs from faecal samples, increasing laboriousness. The protocol developed by Dinkel *et al*., (2011) involves a hybridization probe-based real-time multiplex nested PCR for the simultaneous detection of *E. multilocularis* and host species (carnivores including red fox, *Vulpes vulpes*, corsac fox, *V. corsac*, Tibetan fox, *V. ferrilata* and domestic dog *Canis familiaris*) from faecal samples. The primers for *E. multilocularis* target the mitochondrial 12S RNA gene, and were found by the authors to not cross react with *E. granulosus* (G1), *E. ortleppi*, *E. canadensis* and six *Taenia* species (Dinkel *et al.*, 2011). The protocol developed by Boufana *et al*. (2013b) did not aim to identify host species and was a uniplex PCR. The primers target the NADH dehydrogenase subunit 1 (ND1) mitochondrial gene, and were found to be 100% specific when tested against *E. granulosus* and *E. shiquicus*. In cases where the host species is known, for example where samples are collected from owned domestic dogs, the protocol developed by Boufana *et al*. (2013b) for the detection of *E. multilocularis* is the more practical one to use.

Although both coproELISAs and coproPCRs can be used to detect *Echinococcus* spp. in final hosts, it is important to remember that the two tests are not interchangeable. CoproELISAs measure *Echinococcus* spp. antigens in faecal samples, and as such depend on the presence of worms in the dog intestine, but do not require the presence of eggs. As such coproELISAs may detect pre-patent infections (e.g. Deplazes *et al.*, 1992), and furthermore, *Echinococcus* spp. and *Taenia* spp. antigens have been found to be quite stable in faecal samples (Deplazes *et al.*, 1990; Allan and Craig, 2006), making this a suitable test for samples which are not fresh. As with all assays, however, coproELISAs have detection limits. Antibodies for coproELISA may have different affinities, and diagnostic sensitivity decreases with low worm burdens, as low worm burdens mean lower concentrations of antigens in faecal samples (e.g. Allan and Craig, 2006).

CoproPCR, unlike coproELISA, measures DNA in faecal samples. CoproPCRs can have high analytic sensitivity, but there are problems related to the extraction and PCR of faecal DNA. For example, false positives may occur in cases of coprophagia (Hartnack *et al.*, 2013), or environmental contamination of samples, especially in areas that have high densities of canid faeces. Furthermore, although *Echinococcus* spp. eggs may be stable in the environment for long periods of time under certain climatic conditions, they are susceptible to warm and dry conditions (Veit *et al.*, 1995). DNA is likely to degrade quickly if not properly preserved (e.g. Olson *et al.*, 2005), and when collecting faecal samples from the environment (as opposed to rectally), DNA in these samples may have been degraded. Furthermore, coproPCR includes problems such as presence of DNA inhibitors in faeces (Mathis and Deplazes, 2006), and the presence of non-target DNA (Naidich *et al.*, 2006; Boufana *et al.*, 2008), which may affect results. As such coproPCR could lead to false

negatives. Furthermore, DNA extraction followed by coproPCR is very laborious and costly (Mathis and Deplazes, 2006).

When choosing a diagnostic test, the advantages and disadvantages of coproELISA and coproPCR should be taken into consideration. Several authors recommend a combination of these tests; coproELISA can be used for rapid analysis of large numbers samples (Fraser and Craig, 1997), and can be recommended as a primary diagnostic tool, followed by coproPCR (e.g. Eckert and Deplazes, 2004). CoproELISA measures the more environmentally stable antigens, but is genus rather that species specific, and cannot be used to identify species or strains of *Echinococcus* spp. Therefore, coproELISA positives can be tested further for species using specific coproPCR protocols. This combination allows for screening of large numbers of samples, whilst still being able to identify the species or strains of *Echinococcus* spp. present in a population.

1.4 Cystic and alveolar echinococcosis

1.4.1 Transmission

Of the existing *Echinococcus* species, *E. granulosus, E. canadensis* and *E. multilocularis* are the main causes of human echinococcosis, with *E. granulosus* and *E. canadensis* causing cystic echinococcosis (CE) and *E. multilocularis* causing alveolar echinococcosis (AE). The geographic range of *E. granulosus, E. canadensis* and *E. multilocularis* is very wide, although *E. multilocularis* is restricted to the Northern hemisphere (Eckert and Deplazes, 2004). These three species require intermediate and final hosts. The life cycle of *E. granulosus* is largely domestic, involving domestic livestock (mainly sheep) and domestic dogs. *E. canadensis* may have both sylvatic and domestic lifecycles. For example in Canada, strains G8 and G10 of *E. canadensis* cycle between wild ungulates including elk, *Cervus*

canadensis (Schurer *et al.*, 2013) and grey wolves (Schurer *et al.*, 2014), but in other locations the G6 genotype has been found in livestock, and a domestic cycle appears to be maintained involving domestic species such as camels (Bardonnet *et al.*, 2003; Omer *et al.*, 2010), cattle (Omer *et al.*, 2010; Hailemariam *et al.*, 2012) and sheep and goats (Omer *et al.*, 2010). The life cycle of *E. multilocularis* is largely sylvatic, involving small mammals and wild canids including foxes, although dogs are also excellent final hosts (Eckert and Deplazes, 2004).

The risk to humans from these parasites comes from *Echinococcus* spp. eggs present in the faeces of final hosts (Eckert and Deplazes, 2004). *Echinococcus* spp. eggs may survive in the environment for hundreds of days (Veit *et al.*, 1995). If humans accidentally ingest *Echinococcus* spp. eggs they may develop echinococcosis (Eckert and Deplazes, 2004). Studies in areas of high echinococcosis endemicity have identified risk factors associated with the disease (e.g. Danson *et al.*, 2003; Budke *et al.*, 2005). These include landscape factors such as proximity to suitable host habitat (e.g. Giraudoux *et al.*, 2003), as well as social factors such as owning dogs (Stehr-Green *et al.*, 1988), and especially free-roaming dogs (Kern *et al.*, 2004). In many areas women are at higher risk of infection than men, and people in some occupations such as farmers, are at higher risk than others, such as government employees (Craig *et al.*, 2000).

1.4.2 Pathology

Cystic echinococcosis is typically characterized by the formation of unilocular cysts, usually in the liver, although other organs including the lungs, kidneys, spleen, brain, muscles, heart and bone may also be affected (WHO/OIE, 2001). The initial phases of infection are always asymptomatic, with small encapsulated cysts located in organ sites where they do not cause major pathology, thus causing the infection to often go unnoticed for years (WHO/OIE,

2001). In the later stages of infection, the disease becomes symptomatic, with symptoms depending on the site of infection. If the liver is affected, symptoms commonly include an enlarged liver, jaundice, abdominal pain, and secondary cirrhosis (WHO/OIE, 2001). If the lungs are affected, symptoms include chest pain, chronic coughing, and lung abscesses (WHO/OIE, 2001). Infection in other sites is characterized by pain and the appearance of tumour-like growths (WHO/OIE, 2001). Alveolar echinococcosis is similar to cystic echinococcosis but is often characterized by multivesiculated cysts (Craig *et al.*, 1992). As with cystic echinococcosis, the infection can be asymptomatic for years before pathology develops (WHO/OIE, 2001). The main site of infection is usually the liver, and symptoms of alveolar echinococcosis include jaundice, epigastric pain, fatigue, weight loss, and an enlarged liver.

1.4.3 Treatment

Echinococcosis is difficult to treat, and because the disease can be asymptomatic for long periods of time, the disease is usually in an advanced stage by the time patients seek treatment. Echinococcosis is usually diagnosed by ultrasound, although serological tests are also available and may assist confirmation (WHO/OIE, 2001). The methods of treatment used include chemotherapy, surgery and percutaneous methods (PAIR, Smego Jr and Sebanego, 2005). Chemotherapy involves administering mebendazole or (more commonly) albendazole (Smego Jr and Sebanego, 2005). However, these drugs are not effective in all cases of the disease. One study found that only 73.2% of cystic echinococcosis patients showed a response to albendazole treatment, with cures achieved in only around a third of patients (Horton, 1997). Surgery aims to remove the cysts from the infected organ (usually the liver) and may be conservative (e.g. tube drainage of cysts) or radical, involving resection of (for example) the liver, or even the removal of a complete lobe (Smego Jr and Sebanego, 2005). Cure and survival rates depend on the size and location of the cyst, although these are

often improved by using a combination of chemotherapy and surgery (Ammann, 1991). The prevalence of post-operative long-term recurrence of CE is between 2%-25% (WHO/OIE, 2001). Patients undergoing PAIR usually receive chemotherapy for several days before and after the procedure (Smego Jr and Sebanego, 2005). The procedure for selected CE cases involves three steps: puncture and needle aspiration of the cyst, insertion of a parasiticidal solution (usually hypertonic saline) for about 20—30 minutes, and cyst re-aspiration (Smego Jr and Sebanego, 2005). When PAIR is combined with chemotherapy, over 95% of cystic echinococcosis patients may be cured (Smego Jr and Sebanego, 2005).

1.4.4 Prevention and control

Attempts to control and even eliminate echinococcosis have been carried out all over the world, with differing degrees of success (for examples see Gemmell *et al.*, 1986; Craig and Larrieu, 2006). *E. granulosus* is easier to target than *E. multilocularis* using control programmes because of its largely domestic life cycle. *E. granulosus* control programmes usually include regular praziquantel dosing of dogs, controlled slaughter and meat inspection, reduction of dog populations and education (Gemmell *et al.*, 1986). Control programmes are most efficient in isolated areas, usually islands, with Iceland often cited as an example of a successful echinococcosis eradication programme (Beard, 1973). However, although eradication of *E. granulosus* has been achieved in some locations such as Iceland (Beard, 1973) and Tasmania (see Jenkins *et al.*, 2005), hydatid control programmes require years, if not decades, of commitment. Although initial rates of *Echinococcus* infections may decline rapidly, long term efforts are needed to ensure infection rates don't increase again after an initial 'attack' phase (WHO/OIE, 2001). Control programmes are often difficult to conduct in many areas where echinococcosis is relatively common such as the Tibetan Plateau because of the remoteness of this area, socio-cultural values and the associated logistical difficulties (Budke *et al.*, 2005).

Echinococcus multilocularis is more difficult than *E. granulosus* to control or eradicate due to its largely sylvatic life cycle, although control programs involving praziquantel bait dosing of wild canids (usually foxes) have been conducted (Tsukada *et al.*, 2002; Hegglin *et al.*, 2004), and monitoring of *E. multilocularis* infection rates in wild hosts such as foxes may help reduce risks to humans (Deplazes *et al.*, 2004). Lack of health education is often cited as a risk factor for both CE and AE, and control programmes may include public education campaigns to help reduce risks of *E. multilocularis* and *E. granulosus* to humans (e.g. Eckert and Deplazes, 2004).

1.5 Impact of echinococcosis

Echinococcosis is a serious debilitating and often fatal disease (WHO/OIE, 2001). However, its impact is not limited to human health. Echinococcosis can also be a very large economic burden, with costs to both humans and livestock. Costs to humans include the cost of health care and lost wages due to illness, and costs to livestock include the condemnation of meat, reduced growth of livestock, reduced milk production, and reduced fecundity (Benner *et al.*, 2010). The burden of disease associated with echinococcosis can be calculated using disability adjusted life years (DALYs, Budke *et al.*, 2004). Torgerson *et al.,* (2010) estimate the total number of DALYs due to alveolar echinococcosis per annum for the world at a median of 666,433. The World Health Organization (WHO) estimates the global burdens of cystic and alveolar echinococcosis to result in at least 1.5 million DALYs and possibly considerably more, with cystic echinococcosis estimated to result in losses of US$ 0.5–2 billion to the global livestock industry annually (WHO, 2010). As well as impacts on health and economics, the societal impacts of echinococcosis include social consequences of disability such as unemployment (Torgerson *et al.*, 2003), suffering, and abandonment of farming or agricultural activities by those affected or at risk (Battelli, 2004).

1.6 A neglected zoonotic disease

Despite the high impact of echinococcosis on people, livestock, economies and societies (see also Carabin *et al.*, 2005), echinococcosis is a neglected zoonotic disease (WHO, 2010). There are several potential reasons for this as outlined by Craig *et al.* (2007a), including the fact that it is a non vector-borne zoonotic disease that is not transmissible between humans, making it difficult to target. Also, both CE and AE can be asymptomatic for long periods of time, so that endemic communities and health workers can fail to properly recognize the negative health impacts (Craig *et al.*, 2007a). Furthermore, echinococcosis is difficult to detect and treat (Craig *et al.*, 2007a). Also, human echinococcosis mostly affects remote, poor communities, who are often ethnically or culturally isolated from the general population, and therefore not prioritised by governments (Craig *et al.*, 2007a). Even if the political will to tackle echinococcosis is present, control campaigns are often hindered by logistical problems associated with working in remote areas or by the (semi) nomadic lifestyles of the endemic communities (e.g. Macpherson, 2005).

Despite echinococcosis being a neglected zoonotic disease, efforts are being undertaken to research and control this disease. Recent studies have focused on understanding the different aspects of echinococcosis including its transmission dynamics (e.g. Lahmar *et al.*, 2004; Takumi and van der Giessen, 2005; Robardet *et al.*, 2010; Wang *et al.*, 2010), human risk factors (e.g. Kern *et al.*, 2004), impacts of echinococcosis (e.g. Carabin *et al.*, 2005; Benner *et al.*, 2010) and treatment options (e.g. Smego Jr and Sebanego, 2005). Control programmes are being undertaken in endemic areas, including for example the National Echinococcosis Control Programme in China, launched in 2006 (Chinese Ministry of Health, 2007; Huang *et al.*, 2011).

1.7 *Echinococcus* spp. in Central Asia

1.7.1 Background

Much of Central Asia including Kazakhstan, Kyrgyzstan, Tajikistan, Turkmenistan and Uzbekistan was part of the former USSR (Soviet Union). Since the collapse of the Soviet Union in 1991 these newly independent countries have been through considerable political and economic changes, with governments transitioning from a centralized communist administration to democracies, and economies moving from a centrally planned to a free market economy (Torgerson *et al.*, 2002a). Although echinococcosis did occur in the Soviet Union, it was present at relatively low levels (Torgerson and Budke, 2003). This is possibly due to the Soviet mechanism of agriculture and slaughter, with the rearing of most sheep (the primary intermediate host for *E. granulosus*) taking place on large collectivized farms, and slaughter undertaken in large slaughterhouses under veterinary inspection (Torgerson *et al.*, 2002a). Furthermore, in Soviet times the licensing of domestic dogs and treatment of farm dogs with praziquantel every four months was compulsory (Torgerson *et al.*, 2002a). In contrast, since independence collective farms have broken up into smallholdings, home slaughter has increased, and the dog population has grown (Jenkins *et al.*, 2005; Torgerson *et al.*, 2006). These factors have probably contributed to an increase in both cystic and alveolar echinococcosis in Central Asia over the last few decades (Torgerson *et al.*, 2006; Torgerson *et al.*, 2010).

1.7.2 Re-emergence of echinococcosis in Central Asia

Although detailed data for many areas are lacking, studies from Kazakhstan, and (to a lesser extent) Kyrgyzstan, Tajikistan and Mongolia (which was not officially part of the USSR, although was heavily influenced by it during Soviet times) give a picture of the re-emergence of echinococcosis in Central Asia. For example, during Soviet times pre 1991, human CE

surgical incidence rates in the USSR tended to be relatively low with perhaps at most 1–5 cases per 100,000 per year (Torgerson *et al.*, 2002a). However nowadays in many areas figures suggest the surgical incidence of cystic echinococcosis is now greater than 10 cases per 100,000 (Torgerson *et al.*, 2002a). In Kazakhstan specifically, cystic echinococcosis has increased from 1.4 cases per 100,000 people in 1991 to 5.9 cases per 100,000 people in 2000 (Torgerson *et al.*, 2002b), and in Kyrgyzstan the incidence has increased from 5.4 cases per 100,000 people in 1991 to 18 cases per 100,000 people in 2000 (Torgerson *et al.*, 2003). There are now several high endemic areas in Central Asia, including in southern Kazakhstan, Kyrgyzstan and Tajikistan with incidence rates of up to 13 cases/100,000, 20 cases/100,000 and 27 cases/100,000 respectively (Torgerson *et al.*, 2002a). Echinococcosis is also an increasing health concern in Mongolia (Wang *et al.*, 2001; Ebright *et al.*, 2003). Control programmes are being undertaken in the area, and the World Bank has implemented a project in Kyrgyzstan that includes the implementation of a comprehensive, nationwide testing programme for echinococcosis and the provision of anthelmintic for dogs (World Bank, 2011).

1.8 Aims and structure of this thesis

The aim of this thesis was to study canine echinococcosis in the Alay Valley, southern Kyrgyzstan. In **Chapter 2**, the study site is described in detail, as well as the field and laboratory methods used as part of this study. Canine echinococcosis can be determined by coproELISA (e.g. Allan *et al.*, 1992; Morel *et al.*, 2013) and/or coproPCR (e.g. Abbasi *et al.*, 2003; Dinkel *et al.*, 2011; Boufana *et al.*, 2013b). CoproELISAs are often genus specific (e.g. Craig *et al.*, 1995), whereas coproPCRs may detect only one species of *Echinococcus* (e.g. Dinkel *et al.*, 2011), or even only one strain of *E. granulosus* (e.g. Boufana *et al.*, 2013b). As such several authors recommend a combination of these tests; coproELISA can be used for rapid analysis of large numbers of samples (Fraser and Craig, 1997), and can be

recommended as a primary diagnostic tool, followed by PCR of coproELISA positives to determine the species or strains of *Echinococcus* spp. present in a population (e.g. Eckert and Deplazes, 2004). Here a combination of coproELISA and coproPCR was used. In Chapter 5, all collected faecal samples were tested with both coproELISA and coproPCR. In Chapter 6, only coproELISA positives were tested with coproPCR. In Chapter 7, samples were analysed only by coproELISA. In order to be able to answer epidemiological questions on canine echinococcosis in the Alay Valley, appropriate diagnostic tools first had to be developed. **Chapter 3** describes the development of a new genus specific coproELISA for *Echinococcus* spp.. Specific antibodies were isolated from hyperimmune rabbit sera, and the development and validation of these antibodies is described. Although several sets of primers are available for the detection of *Echinococcus* spp. (e.g. Abbasi *et al.,* 2003; Štefanić *et al.,* 2004; Boufana *et al.*, 2013b), none of these were suitable for areas where *E. granulosus, E. multilocularis* and *E. canadensis* are co-endemic including the study site, and where it is necessary to distinguish between *Echinococcus* species present. **Chapter 4** describes the development of new primers for detection and identification of *E. granulosus* as well as *E. canadensis.*

Echinococcosis is an emerging zoonotic disease in Kyrgyzstan, with human cases increasing rapidly since the collapse of the Soviet Union (Torgerson, 2013; Usubalieva *et al.*, 2013). The World Bank has included echinococcosis as one of the diseases it is targeting through a nationwide project, and it aims to dose all dogs in Kyrgyzstan with anthelmintics (World Bank, 2011). However, to date, little research on echinococcosis has been done in Kyrgyzstan, and particularly research focusing on canine echinococcosis. This current research aimed to assess canine echinococcosis in the Alay Valley of southern Kyrgyzstan. In **Chapter 5**, the prevalence of canine echinococcosis in four communities in the Alay Valley prior to a World Bank echinococcosis intervention campaign was determined. Four

communities were visited, and all owned dogs in three communities were registered and sampled, with approximately 25% of dogs registered and sampled in the fourth community. Faecal samples were analysed for *Echinococcus* spp. using coproELISA and coproPCR. Because domestic dogs are important hosts of *Echinococcus* spp., and pose the main risk for human infection (Budke *et al.*, 2005), it is important to understand the roles, demographics and behaviour of domestic dogs. In **Chapter 6**, in order to understand the role of domestic dogs in the local communities in the Alay Valley, dog owner questionnaires were administered. In addition, dog roaming behaviour was studied with the help of GPS trackers. Furthermore, the environmental contamination of local communities with infected dog faeces was assessed by conducting searches for canid faeces in 50mx50m quadrats. Finally, in order to evaluate the first two years of the World Bank control programme, the method of Lot Quality Assurance Sampling (Dodge and Romig, 1929) was applied in **Chapter 7** to test how well the dosing regime was reaching people and their dogs in the local communities. As well as the four original target communities, six other communities in the Alay Valley (Jaylima, Achyk Suu, Kabyk, Kyzel Eshme, Sary Tash and Archa Bulak) were visited, with a minimum 19 dogs randomly sampled in each community and questionnaires administered to their owners. **Chapter 8**, is a discussion of the results found in Chapters 5, 6 and 7 and overall consideration of the findings and implications of the research.

Chapter 2: General Methods and Materials

2.1 Study Site: Alay Valley, Kyrgyzstan

The fieldwork was conducted in the Alay Valley of southern Kyrgyzstan. Kyrgyzstan was formerly part of the Soviet Union, having gained independence in 1991 (Abazov, 1999). It is bordered by Kazakhstan in the North, Uzbekistan in the west, Tajikistan in the south and China in the east. Kyrgyzstan covers approximately 199,951 km^2, and the country is largely mountainous; 94% of the country is more than 1,000m above sea level (a.s.l). with an average elevation of 2,750m a.s.l. (CIA, 2014). Kyrgyzstan is sparsely populated, with an estimated human population of 5.6 million people (CIA, 2014). Most Kyrgyz people earn their living through agriculture (Abazov, 1999), especially livestock raising (Ronsijn, 2006), although crops including tobacco and cotton, which are exported, are also important (CIA, 2014).

The Alay Valley was selected for the current study based on transmission of *Echinococcus* spp. following reported increases in human cases of echinococcosis from the area (Torgerson, 2013; Usubalieva *et al.*, 2013). The Alay Valley is located in the south of Kyrgyzstan, approximately 50km north of the border of Tajikistan. It is located at approximately 3,000m a.s.l. and surrounded by mountains, including the Pamir Mountains on the border with Tajikistan, and the Alay Mountains in the north (CIA, 2014). The majority of the research was conducted in four communities in the Alay Valley, namely Taldu Suu (39.70°, 72.98°), Sary Mogul (39.68°, 72.89°), Kara Kavak (39.66°, 72.72°) and Kashka Suu (39.64°, 72.67°). These four communities are situated along a main road (A372) that runs east to west, and all four communities are within approximately 17km of each other, with Kashka Suu being the westernmost and Taldu Suu the easternmost (Fig. 2-1). Each of the four communities are

small villages with between ~65 (Kara Kavak) to ~400 households (Sary Mogul). The population of each community ranges from a few hundred to at most ~3,000 people.

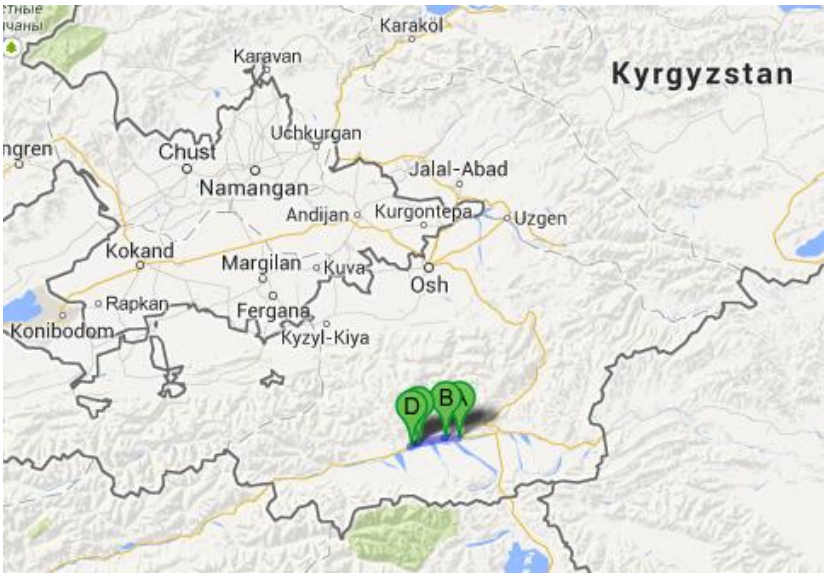

Figure 2-1 The Alay Valley, Kyrgyzstan

Letters A, B, C and D indicate Taldu Suu, Sary Mogul. Kara Kavak and Kashka Suu. (Map from http://maps.google.com)

In addition, six other communities in the Alay Valley were visited (Chapter 7), namely Sary Tash (39.73°, 73.25°), Archa Bulak (39.69°, 73.08°), Kabyk (39.59°, 72.39°), Kyzel Eshme (39.57°, 72.27°), Jaylima (39.62°, 72.59°), and Achyk Suu (39.47°, 72.50°, Fig. 2-2). These were situated along the same road as Taldu Suu, Sary Mogul, Kara Kavak and Kashka Suu (A327) but were located east (Sary Tash and Archa Bulak) and west (Kabyk, Kyzel Eshme, Jaylima and Achyk Suu) of Sary Mogul. These communities were similar in size to Sary Mogul, Taldu Suu, Kara Kavak and Kashka Suu, with similar local customs.

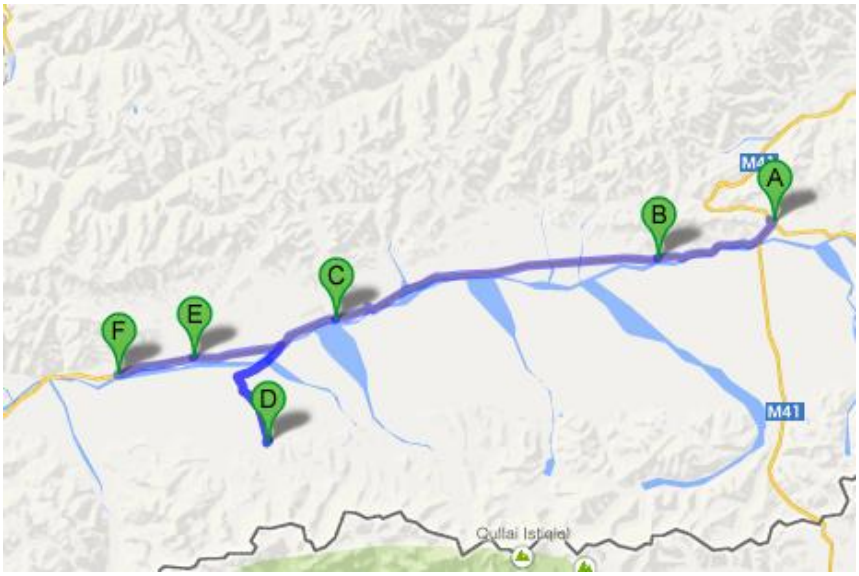

Figure 2-2 The Alay Valley, Kyrgyzstan

Letters A, B, C, D, E and F indicate Sary Tash, Archa Bulak, Jaylima, Achyk Suu, Kabyk and Kyzel Eshme.

(Map from http://maps.google.com)

2.2 Sample Collection

Faecal samples were collected from domestic dogs in each of the ten communities described in section 2.1. Samples were collected by going from house to house in each of the communities and enquiring about the presence or absence of dogs. If dogs were present, they were registered by recording the household members' names, the dog's name, age and sex, and a GPS position of the house was taken using a handheld Garmin® GPS 60. Faecal samples were collected by one of two methods; either rectally by qualified vets (Iskender Ziadinov and/or Alex Mastin), or the dog's owner was asked to indicate where the dog usually defecated and ground samples were collected (Fig. 2-3). Where possible, fresher samples were chosen rather than older samples, as DNA in older faecal samples may degrade (e.g. Deuter *et al.*, 1995). As well as sampling registered dogs at household level, ground faecal samples were also collected from 50mx50m quadrats in open areas within the communities (see Chapter 6). In the six additional communities (i.e. Sary Tash, Archa Bulak,

Jaylima, Achy Suu, Kabyk and Kyzel Eshme) a different sampling frame was used, based on Lot Quality Assurance Sampling theory (Dodge and Romig, 1929). Instead of attempting to sample all dogs in each community, a minimum of 19 dogs were sampled in each community (see Chapter 7 for details).

Each collected faecal sample was divided in two, and a subsample was stored in 35ml universal tubes in 0.3% PBS Tween (Fisher Scientific, Loughborough, UK), with 10% formalin (sourced locally) for use in coproELISA, with a corresponding subsample stored in bijoux tubes/15 ml polypropylene tubes using 70% ethanol (sourced locally) for use in coproPCR. Samples were labelled, covered in parafilm to prevent leaking, and shipped to the University of Salford, United Kingdom, for analysis.

Figure 2-3 Bermet Mytynova (Kyrgyz veterinarian) administering a questionnaire to a dog owner, and author collecting a faecal sample from the ground

2.3 Analysis of faecal samples

2.3.1 CoproELISA

To prepare faecal samples for analysis they were first stored at -80°C for at least four days to remove the risk of infection with *Echinococcus* spp. (WHO/OIE, 2001). Samples were then homogenized using a wooden spatula, shaken, and centrifuged at 2500 r.p.m. (1125G) for 5

minutes using an Eppendorf centrifuge 5804. The resultant supernatant was tested in a coproELISA.

The ELISA used was a sandwich ELISA, which detects antigen between two layers of antibodies (i.e. the capture and conjugate antibody). The ELISA used polyclonal antibodies derived from rabbit serum to detect *Echinococcus* spp. (see Chapter 3). For the ELISA, 4HB 96 well ELISA plates (Fisher Scientific, Loughborough, UK) were coated the day before the ELISA using 100µL of capture antibody diluted in carbonate bicarbonate buffer (Sigma-Aldrich, Dorset, UK, added to all wells except blanks). The plates were covered in cling film and incubated at 4°C overnight.

The next day, plates were washed three times with 0.1% PBS Tween buffer (Fisher Scientific, Loughborough, UK). 100µL of 0.3% PBS Tween was added to each well except blanks. Plates were incubated for 1 hour at room temperature, after which the 0.3% PBS Tween was discarded and plates were patted dry. 50µL of foetal calf serum (Sigma-Aldrich, Dorset, UK) was added to each well except blanks. 50µL of faecal supernatant was added to each well, and mixed by pipetting. All samples were analysed in duplicate. Negative controls from non-endemic and low-endemic areas were used, and known infected samples (e.g. arecoline purge positive samples, or samples spiked with *Echinococcus* spp. whole worm extract) were used as positive controls. After another incubation for 1 hour at room temperature, plates were washed three times with 0.1% PBS Tween buffer, and 100µL of conjugate antibody (diluted in 0.3% PBS Tween) was added to each well, except blanks. After incubating for an hour at room temperature and washing plates three times with 0.1% PBS Tween, 100µL of SureBlue® TMB substrate (Insight Biotechnology, Wembley, UK)

was added to all wells. The plate was incubated for 20 minutes in the dark and then read on a Thermo scientific Multiscan FC platereader at 620nm.

If the controls failed (i.e. negative controls were positive, or vice versa), the whole plate was repeated. If replicates of samples gave OD values that were above and below the cut-off point respectively, these samples were repeated to ascertain whether they were positive or negative. If the repeated OD values of the replicates were again above and below the cut-off, all four replicates were averaged.

2.3.2 CoproPCR

2.3.2.1 DNA extraction

In order to analyse faecal samples using coproPCR, DNA must first be extracted from these samples. A QIAamp® DNA Stool kit (Qiagen, Hilden, Germany) was used for extractions, following the manufacturer's instructions, with the exception of using 1g instead of 0.1g of faeces, and increasing the volume of ASL buffer. This kit is designed especially for the extraction of total DNA from faeces (Qiagen, 2010).

Samples were weighed (~1g) into 50ml polypropylene tubes and 10ml of ASL lysis buffer was added to each sample. After July 2013, a 'blank' was included for every 11 or 23 samples (depending on how many needed to be extracted) which was processed in the same way as the other samples but contained no faeces, to test for contamination. Samples and buffer were vortexed for 1 minute and then incubated for at least five minutes at ≥70°C to help lyse cells and eggs. After the incubation, samples were vortexed for 15 seconds and centrifuged at 5000 r.p.m for two minutes. 1.2ml of supernatant was pipetted into 2ml tubes, and an InhibitEX Tablet was added to each tube. Samples were vortexed for 1 minute and incubated at room temperature for at 1 minute to allow inhibitors to adsorb to the InhibitEX

matrix (Qiagen, 2010). Samples were then centrifuged at full speed for 3 minutes, and the supernatant was pipetted into a new 2ml tube. Samples were again centrifuged at full speed for three minutes, after which 200µL of supernatant was added to a new 1.5ml tube containing 15µL of proteinase K. 200µL of buffer AL was added, and the tubes were vortexed for 15 seconds and incubated at 70°C for 10 minutes. After this incubation, 200µL of ethanol (96-100%) was added to each tube, and tubes were vortexed. The complete lysate was then added to a QIAamp spin column, and centrifuged at full speed for 1 minute. This column allows for DNA to be adsorbed onto the QIAamp silica membrane during the centrifugation step (Qiagen, 2010). DNA bound to the QIAamp membrane was then washed in two centrifugation steps; first 500µL of wash buffer AW1 was added, followed by 1 min of centrifuging at full speed, and then 500µL of wash buffer AW2 was added, followed by 3 min of centrifuging at full speed. By washing using these two wash buffers, complete removal of any residual impurities without affecting DNA binding is ensured (Qiagen, 2010). The final step consists of eluting the DNA from the spin column by adding 200µL of AE, incubating at room temperature for at least 1 minute, and centrifuging at full speed for 1 minute. The resulting DNA was stored at 4°C or at -20°C for long term storage (Qiagen, 2010).

2.3.2.2 PCR Protocol

There are several protocols available for detection of *Echinococcus* spp. in faeces (e.g. Dinkel *et al.*, 2004; Štefanić *et al.*, 2004; Dinkel *et al.*, 2011). Four existing protocols were used, namely a protocol developed by Abbasi *et al.*, (2003) for detection of *E. granulosus sensu lato (s.l.)*, two protocols developed by Boufana *et al* (2013b) for detection of *E. granulosus* G1 and *E. multilocularis* and a generic cestode protocol (von Nickisch-Rosenegk *et al.*, 1999). In addition, a new PCR protocol was developed to detect *E. canadensis* (see Chapter 4).

2.3.2.3 *"Abbasi" PCR protocol for* E. granulosus *sensu lato.*

The protocol developed by Abbasi *et al*., (2003) targets a repeat sequence in the genomic DNA, and is for the detection of *E. granulosus*. Although the original publication discusses the G1 (common sheep strain) of *E. granulosus*, this protocol actually detects several genotypes including G1, G4, G5, G6, G7, and G10 (Boufana *et al.*, 2008, Boufana unpublished data).

A slightly modified protocol was used for the detection of *E. granulosus*, consisting of a 50µL reaction with 5x manufacturers Flexi reaction buffer (Promega, Southampton, UK.), 250 mM of each deoxynucleoside triphosphate (dNTPs; Bioline, London, UK), 1 mM of each primer [1] (Eg2691 5' ACACCACGCATGAGGATTAC 3' and Eg2692 5'ACCGAGCATTTGAAATGTTGC 3'), 2 mM $MgCl_2$ (Promega, Southampton, UK), 2% formamide (VWR, Lutterworth, UK), and 2.5 U of GoTaq polymerase (Promega, Southampton, UK.). The mastermix was overlaid with mineral oil and the thermal cycling profile was as follows: 5 min at 95°C for 1 cycle, followed by 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C for 35 cycles (Boufana *et al.*, 2008) to amplify an *E. granulosus* 133-basepair segment within a repeat unit (Abbasi *et al.*, 2003).

2.3.2.4 *ND1 PCR protocols for* E. granulosus *G1 and* E. multilocularis

The *E. granulosus* G1 protocol developed by Boufana *et al.*, (2013b) targets the NADH dehydrogenase subunit 1 (ND1) mitochondrial gene and is highly specific for this strain of *E. granulosus*. This protocol involves a 50µL reaction with 5x manufacturers Flexi reaction buffer (Promega, Southampton, UK), 200 mM of each deoxynucleoside triphosphate (dNTPs; Bioline, London, UK), 0.3 mM of each primer (Eg1F81, 5' GTT TTT GGC TGC CGC CAG AAC 3' and Eg1R83, 5' AAT TAA TGG AAA TAA TAA CAA ACT TAA TCA

[1] Note: all primers described in this thesis were sourced from Eurofins, Manchester, UK

ACA AT 3'), 2 mM $MgCl_2$ and 2.5 U GoTaq polymerase (Promega, Southampton, UK). The mastermix was overlaid with mineral oil and the thermal cycling profile included 5 min at 94°C for 1 cycle, followed by 36 cycles each consisting of 30 s at 94°C, 50 s at 62°C, and 30 s at 72°C to amplify a species-specific 226 bp fragment in the NADH dehydrogenase subunit 1 (ND1) mitochondrial gene (Boufana *et al.*, 2013b).

The *E. multilocularis* protocol targets the NADH dehydrogenase subunit 1 (ND1) mitochondrial gene, and detects *E. multilocularis* DNA with great analytic specificity (Boufana *et al.*, 2013b). The protocol involves a 50µL reaction with 5x manufacturers Flexi reaction buffer (Promega, Southampton, UK), 200 mM of each deoxynucleoside triphosphate (dNTPs; Bioline, London, UK), 0.3 mM of each primer (EmF19/3, 5' TAG TTG TTG ATG AAG CTT GTT G 3' and EmR6/1, 5'ATC AAC CAT GAA AAC ACA TAT ACA AC 3'), 2 mM $MgCl_2$ and 2.5 U of Hotstart GoTaq polymerase (Promega, Southampton, UK). The mastermix was overlaid with mineral oil and the thermal cycling profile was as follows: 5 min at 94°C for 1 cycle, followed by 30 s at 94°C, 50 s at 53°C, and 30 s at 72°C for 35 cycles to amplify an *E. multilocularis*-specific 207 bp fragment.

2.3.2.5 Generic cestode PCR protocol

The generic cestode protocol targets the 12S rDNA and can be used for the detection of cestode species, including 11 *Taenia* species as well as *E. granulosus* and *E. multilocularis* (von Nickisch-Rosenegk *et al.*, 1999). Although much less specific than the protocols developed by Abbasi *et al.* (2003) and Boufana *et al.* (2013b), this protocol, which was optimised using tissue DNA, is very sensitive. The protocol involves a 100µL reaction with 5x manufacturers Flexi reaction buffer (Promega, Southampton, UK.), 400 mM of each deoxynucleoside triphosphate (dNTPs; Bioline, London, UK), 0.8 mM of each primer (P60F, 5'-TTAA GATA TAT GTG GTA CAG GAT TAG ATA CCC-3' and 5'-AAC CGA GGG

TGA CGG GCG GTG TGT ACC-3'), 2 mM $MgCl_2$ and 2.5 U of GoTaq polymerase (Promega, Southampton, UK.). The mastermix was overlaid with mineral oil and the thermal cycling profile was as follows: 30 seconds at 94°C, 1 minute at 55°C, and 30 s at 72°C for 40 cycles to amplify a 311bp fragment in the mitochondrial 12S rRNA gene (von Nickisch-Rosenegk *et al.*, 1999).

All PCR procedures were carried out in fully equipped molecular laboratories using dedicated equipment at Salford University. Negative controls (PCR grade water) were included in all experiments to monitor for contamination, and positive controls from extracted and sequenced tissue samples were used to test the PCR has worked. A Stratagene Robocycler or Applied Biosystems Veriti thermal cycler was used for all cycling profiles. PCR products were resolved on a 1.5% or 3% (w/v) agarose (Bioline, London, UK) gel in 1x Tris-Borate-EDTA buffer (Severn Biotech, Kidderminster, UK) at 110 V, stained with gel red (Cambridge Biosciences, UK) DNA dye, and visualized using Syngene G:Box gel documentation system.

Where protocols detected several species (for example the von Nickisch-Rozenegk *et al.*, 1999 cestode protocol), it was necessary to sequence positive PCR products in order to identify which species was present. In order to do this, PCR products were sent to Beckman Coulter Genomics in Essex, UK. The sequences were downloaded using FinchTV software (Geospiza Inc, Seattle, USA) and then BLASTed on the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify species.

2.4 Panel samples

2.4.1 Tissue panel samples

This study required several tissue samples, mainly for DNA extraction and use in PCR protocols. In all coproPCR protocols, sequenced DNA from adult worms/cyst material was used as positive controls. Initially, DNA samples of *E. granulosus* G1, *E. multilocularis* and *Taenia hydatigena* were provided by Dr. Belgees Boufana, which had been collected as part of previous studies and archived at Salford University (Boufana *et al.*, 2008; Boufana *et al.*, 2013b).

Tissue DNA from *E. granulosus* G1, G2, G3, *E. equinus*, *E. canadensis* G7, G8, G10, *T. multiceps*, *T. ovis*, *T. crassiceps*, *T. pissiformis*, *T. hydatigena*, *Dipyllidium caninum* and *E. shiquicus* used in Chapter 4 for the development of new PCR protocols was provided by Dr. Boufana, which had been collected as part of previous studies and archived at Salford University (Naidich *et al.*, 2006; Boufana *et al.*, 2008; Boufana *et al.*, 2012; Boufana *et al.*, 2013b; Lett, 2013).

Following fieldwork in Hobukesar County, Xinjiang, China in April 2013, adult *E. granulosus* G1 and *T. hydatigena* were collected from necropsied dogs (see Chapter 7, Appendix 1, and van Kesteren *et al.*, in press). DNA was extracted from these worms, analysed by PCR and sequenced, and subsequently used as PCR controls.

E. multilocularis adult worms were obtained from an arecoline purge wash from Taldu Suu in Kyrgyzstan (for details see Chapter 6). Additional *E. multilocularis* adult worms isolated from necropsied red foxes were provided by Dr. Jenny Knapp at Franche Comtè University

in France. DNA was extracted from these worms, analysed by PCR and sequenced, and subsequently used as PCR controls.

E. canadensis (G6) DNA was obtained from an arecoline purge wash from Kara Kavak, Kyrgyzstan (for details see Chapter 6). DNA was extracted from these worms, analysed by PCR and sequenced, and subsequently used as PCR controls.

2.4.2 Faecal panel samples

For this study, many faecal samples of known status were required, for example for testing the reliability of newly isolated polyclonal antibodies (Chapter 3), testing the analytic sensitivity and specificity of new coproPCR protocols (Chapter 4) and for use in Gaussian or ROC curve panels to determine cut-offs for coproELISA (Chapters 5, 6 and 7). The required samples included samples from dogs known to be infected with *Echinococcus* spp. and dogs known to not be infected with *Echinococcus* spp.

Ideally only 'gold standard' samples should be used. For negative samples these could include samples from a non-endemic area, or samples that were found to be negative by necropsy and thorough investigation of the intestine. For positive samples these could include samples from necropsied dogs, with adult worms collected and analysed with PCR and sequencing. In practice it was not always possible to obtain a suitable number of 'gold standard' samples and other samples had to be included. These include arecoline purge samples (arecoline purging is not always 100% effective, leading to possible false negatives), samples from low endemic areas (Falkland Islands and UK), and in some cases using samples that had been found to be positive for *Echinococcus* spp. with other diagnostics, such

as a different coproELISA and/or molecular methods. Details of the faecal samples used are given below, with details also included in the relevant chapters and appendices.

2.4.2.1 Faecal samples of known negative infection status

Negative samples (Negative #1-#17, see Chapter 3, Appendix 1 and Manchester dog #1-12, see Chapter 3, Appendix 2) were collected from a veterinary practice in Greater Manchester (a non-endemic area) by Matthew Bates (an undergraduate student at Salford University at the time). As canine echinococcosis is not expected to occur in urban areas in the UK, these samples can be used as known negative samples. Further samples from the Falkland Islands (a very low endemic area, see Chapter 3, Appendices 2,4,6) were provided by Dr. Belgees Boufana, after testing these as part of diagnostic services at the University of Salford (see http://www.star.salford.ac.uk/page/Cestode_Diagnostics) and finding them to be coproELISA negative. Further known negative samples were taken from arecoline purge negative and coproELISA negative samples in the Alay Valley (see Chapter 6, and Chapter 7 Appendix 2) and necropsy negative dogs in Hobukesar County, China (see Chapter 7 Appendix 3, and van Kesteren *et al.*, in press).

2.4.2.2 Faecal samples of known positive infection status

Faecal samples of known positive infection status were provided from various sources. Dr. Belgees Boufana provided samples from dogs experimentally infected with *T. hydatigena, T, multiceps, E. equinus,* and *E. granulosus sensu stricto* (Chapter 3, Appendix 1, 3 and Table 4.2 in Chapter 4). Dr. Boufana also provided necropsy confirmed infections from dogs naturally infected with *E. granulosus sensu stricto* (Chapter 3, Appendix 6) and two samples from natural infections in dogs in Kazakhstan (Chapter 4, Table 4-2), confirmed by coproPCR and sequencing (Boufana *et al.*, 2013b).

Samples of known infection with *E. granulosus, E. multilocularis* and *E. canadensis* were obtained from the Alay Valley following arecoline purgation of 20 dogs (see Chapter 6 and Chapter 7 Appendix 2 for details). These samples were used as panels of coproELISA samples of known infection status.

Following fieldwork in Hobukesar County, Xinjiang, China in April 2013, faecal samples were collected from 38 necropsied dogs (see Chapter 7 Appendix 3 and van Kesteren *et al.*, in press). These included 16 samples that were necropsy (and later PCR and sequence) positive for *E. granulosus* G1, with estimated worm burdens ranging from 2 to >10,000. These samples were used as panels of coproELISA samples of known infection status.

2.5 Statistical analysis

All figures were made and all statistical analysis was done using R Statistical Software version 2.15.0 (R Development Core Team, 2012). Details of different statistical tests are given in each chapter. Bar graphs were created using the 'sciplot' package in R (Morales, 2013). Where error bars are given (i.e. in Figures 3-2, 3-3, 3-4) these bars represent the standard error from the mean. Population pyramids were created using the package 'pyramid' in R (Nakazawa, 2013); violin plots were created using the 'vioplot' package (Adler, 2005); Euler diagrams were created using the 'venndiagram' package (Chen, 2015).

Briefly, in Chapter 6, faecal densities within 50x50m quadrats were assessed using a Poisson-normal generalised linear mixed effects model. This was used to model the number of faeces within each quadrat, with effects of 'village' and 'sampling date' (i.e. May or October 2012) included in the model, as well as the log area of the quadrat (not all quadrats were 50x50m). Quadrat ID was included as random variable to account for overdispersion. The accuracy of

iGotU® units used to track dog movements in Chapter 6 was also assessed by comparing these to the positions recorded by handheld Garmin® GPS units in Quantum GIS 1.8.0 (Quantum GIS Development Team, 2012). Analysis of dog movements was conducted by both calculating the dog 'home ranges' and total distances travelled. Home ranges were calculated using Characteristic Hull Polygon methods in R, and maximum distances travelled were calculated by estimating the minimum distance between each relocation point and the start point for each dog recorded in Quantum GIS. Differences in home ranges and median distance moved between male and female dogs were compared using the Wilcoxon rank sum test.

In Chapter 7, Lot Quality Assurance Sampling (LQAS) was used to study the effects of approximately two years of praziquantel dosing on local rates of canine echinococcosis. In order to calculate decision numbers, the hypergeometric distribution was used. Dog population numbers were based on census data or estimated based on village size and average dog densities in the Alay Valley.

Chapter 3: Development and optimization of a coproELISA for the detection of canine *Echinococcus* spp. infections

3.1 Introduction

The enzyme-linked immunosorbent assay (ELISA) was developed after the development of the preceding radioimmunoassay (Adkinson *et al.*, 1988). The earliest radioimmunoassays measured the analyte by competition between ^{131}I-radiolabelled and unlabelled antigen for antibody and involved a separation step to distinguish between bound and free-labelled antigen (Adkinson *et al.*, 1988). It was later found that the efficiency and speed of separation was increased by immobilizing the second antibody on a solid phase such as Sephadex, plastic, or cellulose. The 'sandwich-type' solid phase radioimmunoassays used labelled antibodies rather than labelled antigens (Adkinson *et al.*, 1988). The principle of using enzymes as markers in solid phase tube assays was developed into the enzyme-linked immunosorbent assay (ELISA) in the early 1970s (Engvall and Perlmann, 1972; Adkinson *et al.*, 1988). ELISAs offer a number of advantages over radioimmunoassay, including the fact that the reaction can be read visually without the need for expensive apparatus, and the labelled reagents used are stable and are easily stored for long periods of time without loss of activity. Multiwell microtitre plates are easy to handle and wash and when used with automated readers and multiple well washers allow large numbers of samples to be assayed. A variety of enzyme-labelled antisera of good quality can be purchased commercially and an increasingly wide range of suitable enzyme substrates and chromogens is available (Adkinson *et al.*, 1988).

In order to use ELISAs as a diagnostic tool for antigen detection, antibodies are required. It is possible to obtain antibodies against a very broad range of antigens including proteins, short peptides, carbohydrates, drugs, hormones, nucleic acids etc. (Delves, 1997). For most common antigens, antibodies can be purchased from a commercial company. However, should an antibody against the antigen of interest not be available, it is possible to produce in house antibodies (Delves, 1997). Antibodies include those secreted by a single clone of B lymphocytes (usually from immunised mice), termed monoclonal antibodies, and those produced by a mixture of various B lymphocyte clones (usually from immunised rabbits), termed polyclonal antibodies (Leenaars and Hendriksen, 2005). In making a choice between producing polyclonal or monoclonal antibodies, the desired application of the antibody and the time and money available for production should be considered. A polyclonal antiserum can be obtained within a short time (4-8 weeks) at a relatively low cost, whereas it takes about 3-6 months to produce monoclonal antibodies. Since many research questions can be answered by using polyclonal antibodies, they are often favoured over monoclonal antibodies (Leenaars and Hendriksen, 2005). Polyclonal antibodies can be produced by immunising animals with the desired antigen and harvesting the antibodies from serum. This process includes several steps such as the preparation of the antigen, the selection of the animal species, selecting an injection protocol, and collection of the antibodies (Allan *et al.*, 1992; Leenaars and Hendriksen, 2005).

For most studies the best species to use for immunization is the rabbit if a polyclonal antiserum is required. Rabbits are good responders to a wide range of antigens and, if repeatedly bled following booster immunizations, can yield up to 500ml of serum (Delves, 1997). Rabbits are usually immunised with between 10 and 1000µg of antigen (Delves, 1997). Several sites may be chosen for injection, and the most commonly used routes of injection for polyclonal antibody production are subcutaneous, intradermal, intramuscular,

intraperitoneal, and intravenous (Leenaars and Hendriksen, 2005). Following the first injection with antigen, there is a lag period, followed by the production of detectable amounts of specific IgM in the serum after about 7 days. Specific IgG antibody is then produced after a further 3-4 days, after which the antibody response subsides (Delves, 1997). However, if after a period of time, the antigen is again injected, this 'booster' immunization leads to the production of a secondary immune response which has a much shorter lag period and produces much higher amounts of specific IgG antibody (Delves, 1997). IgG antibodies are detectable 3-4 days following the booster injection, reaching peak levels after 10-14 days, and are produced for a much longer time span than in the primary antibody response. During the production of polyclonal antisera it is common to give several booster injections (Delves, 1997).

In mammals, antibody responses during the experiment can be monitored by obtaining and evaluating a blood sample for antibodies in the serum every few days (Leenaars and Hendriksen, 2005). Once the response appears optimal, the animal can either be bled out or used for repeated immunization and sequential bleeding. Larger volumes of sera can be obtained if the animal is repeatedly given booster injections followed by removal of up to 20% of the total blood volume a few days following each injection (Delves, 1997). Each antiserum should be kept separate until characterized. Quantity, affinity, specificity, and subclass profile of antibodies may differ between serum samples; differences will exist between animals and even between different serum samples from the same animal, as the response may change with time (Hanly *et al.*, 1995).

Once the hyperimmune serum has been obtained from an immunized rabbit, the antibodies need to be purified from the serum. This can be done using a Protein A column. Protein A is a single polypeptide chain of molecular weight 42,000 that contains little or no carbohydrate

and is a major cell wall component of *Staphylococcus aureus* (Goding, 1978). The major feature of Protein A, in terms of immunology, is its high affinity for immunoglobulin, especially IgG, making it very suitable for isolation of IgG from serum (Goding, 1978). The hyperimmune rabbit serum can be run through the column, IgG will bind to the Protein A and the resulting bound antibodies can be eluted and separated (Goding, 1978).

The sandwich ELISA measures the amount of antigen between two layers of antibodies (i.e. the capture and conjugate antibody). Both capture and conjugate antibodies can be obtained from hyperimmune rabbit serum, with the conjugate requiring extra steps. The most important requirement for capture antibodies is that once bound to the solid phase they should have a high binding capacity for relevant antigens (Adkinson *et al.*, 1988). The conjugate antibody can be conjugated in several ways, for example with horseradish peroxidise, alkaline phosphatise and β-galactosidase. Horseradish peroxidise (HRP) has the advantage of having a high turnover rate, being pure, relatively cheap and readily available. Several chromogens may be used with the HRP substrate hydrogen peroxide, including 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB). TMB is sensitive for the detection of low levels of enzyme and has the advantage of being non-mutagenic and non-carcinogenic (Adkinson *et al.*, 1988).

After the capture and conjugate antibodies have been isolated from the rabbit serum, several steps must be undertaken to characterize the antibodies. These include the amount, diagnostic specificity and diagnostic sensitivity (Delves, 1997). Diagnostic sensitivity (Se) in this case can be defined as the proportion of true positives that can be correctly identified by the coproELISA, and diagnostic specificity (Sp) can be correctly identified as the proportion of true negatives that can be detected by the coproELISA (with Se=1 – Sp and vice versa). The amount of antibody present can easily be determined by measuring the optical density using a

spectrophotometer at 280nm and applying a calculation (Delves, 1997). The diagnostic specificity and sensitivity of the antibodies can be tested by setting up an ELISA with known positive and negative samples (Delves, 1997).

In canine echinococcosis, coproantigens can be detected using a coproELISA that includes antibodies specific to *Echinococcus* spp. antigens. Such coproELISAs have been developed and used at Salford University previously (Craig *et al.*, 1995). The antibodies (capture and conjugate) used in the coproELISA are made in house. As part of a three year study on canine echinococcosis in Kyrgyzstan, a reliable coproELISA for the detection of *Echinococcus* spp. was needed. As such, the aim of this chapter was to isolate new antibodies for use in the established coproELISA, and optimise this ELISA for the detection of *E. granulosus* and *E. multilocularis* in canid faeces.

3.2 Methods

3.2.1 Rabbit immunization and selection of sera

Several sera from rabbits previously immunized and bled were stored in a -80°C freezer at Salford University, UK (see for example Feng, 2012). Seven sera (Table 3-1) were selected for an antigen recognition ELISA. These sera were obtained from rabbits immunized in Libya or China between 2 October 2000 and 23 October 2003. Rabbits were immunized with either *Echinococcus granulosus* crude whole worm extract, excretory-secretory (ES) preparations or with a saline wash of intact *E. granulosus* worms.

*Table 3-1Rabbit sera tested in antigen (*E. granulosus *whole worm extract) recognition ELISA*[2]

Rabbit	Serum volume	Date	Inocculated with	Source country
Rabbit 5	50ml	01-Sep-03	RαEgWWE	China
Rabbit 6	70ml	28-Oct-03	RαEgES	Libya
Rabbit 61	22.5ml	02-Oct-00	RαEgWWE	China
Rabbit 75	35ml	12-Oct-00	RαEgWWE	China
Rabbit 91	65ml	26-Jul-02	Saline wash of Eg worms	China
Rabbit 93	70ml	26-Jul-02	RαEgWWE	China
Rabbit 481	50ml	unknown	RαEgWWE	Australia

3.2.2 Testing immunoreactivity of rabbit sera in ELISA

A 4HBX plate (Fisher Scientific, Loughborough, UK) was coated with 1:100 *Echinococcus granulosus* whole worm extract (WWE) diluted in BCB (carbonate bicarbonate) buffer (Sigma-Aldrich, Dorset, UK), with all wells coated except two blanks. The plate was then incubated overnight at 4°C. The next day the plate was washed three times with 0.1% PBSt buffer (1 Phosphate Buffered Saline tablet (Fisher Scientific, Loughborough, UK) per 100ml distilled water, 0.1ml Tween® (Fisher Scientific, Loughborough, UK) per 100ml) and all wells (except blanks) were blocked with 0.3% PBSt (1 Phosphate Buffered Saline (Fisher Scientific, Loughborough, UK) tablet per 100ml distilled water, 0.3ml Tween® (Fisher Scientific, Loughborough, UK) per 100ml) with 5% milk powder. This was then left to incubate for one hour at room temperature. After incubation, the contents of the plate were discarded and the plate was again washed three times with 0.1% PBSt. 200µl of sera from the seven selected rabbits and Normal Rabbit Serum (the control) in 0.3% PBSt and milk solution (1:25 dilution) were pipetted into the first column of wells, and subsequently serially diluted, to give concentrations ranging from 1:25 to 1:25600. After a one hour incubation at room temperature, the plate contents were discarded and the plate was again washed in 0.1%

[2] *RaEgWWE means rabbits inoculated with* E. granulosus *whole worm extract, ES means excretory secretory products*

PBSt. 100µl of alkaline phosphatase labelled conjugate antibody (anti-rabbit IgG alkaline phosphatase, 1:2000 dilution, from Sigma-Aldrich, Dorset, UK) was added to each well except blanks, and incubated for one hour at room temperature. After this incubation the plate was again washed in 0.1% PBSt. 100µl of PNPP (p- nitrophenylphosphatase, Fisher Scientific, Loughborough, UK) was added to each well (including blanks) and the plate was allowed to develop for 20 minutes. The plate was then read using a Tecan® Sunrise plate reader at 405nm (Figure 3-1).

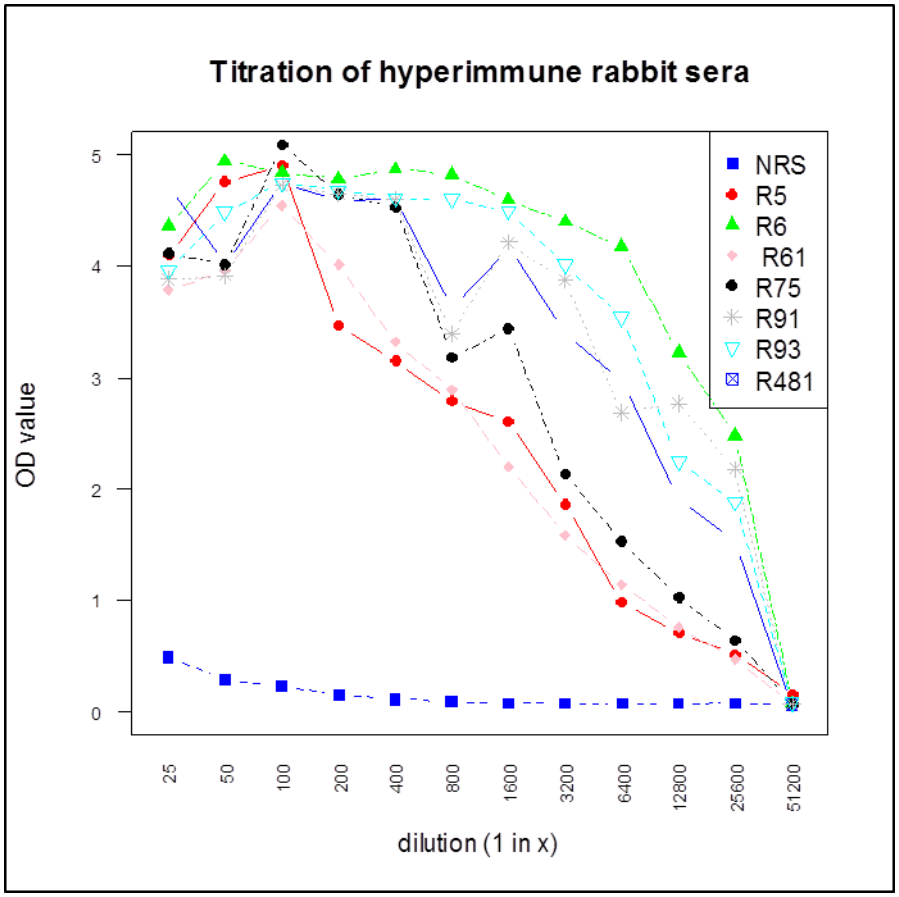

Figure 3-1 Hyperimmune rabbit sera binding to E. granulosus *WWE extract in ELISA* [3]

[3] *Seven different hyperimmune rabbit sera were tested after immunisation. NRS (normal rabbit serum) from a non-immunised rabbit was used as a control.*

3.2.3 IgG purification from rabbit serum and conjugation

The results indicated that all tested rabbit sera reacted with the *E. granulosus* WWE (Fig. 3-1). Rabbit 91 (immunised with a saline wash of adult worms) was the first serum selected for IgG purification. A Protein A Sepharose CL4B (Scientific Laboratories Supplies, Nottingham, UK) column was prepared as follows: 5g of Protein A was washed in excess 0.15M PBS (Phosphate Buffered Saline, Fisher Scientific, Loughborough, UK, 1 tablet per 100ml distilled water) in a 15ml tube. A 30ml syringe was plugged with glass wool and the Protein A was added to the syringe. After flushing the column through with 0.15M PBS, 13ml of Rabbit 91 serum was run through the column, and collected in a 50ml vial. The column was again flushed with 0.15M PBS and then elution buffer was added to the column. 1-1.5ml of elution buffer was collected in cuvettes and measured in a spectrophotometer at 280nm. All cuvettes with an OD value above 0.3 were pooled. The process was repeated with the first run through of Rabbit 91 serum. The pH of the combined elute was checked and $NaHCO_3$ (VWR, Lutterworth, UK) was added until the pooled elute reached a pH of around 7. The column was then flushed with PBS buffer and stored in 10% ethanol at 4°C. To calculate the IgG content in the collected elute, a 1:10 dilution of elute was made in elution buffer, and the IgG content was calculated using the following formula:

(OD 280/13.5) x 10 x (dilution factor) $\rightarrow$ [IgG] mg/ml

This gave an IgG content of 2.4mg/ml. To obtain the desired ~6-8mg/ml concentration the solution was concentrated using an Amicon Stirred Cell and a PM10 membrane, and nitrogen gas. The volume of the elute was reduced from 33ml to 12ml and a new OD reading indicated the IgG content was now 5.78 mgs/ml. The elute was divided in a 2:1 ratio for capture and conjugate. Two pieces of dialysis membrane approximately 7cm in length were boiled in distilled water for 5 minutes and then the IgG designated to become capture and

conjugate were pipette into the membranes, which were tied shut at both ends. The IgG destined to become capture was dialysed overnight in a cold room with 0.15M PBS buffer, the IgG destined to become conjugate was dialysed overnight using 0.01M BCB buffer (1 in 5 dilution of 0.05M BCB buffer; 1.59g Na_2CO_3 and 2.93g $NaHCO_3$ (both from VWR, Lutterworth, UK) in 1 litre of distilled H_2O, pH 9.6).

The next day the capture was aliquoted and stored at -80°C. The IgG destined to become conjugated was stored at 4°C until the next day. In the meantime, a solution of HRPO (peroxidase type vi-a from horseradish, Sigma, Dorset, UK) was made in a ratio of 2:1 (i.e. 4mg HRPO: 8mg/ml IgG where 4mg of HRPO dissolves in 1ml of distilled H_2O). 0.1M sodium periodate ($NaIO_4$, VWR, Lutterworth, UK) was freshly prepared and added to the HRPO solution at a quantity of 200µl/ml. The solution was protected from light degradation by covering the tube in tin foil. The covered tube was placed on an end over end mixer for 20 minutes at room temperature and the mixture was then dialysed against excess 0.001M sodium acetate buffer (0.08203g sodium acetate (Scientific Laboratories Supplies, Nottingham, UK) in 1 litre distilled H_2O, pH 4.4). The next day 0.2M sodium carbonate/bicarbonate buffer (6.4mg Na_2CO_3, 11.7mg $NaHCO_3$, Scientific Laboratories Supplies, Nottingham, UK, in 1 ml distilled H_2O, pH 9.6) was added to the dialysed HRPO solution at a quantity of approximately 20µl/ml in order to raise the pH to 9.6. The HRPO was added to the concentrated IgG solution (0.05M BCB was added to the IgG solution as necessary to equalize the volumes of HRPO and IgG). The two solutions were immediately mixed together in a foil covered tube on an end over end mixer for 2 hours at room temperature. $NaBH_4$ (Scientific Laboratories Supplies, Nottingham, UK) was added to the conjugated solution at a rate of 50µl/ml to reduce any free conjugate remaining. Once added, the solution was left static at 4^oC for 2 hours. The final solution was then dialysed overnight at 4^oC against excess PBS and aliquoted the next day for storage at $–80^oC$.

3.3 Results

3.3.1 Assessment of isolated antibodies

The suitability of new capture and conjugate antibodies needs to be rigorously assessed before these are used to test samples of unknown infection status. The first step in this process is to do a checkerboard titration. An ELISA plate was divided into two halves, for positive (e.g. spiked 1:50 with *E. granulosus* whole worm extract) and negative (not spiked, from a non-endemic area) samples. The capture and conjugate were titrated along the columns or down the rows, for example from a 1:500 to a 1:16000 concentration. When the ELISA was finished and had been read on a plate reader, the O.D. value for the positive sample was divided by the O.D. value of the negative sample to calculate the signal:noise ratio. The higher this number, the more likely that the capture and conjugate antibodies will be suitable. After the first checkerboard it may be desirable to do a second checkerboard at intermediate dilutions (e.g. 1:750 to 1:24000). Once some promising capture/conjugate combinations have been identified, a few of these can be tested again on a plate that is divided into a positive and negative half. In this way promising combinations can be tested with more replicates of the relevant capture/conjugate combinations, which provides greater security that the combinations may be suitable.

The second step was to try to identify a cut-off. This can be done by setting up a panel of known negatives with a few known positives. The cut-off for positive samples is usually determined as the average negative value plus two or three standard deviations (Gaussian approach, e.g. Allan and Craig, 1989; Lahmar *et al.*, 2007b). After this step, a panel of known negatives and positives can be tested with the new capture/conjugate combinations to see if the new antibodies accurately identify positive/negative samples. Samples known to be positive for *Taenia* spp. should be included to test for genus specificity. This panel should

also be tested with the current antibodies (in this case, Rabbit47 antibodies which have been thoroughly validated) for comparison.

3.3.2 Assessment of Rabbit 91 antibodies

In order to assess the suitability of the created R91 (a rabbit immunised with *E. granulosus* adult surface wash) capture and conjugate antibodies, three ELISAs were done. The first was a checkerboard ELISA. A 4HBX ELISA plate (Fisher Scientific, Loughborough, UK) was divided into two halves, for positive and negative samples. R91 capture was diluted 1:500 in BCB buffer and 200µl were added to columns 1 and 7. The capture was then serially diluted in columns 2-6 and 8-12 to give capture concentrations of 1:500 to 1:16000. All wells were coated except two blanks. The plate was then incubated overnight at 4°C, and the next day the ELISA was conducted as described in Chapter 2. On the positive half of the plate, 50µl of a known negative sample spiked with a 1:50 concentration of *E. granulosus* WWE was added. The negative half of the plate contained the same negative sample unspiked. After incubating and washing, 200µl of R91 conjugate (1:250 dilution in 0.3% PBSt) was added to the top row and serially diluted to give conjugate concentrations of 1:250 to 1:32000. The plate was again incubated, washed, and then 100µl of TMB (Insight Biotechnology, Wembley, UK) was added to each well (including blanks). After a 20 minute incubation, the plate was read using a Thermoscientific Multiscan FC platereader at 620nm.

The OD values on the positive side of the plate were divided by the corresponding OD values on the negative side of the plate to calculate the signal to noise ratio. Several capture and conjugate combinations looked promising and two new ELISAs were set up using different combinations of capture and conjugate (Fig. 3-2). The same ELISA protocol was used on two plates to test these combinations (with each row containing replicates of the same

combination), and the positive OD values were again divided by the negative OD values (Fig. 3-2).

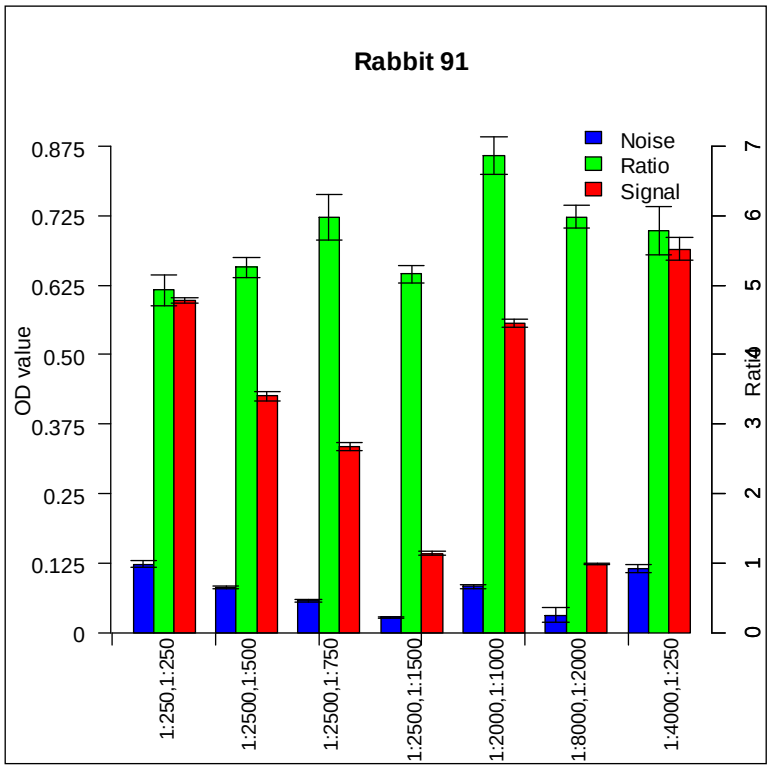

Figure 3-2: Rabbit 91 ratios of signal:noise against E. granulosus *WWE*
The x axis indicates capture/conjugate concentrations. Error bars indicate the standard error from the mean (S.E.M)

The combination with the highest signal to noise ratio was capture 1:2000 and conjugate 1:1000 (average ratio of 6.86), so a negative panel with known negative and positive samples was set up using these concentrations. The cut-off for positive samples is usually determined as the average negative value plus two or three standard deviations (e.g. Allan and Craig, 1989; Lahmar *et al.*, 2007b). A panel of 38 known negative samples (in duplicate) gave an average OD value of 0.0764, with a standard deviation of 0.0428, giving a cut-off of 0.205. However, three known positives (two spiked at 1:100 and one at 1:50 with *E. granulosus* whole worm extract) gave an average OD value of 0.252, with a standard deviation of 0.005. Therefore the difference between the negative cut off point and the high responders was too little, and the R91 capture and conjugate were deemed to be unsuitable for coproELISA testing for *Echinococcus* spp.

3.3.3 Assessment of Rabbit 93 antibodies

The same process as described for Rabbit 91 in section 3 was repeated with 13ml of serum from Rabbit 93 (a rabbit immunised with *E. granulosus* whole worm extract). In a checkerboard the signal to noise ratios were less promising, with the highest value being 4.6. A number of combinations (including Rabbit 91 and Rabbit 93 combinations), were tested but unfortunately the best combination (R93 1:4000/R91 1:1500) yielded a signal to noise ratio of only 3.6 (Fig. 3-3).

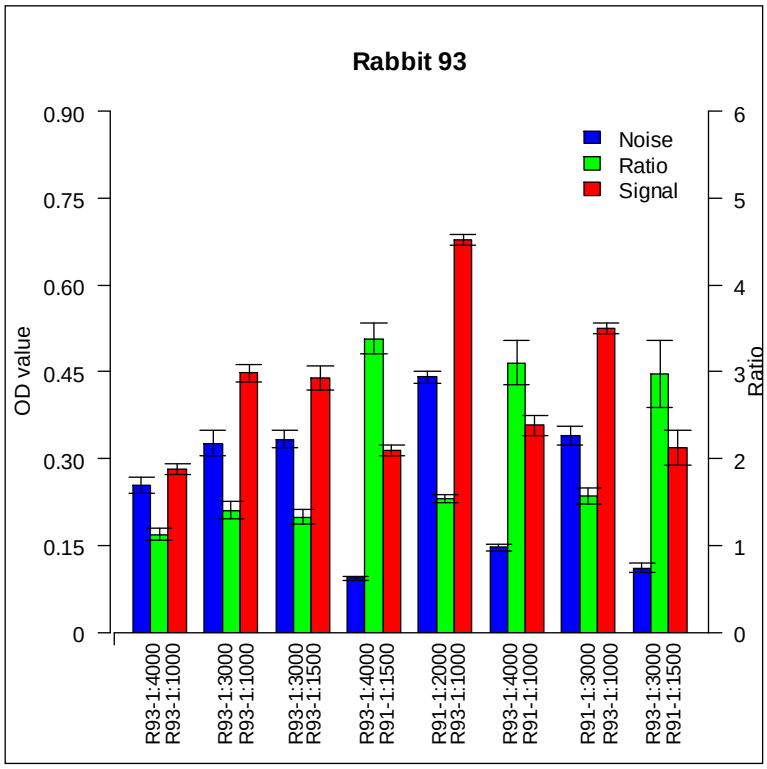

Figure 3-3: Rabbit 93 and 91 ratios of signal:noise against E. granulosus *WWE*
The x axis indicates capture/conjugate concentrations. Error bars indicate the standard error from the mean (S.E.M)

3.3.4 Assessment of Rabbit 5 antibodies

The same process as described for Rabbits 93 and 91 was repeated with 13ml of serum from Rabbit 5 (a rabbit immunised with *E. granulosus* whole worm extract). In a checkerboard the

signal to noise ratios were more promising, with the highest value being 17.38. A number of combinations were tested, yielding high signal to noise ratios (Fig. 3-4).

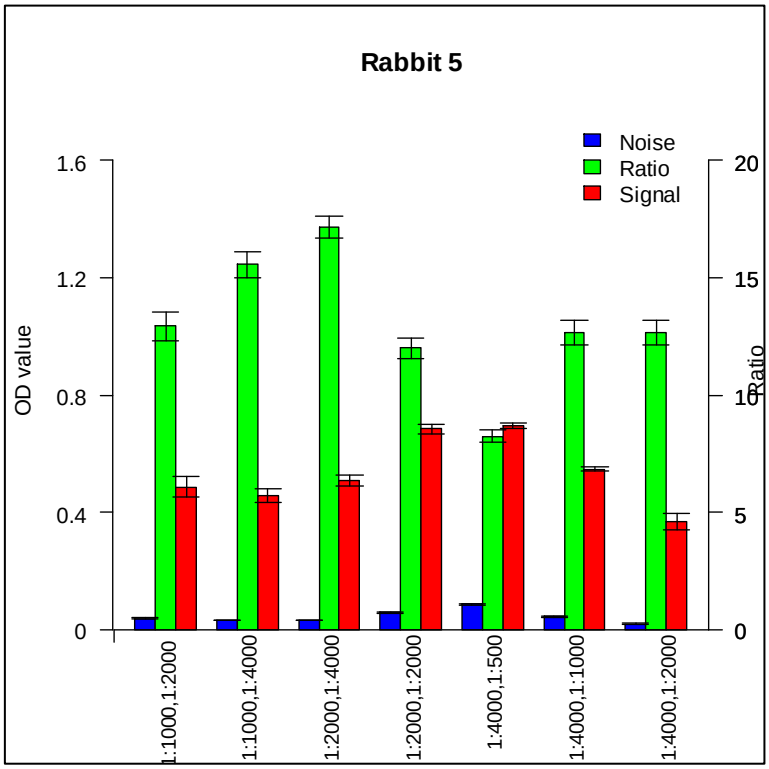

Figure 3-4: Rabbit 5 ratios of signal:noise against E. granulosus *WWE*
The x axis indicates capture/conjugate concentrations. Error bars indicate the standard error from the mean (S.E.M)

A negative panel was set up using R5 capture and conjugate in the following combinations: Capture 1:4000/Conjugate 1:2000, Capture 1:4000/Conjugate 1:1000, Capture 1:1000/Conjugate 1:4000. Ten known negatives and two known positives (natural infections) were used in the panel. The cut-off was determined as the average value for all the negative samples plus three standard deviations. For the first combination the cut-off was determined to be 0.1084, the second combination gave a cut-off of 0.289, and the third combination gave a cut-off of 0.0886 (Table 3-2). Despite the good results found in the checkerboard and panels of negative and spiked samples, when using natural samples the average ratios of positive to negative samples were much lower.

Table 3-2: O.D values for a panel of negative faecal samples (n=10) with different R5 capture/conjugate combinations

	1:4000 Px 1:2000	*1:4000 Px 1:1000*	*1:1000 Px 1:4000*
Mean negatives	0.0409	0.1234	0.0361
St dev negatives	0.0225	0.0552	0.0175
Cut off	0.1084	0.2890	0.0886
Mean positives	0.1965	0.5248	0.1730
St dev positives	0.0305	0.0309	0.0118

3.3.5 Assessment of a combination of antibodies from Rabbit5 and Rabbit 91 in ELISA

A combination of R5 and R91 capture and conjugate antibodies was tested on a checkerboard using spiked and known negative samples. The top half of the plate was coated with R5 capture antibody (concentrations of 1:500 to 1:1600) and the conjugate used was R91 (concentrations of 1:500 to 1:4000). The reverse combination (R91 capture and R5 conjugate) was used on the bottom half of the plate. This also gave good signal to noise ratios. For example R5 capture 1:4000 and R91 conjugate 1:1000 gave a signal to noise ratio of 19.6 and R91 capture 1:2000 and R5 conjugate 1:1000 gave a signal to noise ratio of 19.04.

Two promising combinations of R5 and R91 capture and conjugate (R5 capture 1:2000, R91 conjugate 1:2000 and R5 capture 1:2000, R91 conjugate 1:1500) were selected and used in a panel with 20 known negative samples, two known *Taenia* infections (*T. hydatigena* and *T. multiceps*), one known *E. equinus* infection, three known *E. multilocularis* infections, five natural *E. granulosus* infections, one experimental *E. granulosus* infection and one spiked sample (*E. granulosus* whole worm extract, 1:50). The same panel was tested using Rabbit47 at the tried and tested concentrations (capture 1:4000, conjugate 1:1500) to compare the new R5/R91 concentrations (see Chapter 3, Appendix 1).

The combination of R5 capture (1:2000) and R91 conjugate (1:2000) correctly identified 27/30 samples (90%), with no false positives and three false negatives, based on the cut-off calculated from 19 negatives tested. The R47 combination correctly identified 30/30 samples (100%) based on 19 negatives tested. The R5 capture (1:2000) and R91 conjugate (1:1500) combination appeared to be more promising than the combination of R5 capture (1:2000) and R91 conjugate (1:2000), with 28 out of 30 samples (93.3%) correctly identified with no false positives but two false negatives based on 19 negatives tested. To further test the R5 capture (1:2000) and R91 conjugate (1:1500) combination, another negative panel was set up using 43 samples from non-endemic areas (the Falklands, Manchester, one known negative sample from Wales, see Chapter 3, Appendix 2). This panel was used to calculate the cut-off point, which now was set at 0.0649 (Chapter 3, Appendix 2).

To further test the R5/R91 combination, a panel of known positive infections was tested (n=31). These included samples from the field site in Kyrgyzstan and were found positive through at least two methods including purge positives, previous ELISA positives (tested with R47), PCR positives and/or sequence positives as well as necropsy positive and spiked samples (see Chapter 3, Appendix 3).

The combination of R5 capture (1:2000) and R91 conjugate (1:1500) as tested with 43 known negative samples and 31 known positive samples gave no false positives but two false negatives, giving a diagnostic sensitivity of 93.5% and a diagnostic specificity of 100% (Fig. 3-5). However, these two false negatives likely represented very low worm burdens that may have been below the detection limit of the assay. Taken together, these results suggest that a sandwich ELISA using R5 and R91 antibodies was suitable to use for testing canine echinococcosis in unknown samples.

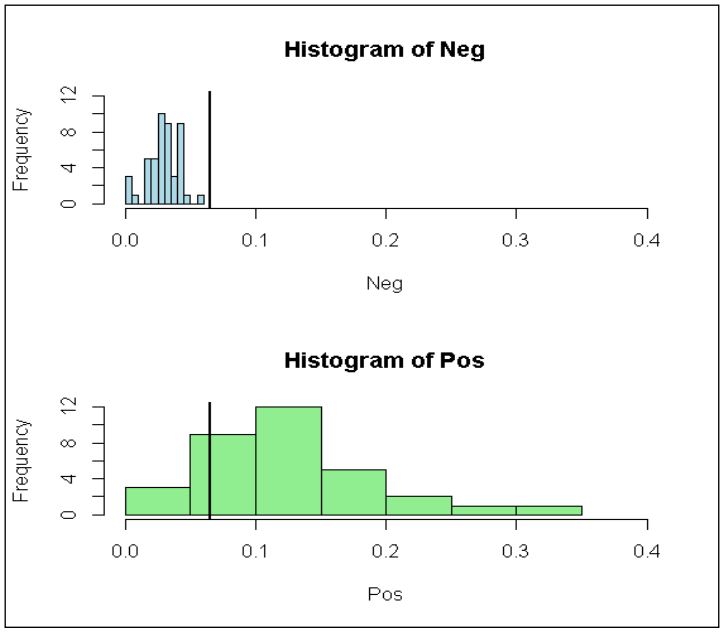

Figure 3-5: Distribution of OD values of the positive (n=32) and negative (n=43) samples tested with R5/R91 antibodies.
The line represents the cut-off at 0.0649

3.3.6 Calibration of R47 antibodies in ELISA

Polyclonal antibodies can be stored at -80°C, and may have a shelf life of several years (http://www.anaspec.com). However, loss of antibody activity has been found in antibodies stored for several years (http://www.labome.com). The in-house assay antibodies were derived from Rabbit47 sera in 1993 and were originally used at capture 1:8000/conjugate 1:4000 combination. However, in 2011 these concentrations failed to produce the expected results and using checkerboards and panels, new combinations were found at capture 1:4000/conjugate 1:2000. In December 2012, the R47 assay was once again failing to produce the expected results.

In order to calibrate the R47 assay, a new checkerboard was set up with capture concentrations ranging from 1:375 to 1:48000 and conjugate concentrations ranging from 1:375 to 1:12000. The combination of capture 1:3000 and conjugate 1:1500 produced the highest signal:noise ratio (15.36) and this combination was tested in panels of known

negative and positive samples. To calculate the cut-off point, the average of all the negatives was included, but those samples that gave poor replicates in OD values (more than 15% difference between the two values) were excluded. Furthermore, one unusually high OD value was excluded. This meant that a total of 35 samples were used to calculate the cut-off point, which now was set at 0.10604 (Chapter 3, Appendix 4).

To further test this R47 combination, a panel of known positive samples was tested. These samples were from the field site in Kyrgyzstan and were found positive through at least two methods including purge positives, previous ELISA positives (tested previously with R47), PCR positives and/or sequence positives. Those samples that gave poor replicates (i.e. 1 OD value above the cut-off point and 1 OD value below the cut-off point) were excluded, leaving 21 known positives (Chapter 3, Appendix 5). This combination of samples lead to 1 false positive and 1 false negative, giving a diagnostic sensitivity of 95.2% and a diagnostic specificity of 97.1%. These results suggest this R47 assay is suitable to use for testing canine echinococcosis in unknown samples.

3.3.7 Evaluation of a commercial *Echinococcus* spp. coproELISA kit

A commercial kit produced by the Lakeview Company in China was evaluated using known positive and negative samples. The kit was tested following the manufacturer's instructions, with the exception that the samples used were not fresh and made up in the provided sample diluents but were stored and extracted in 0.3% PBS Tween buffer. The instructions required for two wells to be kept blank, with only 100µL of sample diluents added to them. Negative and positive controls were provided in the kit, and these were tested in quadruplicate (100µL each). 100µL of each sample was added to wells in duplicate. The plate was then incubated at 37°C for 30 minutes. The plate was washed four times with the provided wash solution, leaving the solution in the wells for 1 minute each time. 50µL of conjugate was added to each

well except blanks, and the plate was again incubated at 37°C for 30 minutes and washed three times as before. 50µL of Substrate A and 50µL of Substrate B was added to each well and the plate was incubated at 37°C for 10 minutes. 50µL of Stop Solution was added to each well, and the plate was read on a plate reader (Thermo Scientific Multiskan FC®) at 450nm.

According to the instructions, the plate results were valid, as the negative controls gave OD values below 0.30, and the positive controls gave OD values above 0.50. The cut-off point was set at 2.1x the average negative OD value, or 0.399. Using this cut-off value, the kit correctly classified 23 samples (53.49%) and incorrectly classified 20 (48.84%) samples (Chapter 3, Appendix 6). With over 48% of samples classified incorrectly, this kit was not deemed to be a viable alternative for the in house ELISA. These results are consistent with another study in which Chinese commercial *Echinococcus* spp. diagnostic kits were evaluated, with Huang *et al*., (2014) finding poor diagnostic sensitivity and specificity in three commercially produced kits.

3.4 Discussion

ELISAs provide a sensitive way of detecting antigens, and the fact that antisera can be raised against most antigen preparations make them practical for a large range of studies. In the current study, antibodies derived from hyperimmune rabbit sera were tested, from rabbits inoculated with *E. granulosus* whole worm extract (R5, R93) or a surface saline wash (R91). Antibodies (IgG) were isolated from the sera using a Protein A column, and capture and conjugate antibodies were prepared. These were then tested for their suitability for use in a coproantigen ELISA. Furthermore, previously isolated antibodies (see Allan *et al.*, 1992) and a commercially available ELISA kit were also evaluated and compared.

The IgG fractions (from R5, R91, and R93) showed different reactivities to *E. granulosus*, as tested in checkerboards with spiked samples (spiked with whole worm extract). Both R5 and R91 rabbit antibodies showed promising results. After further testing, it was found that a combination of R5 capture antibodies and R91 conjugate antibodies showed the most promising results. After testing panels of known positive and negative samples, it was found that an R5/R91 capture and conjugate combination of 1:2000/1:1500 had a high diagnostic sensitivity (100%) and specificity (93.5%), and was found to be suitable for testing canid faeces for *Echinococcus* spp.

Panel testing of the archived R47 antibodies (from a rabbit immunised with *E. granulosus* WWE) was also conducted on known positive and negative samples. Although the newly tested R47 combination (capture 1:3000/conjugate 1:1500) showed good results, the variability in results obtained with R47 antibodies indicate that these antibodies may no longer be appropriate. As the R47 antibodies were isolated more than ten years ago, it may be that they have started to degrade, and this degradation appears to vary between aliquots (larger aliquots may be less susceptible to degradation). The commercial kit tested proved to be unreliable, with roughly half of all samples classified correctly. However, this coproELISA kit did require for samples to be extracted in the provided diluents, and the faecal samples used here were extracted in 0.3% PBS Tween buffer, which may have affected the results.

Following the results given by testing the R5/R91 antibody combination, the new R47 antibody concentrations and the commercial kit it was decided that the R5/R91 antibody combination was the most suitable and should be used to test the samples collected in Kyrgyzstan as part of this study.

3.5 Chapter 3 summary

Canine echinococcosis may be detected by using coproELISAs to detect antigens in faecal samples. This approach has the advantage of being non-invasive, and samples can be analysed relatively easily and affordably (Craig *et al.*, 1995; Allan and Craig, 2006). In order to develop coproELISAs for the detection of *Echinococcus* spp. in faecal samples, antibodies are required. Many ELISAs use polyclonal antibodies, as these often show good affinity to a range of antigens and can be produced more quickly and affordably than monoclonal antibodies (Leenaars and Hendriksen, 2005). Here, previously produced hyperimmune rabbit sera were tested for affinity to *E. granulosus* whole worm extract. Three sera that showed promising results (from Rabbit 91 and Rabbit 93, immunised against *E. granulosus* whole worm extract, and Rabbit 5, immunised against *E. granulosus* surface saline wash) were selected and antibodies were extracted using a Protein A column. Capture and conjugate antibodies were produced from these three sera, and tested for suitability in coproELISA use. This included testing the antibodies with checkerboard ELISAs, and testing the antibodies with panels of known *Echinococcus* spp. positive and negative samples.

Following testing it was found that a combination of R5 capture antibodies (1:2000 dilution) and R91 conjugate antibodies (1:1500 dilution) produced the best results, with panel testing finding a 100% diagnostic specificity and 93.5% diagnostic sensitivity. Previously created polyclonal antibodies (isolated from Rabbit 47 in 1993; antibodies used at Salford University for Cestode Diagnostics, see http://www.star.salford.ac.uk/page/Cestode_Diagnostics) were also calibrated and tested. Although new concentrations of capture (1:3000) and conjugate (1:1500) produced good results (diagnostic sensitivity of 95.2%, specificity of 97.1% as tested with panels of samples of known *Echinococcus* spp. status), concerns over the possible degradation of these antibodies produced in 1993 meant that preference was given to the

newly isolated R5/R91 antibodies. A Chinese commercial coproELISA kit (Lakeview) for diagnosing canine echinococcosis was also evaluated. However, this kit incorrectly classified 48.84% of tested samples and was therefore not considered suitable.

Chapter 4: Development of multiplex and uniplex PCR assays for the differentiation of *E. granulosus sensu stricto*, *E. equinus*, *E. multilocularis* and *E. canadensis* DNA in tissue and faecal samples

4.1 Introduction

Echinococcosis is a zoonotic parasitic disease caused by cestode tapeworms in the genus *Echinococcus*. In Eurasia, echinococcosis may be cystic or alveolar, with cystic echinococcosis usually characterized by unilocular cysts, and alveolar echinococcosis usually characterized by multivesiculated cysts (WHO/OIE, 2001). In both diseases, cysts usually occur in the liver, lungs, or, less commonly in the spleen, kidneys, heart, bone, and central nervous system (Moro and Schantz, 2009). If untreated, echinococcosis is often fatal (Fujikura, 1991; Moro and Schantz, 2009).

Although *E. multilocularis* has long been recognized as a species, the taxonomy of other *Echinococcus* spp. has been subject to much controversy (Tappe *et al.*, 2010, for details see section1.2). The clade of *E. granulosus* G1 (common sheep strain), G2 (Tasmanian sheep strain) and G3 (buffalo strain) is now often referred to simply as *E. granulosus* (Thompson, 2008). Similarly, *E. canadensis* is now recognized as a separate species complex by many researchers (e.g. Tappe *et al.*, 2010), which comprises the G6 (camel), G7 (pig), G8 (cervid), G9 (Polish) and G10 (Fennoscandivanian) strains of *E. granulosus sensu lato* *E. granulosus sensu stricto* (G1,G2,G3) is the main cause of human echinococcosis worldwide (Alvares Rojas *et al.*, 2014) but *E. canadensis* has also been found to be infectious to humans, especially the G6 strain (e.g. Bart *et al.*, 2006). *E. multilocularis* has long been known to be infective to humans and is the cause of alveolar echinococcosis in human patients (Eckert and Deplazes, 2004).

Domestic dogs are final hosts for *E. granulosus* (e.g. Budke *et al.*, 2005), *E. equinus* (e.g. Williams and Sweatman, 1963), *E. multilocularis* (e.g. Budke *et al.*, 2005) and *E. canadensis* (e.g. Bart *et al.*, 2006), and canine echinococcosis can be established by non-invasive analysis of faecal samples. This may involve genus specific coproELISAs (e.g. Jenkins *et al.*, 2000) and/or species/strain specific coproPCRs (e.g. Boufana *et al.*, 2013b). Because *Echinococcus* spp. are closely related, it is important to be able to distinguish different species or strains. For epidemiological purposes it is important to identify the species of *Echinococcus* present, especially as the different species may have different degrees of infectivity to humans and different pathologies. In many areas it appears that *E. granulosus* and *E. canadensis* are co-endemic, such as Xinjiang (Bart *et al.*, 2006), and Tunisia (M'rad *et al.*, 2005), and *E. granulosus*, *E. multilocularis* and *E. canadensis* now appear to be co-endemic in Kyrgyzstan (van Kesteren *et al.*, 2013a, see Chapter 6). Although several coproPCRs are available for the detection of *Echinococcus* spp. (e.g. Abbasi *et al.*, 2003; Dinkel *et al.*, 2011; Boufana *et al.*, 2013b), no test is currently available that allows for easy differentiation of *E. granulosus, E. equinus, E. multilocularis* and *E. canadensis*. This chapter describes the development of PCR protocols that could be used to distinguish these species in both tissue and faecal samples.

4.2 Methods

4.2.1 Primer design

Two sets of primers were designed by Dr. Boufana for the detection of *Echinococcus* spp. using a multiplex PCR following the methods described by Lett (2013). Briefly, relevant mitochondrial genetic sequences were found in the Genbank database (http://www.ncbi.nlm.nih.gov/nucleotide), and transferred to a ClustalW programme (http://www.genome.jp/tools/clustalw/) for comparison of nucleotides by multiple sequence alignment. The first set of primers, EgenF (5'ATT TGG TTG ATT TGA TGG TAG T 3')

and EgenR (5'CCA ACT TAT ATG TCT CAA ATG 3') were used to amplify *E. canadensis*. The second set of primers, Egen1/4F (5' GTT GTC TCT TTA CTA TTT AGT TG 3') and Egen1/4R (5' CAC TTC TGA CAT AGC TAC AGC ACC 3') were used to amplify *E. granulosus, E. equinus* and *E. multilocularis*.

4.2.2 DNA extractions

DNA was extracted from tissue samples using a Qiagen DNEasy blood and tissue kit (QIagen, Hilden, Germany), following the manufacturer's instructions (Qiagen, 2006). Tissue samples used are given in Table 4-1. DNA was extracted from faecal samples using a Qiagen QIAamp® DNA Stool kit, following the manufacturer's instructions, with the exception of using 1g instead of 0.1g of faeces (Qiagen, 2010), and adjusting the lysis buffer volume.

Table 4-1: DNA from cestode tissue samples used in testing PCR primers[4].

Species	Source	Location	DNA ng/µL
E. granulosus (G1)	Sheep cysts	Tunisia	18.4
E. granulosus (G2)	Sheep cysts	Argentina	17.7
E. granulosus (G3)	Buffalo (*Bubalus bubalis*) cysts	Italy	4.4
E. equinus	Horse cyst	United Kingdom	22.7
E. ortleppi	Philippine spotted deer (*Rusa alfredi*) cyst	United Kingdom (translocated from France)	0.2
E. canadensis (G6)	Adult worm	Kyrgyzstan	2.7
E. canadensis (G7)	Pig cyst	Slovakia	2
E. canadensis (G8)	Deer (*Odocoileus virginianus*) cyst	Minnesota, USA	1.1
E. canadensis (G10)	Adult worm	Finland	10.2
Taenia multiceps	Adult worm	United Kingdom	23.5
Taenia ovis	Adult worm	United Kingdom	16.9
Taenia crassiceps	Adult worm	United Kingdom	26.1
Taenia pissiformis	Adult worm	United Kingdom	24.9
Taenia hydatigena	Adult worm	United Kingdom	22.8
Dipyllidium caninum	Adult worm	United Kingdom	1.4
E. multilocularis	Adult worm	Kyrgyzstan	4.5
E. shiquicus	Adult worm	China	21.15

[4] DNA from adult *E. granulosus* G1,G2, G3, *E. equinus, E. ortleppi, E. canadensis* G7, G8, G10, *T. multiceps, T. crassiceps, T. pissiformis, T. hydatigena, D. caninum*, and E. *shiquicus* provided by Dr. Boufana (Naidich *et al.*, 2006; Boufana *et al.*, 2008; 2012; 2013b). DNA from *E. multilocularis* and *E. canadensis* G6 isolated from adult worms in dogs purged with arecoline in the Alay Valley, Kyrgyzstan (see Chapter 6).

4.2.3 *E. granulosus sensu stricto*, *E. equinus* and *E. multilocularis* PCR protocol

To amplify *E. granulosus sensu stricto* (G1,G2,G3), *E. equinus* and *E. multilocularis*, primers Egen1/4F (5'GTT GCT TCT TTA CTA TTT AGT TC 3') and Egen1/4R (5'CAC TTC TGA CAT AGC TAC AGC ACC 3') were used. The reaction volume (total of 50µL) was as follows: 5X GoTaq buffer (Promega, Southampton, UK), 0.15µM of each primer, 200 mM of dNTPs (Bioline, London, UK), 1 Mm $MgCl_2$, 2.5 units of Taq polymerase (Promega, Southampton, UK), and 1µL of tissue DNA or 5µL of coproDNA. The cycling profile was conducted using an Applied Biosystems Veriti 96 well thermal cycler as follows: 5 minutes at 94°C for 1 cycle, followed by 35 cycles of 30 seconds at 94°C, 54°C and 72°C. Resulting PCR products were run on 3% agarose gels (Bioline, London, UK) stained with gel red DNA dye (Cambridge Biosciences UK), at 110v for 2 hours and read at 200ms on a transilluminator using a Syngene G:Box gel documentation system (Cambridge Biosciences, UK).

4.2.4 *E. canadensis* PCR protocol

To amplify *E. canadensis*, primers EgenF (5'ATT TGG TTG ATT TGA TGG TAG T 3') and EgenR (5' CCA ACT TAT ATG TCT CAA ATG 3') were used. The reaction volume (total of 50µL) was as follows: 5X GoTaq buffer (Promega, Southampton, UK), 0.20µM of each primer, 200 mM of dNTPs (Bioline, London, UK), 1 Mm $MgCl_2$, 2.5 units of Taq polymerase (Promega, Southampton, UK), and 1 µL of tissue DNA or 5µL of coproDNA. The cycling profile was conducted using an Applied Biosystems Veriti 96 well thermal cycler as follows: 5 minutes at 94°C for 1 cycle, followed by 35 cycles of 30 seconds at 94°C, 54°C and 72°C. Resulting PCR products were run on 3% agarose gels (Bioline, London, UK), stained with gel red DNA dye (Cambridge Biosciences UK), at 110v for 2

hours and read at 200ms on a transilluminator using a Syngene G:Box gel documentation system (Cambridge Biosciences, UK).

4.2.5 Multiplex PCR protocol

To amplify *E. granulosus sensu stricto* (G1,G2,G3), *E. equinus*, *E. multilocularis* and *E. canadensis*, all four primers were combined in a multiplex PCR. The protocol was as follows: a 50µL reaction volume containing 5X GoTaq buffer (Promega, Southampton, UK),, 0.15µM of each primer (Egen1/4F 5'GTT GCT TCT TTA CTA TTT AGT TC 3', Egen1/4R 5'CAC TTC TGA CAT AGC TAC AGC ACC 3', EgenF 5'ATT TGG TTG ATT TGA TGG TAG T 3', EgenR 5' CCA ACT TAT ATG TCT CAA ATG 3'), 200 mM of each deoxynucleoside triphosphate (dNTPs; Bioline, London, UK), 1 Mm $MgCl_2$, 2.5 units of Taq polymerase (Promega, Southampton, UK), and tissue DNA or 5µL of coproDNA. The cycling profile was conducted using an Applied Biosystems Veriti 96 well thermal cycler as follows: 5 minutes at 94°C for 1 cycle, followed by 35 cycles of 30 seconds at 94°C, 54°C and 72°C. Resulting PCR products were run on 3% agarose gels (Bioline, London, UK) stained with gel red DNA dye (Cambridge Biosciences UK), at 110v for 2 hours and read at 200ms on a transilluminator using a Syngene G:Box gel documentation system (Cambridge Biosciences, UK).

4.2.6 Sequencing

PCR products were sequenced by Beckman Coulter (Essex, UK). Nucleotide sequences were analysed using the FinchTV software package (Geospiza, Seattle, WA) and compared with those in the GenBank database through the use of BLAST biosoftware (www.ncbi.nlm.nih.gov/BLAST/).

4.2.7 Evaluation of analytic PCR tissue DNA specificity

The analytic specificity (defined here as the ability of the coproPCR to detect the species targeted and not detect other, related species) of the multiplex and uniplex assays was tested using DNA extracted from tissue samples of cestodes (Table 4-1). This full panel was tested three times using two different thermocyclers. Because of limited amounts of DNA available, a panel consisting of a subset of these samples (*E. granulosus* G1 from sheep, *E. equinus*, *E. canadensis* G7, *T. multiceps*, *T. ovis*, *T. crassiceps*, *T. hydatigena*, *D. caninum* and *E. shiquicus*) was tested a further four times using two different thermocyclers.

4.2.8 Evaluation of analytic PCR coproDNA specificity

A panel of faecal samples was tested using each uniplex PCR. This panel included eighteen samples of known infection and species status, namely experimental infections with *E. equinus* (n=5 faecal samples), experimental infections with *E. granulosus sensu stricto* (n=8 faecal samples), experimental infections with *T. multiceps* (n=1 faecal sample) and arecoline, PCR and sequence positive samples of *E. granulosus* sensu lato (n=3) and *E. canadensis* G6 (n=1 faecal sample). In addition five samples that were of unknown status but had previously tested positive with other primers for *E. granulosus sensu stricto* (n=2 from Kazakhstan, Boufana *et al.*, 2013b) and *E. granulosus sensu lato* (n=3 from Alay Valley, Kyrgyzstan, Abbasi *et al.*, 2003) and samples of unknown status collected from the Alay Valley that had previously tested positive for *E. multilocularis* using ND1 primers (Boufana *et al.*, 2013b, n=10 faecal samples) were tested. Because of limited amounts of DNA available not every sample could be tested in each PCR (Table 4-2). Subsets of PCR positive samples were sent for sequencing to Beckman Coulter UK (Table 4-2).

Table 4-2: Canid faecal samples (n=33) used in testing PCR primers.[5]

Species	Source	Egen1/4 F&R	Sequenced?	EgenF&R?	Sequenced?
E. equinus #1	Wales (experimental infection)	Positive	Poor sequence	Negative	n/a
E. equinus #2	Wales (experimental infection)	Positive	Poor sequence	Negative	n/a
E. equinus #3	Wales (experimental infection)	Negative	n/a	Negative	n/a
E. equinus #4	Wales (experimental infection)	Negative	n/a	Negative	n/a
E. equinus #5	Wales (experimental infection)	Negative	n/a	Negative	n/a
E. granulosus s.s. #1	Australia (experimental infection)	Positive	*E. granulosus* G1	n/a	n/a
E. granulosus s.s. #2	Australia (experimental infection)	Positive	*E. granulosus* G1	Negative	n/a
E. granulosus s.s. #3	Australia (experimental infection)	Positive	n/a	Negative	n/a
E. granulosus s.s. #4	Australia (experimental infection)	Positive	n/a	Negative	n/a
E. granulosus s.s. #5	Australia (experimental infection)	Positive	n/a	n/a	n/a
E. granulosus s.s. #6	Australia (experimental infection)	Positive	n/a	Negative	n/a
E. granulosus s.s. #7	Australia (experimental infection)	Positive	n/a	Negative	n/a
E. granulosus s.s. #8	Australia (experimental infection)	Positive	n/a	Negative	n/a
E. granulosus s.l. #1	Kyrgyzstan (arecoline purge positive, sequence positive)	Negative	n/a	Negative	n/a
E. granulosus s.l. #2	Kyrgyzstan (arecoline purge positive, sequence positive)	Positive	*E. granulosus* G1	n/a	n/a
E. granulosus s.l. #3	Kyrgyzstan (arecoline purge positive, sequence positive)	Positive	n/a	Positive	None found
E. canadensis G6	Kyrgyzstan (arecoline purge positive, sequence positive)	Negative	n/a	Positive	*E. canadensis*
T. multiceps	Experimental infection	Negative	n/a	Negative	n/a
E. granulosus s.s. A	Kazakhstan (tested with ND1 primers)	Positive	n/a	n/a	n/a
E. granulosus s.s. B	Kazakhstan (tested with ND1 primers)	Positive	n/a	n/a	n/a
E. granulosus s.l. A	Kyrgyzstan (tested with Abassi primers)	Positive	*E. granulosus* G1	Positive	*E. canadensis*
E. granulosus s.l. B	Kyrgyzstan (tested with Abassi primers)	Positive	*E. granulosus* G1	Positive	n/a
E. granulosus s.l. C	Kyrgyzstan (tested with Abassi primers)	Negative	n/a	Negative	n/a
E. multilocularis #1	Kyrgyzstan (tested with ND1 primers)	Negative	n/a	Negative	n/a
E. multilocularis #2	Kyrgyzstan (tested with ND1 primers)	Negative	n/a	Negative	n/a
E. multilocularis #3	Kyrgyzstan (tested with ND1 primers)	Negative	n/a	n/a	n/a
E. multilocularis #4	Kyrgyzstan (tested with ND1 primers)	Negative	n/a	n/a	n/a
E. multilocularis #5	Kyrgyzstan (tested with ND1 primers)	Negative	n/a	n/a	n/a
E. multilocularis #6	Kyrgyzstan (tested with ND1 primers)	Negative	n/a	n/a	n/a
E. multilocularis #7	Kyrgyzstan (tested with ND1 primers)	Negative	n/a	n/a	n/a
E. multilocularis #8	Kyrgyzstan (tested with ND1 primers)	Positive	Poor sequence	Negative	n/a
E. multilocularis #9	Kyrgyzstan (tested with ND1 primers)	Positive	*E. multilocularis*	Negative	n/a
E. multilocularis #10	Kyrgyzstan (tested with ND1 primers)	Positive	*E. multilocularis*	Negative	n/a

4.2.9 Evaluation of analytic PCR sensitivity

DNA from *E. granulosus* G1, *E. equinus* and *E. canadensis* G6 was diluted to a concentration of 20ng/µL (measured using a Thermo Scientific Nanodrop 2000), and then serially diluted to 9.8pg/µL and tested with the multiplex PCR protocol. The analytic sensitivity (defined here as the lowest concentration of DNA that could be detected by the coproPCR) of the uniplex Egen1/4F and Egen1/4R PCR was tested using serial dilutions of

[5]*Faecal samples from dogs experimentally infected with* E. equinus *(#1-#5, see also Lett, 2013),* E. granulosus *sensu stricto (#1-8) and* T. multiceps *as well as two samples from Kazakhstan provided by Dr. Boufana. Remainder of samples collected from the Alay Valley and diagnosed with other coproPCR protocols.*

E. granulosus (G1) and *E. equinus* DNA from 16,600 and 17,600 pg/µL to 1 pg/µL respectively, as well as using serial dilutions of *E. multilocularis* DNA from 14,000 pg/µL to 0.4 pg/µL. The analytic sensitivity of the uniplex EgenF and EgenR PCR was tested using serial dilutions of *E. canadensis* (G6) DNA from 12,700pg/µL to 1pg/µL. All samples were tested in one PCR, run on 3% gels at 110v for 2 hours and read at 200ms on a transilluminator using a Syngene G:Box gel documentation system (Cambridge Biosciences, UK). In addition, to test the analytic sensitivity of the Egen1/F and Egen1/4R protocol, three negative samples (1 gram) spiked with 1000, 10 and 1 and 1 *E. granulosus* eggs retrieved from a naturally infected Chinese dog were tested (provided by B. Boufana, see Boufana *et al.*, 2013b)

4.3 Results

4.3.1 Analytic specificity of the multiplex PCR when tested with cestode tissue DNA

The multiplex PCR was able to detect *E. granulosus* (strains G1, G2, G3), *E. equinus*, *E. canadensis* (G6, G7, G10), and, less effectively, *E. multilocularis*, with diagnostic bands of ~130 base pairs (bp) for *E. canadensis* and diagnostic bands of ~100 bp for *E. granulosus*, *E. equinus* and *E. multilocularis*. No diagnostic band was observed for *E. ortleppi*, strain G8 of *E. canadensis*, *E. shiquicus*, *D. caninum*, nor the *Taenia* species, although *T. multiceps* and *T. ovis* did give fainter bands of a smaller size (Fig. 4-1).

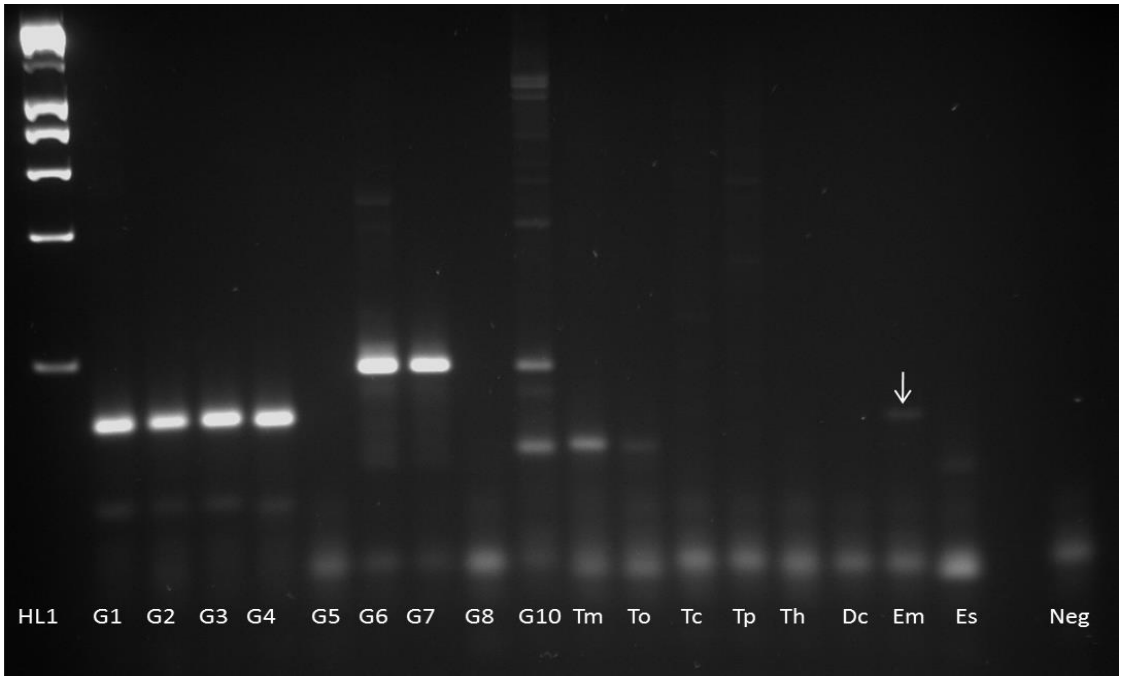

Figure 4-1 PCR products from a panel of cestode tissue samples tested with the multiplex coproPCR.

From left to right: Hyperladder 1, *E. granulosus* G1, *E. granulosus* G2, *E. granulosus* G3, *E. equinus* (G4), *E. ortleppi* (G5), *E. canadensis* G6, *E. canadensis* G7, *E. canadensis* G8, *E. canadensis* G10, *T. multiceps* (Tm), *T. ovis* (To), *T. crassiceps* (Tc), *T. pissiformis* (Tp), *T. hydatigena* (Th), *D. caninum* (Dc), *E. multilocularis* (Em) and *E. shiquicus* (Es). Neg refers to the negative control (PCR grade water).

4.3.2 Analytic specificity of the uniplex PCRs when tested with tissue DNA

Each set of primers (Egen1/4F and Egen1/4R and EgenF and EgenR) was used in a uniplex PCR with the tissue DNA panel. These uniplex reactions showed that the Egen1/4F and Egen1/4R primers amplified *E. granulosus sensu stricto* (G1), *E. equinus*, and, to a lesser extent, *E. multilocularis* (Fig. 4-2) and the EgenF and EgenR primers amplified *E. canadensis* G7 (Fig. 4-3). The primers showed no cross reaction with *T. multiceps*, *T. ovis*, *T. crassiceps*, *T. pissiformis*, *T. hydatigena*, or *D. caninum*. *E. shiquicus* was also not detected by either primer pair.

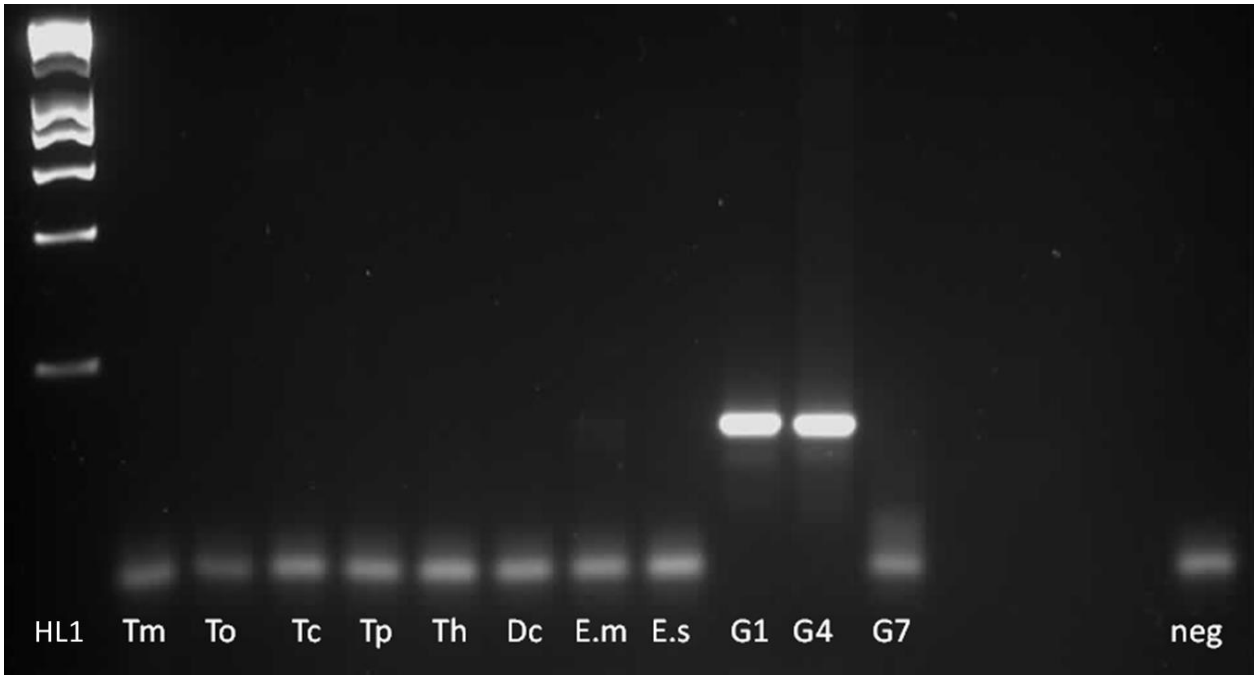

Figure 4-2: Uniplex PCR with primers Egen1/4F and Egen1/4R.

From left to right, Hyperladder 1, *T. multiceps* (Tm), *T. ovis* (To), *T. crassiceps* (Tc), *T. pissiformis* (Tp), *T. hydatigena* (Th), *D. caninum* (Dc), *E. multilocularis* (E.m), *E. shiquicus* (E.s), *E. granulosus* G1 from sheep, *E. equinus* (G4), and *E. canadensis* G7. Neg refers to the negative control (PCR grade water).

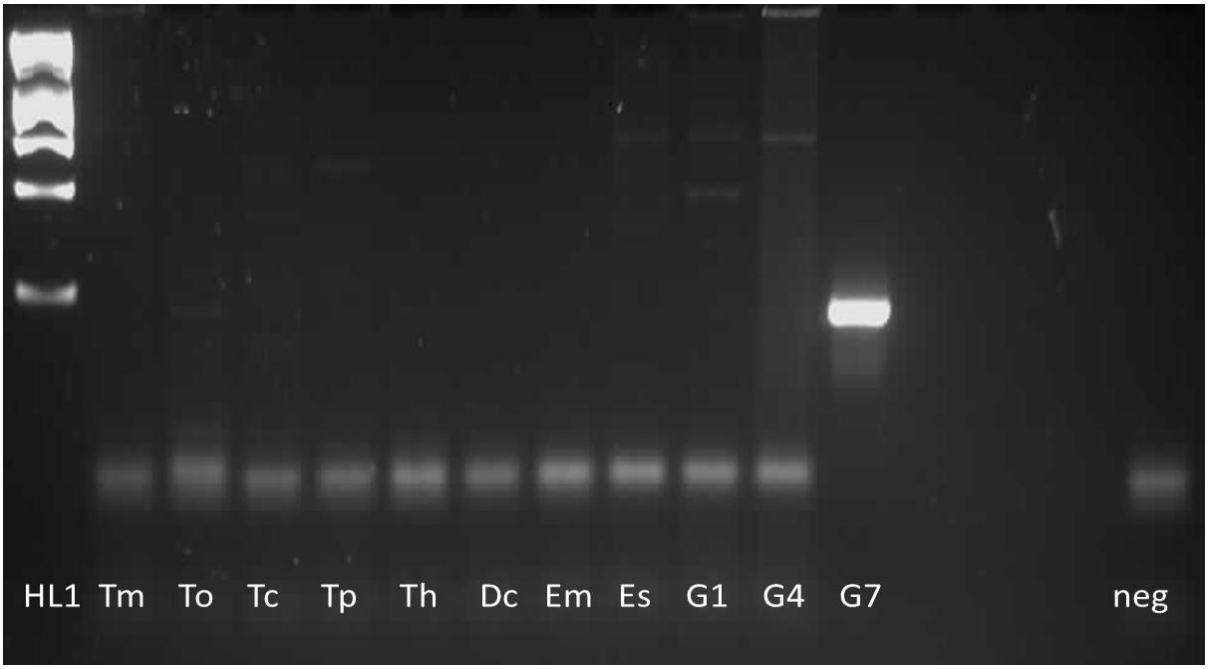

Figure 4-3: Uniplex PCR with primers EgenF and EgenR.

From left to right, Hyperladder 1, *T. multiceps* (Tm), *T. ovis* (To), *T. crassiceps* (Tc), *T. pissiformis* (Tp), *T. hydatigena* (Th), *D. caninum* (Dc), *E. multilocularis* (Em), *E. shiquicus* (Es), *E. granulosus* G1 from sheep, *E. equinus* (G4), and *E. canadensis* G7.

4.3.3 Sequencing results

PCR products from tissue DNA from *E. granulosus* G1, *E. equinus, E. canadensis* G7 and *E. multilocularis* as tested with the multiplex PCR were sequenced (Beckman Coulter, Essex, UK). *E. granulosus* G1, *E. equinus*, and *E. multilocularis* were sequenced with primers Egen1/4F and Egen1/4R, and *E. canadensis* G7 was sequenced using primers EgenF and EgenR. *E. granulosus* G1 gave a 100% match to ncbi database accession number AF297617.1 (*Echinococcus granulosus* genotype 1 mitochondrion, complete genome**)**. *E. equinus* gave a 99% match to ncbi database accession number AF346403.1 (*Echinococcus equinus* mitochondrion, complete genome). *E. canadensis* G7 gave a 99% match to ncbi accession number AB235847.1 (*Echinococcus canadensis* mitochondrial DNA, complete genome, genotype G7). *E. multilocularis* gave a 98% match to ncbi accession number AB018440.2 (*Echinococcus multilocularis* mitochondrial DNA, complete genome).

4.3.4 Analytic sensitivity of the multiplex and uniplex PCRs when tested with tissue DNA

There was evidence that the multiplex PCR had different analytic sensitivities for DNA from *E. granulosus* G1, *E. equinus* and *E. canadensis* G6. Bands were visible for *E. granulosus* G1 and *E. canadensis* G6 even at the lowest concentration of 9.8 picograms, but for *E. equinus* the lowest concentration to give a visible band was 78.1 picograms (Fig. 4-4).

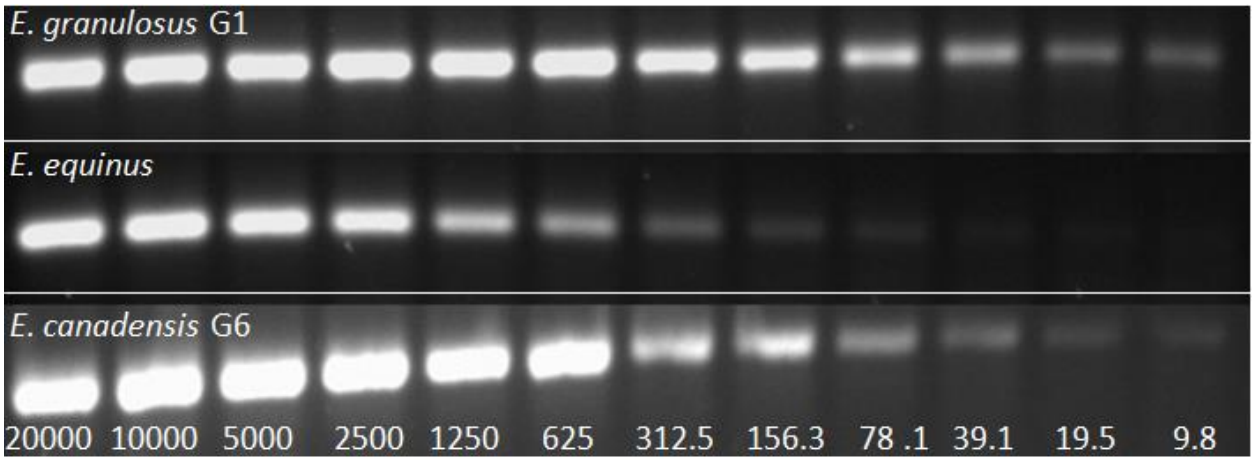

Figure 4-4: Multiplex PCR with serial dilutions of E. granulosus *G1 (top),* E. equinus *(middle) and* E. canadensis *G6 (bottom) from 20,000 to 9.8 pg of DNA*

As well as testing the analytic sensitivity of the multiplex, the analytic sensitivity of the uniplex PCRs was tested using serial dilutions of DNA from *E. granulosus* G1, *E. equinus* (for Egen1/4F and Egen1/4R) and *E. canadensis* G6 and G7 (for EgenF and EgenR). *E. granulosus* G1 DNA was serially diluted from 16,600 pg/µL to 1.01 pg/µL, and *E. equinus* DNA was serially diluted from 17,600 pg/µL to 1.07 pg/µL. For *E. granulosus* G1 a visible band occurred until a dilution of 8.1 pg/µL. The PCR was less sensitive for *E. equinus*, with visible bands up to a dilution of 34.4 pg/µL (Fig. 4-5). In addition the Egen1/4F and R primers gave visible bands for three faecal samples that were spiked with 1000, 10 and 1 *E. granulosus* eggs (data not shown, for details on samples see Boufana *et al.*, 2013b).

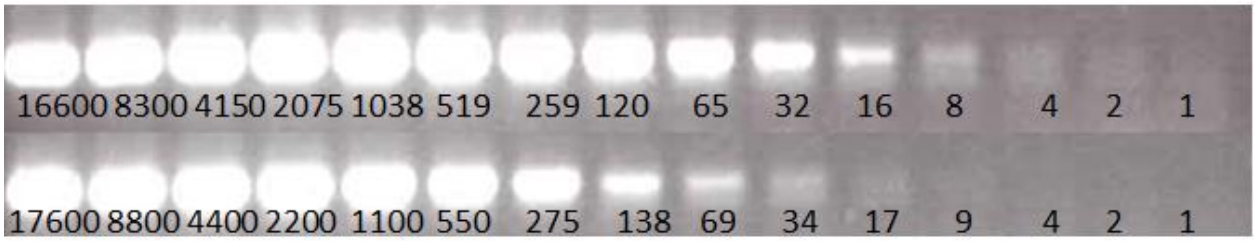

Figure 4-5*: Serial dilutions of* E. granulosus *G1 DNA (top) from 16,600 pg/µL to 1 pg/µL and* E. equinus *DNA (bottom) from 17,600 pg/µL to 1 pg/µL.*
Writing shows dilutions to the nearest pg.

Because the Egen1/4F and Egen1/4R primers appeared to detect *E. multilocularis* DNA, a serial dilution of *E. multilocularis* DNA from a concentration of 14,000 pg/µL to 0.4 pg/µL

was tested in this uniplex PCR. Very faint bands were produced for high concentrations of *E. multilocularis* DNA (14,000, 7,000 and 3,500 pg/µL) but not for lower concentrations (Fig. 4-6).

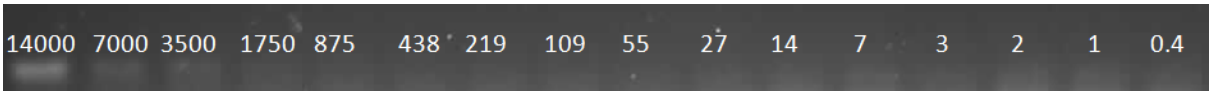

Figure 4-6*: Serial dilution of* E. multilocularis *DNA from 14,000 pg/µL to 0.4 pg/µL. Writing shows dilutions to the nearest pg.*

E. canadensis G6 DNA was diluted from 12,700 pg/µL to 0.78 pg/µL, and *E. canadensis* G7 DNA was diluted from 12,900 pg/µL to 0.78 pg/µL. Both gave visible bands at the highest dilution, although the analytic sensitivity for G6 appeared to be greater (Fig. 4-7).

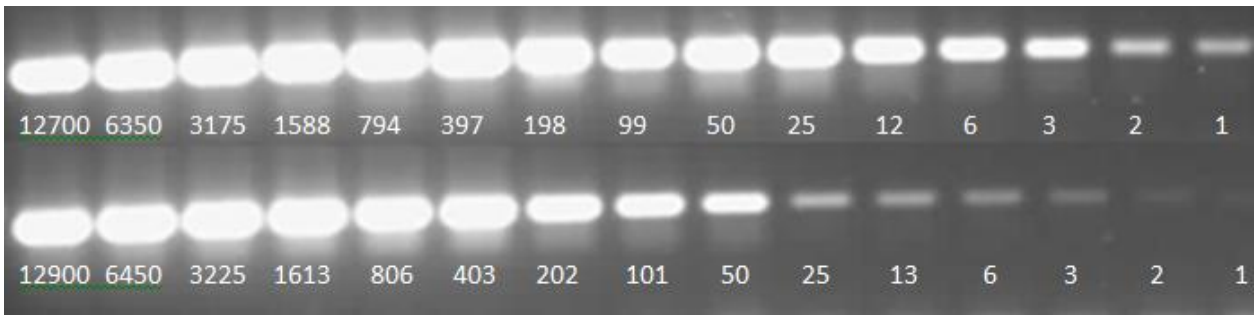

Figure 4-7*: Serial dilutions of* E. canadensis *G6 DNA (top) from 12,700 pg/µL to 1 pg/µL and* E. canadensis *G7 DNA (bottom) from 12,900 pg/µL to 1 pg/µL. Writing shows dilutions to the nearest pg*

4.3.5 Analytic PCR coproDNA specificity

All 33 faecal samples (Table 4-2) were tested with primers Egen1/4F and Egen1/4R and 23 of these were also tested with primers EgenF and EgenR. Of the eighteen samples of known infection and species status, the Egen1/F and Egen1/4R primers detected two of the five *E. equinus* experimental infections, and eight of eight *E. granulosus sensu stricto* experimental infections; two of these were successfully sequenced (by Beckman Coulter, Essex, UK) as *E. granulosus* G1 (100% match to ncbi database accession number AF297617.1, *Echinococcus granulosus* genotype 1 mitochondrion, complete genome). The one confirmed naturally

infected sample of *E. canadensis* (an arecoline purge positive sampled from Kyrgyzstan) was PCR negative with primers Egen1/4F and Egen1/4R but positive with primers EgenF and EgenR and sequenced as *E. canadensis* (99% match to ncbi database accession number AB208063.1, *Echinococcus canadensis* mitochondrial DNA, complete genome).

Of the three arecoline purge *E. granulosus sensu lato* samples (as sequenced with Abbasi *et al.*, (2003) primers) from Kyrgyzstan, one was PCR positive with primers Egen1/4F and Egen1/4R and negative for primers EgenF and EgenR, one was PCR positive for both sets of primers suggesting a mixed infection, but one was negative using both sets of primers. The EgenF and EgenR primers did not detect the *E. equinus* and *E. granulosus sensu stricto* samples tested. Of the unknown samples that had previously tested positive for *E. granulosus sensu lato* (Abbasi *et al.*, 2003), *E. granulosus sensu stricto* (Boufana *et al.*, 2013b) and *E. multilocularis* (Boufana *et al.*, 2013b) using other primers, the Egen1/4F and Egen1/4R primers detected two of two samples tested with ND1 *E. granulosus* primers (Boufana *et al.*, 2013b), and two of three samples tested with the "Abbasi" primers (Abbasi *et al.*, 2003), which were sequenced as *E. granulosus* G1 (100% match to ncbi database accession number AF297617.1, *Echinococcus granulosus* genotype 1 mitochondrion, complete genome). In addition, the Egen1/4F and Egen1/4R primers detected three of ten samples tested with ND1 *E. multilocularis* primers, and two of these were sequenced as *E. multilocularis* (100% match to ncbi database accession number AB018440.2, *Echinococcus multilocularis* mitochondrial DNA, complete genome). The EgenF and EgenR primers did not detect any of the 10 *E. multilocularis* samples, but did detect two of three samples tested with the "Abbasi" primers, suggesting mixed infections. One of these was sequenced as *E. canadensis* (99% match to ncbi database accession number AB208063.1, *Echinococcus canadensis* mitochondrial DNA, complete genome). However the second sample gave a poor sequence and yielded no identifiable BLAST results.

As part of routine testing of faecal samples, some samples that were coproPCR positive were sent for sequencing for further confirmation. In order to try and achieve better sequence results, a sequencing primer was developed (5'-CTCAAATGACAAAATAGCTAG-3') for products resulting from the EgenF and EgenR protocol. The coproPCR was conducted as described above, but samples were sequenced with this primer, in an effort to achieve more accurate results. Ten blindly tested coproELISA and EgenF and EgenR coproPCR positives (all from the Alay Valley, Kyrgyzstan) were sent for sequencing (Beckman Coulter, Essex, UK). Four of these were successfully sequenced as *E. canadensis* (with 99% or 100% matches to ncbi database accession number AB208063.1, *Echinococcus canadensis* mitochondrial DNA, complete genome) but the other six gave unclear sequencing results, even after a second attempt by Beckman Coulter to obtain a clear sequence. Obtaining clear sequences obtained from coproPCR is often difficult due to low DNA quality, presence of contaminants (Boufana *et al.*, 2008) or presence of PCR-inhibitory substances (Mathis and Deplazes, 2006).

4.4 Discussion

E. multilocularis, *E. granulosus* and *E. canadensis* are co-endemic in several regions, and when researching human and/or canine echinococcosis in such areas it is important to be able to differentiate between these zoonotic species. Here sets of primers were optimised to be able to achieve species-specific differentiation. When testing DNA extracted from parasite tissue samples, the multiplex PCR developed was found to be suitable for detecting *E. granulosus sensu stricto* (strains G1,G2,G3), *E. equinus*, *E. canadensis* (G6, G7, G10), and, less effectively, *E. multilocularis*. The different sized bands produced by the two sets of primers also allowed for instant identification of groups of species or strains are present, i.e. the *E. granulosus/E. equinus/E. multilocularis* group, or the *E. canadensis* group, and PCR

products were successfully sequenced using the Egen1/4F and Egen1/4R primers (*E. granulosus* G1, *E. equinus,* and *E. multilocularis*) and the EgenF and EgenR primers (*E. canadensis* G7).

The multiplex PCR was found to have a detection limit of at least 9.8 picograms of *E. granulosus* G1 and *E. canadensis* G6 DNA, but was less sensitive for *E. equinus* DNA, with a detection limit of 78.1 picograms. As faecal samples contain less parasite DNA than DNA extracted from tissue samples, an effort was made to increase the analytic sensitivity of the primers by testing them in uniplex PCRs. In doing so the detection limit of the Egen1/4F and Egen1/4R primers was improved to 8.1 pg/µL for *E. granulosus* G1, and 34.4 pg/µL for *E. equinus*, and the detection limit of the EgenF and EgenR primers was improved to 0.78 pg for *E. canadensis* (G6 and G7). However the Egen1/4F and Egen1/4R showed some reaction with high concentrations of *E .multilocularis* DNA (14,000 to 3,500 pg/µL), suggesting that the primers may also detect *E. multilocularis.*

The uniplex PCRs were tested using a defined panel of dog faecal samples (n=33, see Table 4-2). The Egen1/4F and Egen1/4R primers successfully identified 2 of the 5 experimental *E. equinus* infections, and 8 of the 8 experimental *E. granulosus* infections. A further 2 out of 3 arecoline positive *E. granulosus* infections were detected by the Egen1/4F and Egen1/4R primers. The EgenF and EgenR primers did not detect any of the experimental *E. equinus* or *E. granulosus* infections, but did correctly identify an *E. canadensis* purge positive sample from Kyrgyzstan. A further 5 samples which had previously tested positive for *E. granulosus* G1 using other primers (n=2, Boufana *et al.*, 2013b), and *E. granulosus sensu lato* using other primers (n=3, Abbasi *et al.*, 2003) were also tested. The two *E. granulosus* G1 samples tested positive with primers Egen1/4F and Egen1/4R, and two of three *E. granulosus* sensu

lato samples tested positive with both Egen1/4F and Egen1/4R and EgenF and EgenR primers, with one sample sequenced as *E. granulosus* G1 using the Egen1/4F and Egen1/4R primers, and as *E. canadensis* using the EgenF and EgenR primers, suggesting mixed infections. One of three *E. granulosus sensu lato* samples tested negative with both Egen1/4F and Egen1/4R and EgenF and EgenR primers. Of the ten samples that had previously tested positive for *E. multilocularis* with other primers (Boufana *et al.*, 2008), three were detected by the Egen1/4F and Egen1/4R primers.

Together these results suggest that the uniplex Egen1/4F and Egen1/4R protocol can be used to reliably detect *E. granulosus sensu stricto* in faecal samples. The primers showed a lower affinity with *E. equinus* and *E. multilocularis* DNA, but do also detect these species, thus necessitating sequencing for Egen1/4F and Egen1/4R PCR positives. As this protocol has a limited analytic sensitivity for detecting *E. multilocularis* DNA, it is advisable to use it in combination with the more sensitive ND1 primers developed by Boufana *et al*. (2013b) in areas where *E. granulosus* and *E. multilocularis* are co-endemic. The EgenF and EgenR protocol showed high analytic sensitivity and specificity for *E. canadensis*, and was not found to cross react with *E. granulosus*, *E. equinus* or *E. multilocularis* DNA. The protocol was also used to successfully detect *E. canadensis* DNA in faecal samples, including from two in which *E. canadensis* DNA had not been previously identified.

4.5 Chapter 4 Summary

CoproELISAs can be used to reliably detect *Echinococcus* spp. in faecal samples, but these are usually genus specific (Craig *et al.*, 1995). Therefore, in areas where several species of *Echinococcus* are co-endemic, coproPCR protocols can be used for species specific *Echinococcus* spp. detection. Several coproPCR protocols for the detection of *Echinococcus* spp. in faecal samples have been previously developed and published (Abbasi *et al.*, 2003;

Dinkel *et al.*, 2004; Boubaker *et al.*, 2013; Boufana *et al.*, 2013b). However, the available protocols were not suitable to accurately identify the *Echinococcus* species present in the co-endemic study area, as available protocols either did not allow differentiation between different species/strains (Abbasi *et al.*, 2003), were specific for only one species/strain (Boufana *et al.*, 2013b), or were not suitable or not optimised for copro analysis (Dinkel *et al.*, 2004; Boubaker *et al.*, 2013).

Here, PCR protocols were developed to allow for the identification and differentiation of *E. granulosus, E. equinus*, *E. multilocularis* and *E. canadensis* DNA from tissue and dog faecal samples. A multiplex PCR was developed which could reliably detect *E. granulosus*, *E. multilocularis*, *E. equinus*, and *E. canadensis* DNA from tissue samples, with the advantage that DNA from *E. granulosus, E. multilocularis*, and *E. equinus*, and DNA from *E. canadensis* could be easily distinguished by their different diagnostic band sizes (Fig. 4-1). To increase the analytic sensitivity of the primers, both sets (Egen1/4F, Egen1/4R and EgenF, EgenR) were tested in uniplex PCRs. It was found that, when used as uniplex PCRs, the Egen1/4F and Egen1/4R primers could be used to detect *E. granulosus, E. multilocularis*, and *E. equinus* in faecal samples, although analytic sensitivity for *E. multilocularis* was low (Fig. 4-6). Primers EgenF and EgenR were found to be able to detect *E. canadensis* in faecal samples.

For this study, suitable diagnostic tools were needed to study canine echinococcosis in the Alay Valley in southern Kyrgyzstan, where it was found that *E. granulosus sensu stricto*, *E. canadensis* and *E. multilocularis* are co-endemic (see Chapters 5 and 6). The ND1 protocols developed by Boufana *et al.*, (2013b) for the detection of *E. granulosus sensu stricto* and *E. multilocularis* were found to be most suitable for the detection of these species, as they are

completely specific and do not require sequencing (Boufana *et al.*, 2013b). The newly developed uniplex protocol described here (using primers EgenF and EgenR) was found to be suitable for detection of *E. canadensis* in faecal samples, and was used for testing the samples collected from the Alay Valley (see Chapters 5 and 6).

Chapter 5: Prevalence of canine echinococcosis in the Alay Valley Kyrgyzstan prior to an echinococcosis intervention scheme

5.1 Introduction

Echinococcosis is a public health concern in Kyrgyzstan, and there are concerns the disease may be re-emerging since independence from the Soviet Union in 1991 (Torgerson *et al.*, 2006). During Soviet times, the rearing of sheep (the primary intermediate host for *E. granulosus*) took place on large collectivized farms, slaughter was undertaken in large slaughterhouses under veterinary inspection, and treatment of farm dogs with praziquantel every four months was compulsory (Torgerson *et al.* 2002), which is thought to have resulted in relatively low levels of human echinococcosis (Torgerson *et al.* 2002, 2006). In contrast, since independence collective farms have broken up into small farms, home slaughter has increased, the dog population has grown, and the centralized praziquantel dosing scheme has ceased (Torgerson *et al.* 2006). These changes have been suggested as the cause of higher rates of human echinococcosis (Torgerson *et al.* 2002, 2006). The annual human surgical incidence of cystic echinococcosis (CE) has increased dramatically in Kyrgyzstan (Torgerson *et al.*, 2006). Alveolar echinococcosis (AE) is also thought to be increasing in Kyrgyzstan (Torgerson *et al.*, 2010; Usubalieva *et al.*, 2013), and the Osh Oblast in Kyrgyzstan has some of the highest numbers of AE human cases in the country (Usubalieva *et al.*, 2013).

In 2011, the World Bank considered echinococcosis to be of sufficient concern to implement an intervention programme which included providing anthelmintics for dogs (World Bank, 2011). The Alay Valley in Osh Oblast was selected for a pilot project by the World Bank, but despite this, and despite the increasing numbers of human cases from the Alay Valley, little is known about canine echinococcosis in this area. It is important to establish pre-intervention canine echinococcosis rates in this area, as it is impossible to assess the impact of any control

scheme without baseline data. In order to determine this, four communities in the Alay Valley were investigated prior to the implementation of the World Bank dog dosing scheme, and domestic dog faecal samples were collected and analysed in order to assess canine echinococcosis coproELISA prevalences.

5.2 Methods

5.2.1 Sample collection

The Alay Valley is located in the Osh Oblast in Southern Kyrgyzstan, between the Alay and Pamir Mountains. Four communities in the Alay Valley were selected for this study, namely Taldu Suu (39.70°, 72.98°) Sary Mogul (39.68°, 72.89°), Kara Kavak (39.66°, 72.72°) and Kashka Suu (39.64°, 72.67°, Fig. 2-1, for details see section 2.1). These four communities are situated along a main road (A372), and contain between ~65 (Kara Kavak) to ~400 households (Sary Mogul).

The four communities were visited in May 2012, before the start of the World Bank praziquantel dosing campaign. In Sary Mogul, Taldu Suu and Kara Kavak, all households and dogs were registered, whereas in Kashka Suu, a randomly selected sample of households were registered. Each household that had dogs was registered with the family name, dog name(s), and GPS position recorded. Dog owners were also asked if their dog had received any de-worming treatment in the last six months. Dog faecal samples were collected by one of two methods; either rectally by qualified vets (Iskender Ziadinov or Alex Mastin), or the dog's owner was asked to indicate where the dog usually defecated and ground samples were collected. Where possible, fresher faecal samples were chosen rather than older samples, as DNA in older faecal samples may degrade (e.g. Olson *et al.*, 2005). Each collected sample was divided in two, with a subsample was stored in 35ml universal tubes in 0.3% PBS Tween (Fisher Scientific, Loughborough, UK) with 10% formalin (sourced locally), with a

corresponding subsample stored in bijoux tubes/15 ml polypropylene tubes using 70% ethanol (sourced locally). Samples were labelled, covered in parafilm to prevent leaking, and shipped to Salford, United Kingdom, for analysis.

5.2.2 CoproELISA

After decontaminating at -80°C for at least four days to remove the risk of infection with *Echinococcus* spp. (WHO/OIE, 2001), faecal samples were extracted by homogenizing, shaking and centrifuging at 2500r.p.m (1125G) for 5 minutes using an eppendorf® centrifuge 5804, and collecting the supernatant. All collected faecal samples were analysed for *Echinococcus* spp. coproantigen using a genus-specific sandwich ELISA using the protocol described by Allan *et al*. (1992) and Craig *et al*. (1995, see Chapter 3). Supernatants of two known positives (an arecoline purge positive sample from Kara Kavak, Kyrgyzstan and an antigen spiked sample, spiked 1:100 with *E. granulosus* whole worm extract) were used as positive controls throughout. Three known coproELISA negatives from a low endemic area (the Falkland Islands) were also included as negative controls.

Traditionally the cut-off value for ELISAs such as the one used here is calculated by analysing a panel of known negatives and then setting the cut-off as the average of all negative values plus two or three standard deviations (the Gaussian approach, e.g. Allan and Craig, 1989; Allan *et al.*, 1992). This method was also applied when assessing the antibodies used for the development of the coproELISA described here (see Chapter 3). However, this method takes into account only the distribution of a number of known negatives (often from a low or non-endemic area), and does not take into consideration the true distribution of both negatives and positives from the population being studied (see also Gardner and Greiner, 2006). Receiver operating characteristic (ROC) curves provide an alternative method for calculating a cut-off, and a way of optimising the diagnostic sensitivity and specificity of the

test being used (Gardner and Greiner, 2006). In order to calculate a cut-off with ROC curves, a panel of known positive and negative samples is needed. Ideally this panel should be based on samples from the area being studied, as the distribution of coproELISA OD values may differ between areas. In this case a non-parametric method of using ROC curves was used to account for the non-Gaussian distribution of OD values. This included identifying the OD value from the test panel that gave the best sensitivity and specificity.

Here 19 arecoline purge samples from Kara Kavak and Taldu Suu were used (Table 5-1, for details see Chapter 6 and Chapter 7 Appendix 2). Eight of these were scored macroscopically as purge positive and eleven as purge negative. To increase the sample size for this panel we also included 19 samples from necropsied dogs in Hobukesar County, Xinjiang, China (Table 5-1, see also Chapter 7 Appendix 3 and van Kesteren *et al.*, in press). Although located >1000km northeast of the Alay Valley, Hobukesar County is similar to the Alay Valley in several ways, including the fact that local people are mostly semi-nomadic and communities are pastoral. As such the distribution in coproELISA OD values from dog faecal samples was thought to be comparable with those in the Alay Valley. Rectal samples from 19 necropsied dogs (five necropsy negative and 14 necropsy positive, with estimated worm burdens ranging from 2 to >10,000) were included in the panel.

Using ROC curves, based on our panel of known negative and positive purged and necropsied dogs, the cut-off was set at an O.D value of 0.0763, giving a diagnostic sensitivity of 95%, and a specificity of 81%, with an overall accuracy (defined here as the average of the sensitivity and specificity) of 88%. This cut-off was used in the coproELISA analysis of the faecal samples collected from the four communities in the Alay Valley. The same aliquots of capture and conjugate were used for all plates, and sufficient buffers were made up to analyse all samples, with the same bottle of TMB was used for all plates, to ensure minimum

variation in test conditions. All samples were analysed within three days to minimize variability between batches of samples analysed. Samples were tested in duplicate.

Table 5-1: Samples used for ROC curve analysis. Neg= negative, Pos=positive, E.g = E. granulosus, *E.m*= E.multilocularis

Sample	Source	Method	Status	Worm burden estimate	Further detail
KK1	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
KK2	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
KK3A	Alay Valley	Arecoline	Pos for *Echinococcus*	Low	PCR +ve for *E. g* s.l ("Abbasi" primers)
KK3B	Alay Valley	Arecoline	Pos for *Echinococcus*	Low	PCR +ve for *E. g* s.l ("Abbasi" primers)
KK4	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
KK6	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
KK7	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
KK8	Alay Valley	Arecoline	Pos for *Echinococcus*	High	PCR +ve for *E. g* s.l ("Abbasi" primers)
KK9	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
KK10	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
KK11	Alay Valley	Arecoline	Pos for *Echinococcus*	Medium	PCR +ve for *E. g* s.l ("Abbasi" primers)
KK13	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
KK14	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
KK15	Alay Valley	Arecoline	Pos for *Echinococcus*	Low	PCR +ve for *E. g* s.l ("Abbasi" primers)
KK16	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
KK18	Alay Valley	Arecoline	Pos for *Echinococcus*	Low	PCR +ve for *E. g* s.l ("Abbasi" primers)
KK19	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
IRISBAEV	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
NURIK	Alay Valley	Arecoline	Pos for *Echinococcus*	Low	PCR +ve for *E. g* s.l ("Abbasi" primers)
XP3	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP4	Xinjiang	Necropsy	Pos for *Echinococcus*	~50 worms	Sequenced as *E. granulosus* G1
XP6	Xinjiang	Necropsy	Pos for *Echinococcus*	>5000 worms	Sequenced as *E. g* G1
XP8	Xinjiang	Necropsy	Pos for *Echinococcus*	~20 worms	Sequenced as *E. g* G1
XP9	Xinjiang	Necropsy	Pos for *Echinococcus*	~10 worms	Sequenced as *E. g* G1
XP10	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP13	Xinjiang	Necropsy	Pos for *Echinococcus*	(not recorded)	Sequenced as *E. g* G1
XP14	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP15	Xinjiang	Necropsy	Pos for *Echinococcus*	2 worms	Sequenced as *E. g* G1
XP17	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP18	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP20	Xinjiang	Necropsy	Pos for *Echinococcus*	~ 300 worms	Sequenced as *E. g* G1
XP21	Xinjiang	Necropsy	Pos for *Echinococcus*	~500 worms	Sequenced as *E. g* G1
XP23	Xinjiang	Necropsy	Pos for *Echinococcus*	~100 worms	Sequenced as *E. g* G1
XP24	Xinjiang	Necropsy	Pos for *Echinococcus*	3 worms	Sequenced as *E. g* G1
XP28	Xinjiang	Necropsy	Neg for Echinococcus	n/a	n/a
XP32	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP33	Xinjiang	Necropsy	Pos for *Echinococcus*	>10,000 worms	Sequenced as *E. g* G1
XP38	Xinjiang	Necropsy	Pos for *Echinococcus*	>10,000 worms	Sequenced as *E. g* G1

5.2.3 CoproPCR

All faecal samples were analysed by coproPCR to determine the *Echinococcus* species present in the faecal sample. DNA was extracted from faecal samples using a QIAamp® DNA Stool kit (Qiagen, Hilden, Germany), following the manufacturer's instructions, but using ~1g of faeces, and adjusted the buffer ASL volume. Following findings that *E. multilocularis*, *E. granulosus sensu stricto* and *E. canadensis* are co-endemic in the Alay Valley (van Kesteren *et al.*, 2013a, see Chapter 6), DNA samples were tested for each species using three protocols.

Samples were analysed for *E. multilocularis* using specific primers to amplify a 207bp fragment within the ND1 mitochondrial gene (Boufana *et al.*, 2013b). Samples were tested for *E. granulosus* G1 (common sheep strain) using highly specific ND1 primers to amplify a species-specific 226 bp fragment (Boufana *et al.*, 2013b). Samples were tested for *E. canadensis* using newly developed primers (see Chapter 4). For details on the three PCR protocols, see section 2.3.2 and Chapter 4.

5.3 Results

5.3.1 Validation of the ELISA using ROC curves

Based on the panel of known negatives (12 arecoline purge negative samples from the Alay Valley and 7 necropsy negatives from Hobukesar) and known *Echinococcus* spp. positives (7 arecoline purge positives from the Alay Valley and 12 necropsy positives from Hobukesar), the cut-off was calculated as being 0.0763, giving a diagnostic sensitivity of 95%, and a specificity of 81%, with an overall accuracy of 88% when used to classify the 38 known-status faecal samples used in the panel. This is lower than previously determined for this

assay (see Chapter 3), and is probably due to a difference in distribution of OD values between samples from the Falkland Islands (which were used to calculate the specificity of the assay previously), and the samples from the Alay Valley and Hobukesar (Fig. 5-1).

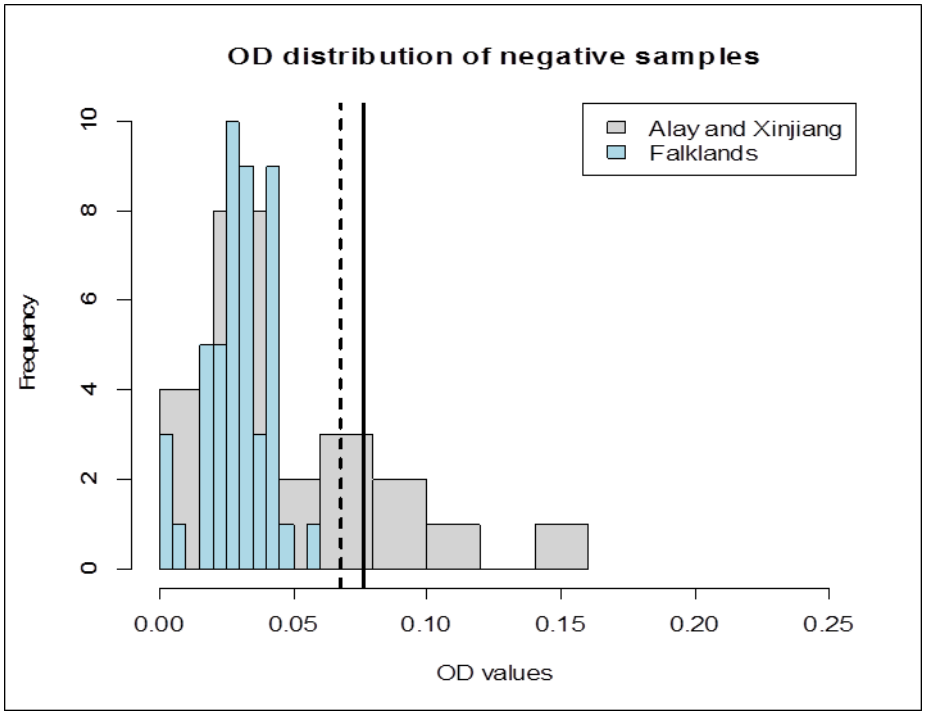

Figure 5-1: Distribution of coproELISA OD values from negative samples collected in the Falkland Islands (n=43) and the Alay Valley (n=14) and Xinjiang (n=7).
The dotted line indicates the previously established cut-off (for details see Chapter 3), and the solid line indicates the ROC curve cut-off.

5.3.2 CoproELISA positives in the Alay Valley

In May 2012, a total 333 dog faecal samples were collected from the four communities in Alay Valley (SM=157, TS=98, KS=43, KK=35). All of these were tested for *Echinococcus* spp. using a coproantigen ELISA with the selected antibodies (capture antibodies from Rabbit 5 and conjugate antibodies from Rabbit 91, see Chapter 3). Out of these, 88 (26.4%) tested positive. ELISA positive rates ranged from 23.3% in Kashka Suu to 28.7% in Sary Mogul, with 24.5% and 25.7% ELISA positives in Taldu Suu and Kara Kavak respectively (Fig. 5-2, note that standard error bars or confidence intervals are not given as census sampling of communities was undertaken).

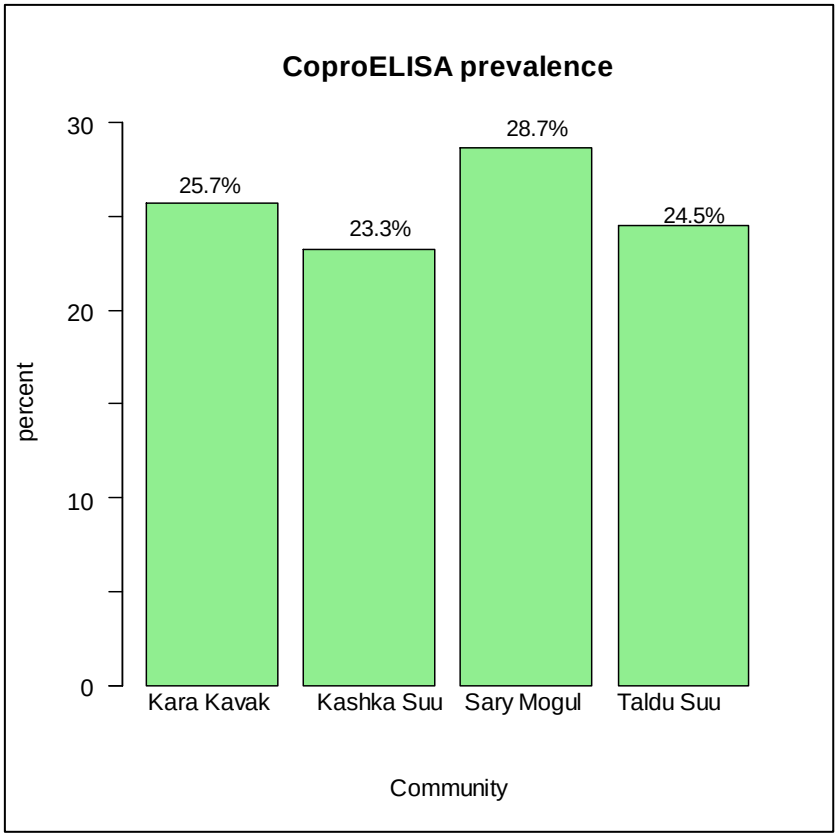

Figure 5-2: CoproELISA prevalences in Kara Kavak, Kashka Suu, Sary Mogul and Taldu Suu in the Alay Valley

5.3.3 CoproPCR results

Of the 333 faecal samples in PBS buffer, 288 (86.5%) could be matched to a subsample in ethanol. Unfortunately 45 samples could not be matched due to labels on either subsample being erased, or samples being lost/broken in transport. Of the 88 ELISA positive samples, 73 (83%) could be matched to an ethanol preserved sub sample, although for 4 samples there was not enough DNA to conduct all three PCRs, so these four samples were tested only for *E. multilocularis* and *E. granulosus*. Of 245 ELISA negative samples, 215 (87.8%) could be matched to an ethanol preserved sub sample.

Of the 73 coproELISA positives, 8 (11%) tested positive for *E. multilocularis* DNA, and 6 (8.2%) tested positive for *E. granulosus* DNA. Of the 69 faecal samples tested for *E. canadensis* DNA, 23 (33.3%) tested positive. The coproPCR positives included mixed

infections of *E. multilocularis* and *E. granulosus* (n=1), *E. multilocularis* and *E. canadensis* (n=3) and *E. granulosus* and *E. canadensis* (n=3). Furthermore, there were 43 samples (58.9%) that did not test positive with any of the three PCRs (see Table 5-2 for total results and Table 5-3 for results per village). Of the 215 coproELISA negatives tested with coproPCR, 34 (15.8%) tested positive for *E. multilocularis* DNA, 28 (13%) tested positive for *E. granulosus* DNA, and 66 (30.7%) tested positive for *E. canadensis* DNA. The coproPCR positives included mixed infections of *E. multilocularis* and *E. granulosus* (n=4), *E. multilocularis* and *E. canadensis* (n=9), *E. granulosus* and *E. canadensis* (n=6), and even *E. multilocularis, E. granulosus* and *E. canadensis* (n=4). Of the coproELISA negatives, 114 (53%) were negative with all three PCRs.

Table 5-2: CoproPCR results of dog faecal samples tested
Note: Em= E. multilocularis, Eg= E. granulosus sensu stricto, Ec= E. canadensis

Category	All negative	Em only	Eg only	Ec only	Em+Eg	Em+Ec	Eg+Ec	Em+Eg+Ec	Total
coproELISA +ves	43	4	2	17	1	3	3	0	73
coproELISA -ves	114	17	14	47	4	9	6	4	215
Total	157	21	16	64	5	12	9	4	288

Table 5-3: CoproPCR results per village
Note: Em= E. multilocularis, Eg= E. granulosus sensu stricto, Ec= E. canadensis

Kara Kavak									
Category	All negative	Em only	Eg only	Ec only	Em+Eg	Em+Ec	Eg+Ec	Em+Eg+Ec	Total
CoproELISA +ves	6	0	0	2	1	0	0	0	9
coproELISA +ves	10	2	1	8	0	0	3	0	24
Total	16	2	1	10	1	0	3	0	33
Kashka Suu									
Category	All negative	Em only	Eg only	Ec only	Em+Eg	Em+Ec	Eg+Ec	Em+Eg+Ec	Total
CoproELISA +ves	6	1	0	3	0	0	0	0	10
coproELISA +ves	20	1	1	5	0	2	0	3	32
Total	26	2	1	8	0	2	0	3	42
Sary Mogul									
Category	All negative	Em only	Eg only	Ec only	Em+Eg	Em+Ec	Eg+Ec	Em+Eg+Ec	Total
CoproELISA +ves	18	1	2	10	0	1	2	0	34
coproELISA +ves	47	8	9	22	2	4	2	1	95
Total	65	9	11	32	2	5	4	1	129
Taldu Suu									
Category	All negative	Em only	Eg only	Ec only	Em+Eg	Em+Ec	Eg+Ec	Em+Eg+Ec	Total
CoproELISA +ves	13	2	0	2	0	2	1	0	20
coproELISA +ves	37	6	3	12	2	3	1	0	64
Total	50	8	3	14	2	5	2	0	84

5.3.4 Praziquantel dosing

A total of 380 dog owners were asked about de-worming their dogs. However, not all dogs could be sampled due to not being able to find a faecal sample, dogs being away in summer pasture, dogs being too young, etc. Of the 380 owners, 78 (20.5%) said their dog had been dosed in the previous six months, but the majority (302, or 79.5%) said they had not dosed their dogs in the previous twelve months. All people who said they had wormed their dog all said the tablets had been obtained from the local veterinarian.

5.4 Discussion

The diagnostic sensitivity of the novel coproELISA, with capture antibodies from a rabbit (R5) immunised with *E. granulosus* whole worm extract, and conjugate antibodies from a rabbit (R91) immunised with an *E. granulosus* surface wash, at 95%, is comparable with that of ELISAs used in other studies. Sensitivity increases with worm burden, and Allan and Craig (2006) found that the sensitivity of coproELISAs was approximately 85% in dogs with worm burdens between 51 and 100 worms, and up to 100% for dogs with burdens of more than 1000 worms. Other studies report sensitivities of 87.5% (Allan *et al.*, 1992), 78.5% (Benito and Carmena, 2005), 100% (Buishi *et al.*, 2005), and 92.6% (Morel *et al.*, 2013). The genus specificity of the current coproELISA, at 81%, was somewhat lower than that reported by other authors. For example, Allan and Craig (2006) found that genus specificities were typically 85% or higher, with reported specificities of 96.5% (Allan *et al.*, 1992), 93.3% (Benito and Carmena, 2005), 98% (Buishi *et al.*, 2005) and 86.4% (Morel *et al.*, 2013). However, few studies reported testing the sensitivity and specificity of their assays using dog faecal samples from the area being studied. For example, Allan *et al.* (1992) assessed their assay using samples from both Turkana, Kenya, and Xinjiang, China. However, the distribution of OD values could have differed between these areas. Similarly, the known

negatives are often from a non-endemic area. For example, Buishi *et al.* (2005) used 25 samples from a non-endemic part of Britain, and calculated their cut-off as 3 standard deviations above the mean of those negatives.

It is very difficult to organise and gather a panel of known positive and negative dog faecal samples from the area being studied. Necropsy is generally considered the 'gold standard', but in the field very low worm burdens can be missed (Allan and Craig, 2006). Similarly arecoline purgation can have a false negative rate of up to 10-20% (Craig *et al.*, 1995). For this reason, samples from non or low endemic areas are often used. However, it was found that distributions of OD values from a low endemic area (the Falkland Islands) were not comparable with distributions of OD values from the Allay Valley (Fig. 5-1). Dogs from areas such as the Falkland Islands are not only very unlikely to have *Echinococcus* spp., but are also unlikely to have other tapeworms such as *Taenia* spp., due to their management and husbandry (Craig and Larrieu, 2006). Although we found that our diagnostic antibodies did not cross react with *Taenia* spp. (see Chapter 3), presence of other tapeworms could affect the distribution of OD values. Furthermore the diet of dogs in the developed world is likely to be different from that in areas such as the Alay Valley. Whereas dogs in areas like the UK are likely to be fed commercial dog food, faecal samples from the Alay could contain plastic, bone, hair, cloth etc. (pers. obs.), which may impact on OD values. When comparing the diagnostic specificity of an assay against samples from a non-endemic area, the specificity obtained may be relatively high, because the samples from a non-endemic area are likely to have very low OD values. For example, the coproELISA assessed in the current study against samples from the Falkland Islands, had a diagnostic specificity of 93.75% (see Chapter 3), but this decreased to 81% when tested from necropsy or arecoline negative samples from highly endemic areas.

The ROC curve for the coproELISA allows for a balance between diagnostic sensitivity and specificity to be chosen. Depending on the question being asked, a preference may be given to a higher sensitivity and lower specificity, or vice versa. Here we chose to prioritize the diagnostic sensitivity as compared to specificity, whilst retaining a high level of accuracy.

The results obtained suggest that levels of coproELISA positive dogs were high in the Alay Valley, with an overall coproELISA positive rate of 26.4%. This is comparable with coproELISA positive rates reported from other areas where human echinococcosis occurs, including Libya (21.6%, Buishi *et al.*, 2005), Australia (29% in New South Wales and 17.5% in Victoria, Jenkins *et al.*, 2006), and Tibet (27.2% in Shiqu County, Moss *et al.*, 2013). Although other canids including red foxes, *Vulpes vulpes* (Ziadinov *et al.*, 2010) and grey wolves, *Canis lupus* (Abdybekova and Torgerson, 2012) may act as hosts for *Echinococcus* spp., domestic dogs pose the greatest risk of human infection due to their close association with humans (Budke *et al.*, 2005). As such, a coproELISA positive prevalence of 26.4% in owned dogs in the Alay Valley poses a significant risk for local people, and may explain why many human cases occur in the area (Usubalieva *et al.*, 2013).

The coproPCR results confirm for the first time that three species of *Echinococcus* are present in the Alay Valley, namely *E. multilocularis*, *E. granulosus* and *E. canadensis*. As three different primer sets and protocols were used for coproPCR, it is difficult to compare the prevalences of each species described here, as the protocols have different sensitivities. However, the results presented here could be used for future monitoring using the same protocols to assess how species prevalences change over time. In this study, the correlation between coproELISA positives and coproPCR positives was poor, with 58.9% of

coproELISA positives being coproPCR negative for all tests, and 47% of coproELISA negatives testing positive with at least one coproPCR protocol.

CoproELISA positives may be negative in coproPCR analysis for several reasons. For example, the infection may have been pre-patent, or the subsample (~1g) extracted for DNA may not have contained any eggs. Furthermore, although *Echinococcus* spp. eggs may be stable in the environment for long periods of time under certain climatic conditions, they are susceptible to warm and dry conditions (Veit *et al.*, 1995). DNA is likely to degrade quickly if not properly preserved (e.g. Olson *et al.*, 2005), and as many of the faecal samples were collected from the environment (with some clearly being older and dry), DNA in these samples may have been degraded. Furthermore the presence of PCR inhibitors and non-target DNA in faecal samples can pose a problem for coproPCR (Mathis and Deplazes, 2006). Conversely, coproELISA negatives may be coproPCR positive for several reasons including very low worm burdens (so that the OD did not exceed the cut-off, i.e. false negatives), or dogs may ingest *Echinococcus* spp. eggs through coprophagia (Hartnack *et al.*, 2013). Faecal samples may also have become contaminated with *Echinococcus* spp. eggs from the environment, as environmental contamination with coproELISA and coproPCR positive samples is high (van Kesteren *et al.*, 2013a, see Chapter 6).

Based on cases of human echinococcosis, the World Bank decided to attempt to control canine echinococcosis in Kyrgyzstan (World Bank, 2011), and dosing started in the Alay Valley in the summer of 2012 (Akjol Gaitanbekov, Sary Mogul local veterinarian, pers. comm.). However, in order to assess whether or not this intervention is successful, it will be important to evaluate it in future. Several intervention campaigns have been conducted and evaluated, on both larger (i.e. national) and smaller (i.e. regional) scales. For example Wei *et*

al. (2005) evaluated the effect of implanting slow release praziquantel bars in dogs in villages in Xinjiang, China. In order to evaluate the effect of this intervention, domestic dogs were tested for *Echinococcus* spp. using coproELISA prior to the intervention, with follow up assessments over four years post intervention (Wei *et al.*, 2005). In addition the authors measured human serum ELISA positive rates and incidence of echinococcosis in children in the area before and after the intervention (Wei *et al.*, 2005). In other areas control programmes have been carried out nationally and over many years. In New Zealand for example, canine echinococcosis was estimated to be between 10%-37% in 1955, based on arecoline data (see Craig and Larrieu, 2006). However in 1959, a National Hydatid Council was launched, and a control scheme including monitoring and dosing of dogs, and quarantine of livestock was conducted. Prevalence of *Echinococcus* spp. in dogs declined to <5% in 1972, and in 2002, New Zealand declared itself to be free of echinococcosis (Craig and Larrieu, 2006). *Echinococcus* spp. control programmes are often costly, and require years or decades of commitment (Craig and Larrieu, 2006). As such it is important to periodically evaluate control programmes, to assess if they are meeting their targets.

The World Bank project aims to control echinococcosis in Kyrgyzstan by providing anthelminthics for dogs (World Bank, 2011), and dosing began in the Alay Valley in the summer of 2012, with the district administrations in Gulcha and Daroot Korgon providing praziquantel to local veterinarians four times a year (Akjol Gaitanbekov, local veterinarian, pers. comm.). However, if in future the World Bank, or the Kyrgyz authorities, or independent scientists want to evaluate the impacts of this intervention, they must have pre-intervention data. This should include data on canine echinococcosis rates to compare later post-intervention canine echinococcosis rates to, but it is also useful to see how much the 'uptake' rate of praziquantel tablets changes with the control scheme. Here pre-intervention coproELISA positive rates in dogs in four communities in the Alay Valley were established,

and an estimate was made for how many people dosed their dogs with praziquantel in the absence of a centralized control scheme.

5.5 Chapter 5 Summary

Echinococcosis appears to be re-emerging in Kyrgyzstan, with increasing numbers of human cases reported from the Alay Valley in the south of the country. In recognition of this public health concern, the World Bank implemented a control scheme that included dosing all owned dogs with praziquantel four times a year by local veterinarians (WorldBank, 2011). However, despite deciding to implement this control programme in the summer of 2012, no data was available on the prevalence of canine echinococcosis in the Alay Valley in the absence of a control scheme. Without pre-intervention data, it is impossible to assess whether or not a control scheme is effective.

Here canine echinococcosis was studied in four communities in the Alay Valley (Kara Kavak, Kashka Suu, Sary Mogul and Taldu Suu), with a total of 333 dogs sampled and questionnaires administered to dog owners (for more details on the results of the questionnaire data see Appendix: Mastin et al, in prep.). All collected faecal samples were analysed by coproELISA, with the cut-off determined using samples from endemic areas (Alay Valley and Xinjiang, China) and applying ROC curves. As such, a cut-off was chosen that gave a coproELISA diagnostic sensitivity of 95% and a specificity of 81%. CoproELISA prevalences in the four communities ranged from 23.3% to 28.7%, with an overall prevalence of 26.4%.

Correlation between coproELISA and coproPCR data was poor, with many (58.9%) of coproELISA positives being coproPCR negative for all tests, and 47.0% of coproELISA

negatives testing positive with at least one coproPCR protocol. However, *E. canadensis*, *E. granulosus* and *E. multilocularis* were all detected in the samples.

The data presented here provides information on canine echinococcosis in the Alay Valley in the absence of a control scheme. This data can be used to evaluate the impact of the World Bank control scheme on canine echinococcosis (Chapter 7, also Mastin *et al.*, in prep.-c), as well as any other future control programmes.

Chapter 6: Dog ownership, dog behaviour and transmission of *Echinococcus* spp. in the Alay Valley, southern Kyrgyzstan[6]

6.1 Introduction

Echinococcosis is a neglected zoonotic disease (WHO, 2010) caused by infection with the larval stage (metacestode) of tapeworms within the genus *Echinococcus* (Eckert and Deplazes, 2004). The most common types of echinococcosis are cystic and alveolar which are mostly caused by *E. granulosus* and *E. multilocularis* respectively (WHO/OIE, 2001), although *E. canadensis* can also cause cystic echinococcosis (Alvares Rojas *et al.*, 2014). The life cycles of *E. granulosus*, *E. canadensis* and *E. multilocularis* involve two mammalian hosts. The adult cestode inhabits the small intestine of a definitive host (usually a canid) and produces eggs which are released into the environment (Eckert and Deplazes, 2004) and may then be ingested by an intermediate host. In the case of *E. granulosus* and *E. canadensis* the intermediate host is usually a herbivore such as a sheep, but may include other herbivore species (e.g. Sweatman and Williams, 1963; WHO/OIE, 2001; M'rad *et al.*, 2005). In the case of *E. multilocularis*, small mammals, including voles (e.g. *Microtus* spp., *Arvicola* spp., Hofer *et al.*, 2000), pika (*Ochotona* spp., Schantz *et al.*, 2003), and Tibetan hare (*Lepus oiostolus*, Xiao *et al.* 2004) may act as intermediate hosts. If the intermediate host is consumed by a definitive host, the cycle is complete. Humans may also inadvertently ingest eggs expelled by the definitive host and develop cystic or alveolar echinococcosis (Deplazes and Eckert, 2001).

In Asia, echinococcosis is a serious public health concern in several areas including the Tibetan Plateau (Budke *et al.* 2005), central China (Craig *et al*. 1992), and Mongolia (Ebright *et al.* 2003; Ito *et al.* 2010). There is concern that echinococcosis is re-emerging in Central

[6] This Chapter was published in Parasitology as van Kesteren, Mastin, Mytynova, Ziadinov, Boufana, Torgerson, Rogan, and Craig (2013). Dog ownership, dog behaviour and transmission of *Echinococcus* spp. in the Alay Valley, southern Kyrgyzstan. See Appendix

Asia following the collapse of the Soviet Union (Torgerson *et al.* 2006), with increases in both cystic (Torgerson *et al.* 2006) and alveolar echinococcosis (Torgerson *et al.*, 2010) recorded. Many echinococcosis cases occur in the Osh Oblast of southern Kyrgyzstan (Torgerson *et al.* 2010; Usubalieva *et al.* 2013).

Although several species of wild canids may be final hosts for *E. granulosus*, such as grey wolves, *Canis lupus* (Abdybekova and Torgerson, 2012) and for *E. multilocularis*, such as red fox, *Vulpes vulpes* (Ziadinov *et al.* 2010), infected domestic dogs, *Canis familiaris*, are considered to pose the greatest zoonotic risk (Budke *et al.* 2005). Domestic dogs are hosts for a number of zoonotic pathogens, and due to their close association with people they may be sources of human infections (Macpherson, 2005). The demography, ecology and behaviour of dogs are therefore relevant in studying diseases that may be spread by them. Furthermore, describing the dog population in a community may help to assess transmission potential, zoonotic risks, and optimization of intervention programmes (Butler and Bingham, 2000). This concept has been recognized in studies relating to rabies (Perry, 1993; Butler and Bingham, 2000; Kitala *et al*. 2001; Macpherson, 2005), but has to date rarely been applied to studies on echinococcosis (but see Vaniscotte *et al.* 2011).

This research aimed to determine the presence of *Echinococcus* spp. in domestic dogs in four communities in the Alay Valley in the Osh Oblast of southern Kyrgyzstan. Further aims included characterizing the domestic dog population in these communities by describing their demographics, roles, husbandry, and roaming behaviour, as well as the levels of environmental contamination with dog faeces. In doing so, the aim was to better understand the role of dogs in *Echinococcus* spp. transmission in these rural communities in southern Kyrgyzstan.

6.2 Materials and Methods

6.2.1 Study site

The Alay Valley is located in the south of Kyrgyzstan, and covers most of the Osh oblast. It is located at an altitude of approximately 3,000m a.s.l. and is bordered by the Pamir Mountains to the south (on the border with Tajikistan), and the Alay Mountains to the north (CIA, 2014). Based on a cluster of human AE cases derived from hospital records reported by Usubalieva *et al.* (2013), four rural communities in the Alay Valley were selected for a study on canine echinococcosis, namely Taldu Suu (39.70°, 72.98°) Sary Mogul (39.68°, 72.89°), Kara Kavak (39.66°, 72.72°) and Kashka Suu (39.64°, 72.67°). These communities are located along a road (A372) that runs through the Alay Valley from east to west.

6.2.2 Household questionnaires about dog ownership and husbandry

Detailed questionnaires were carried out in May 2012. Questionnaires were designed using WHO guidelines (World Health Organization and World Society for the Protection of Animals, 1990). Householders were asked questions about the age, sex, and source of their dogs (see Chapter 6, Appendix 1, for more details on the results of the questionnaire data see Appendix: Mastin *et al*, in prep.). Questions were also asked about the role of the dog, i.e. pet, guard, sheep dog or other. Further questions were included about the diet of the dog, including whether the dog was fed offal or observed eating small mammals, and whether it was ever tied up. On a subsequent visit in October 2012, shorter questionnaires were carried out to ask households if they still owned the previously registered dogs, and if any new dogs had been acquired. If the previously registered dogs were no longer present, the reason for this was asked. Not all questions were answered by all respondents, so that numbers reported are at times less than the total number of dogs registered. All questionnaires were administered in Kyrgyz by native speakers (Bermet Mytynova, Kyrgyz veterinarian based at

the Kyrgyz Veterinary Institute in Bishkek, and Iskender Ziadinov, Kyrgyz veterinarian based at the Institute of Parasitology, University of Zurich).

6.2.3 Faecal quadrats to assess environmental contamination

ArcGIS was used to create shapefiles of the approximate boundaries of the four villages (based on imagery from the SPOT5 satellite, Google Maps, 2012). Within each of these four areas, 17 random points were generated, which were used to define one corner of each quadrat. If the point fell in an inaccessible location (e.g. a house) the nearest possible point was taken. The direction of the quadrat was usually determined by the surrounding buildings, fences, etc. Where there was enough space for the 50x50m quadrat to be done facing several directions, the second hand on a watch was used to determine the direction. Where it was not possible to measure out 50x50m due to the presence of buildings, smaller areas were measured and the size was recorded. Quadrats were searched for presence of faeces by slowly pacing up and down whilst looking at the ground. Canid faeces were identified by their size and shape. In all likelihood faeces found in villages were from domestic dogs, although some may have been from red foxes, and possibly wolves. As dogs, foxes and wolves are all hosts for *Echinococcus* spp. and therefore all pose an infection risk to humans, no effort was made to distinguish between these using DNA identification. The faecal density was calculated as the number of faeces/100m^2. The same 68 quadrats were searched for faeces in May and in October 2012.

During each visit, four quadrats were selected in each village using a random number generator in Microsoft Excel® (Microsoft, Redmond, USA). In all four selected quadrats one third of faecal samples (or at least six, if the total number of faeces was less than 18) were selected by sequentially ordering the samples prior to using an Excel® random number generator. Since these samples were collected from the ground, it is not guaranteed that the

samples collected were from different individual dogs. Subsamples of collected faecal samples were stored in 0.3% PBS Tween (Fisher Scientific, Loughborough UK), buffer with 10% formalin (sourced locally) and 95% ethanol (sourced locally) for coproantigen ELISA and coproPCR analysis respectively, and were shipped to the University of Salford at room temperature.

6.2.4 Coproantigen ELISA

All collected faecal samples were analysed using a genus specific coproantigen ELISA. This allowed for detection of those samples that contained *Echinococcus* spp. antigen, before using coproPCR (see below) to identify whether the samples were positive for *E. multilocularis* or *E. granulosus sensu lato*. Coproantigen ELISA allows for rapid analysis of large numbers samples and can detect pre-patent infections (Fraser and Craig, 1997), and can be recommended as a primary diagnostic tool, followed by PCR (e.g. Eckert and Deplazes, 2004). Samples were first stored at -80°C for a minimum of five days in order to kill off any *Echinococcus* spp. infective eggs (WHO/OIE, 2001). Samples were then defrosted and homogenized with wooden spatulas, shaken and centrifuged at 2500 r.p.m. (1125G) for five minutes using an Eppendorf centrifuge 5804. Supernatants were decanted into bijoux tubes and stored at -20°C until used for analysis. A genus-specific sandwich ELISA using the protocol described by Allan *et al*. (1992) and Craig *et al*. (1995) was used to test for *Echinococcus* spp. coproantigen (see Chapter 3). Supernatants of two known positives (an arecoline purge positive sample from Kara Kavak and an antigen spiked sample, spiked 1:100 with *E. granulosus* whole worm extract) were used as positive controls throughout. Two known negatives from non-endemic and low endemic areas (Manchester, UK and the Falkland Islands) were also included as negative controls.

6.2.5 CoproPCR

CoproDNA was extracted from ~1g of dog faeces using the QIAamp® DNA Stool Mini Kit following the manufacturer's instructions. The extracted DNA was used to test for *E. granulosus* using two sets of primers. Initially, samples were tested for *E. granulosus* G1 (common sheep strain) using highly specific ND1 primers to amplify a species-specific 226 bp fragment (Boufana *et al.* 2013) . However, because the strains of *E. granulosus sensu lato* present in this area are unknown, another protocol was used to detect *E. granulosus sensu lato* by amplifying a 269bp tandem repeat region (Abbasi *et al.* 2003) using modifications described by Boufana *et al.* (2008). DNA samples were also tested for *E. multilocularis* using PCR-specific primers (Boufana *et al.* 2013) to amplify a 207bp fragment within the ND1 mitochondrial gene. For details on each PCR protocol, see section 2.3.2.

6.2.6 Arecoline purges

Twenty dogs (16 in Kara Kavak and 4 in Taldu Suu) were voluntarily brought by their owners and dosed with a 0.4% solution of arecoline hydrobromide (sourced locally) in water (7mg arecoline/kg body weight) by Iskender Ziadinov (Kyrgyz veterinarian), and were restrained safely by their owners until they purged. The purges were examined in the field using a handheld magnifying glass and scored for presence/absence of *Echinococcus* spp. and *Taenia* spp. based on gross morphology by an experienced fieldworker (P.S. Craig). Subsamples of these purges were stored in 0.3% PBS Tween (Fisher Scientific, Loughborough, UK) buffer with 10% formalin (sourced locally) and 95% ethanol (sourced locally) for coproantigen and coproPCR analysis respectively, and shipped to the University of Salford at room temperature.

6.2.7 Dog movement

A total of 40 dogs (11 from Sary Mogul, 14 from Taldu Suu, 12 from Kashka Suu, and three from Kara Kavak) were fitted with iGotU® GPS trackers. The iGotU® unit is a GPS tracker that can record GPS positions at programmed intervals (www.i-gotu.com). These units were attached to regular dog collars using ziplock bags and adhesive tape. The accuracy of the GPS units was validated by both leaving units in set locations (stationary recording) and by moving units along a path (dynamic recording). These activities were undertaken in both the Alay Valley and in relatively sparsely built-up areas in the United Kingdom (adjacent to South Park, Macclesfield for stationary recording, and Peel Park, Salford for dynamic recording). In both cases, a Garmin® GPS60 unit was used for comparison.

Dogs were selected for GPS collaring in the field during dog registration, and with owner consent. Selection could not be completely random, as only those dogs present and tame enough to be handled were selected. Although an effort was made to track each selected dog for 24 hours this was not always possible due to field logistics and limitations in battery life. In addition, a number of GPS trackers could not be retrieved. Dogs were tracked for between 1.5 and 47 hours (mean=20 hours, SD=9 hours), and trackers were set to record GPS positions every five minutes, with between 25 and 380 positions recorded per dog (mean=156, SD=81). Dogs were recorded for a total of 787 hours, with a total of 6,256 GPS points recorded. However, dogs with fewer than 50 points recorded (n=3) were removed from further analysis, leaving a total of 37 dogs. Of these 37, 26 were male (SM=7, TS=11, KS=5, KK=3) and 7 were female (SM=4, KS=3). For four dogs the sex was not recorded.

6.2.8 Data analysis

Data were analysed and figures were made using R statistical software version 2.15.2 (R Development Core Team, 2012). In order to analyse the quadrat data, the glme function in the lme4 package (Bates *et al*. 2012) was used to create a Poisson-normal generalised linear mixed effects model. This was used to model the number of faeces within each quadrat and investigate the effect of village and date of visit. The log area of the quadrat was included as an offset variable in the model, and the quadrat ID was included as a random effect in order to account for overdispersion. Models were created including both village-specific and overall random effects, and were compared using a likelihood ratio test. In order to assess for overdispersion in the final model, the ratio of the sum of squared Pearson residuals to the residual degrees of freedom was calculated, with a value of greater than one used to suggest overdispersion.

The accuracy of the iGotU units used for monitoring dog movements was tested using both stationary and dynamic recordings. The accuracy of the stationary units was estimated by calculating the distance recorded by the units from the true location (as determined by the Garmin® GPS60 unit), using the 'Hub Distance' tool in the MMQGIS add-on (http://michaelminn.com/linux/mmqgis) for Quantum GIS 1.8.0 (Quantum GIS Development Team, 2012). For the dynamic data, all points were matched to the nearest time point recorded by the Garmin® unit, and the distance between these points was estimated using the 'Hub Lines' tool in the MMQGIS add-on for Quantum GIS.

Analysis of dog movements was conducted in order to characterise both the size of the 'home range' of these animals and the total distances travelled from the household. The R package 'adehabitatHR' (Calenge, 2006) was used for the estimation of home range size. For this, the

characteristic hull polygon (CHP) method first developed by Downs and Horner (2009) was used, due to the recognised limitations of the usual minimum convex polygon (MCP) and kernel density methods which have been used historically. The total areas of these home ranges were estimated using Quantum GIS 1.8.0, and exported to R for further analysis.

The 'Hub Lines' tool in the MMQGIS add-in for Quantum GIS was used to estimate the minimum distance between each relocation point and the start point for each animal. Violin plots were created using the 'vioplot' package' (Adler, 2005), and confidence intervals for the village-specific median distance travelled (calculated from the median distances travelled per dog) were bootstrapped from the data using the 'boot' package (Canty and Ripley, 2012) with 1000 replications. Researchers conducting household surveys carried Garmin GPS units with tracking mode enabled. This data was analysed using the MCP method in the Geoprocessing Tools of Quantum GIS, in order to identify the boundaries of the villages under study. These were then used to estimate the number and proportion of dog relocations which were outside the village boundaries. Differences in home ranges and median distance moved (per dog) between male and female dogs, were compared using the Wilcoxon rank sum test.

6.3 Results

6.3.1 Dog owner questionnaire data

A total of 644 households were registered in the four communities, with a combined population of 3,677 people (Table 6-1). Questionnaire data revealed that between 38.0% and 74.4% of households in the four communities had at least one dog, with a total estimated dog population of 393, or 1 dog for every 9.36 people (although this does not include the total dog population in Kashka Suu, where only a sample of dogs was taken).

Table 6-1. Characteristics of the populations under investigation in the Alay Valley

	Community			
	Sary Mogol	**Taldu Suu**	**Kashka Suu**	**Kara Kavak**
Number of households registered	368	125	86	65
Total number of people	2173	588	518	398
Total number of dogs reported	178	119	50	46
% of households with at least one dog	38%	74%	51%	52%
Total number of dogs registered	155	115	49	38

Reported dog ages ranged from 2 weeks to 15 years, with a median age of two years. Males represented around 77% of the total dog population. Fig. 6-1 shows the population pyramid for all dogs sampled.

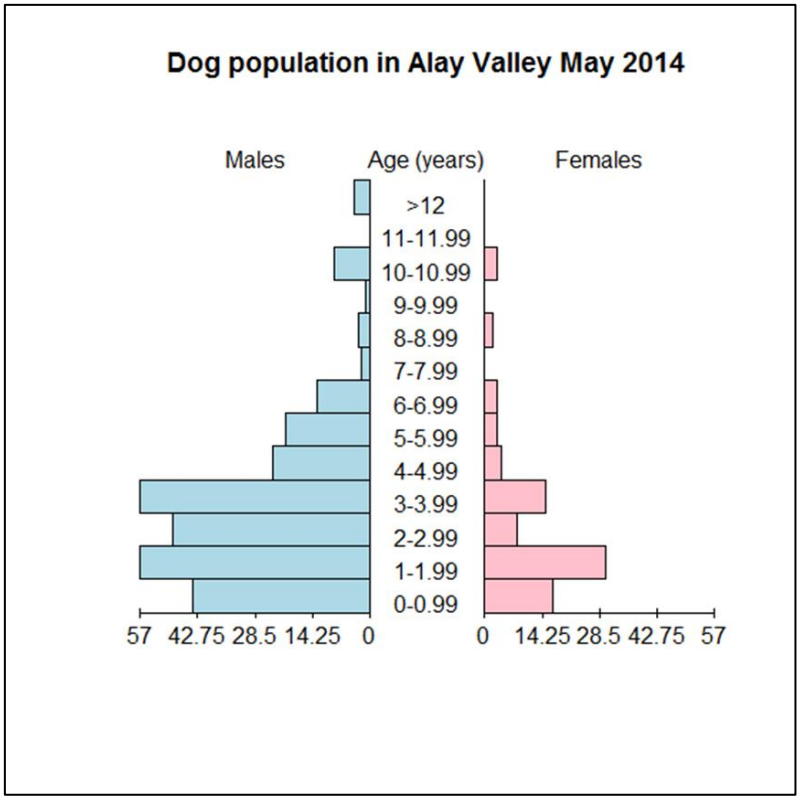

Figure 6-1. Population pyramid for all dogs sampled in May 2012 (n=383).

People in all four communities believed there were un-owned ‘stray’ dogs in their village (SM: 45.9%, TS: 8.8%, KK: 4.6%, KS: 14.0%) although few people who reported a stray dog population had any idea of the size of this. 357 dogs were registered, with questions asked about their role and management. Around 75% of dogs for which this question was answered were described as pets, although many of these were also described as guard dogs, as shown in Fig. 6-2.

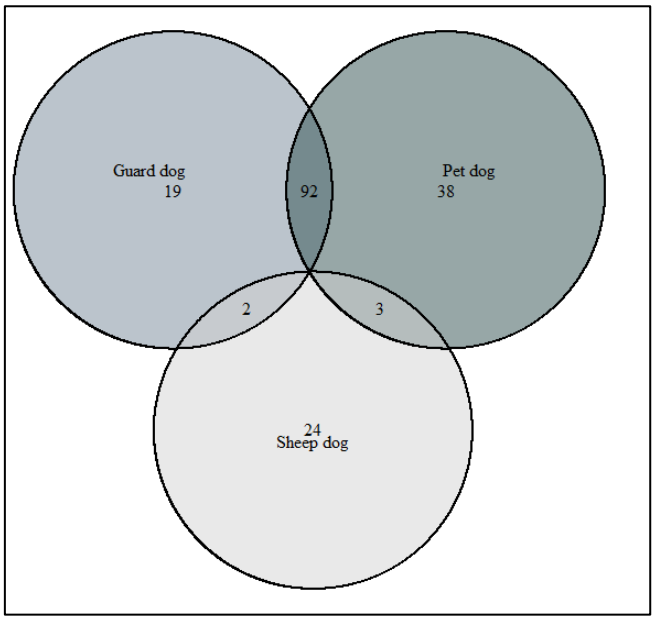

Figure 6-2. Euler diagram of the reported uses of dogs registered in the study. Numbers represent total number of dogs in each category.

The proportion of dogs which were never restrained and free to roam at will was higher in Taldu Suu (110/114 = 96%) than in the other villages (232/265 = 88%, Chi square *P*=0.01). Of the remaining dogs, most were always chained (see Fig. 6-3).

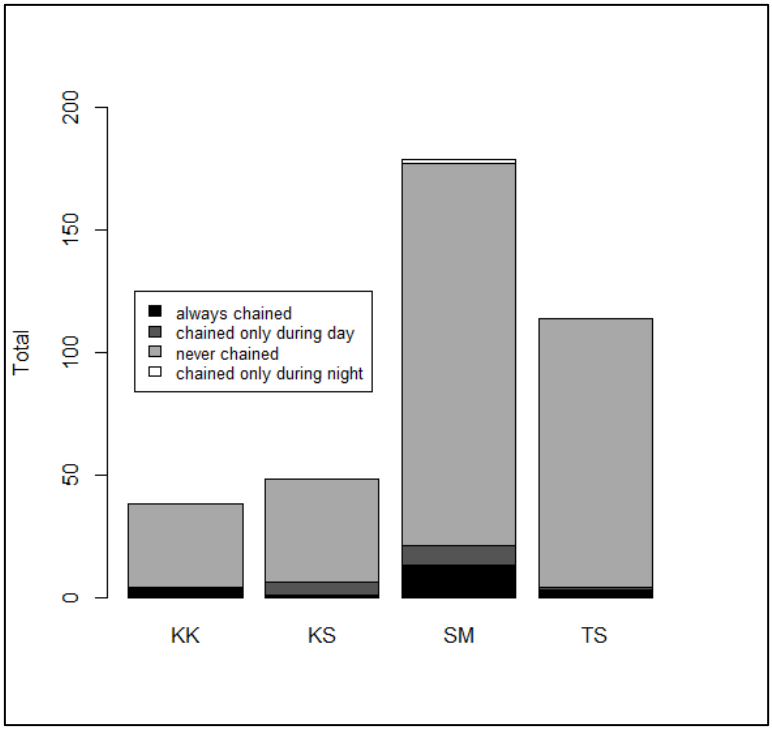

Figure 6-3. Stacked bar chart of frequency of dog restraint in the study villages.
Note: KK= Kara Kavak, KS= Kashka Suu, SM= Sary Mogul, TS= Taldu Suu

Dogs were all fed by household members, and were most often fed table scraps, although offal was also reported to be commonly fed (Fig. 6-4). Dogs were rarely observed eating rodents, although this was occasionally reported, especially in Kashka Suu and Taldu Suu (Fig. 6-4).

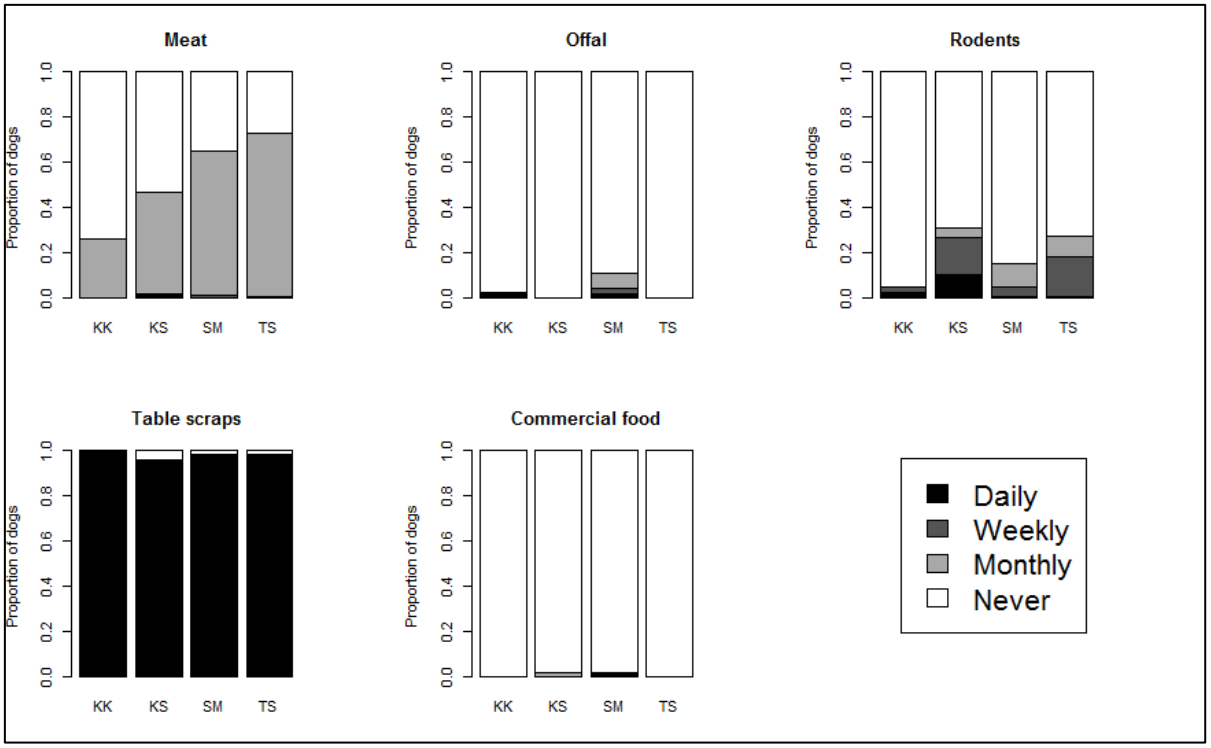

Figure 6-4. Stacked bar charts of proportion of dogs fed different food types and reported frequencies of feeding.

In May 2012, a total of 222 owned dogs were thought to be present in Sary Mogul; 141 in Taldu Suu; and 41 in Kara Kavak. Based on crude estimates of numbers of households from remote sensing data, it is assumed that around 25% of households in Kashka Suu were visited – suggesting a total owned dog population of around 200 in this village.

In October, the owned dog population was found to have reduced in all three of these villages – to 121 dogs in Sary Mogul; 126 in Taldu Suu; and 36 in Kara Kavak. The majority of this difference resulted from the loss of dogs (usually either as missing dogs, through accidental death or by culling), although some dogs moved to mountain pastures. Between the two visits, a total of 52 new dogs (usually puppies) were obtained in Sary Mogul, 36 in Taldu Suu and four in Kara Kavak (although it should be noted that in Kara Kavak, only those households which previously had dogs were revisited in October 2012). Using the data from the census villages of Sary Mogul and Taldu Suu, this suggested that per owned dog present in May, the rate of removal over the five months between visits was around 0.7 in Sary

Mogul and 0.4 in Taldu Suu, whereas the replacement rate was around 0.2 in Sary Mogul and around 0.3 in Taldu Suu. It is also important to note that the estimates for Taldu Suu were made prior to the second visit of the person responsible for dog culling, whereas those for Sary Mogul were made after this visit.

6.3.2 Quadrats for assessing faecal environmental contamination

It was often (42/68 quadrats) not possible to measure out 50x50m quadrats due to the presence of buildings, etc. In these cases smaller areas (mean=1660.7m^2, SD=588.6m^2) were measured and the size was recorded, with faecal densities calculated as faeces/100m^2. Canid faecal densities ranged from a median of 0.45 faeces/100m^2 in Kara Kavak to 1.20 faeces/100m^2 in Kashka Suu in May; and from a median of 0.22 faeces/100m in Sary Mogul to 0.60 faeces/100m in Kashka Suu in October. The Poisson-Normal GLMM found no evidence that random effects were village-specific, and found no evidence of any interaction between village and date of visit. The final model including quadrat ID as an overall random effect showed no evidence of overdispersion. There was strong evidence of a significant difference between faecal contamination in Kashka Suu and all other villages (Wald P<0.001). Compared to Kashka Suu, the density of faeces in Sary Mogul was 0.46 (95% confidence interval 0.37-0.57); in Taldu Suu 0.57 (0.46-0.70); and in Kara Kavak 0.42 (0.34-0.52). Additionally, there was very strong evidence of a reduction in faecal contamination between visits (Wald P <0.001), with the density of faeces in October being 0.53 of that in May (95% CI 0.50-0.56). Fig. 6-5 shows the crude estimates of the faecal densities amongst the different villages over the two visits.

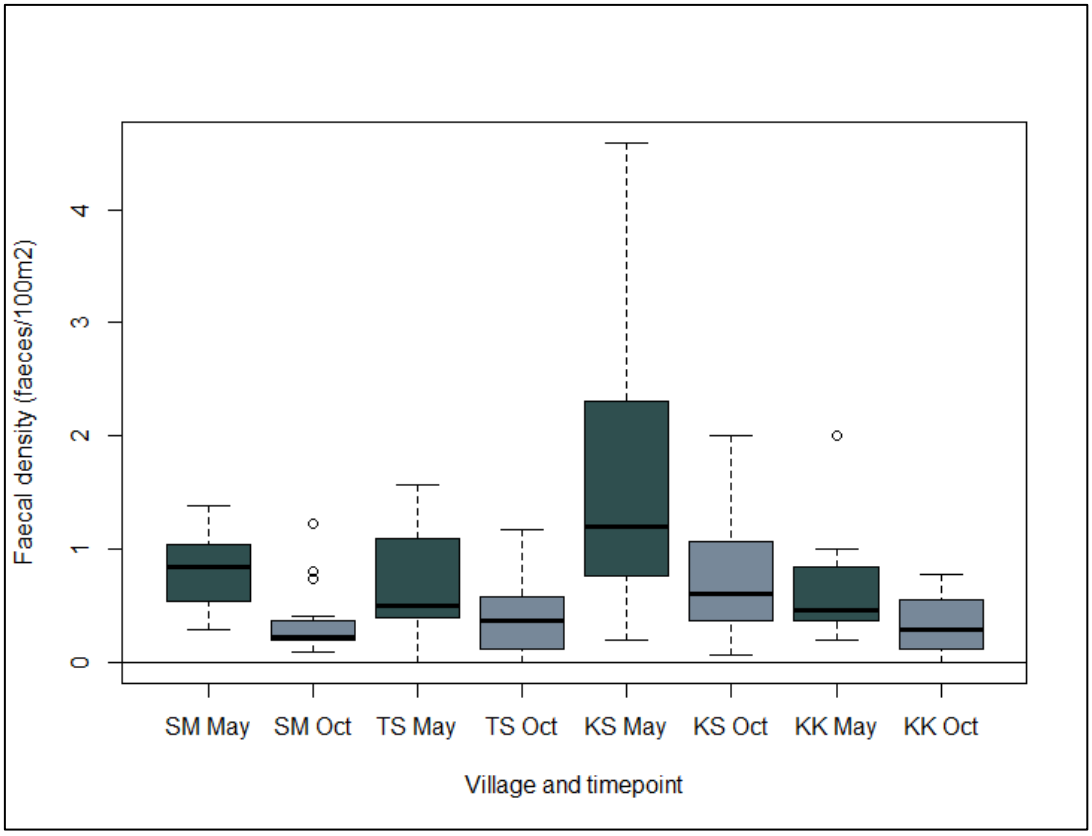

Figure 6-5. Box plot of canid faecal densities amongst the different villages visited in May and October 2012
(KK=Kara Kavak, KS= Kashka Suu, SM= Sary Mogul, TS=Taldu Suu).

6.3.3 CoproELISA results of faecal quadrat samples

In May 2012 a total of 104 faecal samples were collected from the quadrats in the four villages (KK=24, KS=28, SM=28, TS=24), of which 7 (6.7%) tested positive for *Echinococcus* spp.. ELISA positives ranged from 1/28 in Kashka Suu to 3/24 in Taldu Suu, with 1/24 and 2/28 ELISA positives in Kara Kavak and Sary Mogul respectively. In October 2012 a total of 100 ground faecal samples were collected (KK=24, KS=24, SM=28, TS=24) of which 18 (18%) tested positive. ELISA positives ranged from 2/24 in Kara Kavak to 8/24 in Taldu Suu, with 3/24 and 5/28 ELISA positives in Kashka Suu and Sary Mogul respectively.

6.3.4 PCR results of faecal quadrat samples

All 25 ELISA positive samples that had been collected from the faecal quadrats were analysed for *E. multilocularis*, *E. granulosus* G1, and *E. granulosus sensu lato* using coproPCR. Three samples (1 from KS, 1 from SM, 1 from TS) tested positive for *E. granulosus sensu lato*, and four samples (1 from KK, 1 from SM and 2 from TS) tested positive for *E. multilocularis.* One of these (a sample collected from a quadrat in Sary Mogul in October) was a mixed infection, testing positive for both *E. multilocularis* and *E. granulosus sensu lato*. All coproPCR positive samples were collected in October; all seven ELISA positive samples collected in May were coproPCR negative. The remaining 18 ELISA positive samples that were analysed with coproPCR were PCR negative.

6.3.5 Arecoline purge data

Of the 20 arecoline purges, eight were scored macroscopically as *Echinococcus* spp. positive in the field. Of these eight positive samples, three also harboured *Taenia* spp.. In addition, seven faecal samples were scored macroscopically as *Taenia* spp. positive but *Echinococcus* spp. negative. All 20 purges were analysed using coproELISA. Seven of the eight *Echinococcus* spp. purge positive samples were also coproELISA positive (five from Kara Kavak and two from Taldu Suu). Additionally, one sample (from Kara Kavak) that had not been scored as *Echinococcus* spp. positive in the field (but was scored as *Taenia* spp. positive), was coproELISA positive. The remaining six *Taenia* spp. purge positives were coproELISA negative. The eight coproELISA positive faecal samples were analysed for *Echinococcus* species using coproPCR. Three of these tested positive for *E. granulosus* G1 using the ND1 primers (Boufana *et al.* 2013) , and all eight tested positive for *E. granulosus sensu lato* using the "Abbasi" primers (Abbasi *et al.* 2003; Boufana *et al.* 2008). One sample from Taldu Suu also tested PCR positive for *E. multilocularis* (Boufana *et al.* 2013) ,

indicating a mixed infection. PCR products from the eight coproPCR positive samples were sequenced using the "Abbasi" primers (Beckman Coulter Genomics, Essex, UK). BLAST search gave 99% match to *E. granulosus* (accession number DQ157697, *Echinococcus granulosus* repeat region sequence) with no specification of genotype. One sample for which there was sufficient DNA present (from Kara Kavak) was further analysed and sequenced using generic cestode primers (von Nickisch-Rosenegk *et al.* 1999). BLAST search gave 99% match to *E. canadensis* (NCBI accession number AB794685).

6.3.6 Dog movement data

Four stationary iGotU® GPS loggers were evaluated simultaneously, over a period of 12 hours in the UK (633 recorded points). The distance recorded from the true location for the UK loggers ranged from 0m to 206m, with 95% of recorded locations for each logger being less than 45m. The stationary logger left for three and a half hours in Kyrgyzstan (total 40 points) recorded a difference of 0 to 32m, with a median of 5m.

Of the two dynamic GPS recordings made in Kyrgyzstan (a total of 155 points), the median difference from the true location was 16m, with 95% of readings being within 70m of the true value. The dynamic recordings made in the UK (35 points) showed a median difference of 31m with 95% of readings being within 90m of the true value.

Table 6-2 shows the median 95% characteristic hull polygon areas and distance travelled from the start location for those dogs monitored in each village. Dogs with less than 50 points recorded were excluded from analysis. A significant difference in distance travelled was found between villages (Kruskal Wallis test $P<0.001$), which was present when each individual village was compared with each other using a pairwise Wilcoxon test with Holm-

Bonferroni correction ($P<0.001$ in all cases). The same overall effect was also found when individual dogs were accounted for by comparing the median distance travelled (Kruskal Wallis test $P=0.004$), although with this analysis there was only a significant difference found between Taldu Suu and Kara Kavak or Kashka Suu (Wilcoxon with Holm-Bonferroni correction $P=0.02$ and $P=0.01$ respectively). No difference was found in the size of the home ranges between villages ($P=0.13$). There was also no difference in the size of the home range between males and females, either overall (Wilcoxon rank sum, $P=0.50$) or within those villages with both sexes represented (SM $P=0.53$; KS $P=0.25$). There was also no difference in the median distance travelled (per dog), according to sex (overall Wilcoxon rank sum $P=0.85$; SM only $P=0.41$; KS only $P=0.25$). Village areas based on MCP methods are shown in Table 6-2. Of all 37 dogs studied, 22 (59%) left the village boundary at least once during monitoring (note that figures in brackets relate to the bootstrapped confidence interval for the median). No difference was found in this proportion between different villages (Chi square test $P=0.73$).

Table 6-2. Description of dog movements and home range sizes for the dogs monitored with iGotU® GPS collars in the study.

Village	Village area (km^2)	Number of points	Number of dogs monitored	Median home range (m^2)	Median of median distance travelled per dog (m)
SM	3.32	1,494	11	22,650	39 (31-84)
TS	1.63	2,459	13	15,700	20 (20-29)
KS	1.16	1,637	10	37,490	46 (28-308)
KK	0.81	666	3	29,730	62 (58-629)
Overall	**-**	***6,256***	***37***	***22,650***	***35 (28-48)***

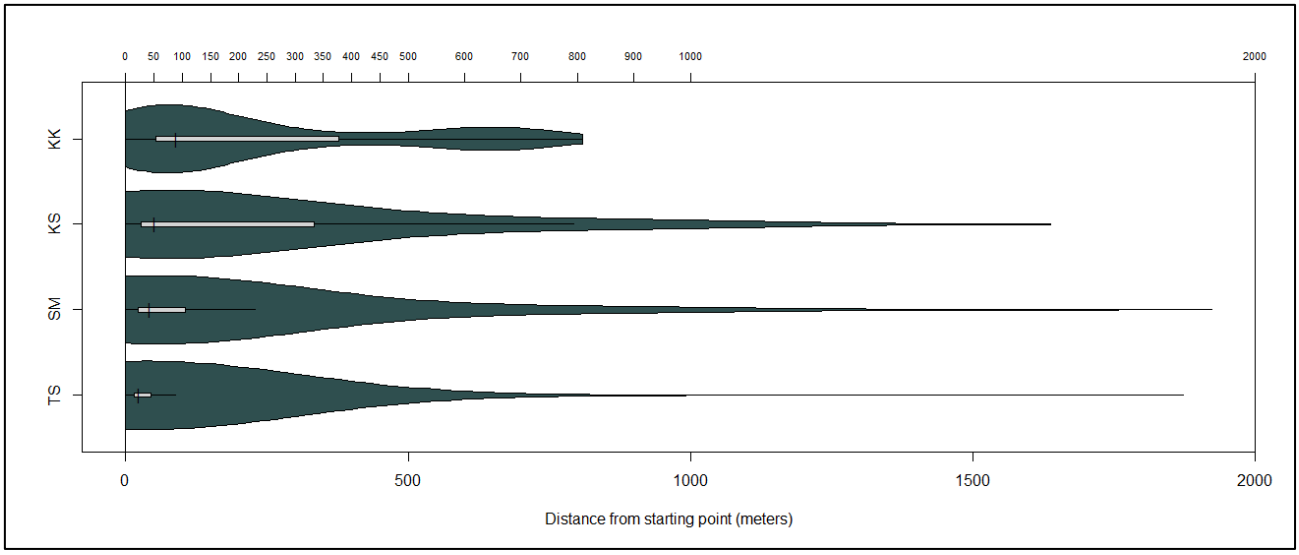

Figure 6-6. Violin plot of distances travelled by dogs from each village.
The light bar represents the interquartile range, the horizontal black line represent the distances to the 'inner fence' (1.5 times the interquartile range), and the vertical black lines represent the median. The dark grey areas represent the probability density.

6.4 Discussion

Echinococcosis is a national public health concern in Kyrgyzstan (Torgerson *et al.* 2002; Usubalieva *et al.* 2013). However no studies of canine echinococcosis have been undertaken in the Alay Valley of Osh oblast in the south-west of the country. Furthermore no information exists in Kyrgyzstan about environmental faecal contamination and behaviour of dogs in relation to transmission of *Echinococcus* spp..

The faecal samples collected and analysed from arecoline purged dogs confirmed that *Echinococcus* spp. are present in domestic dogs in the Alay Valley. This expands the known distribution of canine echinococcosis in southern Kyrgyzstan (Ziadinov *et al.* 2008). The ELISA positive arecoline purges (8/20) were all tested using coproPCR and the results showed that *E. granulosus sensu lato* (8/8), including *E. granulosus* G1 (3/8), as well as *E. canadensis* (1/8), and *E. multilocularis* (1/8) were present in domestic dogs in the Alay Valley. Regarding environmental contamination, of the ELISA positive faecal samples collected from the faecal quadrats (25/204), three samples tested DNA positive for *E.*

granulosus sensu lato, and four samples tested DNA positive for *E. multilocularis.* For 72% of ELISA positive samples however, the coproPCR analysis (for *E. granulosus* and *E. multilocularis*) yielded a negative result. This is likely due to the fact that many of the ground faecal samples collected from the quadrats were not fresh, and DNA in faeces is known to degrade over time unless preserved properly (e.g. Olson *et al.* 2005). Although these data confirm the presence of *Echinococcus* spp. in dogs in the Alay Valley, the data presented here are not sufficient to infer canine infection rates with *Echinococcus* spp., or to assess seasonality of canine echinococcosis. Further surveys have been undertaken to more accurately assess canine echinococcosis in the owned dog populations in the Alay Valley (see Chapter 5).

Between 38.0% and 74.4% of households in the four communities surveyed in the Alay Valley owned at least one dog. Male dogs were more commonly kept than females, as is often the case in rural communities (e.g. Butler and Bingham, 2000), and this may be related to males being seen as better guard/sheep dogs, or may be due to people not wanting to deal with pups. Questionnaire analysis revealed that almost all owned dogs were free to roam, with very few dogs being leashed. In addition, the dog population in all four communities appeared to have a high turnover. The local municipality in Gulcha (district administrative capital) arranges for dogs to be culled at least once a year in order to control dog population numbers (Akjol Gaitanbekov, Sary Mogul veterinarian, pers. comm.), and a decline in dog numbers was observed between May and October. Although many people acquired new dogs between visits, few of these did so to replace dogs which were lost or died. As such, based on current observations, the dog population in these southern Kyrgyz communities appears to be quite dynamic, with changes in dog numbers and dog ownership.

Dog faecal contamination in all four villages was found to be high, with an overall faecal density of 77.6 and 41.3 faeces/hectare in May and October respectively. This was higher than the faecal contamination reported in highly endemic *Echinococcus* spp. rural Tibetan communities in western China (Vaniscotte *et al.* 2011). The overall density of dog faecal contamination in the communities in the Alay Valley was significantly lower in October than in May. This may be because of the dog culling that took place before and in October, or may be due to the fact that faeces degraded faster in the warmer months between May and October than in the months before May. However, as only two sampling times were included, it is not conclusive that dog faecal environmental contamination is always higher during spring than autumn. Dog culling may take place twice a year, and may take place at different times of the year (Akjol Gaitanbekov, pers. comm.), and this will clearly affect dog faecal densities. Dog faeces present a risk to humans, and *Echinococcus* spp. eggs may survive in the environment for hundreds of days (Veit *et al.* 1995). The majority of dogs in these communities were free roaming, and as a result even gardens belonging to families that did not own dogs, or areas surrounding dog-free households were often contaminated with dog faeces (pers. obs.). Faecal contamination was also notably higher in Kashka Suu than in the other three villages, which is probably due to a higher dog density in this village.

The stationary and dynamic GPS recordings by the iGotU® units suggest that these GPS loggers can be used to monitor dog movements with reasonable accuracy. Although the battery life of the iGotU® units was limited and several units switched off prior to collection, the iGotU® units have several advantages over conventional GPS animal monitoring units. These benefits include a very small size (20g), frequent recording capacities, and being very affordable. As was found in community dogs in western China (Vaniscotte *et al.* 2011), dogs mostly stayed within a few hundred metres of their owners' homes (median 11-931m), with median home ranges between 15,700-37,490m^2. However, Alay Valley dogs were found to

roam up to 2km away from their owners' home, and most (59%) left the village boundary. Furthermore, these estimates of dog movements are probably conservative as only dogs that were present and tame enough to be handled could be included (i.e. those dogs that accompany livestock to pasture during the day were not included and aggressive dogs were not included but may have been more active than tamer dogs). There were significant differences in the distances travelled by dogs between different villages, with dogs in Taldu Suu generally travelling shorter distances than those in other villages. Although there was no evidence of any significant difference in the sizes of their core home ranges, this may be a result of the relatively small sample size, as the general trend in home range size was similar to that of median distance travelled (Table 6-2). In addition, although previous studies have found that male dogs generally move further than females (e.g. Vaniscotte *et al.* 2011), no evidence of a sex difference was found here. Although over 88% of people in all villages reported never feeding their dogs offal and only few people (5-39% per village) reported seeing their dogs eat rodents, the fact that dogs roamed freely and moved outside of their communities meant that people could not be sure of what their dogs were eating. Dogs were observed eating offal on several occasions (pers. obs.), and are likely to consume small rodents in or around villages.

Kyrgyzstan became independent around the time of the collapse of the Soviet Union in 1991, and has since been through considerable changes, including changes in sheep rearing practices, slaughter practices and praziquantel dosing of domestic dogs (Torgerson *et al.* 2002). Since independence, collective farms have broken up into small farms, home slaughter has increased, and the dog population has grown (Torgerson *et al.* 2006), which has been suggested as the cause of higher rates of human echinococcosis (Torgerson *et al.* 2002, 2006). The current data show that dogs are common in rural communities in the Alay Valley in southern Kyrgyzstan. The majority of dogs roamed freely, and dogs may roam several

kilometres away from their owners' home, thus being able to scavenge offal and consume rodents, putting them at risk of infection with *E. granulosus, E. canadensis* and *E. multilocularis.* The free roaming dogs also defecate wherever they roam, thus putting people in the community at a potential risk of infection with *Echinococcus* spp..

The first reports were made confirming the presence of *E. multilocularis, E. granulosus* G1 and *E. canadensis* in dogs in the Alay Valley, Osh oblast, Kyrgyzstan. Attempts to control and even eliminate echinococcosis have been carried out in several different locations, with differing degrees of success (Gemmell *et al.* 1986; Craig and Larrieu, 2006). The World Bank proposed an *Echinococcus* control programme for Kyrgyzstan, which included providing anthelminthics for dogs (World Bank, 2011), and indeed this programme was implemented in summer 2012. However, hydatid control programmes will benefit from being informed by an understanding of dog population size, basic dog ecology and dog behaviour. Collecting data such as that presented here can improve the efficacy of intervention programmes. Further studies to gain knowledge on dog population turnover and infection and re-infection rates will be beneficial, especially to determine optimal cost-benefits of dog dosing schedules.

6.5 Chapter 6 Summary

Echinococcosis is re-emerging in Kyrgyzstan, with numbers of human cases having increased substantially since the collapse of the Soviet Union in 1991, and increasing numbers of cases being reported from the Alay Valley. Due to their close proximity to people, domestic dogs play an important role in the transmission of *Echinococcus* spp.. It is therefore relevant to study the demography, ecology and behaviour of dogs in endemic areas. Dog demographics, roles of dogs, dog movements and faecal environmental contamination were assessed in four rural communities in the Alay Valley, southern Kyrgyzstan.

Arecoline purge data from twenty owned dogs in the Alay Valley revealed for the first time that *E. granulosus, E. canadensis* and *E. multilocularis* are co-endemic and occur in domestic dogs in the Alay Valley. Household surveys revealed that many households had dogs, and the dog population was large, with an estimated 1 dog for every 9.36 people. Dogs played various roles in the communities, including as pets, guard dogs, sheep dogs or combinations of these. Few people reported feeding their dogs offal, or observing their dogs eating small mammals. However, almost all dogs were free to roam so that owners could not be certain of what their dogs were eating. GPS data revealed that many dogs moved outside their communities, thus being able to scavenge offal and consume rodents. Faecal environmental contamination was high, with higher densities of faeces in May than in October. High environmental faecal densities present a significant infection risk to the local communities.

Chapter 7: Use of Lot Quality Assurance Sampling (LQAS) to evaluate the impact of two years of intervention on canine echinococcosis in the Alay Valley, Kyrgyzstan

7.1 Introduction

Echinococcosis is a neglected zoonotic disease (WHO, 2010) caused by infection with the larval stage of cestode tapeworms in the genus *Echinococcus* (Eckert and Deplazes, 2004). The most common types of echinococcosis are cystic and alveolar which are caused by *E. granulosus*/*E. canadensis* and *E. multilocularis* respectively (WHO/OIE, 2001). Both diseases are characterized by the formation of cysts, usually in the liver or lungs,, with cystic echinococcosis usually characterized by unilocular cysts, and alveolar echinococcosis usually characterized by multivesiculated cysts (WHO/OIE, 2001). Although the initial phases of infection are always asymptomatic and often go unnoticed for years (WHO/OIE, 2001), the disease becomes symptomatic in the later stages of infection.. If untreated, echinococcosis is often fatal (Fujikura, 1991; Moro and Schantz, 2009).

Echinococcosis affects communities worldwide, although the burden of the disease varies greatly in different locations (WHO/OIE, 2001). Echinococcosis is relatively common in Central Asia (Torgerson *et al.*, 2006; Torgerson, 2013), and it is a public health concern in Kyrgyzstan (Torgerson *et al.*, 2006). There are concerns the disease may be re-emerging, and human cases of both cystic and alveolar echinococcosis have increased greatly since Kyrgyzstan's independence from the Soviet Union in 1991 (Torgerson *et al.*, 2006; Usubalieva *et al.*, 2013).

In 2011, the World Bank considered echinococcosis to be of sufficient concern to implement an intervention programme which includes providing anthelmintics for dogs (World Bank,

2011), and dosing of domestic dogs began in the summer of 2012, with an aim to dose all owned dogs four times a year (Akjol Gaitanbekov, local veterinarian, pers. comm.). When implementing control programmes, it is important to evaluate how well these are meeting their targets (Schantz *et al.*, 1995; Schantz, 1997). However, as echinococcosis often affects rural and relatively remote communities (Craig *et al.*, 2007a), the same challenges associated with implementing the control scheme affect the evaluation of the control scheme. As such, relatively quick and easy evaluation tools to assess the impact of echinococcosis control schemes are needed (see also van Kesteren *et al.*, in press).

Originally developed for industry (Dodge and Romig, 1929), LQAS provides a statistically robust method of interpreting data despite requiring a relatively small sample size. LQAS was originally used to evaluate industrially produced goods; in these cases manufacturers aim to ensure a certain standard of quality, whilst keeping the economic costs of evaluation to a minimum (Dodge and Romig, 1929). To achieve this, a small representative sample of goods can be inspected, and if the number of defective goods exceeds a predetermined 'allowable number' (based on production standards and sample size) then the lot is rejected (Dodge and Romig, 1929). If this 'allowable number' of defects is not reached, the lot of goods can be classified as being of acceptable quality (Dodge and Romig, 1929). LQAS methodology has been adapted and simplified for application in field studies (Valadez *et al.*, 2002), and has been applied to studies related to healthcare. In healthcare LQAS can be used to assess, for example, whether or not a vaccination campaign has had an 'acceptable' coverage, or whether a disease prevalence is 'unacceptable' and requires intervention (for a review of LQAS studies in healthcare see Robertson and Valadez, 2006). In order to evaluate the World Bank echinococcosis intervention programme, ten communities in the Alay Valley were visited in April 2013 and April 2014 and LQAS methodology was applied to assess praziquantel dosing compliance and canine echinococcosis.

7.2 Methods

7.2.1 Communities

Ten communities in the Alay Valley were selected as part of this study. All were situated along a road (A327) that runs through the Valley from west to east. The communities sampled were (from west to east): Kyzyl Eshme (39.57°, 72.27°), Kabyk (39.59°, 72.39°), Achyk Suu (39.47°, 72.50°), Jaylima (39.62°, 72.59°), Kara Kavak (39.66°, 72.72°), Kashka Suu (39.64°, 72.67°), Sary Mogul (39.68°, 72.89°), Taldu Suu (39.70°, 72.98°), Archa Bulak (39.69°, 73.08°) and Sary Tash (39.73°, 73.25°, Figs. 2-1 and 2-2 in Chapter 2). All communities were small villages with up to ~400 households, and populations of between a few hundred to at most ~3,000 people.

7.2.2 Establishing a pre-intervention canine coproELISA prevalence

Four communities (Taldu Suu, Sary Mogul, Kara Kavak and Kashka Suu) were visited in May 2012, prior to the start of the World Bank intervention programme (see Chapter 5). All households were visited in Taldu Suu, Sary Mogul, and Kara Kavak, and all dogs present were sampled. In Kashka Suu, a randomly selected sample of households were registered (estimated to represent 25% of all households), and dogs in these households were sampled. As such, the number of samples collected and analysed from each community was as follows: Kara Kavak=35, Kashka Suu=43, Sary Mogul=157, Taldu Suu=98 (see Chapter 5).

7.2.3 Lot Quality Assurance Sampling: faecal sample and questionnaire data collection

A Lot Quality Assurance Sampling (LQAS) frame was adopted to evaluate canine echinococcosis in April 2013. A minimum of 19 dogs were sampled in Achyk Suu, Archa Bulak, Kabyk, Kyzyl Eshme, Jaylima and Sary Tash (a sample size of 19 minimizes the risk of type A and B errors, see Chapter 7, Appendix 1 taken from Valadez *et al.*, 2002). To select

sampled dogs, a GPS coordinate for each community was determined using Google Earth images, aiming to find a coordinate relatively central to each community. This point was taken as a start point. Upon arriving at this point, the second hand on a watch was used to determine a direction in which to walk. Alternate houses on this route were visited and if dogs were present they were sampled and questionnaires were administered to their owners to ask when their dog had last been dosed. The age and sex of each dog was recorded, as well as its GPS position. If a dead end or the end of the community was reached, the second hand of the watch was again used to determine a new walking direction and the same approach was used, until a minimum of 19 dogs had been sampled, with some additional dogs sampled if time allowed for it (however one sample collected from Achyk Suu in 2013 was lost in transport between the Alay Valley and the laboratory in Salford).

In the remaining four communities (Taldu Suu, Sary Mogul, Kara Kavak and Kashka Suu), more extensive sampling was undertaken as part of another study, with 85, 69, 31 and 59 dogs sampled respectively in 2013 and 102, 84, 31, and 45 respectively in 2014 (Mastin *et al*., in prep). For these communities, the LQAS sampling approach was mimicked upon return to Salford by selecting a theoretical start point in the centre of the community, choosing a direction using a watch and selecting 19 sampled households in that direction from the 'start point', and including any dogs in these households. As such the number of samples analysed per community was as follows (shown as 2013/2014): Kyzyl Eshme=19/19, Kabyk=19/19, Achyk Suu=18/19, Jaylima=19/21, Kara Kavak=21/19, Kashka Suu=19/19, Sary Mogul=19/19, Taldu Suu=19/19, Archa Bulak=19/19 and Sary Tash=19/19.

Dog owners were asked about the age and sex of their dogs, and when their dog was last dosed with praziquantel. In 2014, dog owners were also asked if they had heard of echinococcosis, and if they knew what caused the disease. Questionnaires were administered

in Kyrgyz by a native speaker (Bermet Mytynova, Kyrgyz veterinarian based at the Kyrgyz Veterinary Institute in Bishkek). Faecal samples were collected from around the dog owner's homes and subsamples were stored in 0.3% PBS Tween (Fisher Scientific, Loughborough, UK) with 10% formalin (sourced locally) Samples were shipped to the University of Salford, UK at room temperature.

LQAS was used to determine whether the control programme was reaching people in the communities. Although praziquantel dosing schemes may aim to reach all owned dogs, it is unrealistic to assume a 100% compliance rate, with rates of 59% to 80% previously reported from Kenya and China (see Torgerson, 2003). The World Bank aims to dose dogs four times a year, and mathematical models have shown that with dosing every 3-4 months, a compliance rate of 75% can be effective in reducing echinococcosis (Torgerson, 2003; Torgerson and Heath, 2003). For this reason we set our criterion at 75% of dogs dosed in the four months prior to our visit. Because dog owners could often not remember the exact day of dosing, only the month was noted and all dosings in January, February, March and April were included as being within four months prior to our visit (samples were collected between 6 and 20 April 2013 and 5 and 12 April 2014). Where the latest dosing was not known, it was assumed the dog had not been dosed in the previous four months.

7.2.4 Choosing LQAS decision numbers

Although simplified field manuals including decision numbers are available for LQAS sampling (Valadez *et al.*, 2002, see Chapter 7, Appendix 1 for decision number tables), it is possible to calculate decision numbers more accurately if the population size and exact prevalence are known (note that the simplified tables allow estimation of prevalence to the nearest 5%). This can be done using the hypergeometric distribution, which accounts for the fact that the probability of selecting a (in this case) coproELISA positive dog from the

population changes as dogs are sampled without replacement (Lemeshow and Taber, 1991). As such the decision number can be calculated using the following formula (from Lemeshow and Taber, 1991):

$$P(d \le d^*) = \sum_{d=0}^{d^*} \frac{\binom{NP_0}{d}\binom{N(1-P_0)}{n-d}}{\binom{N}{n}}.$$

Where N= the total dog population size in a community, P_0= the prevalence threshold, n=the number of dogs sampled and d*= the decision number-1. The decision number must be an integer and should be the lowest possible integer at which P exceeds or equals 10%. This means that the cumulative probability of obtaining d* positive samples (given the parameters N, n and P_0) is lower than 10%. If d* or fewer positive samples are obtained, this is interpreted as some evidence of a reduction in parameter P_0.

In order to calculate decision numbers, variables N, n and P_0 must be determined. In order to calculate N (the total dog population size in each of the communities) data collected from census data in Sary Mogul, Taldu Suu and Kara Kavak was used, and extrapolated from the randomly sampled houses in Taldu Suu. In May 2012, all households in Sary Mogul, Taldu Suu and Kara Kavak were visited and all dogs registered. In Kashka Suu an estimated quarter of households were visited, so that the total dog population can be estimated by multiplying this number by four. The size of these four villages was estimated broadly using the 'measure distance' tool in Google Earth, to select the area that contained most of the houses (Fig. 7-1). The number of dogs (from census data) was then divided by the area of the villages to give an estimate of number of dogs/100m^2 of village. This gave an average of 1.56 dogs/100m^2 (SE=0.30). The sizes of the other six communities were then estimated using Google Earth and the dog population estimated using the average dog density of 1.56 dogs/100m^2 (Table 7-1). It is worth noting that dog population numbers in the Alay Valley do fluctuate due to a bi-

annual dog culling campaign (Akjol Gaitanbekov, local veterinarian, pers. comm.). The data from May 2012 was collected just prior to dog culling and as such the dog population numbers shown in Table 7-1 are estimates extrapolated from populations that had not recently been culled (culling occurs somewhat randomly during the year, and varies per village).

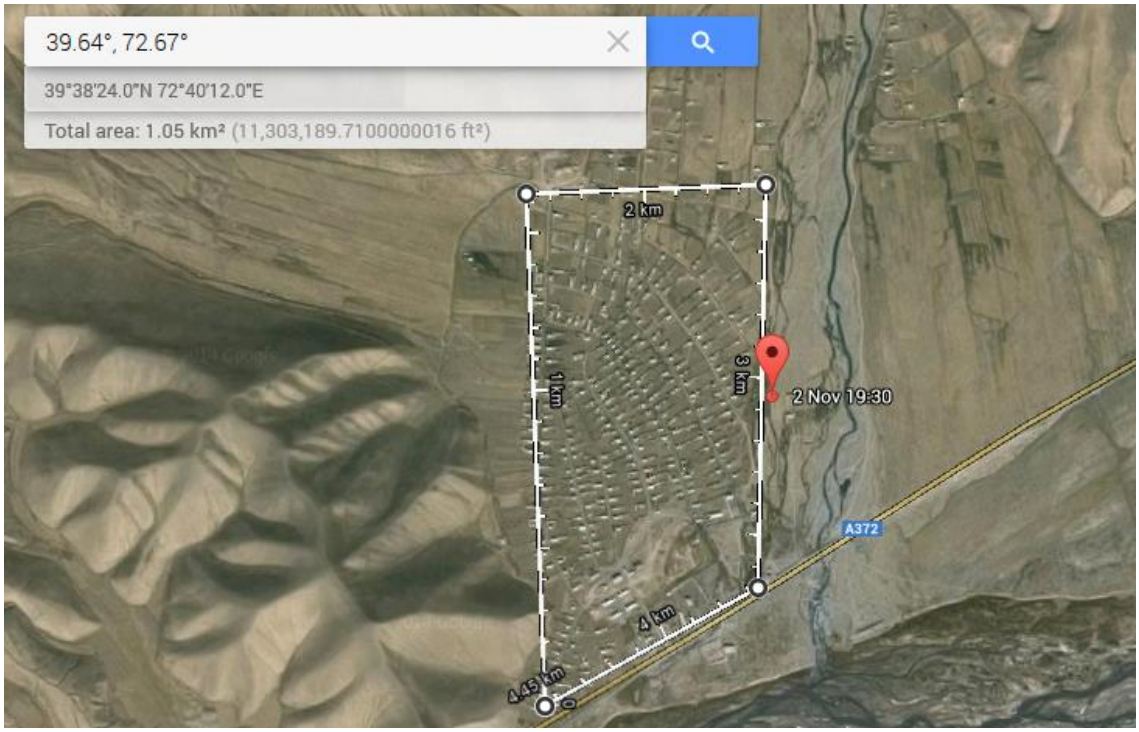

Figure 7-1: Example of how village areas were estimated in Google Earth.
Village shown is Kashka Suu, boundaries are drawn according to contain most houses in the community and the total area size is shown in the box on the left.

Table 7-1: Estimated dog populations in the 10 communities sampled.

Village	Estimated area m^2	Dogs (from census or estimated to nearest 5)
Taldu Suu	367864	98
Sary Mogul	1210000	157
Kara Kavak	312322	35
Kashka Suu	1050000	120
Archa Bulak	159300	25
Sary Tash	558879	90
Kabyk	293154	50
Kyzyl Eshme	675193	105
Achyk Suu	608317	95
Jaylima	172641	30

After determining N, n and P_0 had to be determined. P_0 was set at the pre-intervention prevalence as calculated from the samples collected from Sary Mogul, Taldu Suu, Kashka Suu and Kara Kavak in 2012, and n was determined as 18, 19 or 21 (the number of dogs sampled in each community in each year). For praziquantel dosing the P_0 was set at 75%, for coproELISA prevalence the average pre-intervention prevalences, calculated from samples collected in Sary Mogul, Taldu Suu, Kashka Suu and Kara Kavak were used (for decision numbers see Results).

7.2.5 CoproELISA

After decontaminating at -80°C for a minimum of four days (WHO/OIE, 2001), faecal samples were extracted by homogenizing, shaking and centrifuging at 2500r.p.m (1125G) for 5 minutes using an eppendorf® centrifuge 5804, and collecting the supernatant. All collected faecal samples were analysed for *Echinococcus* spp. coproantigen using a genus-specific sandwich ELISA using the protocol described by Allan *et al*. (1992) and Craig *et al*. (1995, see Chapter 3 for details). Supernatants of two known positives (an arecoline purge positive sample from Kara Kavak or a necropsy positive sample from Hobukesar County, Xinjiang, China, and an antigen spiked sample, spiked 1:100 with *E. granulosus* whole worm extract) were used as positive controls throughout. Three known negatives from a very low endemic area (Falkland Islands) were also included as negative controls.

The 'pre-intervention' samples collected in May 2012 and the LQAS samples collected in April 2013 and April 2014 were analysed in two lots. Aliquots of capture and conjugate antibody were pooled to a sufficient volume to test all the samples in each lot and mixed. Sufficient BCB, wash buffer (0.1% PBS Tween) and blocking buffer (0.3% PBS Tween) was made up to analyse all the samples in each lot, and the same bottle of TMB was used for all plates, to ensure minimum variation in test conditions.

Prior to analysing the collected faecal samples in each of the two lots, a panel of known positive and negative dogs was tested with the antibodies. These panels included arecoline purge samples from dogs in Taldu Suu and Kara Kavak (total n=20 of which 12 were negative by examination with a magnifying glass and subsequent coproELISA, and 8 were positive by examination with a magnifying glass and subsequent coproELISA, see Chapter 7, Appendix 2). The panels also included 36 samples from necropsied dogs in communities in Hobukesar County, Xinjiang China. These included 16 necropsy positives (with estimated worm burdens between 2 and >10,000), and 20 necropsy negative samples (see Chapter 7, Appendix 3 for details). For the May 2012 samples, the panel consisted of the 20 arecoline purge samples as well as 19 samples collected from necropsied dogs in Hobukesar County (including 12 necropsy positive with worm burdens ranging from 2 to >10,000 and 7 necropsy negative samples). For the 'LQAS' lot of samples the panel consisted of seven arecoline purge samples (3 positive and 4 negative) and 35 samples collected from necropsied dogs in Hobukesar County, fifteen of which were necropsy positive, with worm burdens ranging from 2 to >10,000, and ten samples were necropsy negative. Because the communities from which the dogs were sampled in Hobukesar are culturally similar to the Alay Valley (i.e. both locations include relatively small rural communities of semi nomadic people whose livelihoods are largely based on animal husbandry) the samples from Hobukesar are appropriate for use in a panel for the Alay Valley.

The faecal sample panels were analysed prior to analysing the field samples and a cut-off determined using ROC curves (Gardner and Greiner, 2006). Normally ROC curves are used to maximise diagnostic sensitivity and/or specificity, depending on the aims of the study. In this case, the aim was to compare the pre-intervention data to the data collected approximately one and two years after the start of the intervention campaign, rather than to

estimate true coproELISA prevalences. As such cut-offs were chosen to give similar sensitivities and specificities between the two lots of samples (i.e. those collected in May 2012 and those collected in April 2013 and April 2014). For the pre-intervention samples (May 2012) a cut-off was chosen that gave a diagnostic sensitivity of 90% and a specificity of 86%. For the 'LQAS' samples (April 2013 and April 2014) a cut-off was chosen that gave a diagnostic sensitivity of 89% and a specificity of 88%. (Note that for this reason the pre-intervention coproELISA prevalence described here is slightly different from that described in Chapter 5).

The coproELISA described here is genus-specific for *Echinococcus* spp., but *E. granulosus, E. multilocularis* and *E. canadensis* are co-endemic in the Alay Valley (Chapter 6, van Kesteren *et al.,* 2013). For the pre-intervention samples (n=333) the results from three species specific coproPCR protocols are given in Chapter 5. For the LQAS samples the number of samples collected per village was small (n=18-21) with low numbers of ELISA positives and poor correlations between coproELISA and coproPCR data. For these reasons, coproPCR data was not considered to be informative in this case.

7.3 Results

7.3.1 Pre-intervention coproELISA prevalence

The samples collected in May 2012 (n=333) found an average coproELISA prevalence of 23.7%, with a range from 21.4% in Taldu Suu to 25.7% in Kara Kavak, as determined using the cut-off from the ROC panel (Table 7-2). The P_0 for coproELISA prevalence was therefore set at 23.7%

Table 7-2: Pre-intervention coproELISA prevalences in four communities in the Alay Valley

Community	CoproELISA +ve	CoproELISA -ve	Total
Kara Kavak	9 (25.7%)	26 (74.3%)	35
Kashka Suu	10 (23.3%)	33 (76.7%)	43
Sary Mogul	39 (24.8%)	118 (75.2%)	157
Taldu Suu	21 (21.4%)	77 (76.8%)	98
Total	79 (23.7%)	254 (76.3%)	333

7.3.2 Dog demographics and praziquantel dosing in April 2013 and April 2014

A total of 191 dogs were sampled in April 2013 (when accounting for one sample lost in transport). The majority of these (157 or 82.2%) were male, with 28 females (14.7%). For six dogs (3.1%) the sex was not recorded. Most dogs were younger than five years (131, or 69.3%, see Fig. 7-2), although age of 8 male and 2 female dogs was not recorded, and for 6 dogs neither age nor sex was recorded.

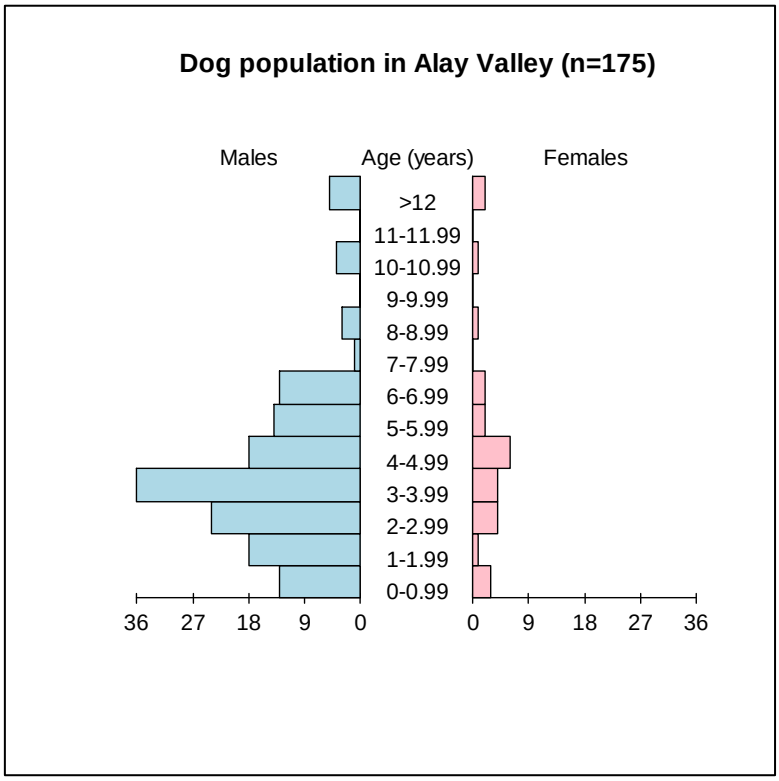

Figure 7-2: Dog demographics in the Alay Valley in April 2013, based on LQAS sampling of ten communities
(Note: age and/or sex of 16 dogs not recorded)

A total of 192 dogs were sampled in April 2014. The majority of these (156 or 81.3%) were male, with 35 females (18.2%). The sex of one dog (0.5%) was not recorded. Most dogs were younger than five years (156 or 81.3%) %, see Fig. 7-3), and for 5 dogs the age/sex was not recorded.

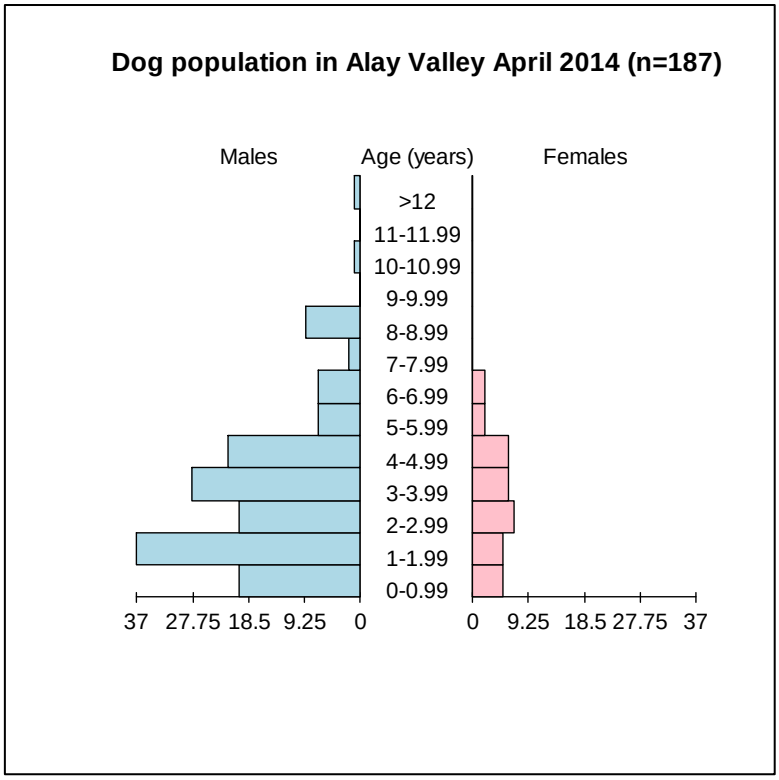

Figure 7-3: Dog demographics in the Alay Valley in April 2014, based on LQAS sampling of ten communities
(Note: age and/or sex of 5 dogs not recorded)

In 2013, the majority of dog owners reported dosing their dog at different times in the seven months before sampling (141, or 73.8%, Fig. 7-4), with one person reportedly dosing their dog 11 months before sampling (0.52%). However 39 dog owners (20.42%) reported never dosing their dogs, and a further 10 owners (5.24%) did not know when their dog had last been dosed, if ever (Fig. 7-4). In 2014, 152 dog owners (79.2%, Fig. 7-4) reported dosing their dog at different times in the seven months before sampling, with four dogs (2.1%) being dosed between 7 and 8 months prior to sampling. In 2014, 23 dog owners (12%) reported

never dosing their dogs and for a further 13 dogs (6.8%), the latest dosing was not known (Fig. 7-4).

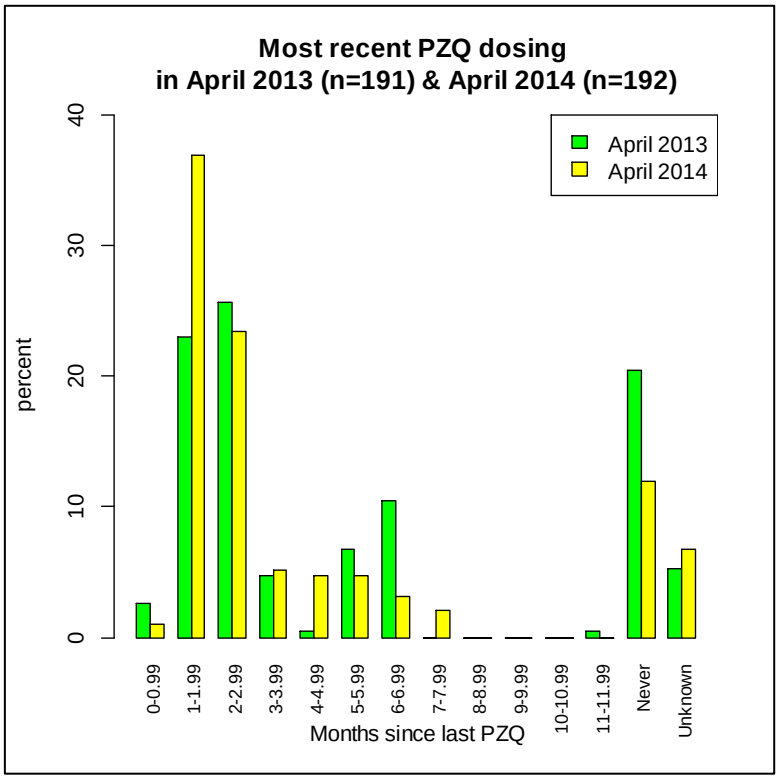

Figure 7-4: Most recent praziquantel dosing for dogs in the Alay Valley in April 2013 and April 2014

7.3.3 Local knowledge of echinococcosis

In 2014, dog owners were asked if they had heard about human echinococcosis (yes/no question) and what they thought caused human echinococcosis (open question). A total of 149 dog owners were asked these questions (a total of 192 dogs were sampled but some owners had multiple dogs, and for five dogs the owners did not answer these questions). For the cause of echinococcosis, answers were classified as either 'correct', 'incorrect' or 'partially correct'. 'Correct' answers included: dog faeces, foxes, wolves, and contact with dogs. If owners correctly identified dogs and dog faeces as possible sources of infection but also listed incorrect sources such as sheep or mice, these were classed as 'partially correct'. If owners said they didn't know what caused echinococcosis, or gave wrong responses, for example 'livers' then the answer was classed as 'incorrect'. Out of the 149 respondents, 126

(84.6%) had heard of echinococcosis, and 93 of these (78.3%) correctly identified causes of echinococcosis, with a further 13 respondents (10.3%) giving partially correct responses. 23 dog owners (15.4%) had not heard of echinococcosis and could not correctly identify its causes, but of the respondents who had heard of echinococcosis, 20 could also not correctly identify its causes. As such a total of 43 dog owners (28.9%) could not correctly identify causes of echinococcosis.

7.3.4 Using LQAS to evaluate PZQ dosing

Although the majority of dogs were dosed in the four months prior to sampling in 2013 (109, or 56.5%), there were marked differences between villages with none of 19 dogs dosed in the previous four months in Sary Mogul in 2013, and 16 out of 19 dogs (84.2%) dosed in Jaylima and Kabyk in 2013 (Table 7-3, with communities in green indicating that the LQAS requirements were met, and communities in red indicating LQAS requirements were not met). Six out of ten communities (Archa Bulak, Kara Kavak, Kashka Suu, Kyzyl Eshme, Sary Mogul, Sary Tash) did not meet the decision number set according to LQAS requirements, whereas four others (Achyk Suu, Jaylima, Kabyk and Taldu Suu) did. This suggests that the praziquantel dosing scheme failed to reach at least 75% of owned dogs in six of the ten communities in 2013.

Table 7-3: Dogs dosed in the four months prior to sampling in each of the ten communities in April 2013 and April 2014.

2013				2014			
Community	Dosed in last 4 months	Not dosed in last 4 months	Decision number	Community	Dosed in last 4 months	Not dosed in last 4 months	Decision number
Achyk Suu	13	5	11	Achyk Suu	16	3	12
Archa Bulak	11	8	13	Archa Bulak	17	2	13
Jaylima	16	3	13	Jaylima	19	2	14
Kabyk	15	4	12	Kabyk	14	5	12
Kara Kavak	10	11	14	Kara Kavak	16	3	12
Kashka Suu	10	9	12	Kashka Suu	5	14	12
Kyzyl Esme	12	7	12	Kyzyl Esme	4	15	12
Sary Mogul	0	19	12	Sary Mogul	13	6	12
Sary Tash	7	12	12	Sary Tash	17	2	12
Taldu Suu	15	4	12	Taldu Suu	16	3	12

In 2014, the number of dogs dosed no more than four months prior to sampling was higher than in 2013 (128, or 66.7%). Dosing compliance rates also seemed to have improved with 8/10 communities (Achyk Suu, Archa Bulak, Jaylima, Kabyk, Kara Kavak, Sary Mogul and Taldu Suu) meeting the decision number set according to LQAS requirements (Table 7-3,). Only two communities (Kashka Suu and Kyzyl Eshme) did not meet our decision numbers. This suggests that the praziquantel dosing scheme was reaching more owned dogs in 2014 than in 2013.

7.3.5 Using LQAS to evaluate the impact of two years of intervention on coproELISA prevalence

In order to evaluate whether the coproELISA prevalence had decreased following the start of the intervention programme, LQAS methodology was used. As the pre-intervention prevalence was determined to be 23.7%, we set P_0 at 23.7% and aimed to identify those villages that had achieved a reduction on their coproELISA prevalence. Decision numbers were calculated using the hypergeometric distribution formula (see above).

In 2013, five communities (Archa Bulak, Kara Kavak, Kashka Suu, Sary Mogul and Sary Tash) met our decision number set according to LQAS requirements, providing evidence that in these communities the coproELISA prevalence in dogs was less that 23.7% (Table 7-4, with communities in green indicating that the LQAS requirements were met, and communities in red indicating LQAS requirements were not met).

Table 7-4: CoproELISA prevalences in the ten communities sampled in April 2013 and April 2014.

2013				2014			
Community	CoproELISA +ve	CoproELISA -ve	Decision number	Community	CoproELISA +ve	CoproELISA -ve	Decision number
Achyk Suu	4	14	2	Achyk Suu	5	14	3
Archa Bulak	1	18	3	Archa Bulak	0	19	3
Jaylima	6	13	3	Jaylima	2	19	4
Kabyk	4	15	3	Kabyk	4	15	3
Kara Kavak	1	20	3	Kara Kavak	2	17	3
Kashka Suu	1	18	2	Kashka Suu	5	14	2
Kyzyl Esme	3	16	2	Kyzyl Esme	7	12	2
Sary Mogul	0	19	2	Sary Mogul	4	15	2
Sary Tash	1	18	2	Sary Tash	0	19	2
Taldu Suu	2	17	2	Taldu Suu	4	15	2

In 2014, four communities (Archa Bulak, Jaylima, Kara Kavak and Sary Tash) met our decision number set according to LQAS requirements, providing evidence that in these communities the coproELISA prevalence in dogs was less that 23.7% (Table 7-4).

7.4 Discussion

Echinococcosis is a serious disease that can be seriously debilitating and fatal (WHO/OIE, 2001). However, the impact of echinococcosis is not limited to human health, and infection of livestock with *Echinococcus* spp. can lead to the condemnation of meat, reduced growth of livestock, reduced milk production, and reduced fecundity (Benner *et al.*, 2010), which has

an economic impact on rural communities. Echinococcosis appears to be re-emerging in Kyrgyzstan (Torgerson *et al.*, 2003). A World Bank echinococcosis control programme aimed to address this through dosing owned dogs with praziquantel (World Bank, 2011). However, echinococcosis is very difficult to control or eliminate (WHO/OIE 2001) and to date only Iceland, New Zealand and Tasmania have declared elimination status for *Echinococcus* spp. after several decades of intervention (Craig and Larrieu, 2006). Control programmes may include several measures to try to reduce or eradicate echinococcosis, including education campaigns, praziquantel dosing of dogs, controlled slaughter of livestock (Gemmell *et al.*, 1986), and vaccination of sheep, the main intermediate host for *E. granulosus* (Barnes *et al.*, 2012). Although control programmes have been successful in islands including Iceland, New Zealand and Tasmania (Craig and Larrieu, 2006), continental areas present greater challenges for control of echinococcosis. This is especially true for regions that are relatively remote and where people are nomadic or semi-nomadic (e.g. Schantz *et al.*, 2003). In these cases frequent praziquantel dosing of domestic dogs (standard recommended dosing every six weeks) may not be practically feasible (Gemmell *et al.*, 1986; Lembo *et al.*, 2013).

Echinococcosis control programmes often require years, if not decades, of commitment. Although initial rates of *Echinococcus* spp. infections may decline rapidly, long term efforts are needed to ensure infection rates don't increase again after an initial 'attack' phase (WHO/OIE, 2001). Echinococcosis control programmes are likely to face many challenges, especially in remote, less developed areas, and especially where communities are (semi) nomadic. The Alay Valley is relatively remote and located between the Alay and Pamir Mountains. The area is rural and sparsely populated, with limited infrastructure. In addition, many people travel to summer pasture. These factors mean the praziquantel dosing scheme faces several challenges in this area.

The implementation of echinococcosis control programmes is costly in terms of both money and human resources, but challenges associated with control programmes are well known, and in many places control programs have not had the long term success hoped for (for examples see Craig and Larrieu, 2006). As such, it is important to evaluate the real impact of control programmes, and not simply to judge these by measures such as 'money invested' or 'praziquantel tablets distributed'. This requires reliable pre-intervention data on (depending on the questions being asked) dog infection rates and human echinococcosis cases, in order to assess the pre and post intervention data. However, if implementing control programmes is difficult due to factors such as limited human resources, funds, laboratory resources, remoteness of communities and other logistical challenges, then the evaluations of control programmes are likely to suffer from the same challenges. As such, relatively quick and easy tools to evaluate echinococcosis control programmes are highly desirable. In this chapter, Lot Quality Assurance Sampling (LQAS) was used, as a relatively quick and easy tool to evaluate the impact of two years of the World Bank praziquantel dosing scheme on canine echinococcosis in ten communities in the Alay Valley, southern Kyrgyzstan.

In order to evaluate the impact of the intervention programme in the Alay Valley, a total of 333 owned dogs were sampled in four communities (Kara Kavak, Kasha Suu, Sary Mogul and Taldu Suu) prior to the World Bank dosing scheme (starting in 2012). Faecal samples were tested with coproELISA and, using a cut-off determined using ROC curves, the overall prevalence was found to be 23.7%. In April 2013 and April 2014 (approximately 9 and 21 months after the start of the dosing scheme), ten Alay Valley communities were visited. In these communities two measures were tested. One was the most recent reported praziquantel dosing of owned dogs, and the second was the coproELISA prevalence. Based on pre-intervention coproELISA results, the threshold for coproELISA prevalence was set at 23.7%,

and based on previous studies (Torgerson, 2003), the dosing threshold was set at 75% of dogs dosed in the previous 3-4 months. In 2013, four of the ten communities showed evidence of reaching a 75% dosing target, and in 2014 this number had increased to eight out of ten. This suggests that in 2014, the dosing scheme was reaching at least 75% of owned dogs in most communities sampled. Furthermore, in 2014 a majority of dog owners (84.6%) had heard of human echinococcosis and could describe its causes (78.3%).

The coproELISA prevalence data gave less clear results. In 2013, five communities did not exceed the LQAS decision number, providing evidence that the coproELISA prevalence in these communities was less than the pre-intervention estimate of 23.7%. However, in 2014, this had decreased to four out of the ten communities sampled, despite the higher number of dogs being reportedly dosed with praziquantel. Of the 33 coproELISA positive dogs in 2014, 15 (45.5%) had not been dosed between January and April 2014, and were either never dosed at all (n=6), dosed prior to January 2014 (n=6), or the last dosing was not known (n=3). However, 18 coproELISA positive dogs (54.5%) had been dosed between January and April 2014. Although praziquantel is highly effective in treating canine echinococcosis, it provides no protection against reinfection, and if dogs continue to have access to offal and/or small mammals, they may become re-infected with *E. granulosus*, *E. canadensis* or *E. multilocularis* respectively after dosing. The infection pressure to dogs depends on several factors including, for *E. granulosus* and *E. canadensis*, the prevalence of infection in sheep and other herbivores and the frequency of feeding offal to dogs (Torgerson and Heath, 2003), and for *E. multilocularis*, the presence, population size and infection rates of intermediate hosts (see Inoue *et al.*, 2007). Therefore, even if dogs were correctly dosed, they may still be subject to high reinfection pressures. This could also explain the poor correlation between reported praziquantel dosing and coproELISA prevalence in 2013.

Deworming dogs using praziquantel is generally considered to eventually reduce the infection pressure to dogs although this takes time (e.g. Torgerson and Heath, 2003). Praziquantel dosing will lead to a decrease in *Echinococcus* spp. infections in dogs, which will result in a lowered infection pressure in sheep (and other livestock), and the numbers of cysts in sheep (and other livestock) will begin to decline. However, this will not change the infection pressure in dogs for some time because of the longevity of cysts in the older sheep (Torgerson and Heath, 2003). Similarly, although the lifespan of voles and other small mammals is much shorter than that of sheep (usually between a few months and 1.5 years, e.g. Bobek, 1969; Devevey and Christe, 2009), it will take some time for small mammals that were infected before dosing began to die off. Furthermore, *E. multilocularis* is expected to be less vulnerable to dog dosing campaigns due to its sylvatic lifecycle (Eckert and Deplazes, 2004).

As such, after just two years of intervention it may be that the infection pressure is still high, and dogs may be getting re-infected even if they are dosed several times a year. It is likely that the dosing scheme will have to be conducted for a longer period before infection pressures decrease and the re-infection rate of dosed dogs decreases. Nevertheless the data collected here suggests that the praziquantel dosing scheme is reaching at least 75% of people in 8/10 communities, and that the coverage seems to have increased between 2013 (when 75% of people reported dosing their dogs in 4/10 communities) and 2014 (when this had increased to 8/10 communities). CoproELISA prevalences also showed evidence of decreasing from pre-intervention prevalences with 5/10 and 4/10 communities showing evidence of reduced coproELISA prevalence in 2013 and 2014 respectively. This is concurrent with the larger scale studies undertaken in Sary Mogul, Taldu Suu, Kara Kavak, and Kashka Suu, where all dogs registered in May 2012 (n=333) have been followed up and sampled every spring (April/May) and autumn (September/October) until September 2014

(Mastin *et al.*, in prep.-c). This larger sample size also showed evidence that coproELISA prevalence had been reduced since the start of the World Bank praziquantel dosing campaign in 2012.

The echinococcosis control programme in the Alay Valley is in the early stages, and mixed results are to be expected so soon (9 and 21 months) after the programme was first implemented. However, control programmes that include praziquantel dosing of dogs should include a baseline survey and surveillance to monitor progress (WHO/OIE, 2001). In the longer term, control programmes can be evaluated through the surveillance of human echinococcosis (for example through ultrasound screening surveys) and surveillance of CE in the intermediate hosts (for example through slaughterhouse inspections, Gemmell and Schantz, 1997; WHO/OIE, 2001). However, because of the slow growth of cysts in human patients (Moro and Schantz, 2009) and the fact that small cysts can be missed by ultrasound screening (Craig *et al.*, 2003), it can take several years before changes in human prevalences of echinococcosis can be detected. The ethics of community ultrasound surveys should also be considered, especially in areas where opportunities to follow up on patients and/or treatment options may be limited (Macpherson *et al.*, 2003). In slaughterhouse inspections, small cysts may be missed (e.g. Liu *et al.*, 1993) or livestock may not be slaughtered until they are older, so changes in prevalences can take years to detect. Additional challenges include the fact that in developing countries it can be difficult to trace the origin of livestock brought to slaughterhouses, and slaughterhouses may not be used in favour of home slaughter (e.g. Buishi *et al.*, 2005).

Surveillance of echinococcosis in domestic dogs allows for a more immediate evaluation of a control programme. This has the added benefit that dogs can be sampled and tested for *Echinococcus* spp. non-invasively through coproELISA analysis of ground faecal samples

(e.g. Pierangeli *et al.*, 2010). Echinococcosis is asymptomatic in dogs, and can be easily and relatively affordably treated with praziquantel (Eckert and Deplazes, 2004), thus avoiding some of the ethical concerns with diagnosis of human cases. These advantages mean that measuring canine echinococcosis rates provides a pragmatic method of evaluating control programmes. However, evaluations of control programmes are likely to be subject to the same challenges as the control programme itself, such as lack of resources, remoteness of communities, and lack of expertise/infrastructure (Craig *et al.*, 2007b). As such, relatively quick and easy tools to assess whether or not control programmes are meeting their targets are desirable. Here, Lot Quality Assurance Sampling was adopted to assess the impact of two years of quarterly praziquantel dosing by the World Bank. Results showed that by 2014, targets of 75% of dogs being dosed were reached in 8/10 communities sampled, and 5/10 and 4/10 communities showed evidence of a reduction in canine coproELISA prevalences in 2013 and 2014 respectively.

7.5 Chapter 7 Summary

Echinococcosis is a re-emerging zoonotic disease in Kyrgyzstan. In 2012, the World Bank implemented an echinococcosis control scheme that includes dosing all owned dogs in Alay Valley communities with praziquantel four times a year. Control programmes often require large investments of money and resources, and as such it is important to evaluate how well these are meeting their targets (Schantz *et al.*, 1995; Schantz, 1997). In order to do this, it is crucial that pre-intervention data are collected (Schantz *et al.*, 1995; Schantz, 1997). However, problems associated with trying to control echinococcosis are well known, and include remoteness of communities, semi-nomadic customs in affected communities, and lack of funds and trained personnel etc. (Macpherson, 2005; Craig and Larrieu, 2006; Craig *et al.*, 2007a). These same problems apply to efforts to evaluate control schemes, and therefore relatively quick and easy assessment tools are highly desirable.

Here Lot Quality Assurance Sampling (LQAS) was used to assess the impacts of approximately two years of the World Bank echinococcosis control programme. Although originally developed for industry (Dodge and Romig, 1929), LQAS has been adapted for use in healthcare studies (Robertson and Valadez, 2006) and was applied here to test praziquantel dosing compliance and coproELISA prevalence in the Alay Valley. Ten communities were visited, with between 18 and 21 dogs sampled in each community, and questionnaires administered to dog owners. Based on sampling prior to the World Bank control programme, the pre-intervention coproELISA prevalence was set at 23.7%, and the threshold for dosing compliance was set at 75% based on previous studies (Torgerson, 2003; Torgerson and Heath, 2003).

In April 2013, approximately nine months after the start of the World Bank dosing scheme, 4/10 communities sampled showed evidence of having met the 75% dosing compliance target; in April 2014 (approximately 21 months after the start of the dosing scheme) this increased to 8/10 communities. In April 2013 and April 2014, 5/10 and 4/10 communities respectively showed evidence of having a coproELISA prevalence that was reduced from pre-intervention levels (23.7%). Echinococcosis takes years if not decades of sustained intervention to be effectively controlled, and it is likely that after only 21 months of dosing *Echinococcus* spp. infection pressure to dogs in the Alay Valley was still high. Nevertheless, these results show that dosing compliance targets (of 75%) are being met in most communities in the Alay Valley, and data suggest that coproELISA prevalences have decreased from pre-intervention levels.

Chapter 8: General Discussion

8.1 Discussion

Echinococcosis is a neglected zoonotic disease that occurs virtually world-wide (WHO, 2010). As well as the direct effects of the illness on individuals and their communities, *Echinococcus* spp. impact on livestock and associated economies (Carabin *et al.*, 2005). There are concerns that echinococcosis is re-emerging in Kyrgyzstan since the collapse of the Soviet Union, with many cases occurring in the Osh Oblast of southern Kyrgyzstan (Torgerson *et al.*, 2006; Usubalieva *et al.*, 2013). The World Bank considered echinococcosis to be of sufficient concern to start a praziquantel dosing campaign in 2012 (WorldBank, 2011). However, despite this, little is known about canine echinococcosis in Kyrgyzstan, and especially in the Osh Oblast area of the country.

Domestic dogs are final hosts for *Echinococcus* spp. including *E. granulosus*, *E. multilocularis* (see Thompson and McManus, 2002) and *E. canadensis* (Nakao *et al.*, 2013b), and, due to their close association with humans, are often the main source of human infections with *Echinococcus* spp. (Budke *et al.*, 2005). Therefore, it is important to consider local domestic dog populations when studying echinococcosis. This thesis therefore aimed to study canine echinococcosis in the Alay Valley, in Osh Oblast, southern Kyrgyzstan. This study had four main aims namely: 1. To develop appropriate coproELISA and coproPCR tools to diagnose canine echinococcosis. 2. To assess canine echinococcosis in four communities in the Alay Valley prior to intervention. 3. To study the roles and ecology of domestic dogs in the Alay Valley and lastly: 4. To assess the impact of nearly two years of praziquantel dosing on canine echinococcosis in the Alay Valley.

The first aim undertaken was the development of a suitable coproELISA, and, as described in Chapter 3, polyclonal antibodies from previously obtained rabbit sera were extracted and optimized to create a sandwich coproELISA that could reliably detect *Echinococcus* spp. antigens in domestic dog faecal samples. Following the discovery from adult worms recovered from arecoline purges that *E. canadensis* occurs in domestic dogs in the Alay Valley, it also became necessary to be able to detect this species, and in Chapter 4, two new sets of primers were optimized for the PCR detection of *E. granulosus sensu stricto*, *E. equinus*, *E multilocularis* and *E. canadensis* in both tissue and faecal samples. In Chapter 5, the canine echinococcosis coproELISA prevalence in four communities in the Alay Valley prior to the World Bank praziquantel dosing scheme was assessed, as well as determining the species of *Echinococcus* present using coproPCR. In Chapter 6 the ecology of domestic dogs in four communities in the Alay Valley was investigated, including their roles in the communities, husbandry, and roaming behaviour. Environmental contamination with dog faeces was also assessed, as this can pose an infection risk to local people. Lastly, in Chapter 7 the impact of approximately two years of the World Bank praziquantel dosing scheme on canine echinococcosis in the Alay Valley was assessed.

CoproELISAs are commonly used to detect canine echinococcosis (Allan *et al.*, 1992; Craig *et al.*, 1995; Benito and Carmena, 2005; Allan and Craig, 2006; Morel *et al.*, 2013). CoproELISA results are numerical O.D values, and to classify samples as 'positive' or 'negative', a cut-off is required. Many authors use the Gaussian approach to determine the cut-off, which requires testing a panel of known negative samples and calculating the cut-off as the mean O.D value plus two or three standard deviations (Allan and Craig, 1989; Allan *et al.*, 1992; Lahmar *et al.*, 2007b), and this approach was originally used for the genus specific coproELISA developed in Chapter 3. The cut-off was determined using the Gaussian approach based on dog faecal samples collected from the Falkland Islands (a very low

endemic area) and after testing the coproELISA with the Gaussian cut-off using known positive and negative samples, the coproELISA was found to have very good diagnostic sensitivity (93.5%) and specificity (100%).

The Gaussian approach was used to study coproELISA prevalence of environmental faecal samples described in Chapter 6 (chronologically the first chapter to be completed). However, following more ELISA testing of samples from endemic areas, it became clear that the distribution of coproELISA O.D values from the samples collected from endemic areas, such as Kyrgyzstan and Xinjiang did not show the same distribution profile as the samples from the Falkland Islands and the known positives used to optimize the coproELISA (Fig. 5-1, Chapter 5). The problem with the Gaussian approach is that this method takes into account only the distribution of a number of known negatives (often, including in this case, from non- or very low- endemic areas), and does not take into consideration the true distribution of both negatives and positives from the population being studied (Gardner and Greiner, 2006). Furthermore, there appeared to be some variability in the performance of the coproELISA. The same set of samples tested on different days could give somewhat different results. This may have been due to variations in antibody concentrations in different aliquots, or minor variations in conditions such as the temperature of the laboratory. Some protocols account for such variability by including standard curves on the ELISA plates and calculating concentrations of the analyte being measured from these standard curves (e.g. van Kesteren *et al.*, 2012; van Kesteren *et al.*, 2013b). Similarly other protocols, such as that for the commercial Lakeview coproELISA kit described in section 3.3.7, correct for such variations by calculating the cut-off in relation to a given control (for this kit, the cut-off was calculated based on the provided negative control). For the coproELISA used in this thesis, the cut-off was initially calculated using the Gaussian approach, although it was later concluded that the application of the Gaussian approach of determining the cut-off for testing all field samples

may be limited. Instead a ROC curve approach was adopted, and the effects of minor variations in conditions such as laboratory temperatures on the performance of the coproELISA were addressed by testing all samples in 'batches' as described below.

An alternative to the Gaussian approach is the use of ROC curves (Gardner and Greiner, 2006). ROC curves take into consideration the distribution of both positives and negatives, and allow for some flexibility in choosing the cut-off to maximise diagnostic sensitivity of specificity (Gardner and Greiner, 2006). Several studies on canine echinococcosis have used ROC curves to determine cut-offs, rather than the Gaussian approach (Benito and Carmena, 2005; Morel *et al.*, 2013), and this approach was also adopted in Chapters 5 and 7 (chronologically completed after Chapter 6), using panels of samples of known status from Kyrgyzstan or from a comparable region in Xinjiang, China (see Chapter 7, Appendix 2 and 3). To further optimize the reliability of the coproELISA, all faecal samples were tested in 'batches' (for example all samples collected from one field season were tested in a single batch), with enough antibodies and reagents pooled to test all samples in each batch. All samples in each batch were tested within three or four days of each other, to minimize variability in test conditions such as lab temperatures. A new ROC panel was done for each batch of samples, using the same pooled antibodies and buffers, to maximize reliability.

Despite attempting to minimize problems with coproELISA classification through using ROC curves and testing samples in batches, problems associated with dichotomously classifying continuous data (in this case, coproELISA O.D values) as positive or negative remain. Using ROC curves, it is possible to choose a certain diagnostic specificity and sensitivity. For example, in Chapter 5, a cut-off was chosen that maximized diagnostic sensitivity (95%) and specificity (81%), i.e. the 'optimal' cut-off was chosen that would give the highest accuracy. In Chapter 7, to be able to compare two different sets of data (the pre-

intervention results and the LQAS results) two cut-off values were chosen that were roughly comparable and gave sensitivities of 90% and 89% and specificities of 86% and 88% respectively. However, the ROC curve cut-offs were chosen based on single ELISA plate panels of at most 42 samples. Furthermore, a sensitivity of 90% may mean that up to 10% of samples classified as 'positive' may be false positives, and a specificity of 89% may mean that 11% of samples classified as 'negative' may be false negatives.

Another problem associated with coproELISA is that it is generally agreed that the diagnostic sensitivity of coproELISAs decreases with low worm burdens, as low worm burdens mean lower concentrations of antigens in faecal samples (e.g. Allan and Craig, 2006). Previous studies have found that coproELISAs could not reliably detect worm burdens below 20 (Craig *et al.*, 1995), or that the test was less reliable with burdens below 50-100 worms (Lembo *et al.*, 2013), and an effective detection limit above 50 worms is often assumed for coproELISAs (e.g. Allan and Craig, 2006). However, *Echinococcus* spp. infections are usually overdispersed in canid hosts, with most individuals harbouring a few worms, and some having many thousands of worms (e.g. Gemmell, 1990; Hofer *et al.*, 2000). As such, most dogs will harbour few worms, and these are the ones likely to be missed by the coproELISA. Furthermore, echinococcosis control programmes that involve dosing canids with praziquantel may quickly reduce average worm burdens per canid host (Takumi and van der Giessen, 2005), which may mean that detecting canid infections with coproELISAs becomes more difficult in a control scheme than in a natural situation.

The issues surrounding coproELISAs can be somewhat mitigated by, for example, using samples from endemic areas and ROC panels to determine cut-offs, and being aware of test limitations. Efforts were made to adopt these approaches in this study. Furthermore, when comparing data over time (as in Chapter 7), the exact diagnostic sensitivity and specificity of

the test may be less important, as the aim is not to determine absolute infection rates, but to study changes over time. In such cases, if the samples are collected and preserved in the same way, and analysed in the same way in the same laboratory and by the same researcher, results can be compared, even if there are test limitations. However, the coproELISA limitations cannot be completely avoided, and it is important to bear these in mind when interpreting coproELISA data.

CoproELISAs are usually genus specific (Christofi *et al.*, 2002; Allan and Craig, 2006), and as such in co-endemic areas coproPCR is often used to determine the species of *Echinococcus* spp. present (e.g. Moss *et al.*, 2013). Different species of *Echinococcus* were found to be present in the Alay Valley (i.e. *E. granulosus* G1, *E. multilocularis* and *E. canadensis*, see Chapters 5 and 6), and it was necessary to analyse the samples with coproPCR to determine the species present. Although published protocols were available for the detection of *E. granulosus* and *E. multilocularis*, these had their limitations. The protocol developed by Abbasi *et al*. (2003) detected *E. granulosus sensu lato* in canid faeces. Although the original publication discussed the G1 (common sheep strain) of *E. granulosus*, this protocol actually detects several genotypes including G1, G4, G5, G6, G7, and G10, and does not allow for differentiation between these strains (Boufana *et al.*, 2008, Boufana unpublished data). Based on arecoline purges and molecular analyses of adult worms from purges it was found that *E. granulosus* G1, *E. canadensis* and *E. multilocularis* are co-endemic in the Alay Valley (Chapter 6, van Kesteren *et al.*, 2013). Thus the applicability of the Abbasi *et al.* (2003) protocol for the Alay Valley faecal samples was limited. Instead another protocol, specific for *E. granulosus* G1, was used (Boufana *et al.*, 2013b). However, although more analytically specific, this protocol was less analytically sensitive, and the authors recommended ethanol precipitating DNA to improve analytic sensitivity. For this study, due to the large number of samples involved, the laboriousness of the DNA extractions

and the need to PCR each sample three times (to test for *E. granulosus* G1, *E. canadensis* and *E. multilocularis*), it was not feasible to ethanol precipitate every sample, a process that greatly increases the laboriousness of the work. The same is true for the protocol used to detect *E. multilocularis* (Boufana *et al.*, 2013b).

No suitable previously published protocols were available to detect *E. canadensis* in faecal samples, as previous protocols were not optimized for coproPCR (Dinkel *et al.*, 2004; Boubaker *et al.*, 2013). Therefore, a new PCR protocol was developed to detect *E. canadensis* in the collected dog faecal samples. Primers previously designed by Dr. Boufana were optimized and assessed. These included a set (Egen1/4F and Egen1/4R) that could detect *E. granulosus sensu stricto*., *E. equinus* and, with less analytic sensitivity, *E. multilocularis* (Chapter 4). However, the fact that these primers required sequencing to determine the species present, and the fact that sequencing PCR products obtained from faeces is often difficult (Boufana *et al.*, 2008; pers. obs.), meant that they offered no real advantage over the specific *E. granulosus* G1 primers developed by Boufana *et al.* (2013b), and as such were not used to test the samples collected as part of this study.

Primers EgenF and EgenR were used to detect *E. canadensis*. The primers showed good analytic specificity when tested in tissue panels using DNA extracted from other adult cestodes, and showed high analytic sensitivity when tested with serially diluted *E. canadensis* tissue DNA (Chapter 4). However, optimizing the protocol for faecal samples was challenging due to the low number of confirmed natural *E. canadensis* positive samples from domestic dogs. The only true known sample was an arecoline purge sample from the Alay Valley, and *E. canadensis* DNA concentrations in an arecoline purge sample are likely to be higher than in non-purge faecal samples collected from naturally infected dogs. A further sample that had tested positive with the Abbasi *et al.* (2003) protocol was found to be

coproPCR and *E. canadensis* sequence positive with primers EgenF and EgenR, and in addition a further four samples that were tested blindly and were coproPCR positive were successfully sequenced as *E. canadensis*. However, another six coproPCR positive samples did not yield clear genetic sequences, and ideally a much larger panel of known samples (for example from necropsy or arecoline purgation and PCR of adult worms) should be available to test these primers.

Further challenges associated with the coproPCR protocols included the fact that data obtained from the different PCR protocols are difficult to compare, and also the poor correlation between coproELISA and coproPCR data. Although the three PCR protocols used here appear to be completely specific to their targets (i.e. *E. granulosus/E. multilocularis/E. canadensis*), as tested using defined DNA specificity panels (see Chapter 4 and Boufana *et. al.*, 2013b), the sensitivities are less easy to quantify. Serial dilutions of tissue DNA are often used to calculate the analytic sensitivity of PCRs (as done in Chapter 4), but it is not possible to translate a known amount of tissue DNA to the equivalent amount of target DNA within a faecal sample. Although the total concentration of DNA extracted from faeces could be measured using a Thermo Scientific Nanodrop 2000 (as done with the tissue samples), it is important to remember that a faecal sample from an *Echinococcus* spp. infected dog will contain many kinds of DNA besides that of *Echinococcus* spp., including host DNA, DNA from prey items, and DNA from bacteria, fungi and other parasites present in the sample (including those that establish on the sample post-defecation). In addition faeces often contain PCR inhibitors, adding to the challenges of coproPCR (Mathis and Deplazes, 2006). *Echinococcus* spp. worm burdens in final hosts will also affect the outcome of coproPCRs, with higher worm burdens leading to higher numbers of eggs in faeces. As such, if out of 100 collected samples 50 test positive for *E. granulosus* G1 and 50 test positive for *E.*

multilocularis, it would be overly simplistic to say that the distribution of infection with these two species is exactly 50%.

The poor correlation between coproELISA and coproPCR data was another problem. In Chapter 5, 333 dog faecal samples collected from four communities in the Alay Valley were analysed with both coproELISA and coproPCR (although not all ELISA and PCR samples could be matched, so that a total of 288 samples were tested with coproPCR). Of the 73 coproELISA positives tested with the three coproPCR protocols, 43 (58.9%) were coproPCR negative for all protocols. Conversely for the 215 coproELISA negative samples tested with coproPCR, 101 (47.0%) tested positive with at least one protocol. The failure of a proportion of coproELISA positives to test positive for any of the coproPCR protocols could be due to various factors. These may include low worm burdens (and therefore low numbers of eggs in faeces), degradation of DNA (although *Echinococcus* spp. eggs are very stable under cool and humid conditions, they are susceptible to warmer and drier conditions (Veit *et al.*, 1995), and DNA may degrade unless properly preserved, (Olson *et al.*, 2005)), very low concentrations of target DNA, or failure of DNA to amplify to due to the presence of PCR inhibitors. Conversely, false positives may occur with coproPCR due to coprophagia (Hartnack *et al.*, 2013) or due to cross contamination of samples, which may happen in the field (faecal densities were high and many collected samples were at least several days old, so they may have been contaminated with eggs from other samples) or in the lab, although every effort was made to control for this by including 'blanks' during the extractions and PCRs, and using negative controls in each PCR. Alternatively coproELISA negative samples may be coproPCR positive if worm burdens were low (below the detection limit of the coproELISA) but eggs were present in the faecal sample. Poor correlations between coproELISA and coproPCR results have also been found in other studies. For example Moss *et al.* (2013) found that 17/58 coproELISA positives were coproPCR negative, with a further

20 coproELISA negatives tested being PCR positive for *E. granulosus* (n=1) or *E. multilocularis* (n=19).

Problems associated with diagnosis of canine echinococcosis have been recognized by several authors (e.g. Mathis and Deplazes, 2006; Hartnack *et al.*, 2013). Only necropsy and thorough examination of intestines can be considered truly 'gold standard' (Allan and Craig, 2006), but this is often infeasible as a surveillance tool as it would require euthanizing large numbers of dogs, which is usually undesirable due to the roles they play in communities or due to local cultural beliefs (Hartnack *et al.*, 2013). Similarly, arecoline purgation (often considered the second best option, after necropsy) is problematic due to the fact that it requires trained personnel, and is labour intensive and biohazardous (Craig *et al.*, 1995). As such, coproELISA and coproPCR are often used, but these may have limitations. For coproELISA these include detection limits, i.e. low worm burdens may be missed, and limited diagnostic sensitivity and specificity (Allan and Craig, 2006). For coproPCR these may include problems with PCR inhibitors, low quantities of target DNA and contamination issues (Mathis and Deplazes, 2006). These test limitations need to be considered, and several authors have attempted to mitigate these, for example using latent class analysis (Hartnack *et al.*, 2013), or other mathematical modelling approaches to interpret coproELISA/coproPCR data (Mastin *et al.*, in prep.-b; Mastin *et al.*, in prep.-a).

Differences in sensitivities in coproPCR protocols and the poor correlation of coproELISA and coproPCR results meant that data were difficult to interpret. In addition, in terms of surveillance, the practicalities of canine echinococcosis diagnostic tests need to be considered (see also Mathis and Deplazes, 2006). This study aimed to assess, amongst other things, pre-intervention canine echinococcosis in the Alay Valley (Chapter 5), as well as the effects of nearly two years of praziquantel dosing (Chapter 7). However, this research was part of a

larger Wellcome Trust project to study canine echinococcosis in the Alay Valley, with ~2,000 samples collected from the Alay Valley between 2012 and 2014 (Mastin *et al.*, in prep.-c). All samples were analysed by coproELISA, which provided a relatively quick, easy and affordable method of testing samples. In contrast, the faecal DNA extraction protocol was laborious and time consuming (Qiagen, 2010), as well as expensive (£140.40 per kit, which can process 50 samples, www.qiagen.co.uk). Each sample needed to be tested with coproPCR three times (for *E. granulosus* G1, *E. multilocularis* and *E. canadensis*), which was also time consuming and costs associated with PCR (especially Taq) were also very high. As such, it was not feasible to extract and PCR every sample collected. All pre-intervention samples were DNA extracted and analysed with coproPCR (Chapter 5), and for all subsequently collected samples, only coproELISA positives plus one third of negatives were DNA extracted and analysed by coproPCR. However, this was still very laborious and costly (>800 faecal samples DNA extracted, with 2,400 associated coproPCR tests).

The applicability of this methodology (coproELISA analysis of all samples followed by coproPCR of all samples or coproELISA positives and 30% of coproELISA negatives) is limited in many areas where echinococcosis occurs, especially as such areas (including Kyrgyzstan) often lack the funds and trained personnel to carry out such testing. The difficulty of interpreting the results adds to this problem: how to interpret a coproELISA negative sample that is coproPCR positive and vice versa? As such recommendations for future surveillance would be to test all samples with coproELISA (which is relatively quick, easy and affordable), and if species specific data are required, DNA extract and test only coproELISA positive samples (see also Mathis and Deplazes, 2006). Although it is likely that many of these will be coproPCR negative (Chapter 5, see also van Kesteren *et al.*, in press.), and that it is difficult to estimate true prevalence of species present from coproPCR data, the data can be used to monitor situations over time, as long as the same protocols are used.

Despite limitations in diagnostic testing, coproELISA and coproPCR can be used to detect canine echinococcosis. In the current study, every effort was made to maximize test reliability, including extracting and optimizing new polyclonal antibodies for coproELISA and thoroughly testing these (Chapter 3), using ROC curves (based on samples from endemic areas) rather than Gaussian approaches to determine cut-offs and analysing samples in batches to minimize variability in test conditions (Chapters 5 and 7), and optimizing new primers to detect *E. canadensis* in faecal samples (Chapter 4). Once these diagnostic tools were optimized they were applied to study canine echinococcosis in the Alay Valley, southern Kyrgyzstan.

In 2012, the World Bank began an echinococcosis control scheme in the Alay Valley, which included dosing owned dogs with praziquantel (World Bank, 2011). Echinococcosis control schemes frequently include praziquantel dosing of domestic dogs (for a review see Craig and Larrieu, 2006), for example in the Falkland Islands (Reichel *et al.*, 1996) and in China (Chinese Ministry of Health, 2007).

In the Alay Valley, praziquantel dosing of domestic dogs appeared to be done through local chains of administration. Each Alay Valley community has its own government veterinarian, with larger communities, for example Sary Mogul, sometimes having two veterinarians, and smaller communities sometimes 'sharing' a veterinarian with a neighbouring community. For example, Kara Kavak shares a veterinarian with Kashka Suu (Turdumammat Sultanov, Taldu Suu government veterinarian, pers. comm.). In addition, private veterinarians may be present in communities (T. Sultanov, pers. comm.). Government veterinarians receive their salaries and are managed from administrative capitals (for the eastern Alay Valley this is Gulcha, for

the western Valley it is Daroot Korgon). Periodically, the village veterinarians are called for meetings in the administrative capitals and provided with instructions, equipment and payment. For praziquantel, village veterinarians are provided with tablets (paid for by the World Bank) and instructed to dose all owned dogs four times a year, i.e. once in winter, once in spring, once in summer and once in autumn (T. Sultanov, pers. comm.). The veterinarians then travel door to door in their communities to dose dogs. This use of local chains of administration and local government veterinarians provides a good approach to dosing domestic dogs, as compared with trying to set up an external dosing system. The local veterinarians are often well known in the small communities and have a good knowledge of dog ownership in their communities, especially the smaller ones (pers. obs.).

Dog dosing frequencies of every six weeks are often recommended during a control programme (Gemmell *et al.*, 1986; Lembo *et al.*, 2013), with the aim of preventing *Echinococcus* spp. from reaching patency even in the case of immediate reinfection, and therefore preventing the release of any eggs from dogs. If this dosing regime is carried on for a sufficient time period to allow for previously infected intermediate hosts such as sheep or small mammals to be removed from the population, the transmission cycle of *Echinococcus* can be broken (Economides *et al.*, 1998; Torgerson and Heath, 2003). However, although recommended and often aimed for, these dosing frequencies are often not achieved in *Echinococcus* endemic areas (Economides *et al.*, 1998; Craig and Larrieu, 2006). As such, it may be preferable to set more realistic goals; even if it is not feasible to eliminate echinococcosis from a certain area, reductions in transmissions to humans can be achieved with more modest dosing frequencies, thus reducing the echinococcosis public health concern. For example, mathematical models have suggested that dosing frequencies can be reduced to once every 3 months and still reduce prevalence rates in dogs and livestock to less

than 1% within 10–15 years (Torgerson, 2003). Therefore the World Bank scheme, which aims to dose all owned dogs four times a year, is practical in remote communities such as those in the Alay Valley, whilst theoretically still being capable of achieving a reduction in echinococcosis. However, in order to evaluate how well this control scheme is working, it is critical to have data from before the start of dosing (see also Schantz *et al.*, 1995; WHO/OIE, 2001).

In May 2012, just prior to the start of the World Bank praziquantel dosing scheme four communities in the Alay Valley were visited, namely Taldu Suu, Sary Mogul, Kara Kavak and Kashka Suu. A total of 333 dogs were sampled in these four communities, and these samples were analysed using coproELISA and coproPCR. CoproELISA prevalence ranged from 23.3% in Kashka Suu to 28.7% in Sary Mogul, with 24.5% and 25.7% coproELISA positives in Taldu Suu and Kara Kavak respectively. These prevalences are similar to those in other areas where echinococcosis is a public health concern such as Shiqu County on the Tibetan Plateau (coproELISA prevalence of 21% prior to intervention, Moss *et al.*, 2013), rural New South Wales, and Victoria, Australia (coproELISA prevalences of 29% and 17.5% respectively, Jenkins *et al.*, 2006) and north west Libya (coproELISA prevalence of 21.6% prior to intervention, Buishi *et al.*, 2005).

In many areas where human echinococcosis occurs, there is usually only species of *Echinococcus* present (usually *E. granulosus*, for example in Australia, Jenkins, 2006), and therefore coproPCR to determine the species of *Echinococcus* is not necessary. However, in other areas, such as the Tibetan Plateau, *E. granulosus*, and *E. multilocularis* (Budke *et al.*, 2005) and the more recently discovered *E. shiquicus* (Boufana *et al.*, 2013a) are all co-endemic, thus requiring the need for coproPCR (Moss *et al.*, 2013). As the Alay Valley was found to be co-endemic for *E. granulosus*, *E. multilocularis* and *E. canadensis* (Chapter 6),

coproPCR was needed. Of the coproELISA positives, 11% tested positive for *E. multilocularis* DNA, 8.2% tested positive for *E. granulosus* DNA and 33.3% tested positive for *E. canadensis*. However, many coproELISA positive samples (58.9%) were coproPCR negative. Similar results were found by Moss *et al.,* (2013) in the co-endemic area of Shiqu County on the Tibetan Plateau, where 3.6% of samples were coproPCR positive for *E. granulosus* DNA and 11% were coproPCR positive for *E. multilocularis* DNA, but the remainder of coproELISA positives were coproPCR negative, with dogs not tested for *E. shiquicus* (Moss, 2011; Moss *et al.*, 2013).

In Taldu Suu, Sary Mogul, Kara Kavak and Kashka Suu, dog owners were asked if they had de-wormed their dogs in the previous six months. The majority (79.5%) of owners had not de-wormed their dogs in the previous six months, but 20.5% had. Those owners who had de-wormed their dogs all said they had obtained the tablets from the local veterinarian. These results provide some data on the canine echinococcosis situation in the Alay Valley in the absence of any control scheme. Although only four communities were sampled, these may be assumed to be representative of the Alay Valley, and later visits to six other communities in the Alay Valley confirmed that all communities in the Alay Valley are similar in terms of local culture and livelihoods. The pre-intervention data obtained here can be used to monitor the effectiveness of the World Bank control programme, or any other future control programmes.

In May 2012 and October 2012, the roles of domestic dogs in the four study communities in the Alay Valley (Taldu Suu, Sary Mogul, Kara Kavak and Kashka Suu) were investigated. Domestic dogs are the main source of human infection with *Echinococcus* spp. (Budke *et al.*, 2005), and therefore the demography, ecology and behaviour of dogs are relevant in studying

diseases that may be spread by them, including echinococcosis. Describing dog populations in communities may also help to assess transmission potential, zoonotic risks, and optimization of intervention programmes (Butler and Bingham, 2000). This concept has been recognized in studies relating to rabies (Perry, 1993; Butler and Bingham, 2000; Kitala *et al*. 2001; Macpherson, 2005), and should also be applied to studies on echinococcosis (Watson-Jones and Macpherson, 1988; Vaniscotte *et al.*, 2011).

Four aspects relating to dog demography, ecology and behaviour in the Alay Valley were included. The first was basic demography, i.e. the ages and genders of the dog population present. The second was dog husbandry, including what dogs were fed, if they were ever observed eating rodents, and whether or not they were ever restrained. These two topics were explored through dog owner questionnaires. Dog roaming behaviour was also investigated, as this may be relevant for disease transmission. For example, dogs that roam outside of villages may be more likely to consume small rodents, and if dogs roam away from their owner's houses they may defecate in other parts of the community, potentially putting people at risk of *Echinococcus* spp. infection. Dog roaming behaviour was studied using iGotU GPS trackers, which were tested as part of the study. Finally an assessment of the faecal contamination of communities in the Alay Valley was undertaken, as this may give an indication of infection risk.

Questionnaire data revealed a total estimated dog population of 393 in the four Alay Valley communities, or 1 dog for every 9.36 people. Dogs were reportedly aged between 2 weeks and 15 years, with a median age of two years, and males represented around 77% of the total dog population. There are often more male than female dogs in rural communities, which may be related to males being perceived as being better guard dogs, or people not wanting to deal with pups. Dogs in such communities are also usually young, as life expectancies are

often short (Butler and Bingham, 2000; Kitala *et al.*, 2001). In the Alay Valley, dogs played different roles in the communities, including as pets, sheep dogs and guard dogs. Dogs in rural communities are often used as guard dogs, although dogs are less commonly referred to just as pets (Butler and Bingham, 2000; Kitala *et al.*, 2001; Vaniscotte *et al.*, 2011). Most dogs (>88% in all communities) were never restrained and free to roam at will. This is similar to reports from rural communities in Zimbabwe (Butler and Bingham, 2000) but unlike dogs in rural communities in the Tibetan Plateau, which are usually always tied up or tied during the day and released only at night (Moss, 2011). Dogs were most often fed table scraps, although offal was also reported to be commonly fed. Dogs were rarely observed eating rodents.

GPS data showed that dogs mostly stayed close to their owner's homes, but could roam up to 2km away from their owner's homes. This is similar to results found by Vaniscotte *et al.*, (2011) in Tibetan rural communities, where dogs mostly stayed close to their release points, with 95% of dogs spending 80% of their time at a maximum distance of 115m from their owners' houses, although dogs could move up to 1,500m away. Faecal sample quadrats showed that dog faeces occurred at high densities in the four communities. Faecal densities ranged from 0.45 faeces/100m^2 to 1.20 faeces/100m^2 in May; with lower densities of between 0.22 faeces/100m and 0.60 faeces/100m in October. This faecal density is higher than the average faecal density observed by Vaniscotte *et al.*, (2011), although that study included quadrats located outside of villages, with faecal densities higher inside villages. All our quadrats were conducted inside villages, where high faecal densities probably correlate with high numbers of dogs which generally stay near their owner's houses. In May 6.7% of collected faecal samples were coproELISA positive for *Echinococcus* spp.; in October this was 18%.

Echinococcosis control programmes often require large investments of time and resources, and as such it is important to assess how well these are meeting their targets. Many echinococcosis control programs have been conducted in areas such as Iceland, Cyprus, and parts of Africa and South America (for a review see Craig and Larrieu, 2006). Control programmes often include praziquantel dosing of domestic dogs (e.g. Chinese Ministry of Health, 2007), and may also include other measures such as vaccination of sheep, the main intermediate host for *E. granulosus* (Larrieu *et al.*, 2013), public health education (Beard, 1973), culling of stray dogs and slaughterhouse surveillance (Economides *et al.*, 1998).

Surveillance methods include those that target livestock, people and dogs. For example incidence of echinococcosis in livestock can be measured using either active or routine slaughterhouse surveillance (Economides *et al.*, 1998). In humans prevalences can be measured using ultrasound surveys (Lembo *et al.*, 2013). However dogs are often the easiest to test for surveillance purposes as this can be done non-invasively using copro-tests and in the case of positive diagnosis dogs can be easily treated with praziquantel. As such, many surveillance programmes have targeted domestic dog populations. This can involve culling and examination of intestines (see Beard, 1973), but more frequently involves arecoline purging (Gemmell, 1990; Economides *et al.*, 1998) or coproELISA testing (Christofi *et al.*, 2002).

Several evaluations of control programmes have required testing large numbers of dogs, especially as in an effective control scheme both the prevalence of canine echinococcosis and worm burdens are expected to decrease. For example Christofi *et al.*, (2000) tested 6,551 dogs in Cyprus with coproELISA, and Economides *et al.*, (1998), describe arecoline programmes in Cyprus in 1993 in which 2,941 dogs in 48 villages were purged with arecoline. However, because echinococcosis is commonly a disease of remote, marginalised

communities (Craig *et al.*, 2007a), surveillance is often hindered by logistical difficulties, and sampling and testing thousands of dogs is not always feasible. As such, relatively quick and efficient methods of evaluating control schemes are desirable. Lot Quality Assurance Sampling potentially provides such a tool, as it requires a relatively small sample size whilst remaining statistically robust (Dodge and Romig, 1929).

In April 2013 and April 2014, ten communities in the Alay Valley were visited to attempt to evaluate the impact of approximately two years of the World Bank praziquantel dosing scheme on canine echinococcosis. Lot Quality Assurance Sampling (LQAS) provides a relatively quick and easy but statistically robust method of testing whether certain 'targets' are being met, and this method has been applied to studies related to human health (Robertson and Valadez, 2006). LQAS methodology was used to study canine echinococcosis in the Alay Valley, with an aim to detect a decrease from pre-intervention coproELISA prevalences. As such, the samples collected from Sary Mogul, Taldu Suu, Kara Kavak and Kashka Suu in May 2012 were used to determine the pre-intervention coproELISA prevalence. Samples collected from the ten communities (including the original four) in April 2013 and April 2014 were then analysed and coproELISA prevalences were compared. In order to be able to compare the different lots of samples, ROC curves were used to determine cut-offs that gave comparable sensitivities and specificities. Praziquantel dosing was also assessed, with a target of 75% chosen, as based on mathematical models to simulate echinococcosis control (Torgerson, 2003). Decision numbers were calculated using the hypergeometric distribution and estimated dog population sizes in each community.

Results from administering questionnaires to dog owners revealed that in 2013, the 75% dosing target was met in 4/10 communities, and in 2014 this increased to 8/10 communities. The coproELISA results suggested that a reduction from baseline prevalences had been

achieved in 5/10 and 4/10 communities in 2013 and 2014 respectively. Furthermore, in 2014, 84.6% of questionnaire respondents had heard of echinococcosis, with 78.3% of these being able to correctly identify the causes of echinococcosis, and a further 10.3% giving partially correct responses.

This evaluation was undertaken soon after the start of the World Bank dosing programme (approximately 9 and 21 months after its start) when 'teething problems' with the implementation of the control programme are to be expected. For example in the first evaluation it seemed the dosing scheme was not managing to reach at least 75% of dogs in 6/10 communities, but this seems to have improved in 2014. Similarly, so soon after the start of a control programme the system is being disturbed from its equilibrium, and a dosing period of 21 months is not sufficient to eliminate the re-infection pressure to dogs. Nevertheless, the results suggest progress, with many dog owners reporting dosing their dogs and evidence for reductions in coproELISA prevalences, something also found by analysis of larger numbers of samples from Kara Kavak, Kashka Suu, Sary Mogul and Taldu Suu (Mastin *et al.*, in prep.-c).

The trialling of a new methodology for evaluating echinococcosis control schemes is valuable, as LQAS can be a practical method for evaluating how well the control programme is meeting its targets. As ground faecal samples can be collected this means that (unlike with arecoline purging, which is biohazardous, or rectal sampling, which is laborious, potentially dangerous and requires some training) relatively untrained personal can easily collect the data. The LQAS sampling frame is also relatively simple (going to the centre of a community, looking at a watch to choose a direction and sampling alternate houses whilst asking about dog dosing), and can be simplified further if in future, rather than trying to

accurately calculate the decision number using population estimates, the simplified methodology described by Valadez *et al.*, (2002) and associated decision number tables could be applied (see Chapter 7, Appendix 1). The relative ease of collecting ground faecal samples using LQAS means that such evaluations could potentially be carried out in future by the local veterinarians, with samples sent to laboratories for testing.

Currently the lack of suitable diagnostic laboratories in Kyrgyzstan is a problem for canine echinococcosis surveillance (B. Mytynova, Kyrgyz Veterinary Institute, pers. com). Although laboratories that are capable of ELISA testing exist in both Osh and Bishkek (pers. obs.), the lack of suitable coproELISA antibodies remains a problem. Commercial kits are available, but have been shown to have poor accuracies (Huang *et al.*, 2014). As such if the World Bank or the Kyrgyz authorities wish to continue to monitor canine echinococcosis in future, capacity building should be a priority. Local chains of veterinary authority already seem reasonably well placed for conducting praziquantel dosing, and, with suitable capacity building, coproELISA surveillance could also be achieved within Kyrgyzstan.

8.2 Conclusions and further studies

There is evidence that human echinococcosis (including AE and CE) is re-emerging in Kyrgyzstan (Torgerson *et al.*, 2003; Torgerson, 2013; Usubalieva *et al.*, 2013), and results from four communities in the Alay Valley, southern Kyrgyzstan, prior to a control programme found high coproELISA prevalences in owned domestic dogs, with an average of 26.4%. For the first time, three species were found to be present in the Alay Valley, namely *E. multilocularis*, *E. granulosus* and *E. canadensis*. All three species are zoonotic, with *E. granulosus* being the main cause of human cystic echinococcosis globally (Alvares Rojas *et al.*, 2014) and *E. multilocularis* causing alveolar echinococcosis (WHO/OIE, 2001). *E canadensis* also causes cystic echinococcosis, although it is considered to be less pathogenic than *E. granulosus* (Alvares Rojas *et al.*, 2014). There is also evidence that *E. canadensis* is more likely than *E. granulosus* to cause cysts in lungs (e.g. Omer *et al.*, 2010) or the brain (e.g. Sadjjadi *et al.*, 2013). High prevalences of these co-endemic species in domestic dogs pose a risk to humans. This risk has been recognized by the World Bank, which started dosing domestic dogs with praziquantel through local veterinary channels in 2012. However, this control programme has not followed the recommended guidelines suggested by experts such as the WHO (WHO/OIE, 2001) and academics in the field (e.g. Schantz, 1993), which include the need to establish pre-intervention prevalences and surveillance in order to evaluate echinococcosis control programmes.

Here, after developing appropriate coproELISA (Chapter 3) and coproPCR (Chapter 4) tools for diagnosis, pre-intervention coproELISA prevalences of canine echinococcosis were established (Chapter 5). These can be used to evaluate the effect of the World Bank praziquantel dosing scheme. The demographics, roles, husbandry and roaming behaviour of domestic dogs in the Alay Valley were studied, as these have an impact on disease spread by

domestic dogs, including echinococcosis (Chapter 6). An evaluation of the first 21 months of the control programme using Lot Quality Assurance Sampling (LQAS) suggested that the control programme was having an effect in local communities with 8/10 communities reaching a 75% dog dosing target in 2014, and 5/10 and 4/10 communities showing a reduction in coproELISA prevalences in 2013 and 2014 respectively (Chapter 7). These early results are encouraging, and it is hoped that echinococcosis control efforts will continue in the Alay Valley, as long term commitments are required.

It is considered that this research will contribute to a better understanding of echinococcosis in the Alay Valley of Kyrgyzstan. The tools developed here could be used for future studies on canine echinococcosis and the pre-intervention data can be used to assess the impact of longer term control efforts on canine echinococcosis in the Alay Valley. LQAS methods could also provide a relatively easy method of assessing control programmes, and it is recommended that surveillance methods based on this research are established within Kyrgyzstan.

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Appendices

10.1 Chapter 3 Appendices

10.1.1 Chapter 3 Appendix 1

Panel of known positive and negative samples tested with R5/R91 and R47 capture and conjugate. Note: Negatives #1-#17 were collected from a veterinary practice in Greater Manchester by Matthew Bates. Dr. Boufana provided samples from dogs experimentally infected with T. hydatigena, T, multiceps, E. equinus, *and* E. granulosus *sensu stricto. (including 'Aus low/med/high', with worm burdens qualitatively assessed) as well as necropsy positive samples from foxes naturally infected with* E. multilocularis *and a dingo infected with* E. granulosus. *'Meg spike' indicates a sample from Manchester spiked with 1:100* E. granulosus *whole worm extract.*

	cap R5 1:2000. Px R91 1:2000		capR47 1:4000, px R47 1:1500		Cap R5 1:2000, px R91 1:1500		
Sample	**Mean OD**	**Pos or neg**	**Mean OD**	**Pos or neg**	**Mean OD**	**Pos or neg**	**Known value**
Negative 1	0.015	Neg	0.055	Neg	0.025	Neg	Neg
Negative 2	0.061	Neg	0.070	Neg	0.026	Neg	Neg
Negative 3	0.025	Neg	0.061	Neg	0.025	Neg	Neg
Negative 4	0.014	Neg	0.056	Neg	0.009	Neg	Neg
Negative 5	0.012	Neg	0.070	Neg	0.003	Neg	Neg
Negative 6	0.025	Neg	0.061	Neg	0.028	Neg	Neg
Negative 7	0.028	Neg	0.070	Neg	0.027	Neg	Neg
Negative 8	0.024	Neg	0.062	Neg	0.033	Neg	Neg
Negative 9	0.008	Neg	0.043	Neg	0.001	Neg	Neg
Negative 10	0.026	Neg	0.069	Neg	0.017	Neg	Neg
Negative 11	0.029	Neg	0.068	Neg	0.021	Neg	Neg
Negative 12	0.019	Neg	0.069	Neg	0.004	Neg	Neg
Negative 13	0.067	Neg	0.094	Neg	0.062	Neg	Neg
Negative 14	0.035	Neg	0.125	Neg	0.034	Neg	Neg
Negative 15	0.050	Neg	0.115	Neg	0.059	Neg	Neg
Negative 16	0.029	Neg	0.087	Neg	0.043	Neg	Neg
Negative 17	0.032	Neg	0.115	Neg	0.047	Neg	Neg
T.hydatigena	0.036	Neg	0.111	Neg	0.043	Neg	Neg
T.multiceps	0.020	Neg	0.087	Neg	0.016	Neg	Neg
E. equinus	0.053	Neg	0.288	Pos	0.091	Pos	Pos
E. multilocularis	0.040	Neg	0.190	Pos	0.033	Neg	Pos
E. multilocularis	0.051	Neg	0.259	Pos	0.058	Neg	Pos
E.multilocularis	0.137	Pos	0.407	Pos	0.212	Pos	Pos
AUS low	0.113	Pos	0.319	Pos	0.118	Pos	Pos
AUS med	0.134	Pos	0.393	Pos	0.189	Pos	Pos
AUS high	0.169	Pos	0.614	Pos	0.237	Pos	Pos
Dingo	0.116	Pos	0.556	Pos	0.166	Pos	Pos
Meg spike	0.310	Pos	0.954	Pos	0.335	Pos	Pos
Aus exp inf	0.123	Pos	0.542	Pos	0.185	Pos	Pos
Dog 8 E.g	0.111	Pos	0.566	Pos	0.139	Pos	Pos
Average all neg	0.029		0.078		0.027		
Stdev	0.016		0.024		0.018		
Cutoff	0.076		0.149		0.080		
Correct	27		30		28		
Incorrect	3		0		2		

10.1.2 Chapter 3 Appendix 2

Panel of known negative samples tested with R5/R91 capture (1:2000) and conjugate (1:1500). Note: Samples called 'Manchester dog #1-#12' were collected from a veterinary practice in Greater Manchester by Matthew Bates. Dr. Boufana provided coproELISA negative samples from the Falkland Islands, as well as one coproELISA negative sample from Wales.

Sample	Average OD	Sample	Average OD
Tia (Falklands)	0.0352	Chanti (Falklands)	0.0415
Storm (Falklands)	0.0180	Georgia (Falklands)	0.0444
Rikki (Falklands)	0.0280	Georgie (Falklands)	0.0279
Bella (Falklands)	0.0416	Dixie (Falklands)	0.0181
Buster (Falklands)	0.0554	Bagley (Falklands)	0.0270
Barney (Falklands)	0.0385	Duel (Falklands)	0.0321
Jasmine (Falklands)	0.0250	Pebbles (Falklands)	0.0311
Bramble (Falklands)	0.0347	Shrek (Falklands)	0.0294
Nambe (Falklands)	0.0323	Calvin (Falklands)	0.0293
Ninja (Falklands)	0.0167	Manchester dog1	0.0246
Shelby (Falklands)	0.0329	Manchester dog2	0.0260
Tess (Falklands)	0.0287	Manchester dog3	0.0250
Berkeley (Falklands)	0.0466	Manchester dog4	0.0089
Nelson (Falklands)	0.0350	Manchester dog5	0.0033
Meg (Falklands)	0.0400	Manchester dog6	0.0282
Dylan (Falklands)	0.0409	Manchester dog7	0.0270
Wolf (Falklands)	0.0332	Manchester dog8	0.0330
Bute (Falklands)	0.0239	Manchester dog9	0.0006
Ruby (Falklands)	0.0407	Manchester dog10	0.0166
Romeo (Falklands)	0.0444	Manchester dog11	0.0205
Jet (Falklands)	0.0173	Manchester dog12	0.0041
Keechi (Falklands)	0.0409	Dude (Welsh)	0.0338
Connor (Falklands)	0.0414	**Average**	**0.0296**
Cassidy (Falklands)	0.0441	**Stdev**	**0.0118**
Rufus (Falklands)	0.0251	**Cutoff**	**0.0649**

10.1.3 Chapter 3 Appendix 3

Panel of known positive samples tested with R5/R91 capture (1:2000) and conjugate (1:1500). Details of sample status are given in 'Known value' column. Samples SMB10101, KK#15, SMB06401, SMB11801, KK#8A, SMB02401, KK#6, Nurik, KK#3A, KK#11 and KK#3 are from the Alay Valley, Kyrgyzstan. Samples 'Dog 2,3,9,11,13,15,16,17', 'Sable dog 2, 'E. multilocularis', 'Aus med/low/high/exp inf' and 'Dingo' and were provided by Dr. Boufana. 'Meg spike' indicates a sample from Manchester spiked with 1:100 E. granulosus *whole worm extract. 'Abbasi' indicates samples that were coproPCR positive with the Abbasi et al (2003) protocol, 'sequen pos' indicates samples that were successfully sequences as* E. granulosus *sensu lato with the 'Abbasi' primers.*

Sample	Average OD	Pos/Neg	Known value
SMB10101	0.0841	Pos	ELISA,Abbasi,sequen pos
KK#15	0.0816	Pos	ELISA,Abbasi,sequen pos
SMB06401	0.1076	Pos	ELISA,Abbasi,sequen pos
SMB11801	0.1007	Pos	ELISA,Abbasi,sequen pos
KK#8A	0.1137	Pos	ELISA&Abbasi pos
SMB02401	0.1398	Pos	ELISA&Abbasi pos
KK#6	0.0746	Pos	ELISA&Abbasi pos
NURIK	0.0944	Pos	ELISA,Em,Abb,sequen pos
KK#3A	0.1092	Pos	ELISA and Abbasi pos
KK#11	0.0699	Pos	ELISA positive
KK#3	0.1902	Pos	ELISA and Abbasi pos
DOG2	0.1972	Pos	necropsy positive
DOG3	0.1167	Pos	necropsy positive
DOG9	0.1315	Pos	necropsy positive
DOG11	0.0920	Pos	necropsy positive
DOG13	0.1340	Pos	necropsy positive
DOG15	0.0773	Pos	necropsy positive
DOG16	0.1288	Pos	necropsy positive
DOG17	0.1340	Pos	necropsy positive
SABLE DOG 2	0.2702	Pos	Known positive
T.hydatigena	0.043	Neg	Negative for *Echinococcus* spp.
T.multiceps	0.016	Neg	Negative for *Echinococcus* spp.
E. equinus	0.091	Pos	Positive for *E. equinus*
E. multilocularis (7 worms)	0.033	Neg	necropsy positive
E. multilocularis (7 worms)	0.058	Neg	necropsy positive
E.multilocularis Fox	0.212	Pos	necropsy positive
AUS low	0.118	Pos	Spiked sample
AUS med	0.189	Pos	Spiked sample
AUS high	0.237	Pos	Spiked sample
Dingo	0.166	Pos	necropsy positive
Meg spike	0.335	Pos	Spiked sample
Aus exp inf	0.185	Pos	necropsy positive
Dog 8 E.g	0.139	Pos	necropsy positive

10.1.4 Chapter 3 Appendix 4

Panel of known negative samples tested with R47 capture (1:3000) and conjugate (1:1500). Dr. Boufana provided coproELISA negative samples from the Falkland Islands

Sample	Average OD	Sample	Average OD
Tia (Falkands)	0.08495	Bute (Falkands)	0.02750
Storm (Falkands)	0.03145	Jet (Falkands)	0.02870
Jack (Falkands)	0.02430	Keechi (Falkands)	0.05290
Ben (Falkands)	0.02715	Connor (Falkands)	0.10750
Rikki (Falkands)	0.06255	Cassidy (Falkands)	0.06235
Poppy (Falkands)	0.02115	Rufus (Falkands)	0.05550
Bella (Falkands)	0.05540	Chanti (Falkands)	0.07225
Buster (Falkands)	0.05080	Georgia (Falkands)	0.06125
Jasmine (Falkands	0.02650	Marley (Falkands)	0.06180
Bramble (Falkands	0.05215	Missy (Falkands)	0.06770
Nambe (Falkands)	0.05120	Georgie (Falkands)	0.03955
Ninja (Falkands)	0.03765	Dixie (Falkands)	0.05850
Shelby (Falkands)	0.04300	Duel (Falkands)	0.04325
Tess (Falkands)	0.04935	Pebbles (Falkands	0.05230
Berkely (Falkands	0.06735	Calvin (Falkands)	0.04920
Nelson (Falkands)	0.06080		
Monty (Falkands)	0.06915	**Average**	**0.05140**
Meg (Falkands)	0.05955	**Stdev**	**0.01821**
Dylan (Falkands)	0.04740	**Cutoff**	**0.10604**
Wolf (Falkands)	0.03690		

10.1.5 Chapter 3 Appendix 5

Panel of known positive samples tested with R47 capture (1:3000) and conjugate (1:1500). Samples SMB10101, KK#15, SMB06401, SMB11801, KK#8A, SMB02401, KK#6, Nurik, KK#3A, KK#11, KK#3 and TS237 are from the Alay Valley, Kyrgyzstan. Samples 'Dog 2,3,9,11,13,15,16,17' and 'Sable dog 2' were provided by Dr. Boufana. 'PCR Abbasi' indicates samples that were coproPCR positive with the Abbasi et al (2003) protocol, 'seq positive' indicates samples that were successfully sequences as E. granulosus *sensu lato with the 'Abbasi' primers. Sample 'Nurik' was also successfully sequenced as* E. multilocularis *with the ND1 primers (Boufana et al., 2013b).*

Sample	Average C	Value	True value
SMB10101 (Kyrgyzstan)	0.12950	Pos	ELISA, PCR Abbasi and seq positive
KK#15 (Kyrgyzstan)	0.17675	Pos	ELISA, PCR Abbasi and seq positive
SMB06401 (Kyrgyzstan)	0.13065	Pos	ELISA, PCR Abbasi and seq positive
SMB11801 (Kyrgyzstan)	0.19725	Pos	ELISA, PCR Abbasi and seq positive
KK#8A (Kyrgyzstan)	0.19780	Pos	ELISA and PCR Abbasi positive
SMB02401 (Kyrgyzstan)	0.23630	Pos	ELISA and PCR Abbasi positive
KK#6 (Kyrgyzstan)	0.15170	Pos	ELISA and PCR Abbasi positive
NURIK (Kyrgyzstan)	0.19075	Pos	ELISA,PCR *E. multilocularis* , PCR Abbasi, and sequence positive
TS237 (Kyrgyzstan)	0.09200	Neg	ELISA, PCR Abbasi and seq positive
KK#3A (Kyrgyzstan)	0.17735	Pos	ELISA and Abbasi pos
KK#11 (Kyrgyzstan)	0.16465	Pos	Purge and ELISA positive
KK#3 (Kyrgyzstan)	0.26400	Pos	ELISA and PCR Abbasi positive
DOG2 (Tunisia)	0.56020	Pos	necropsy positive
DOG3 (Tunisia)	0.48470	Pos	necropsy positive
DOG9 (Tunisia)	0.54075	Pos	necropsy positive
DOG11 (Tunisia)	0.31975	Pos	necropsy positive
DOG13 (Tunisia)	0.40380	Pos	necropsy positive
DOG15 (Tunisia)	0.27075	Pos	necropsy positive
DOG16 (Tunisia)	0.52925	Pos	necropsy positive
DOG17 (Tunisia)	0.49975	Pos	necropsy positive
SABLE DOG 2 (Tunisia)	0.62840	Pos	necropsy positive

Chapter 3 Appendix 6

Panel of known positive samples tested with a commercial Echinococcus *spp. ELISA kit. Samples SMB10101, KK#15, SMB06401, SMB11801, KK#8A, SMB03401, KK#6, Nurik, KK#3A, KK#11, KK#3 are from the Alay Valley, Kyrgyzstan. Samples 'Dog 2,3,9,11,13,1 5,16,17' and 'Sable dog 2' as well as coproELISA negative samples from the Falkland Islands were provided by Dr. Boufana. 'Abbasi pos' indicates samples that were coproPCR positive with the Abbasi et al (2003) protocol, 'sequen pos' indicates samples that were successfully sequences as* E. granulosus *sensu lato with the 'Abbasi' primers. Sample 'Nurik' was also successfully sequenced as* E. multilocularis *with the ND1 primers (Boufana et al., 2013b). Purge indicates arecoline purge positive samples.*

Sample	Average OD	pos/neg	True classification	Correct classification?
KK#3	0.15735	Neg	ELISA and Abbasi pos	No
SMB06401	0.1032	Neg	ELISA,Abbasi,sequen pos	No
SMB02401	0.12575	Neg	ELISA&Abbasi pos	No
KK#15	0.2053	Neg	ELISA,Abbasi,sequen pos	No
SMB02301	0.29575	Neg	ELISA negative	Yes
SMB10101	0.41905	Pos	ELISA,Abbasi,sequen pos	Yes
KK#3A	0.1377	Neg	ELISA and Abbasi pos	No
KK#11	0.16125	Neg	ELISA positive	No
KK#6	0.13785	Neg	ELISA&Abbasi pos	No
SMB03401	0.08275	Neg	ELISA negative	Yes
SMB11801	0.39575	Neg	ELISA,Abbasi,sequen pos	No
NURIK	0.7586	Pos	ELISA,Em,Abb,sequen pos	Yes
TASHA	0.16465	Neg	Falklands negative	Yes
GIN	0.5763	Pos	Falklands negative	No
ELLIE	0.1667	Neg	Falklands negative	Yes
JAFF	0.6151	Pos	Falklands negative	No
PIPPA	0.25485	Neg	Falklands negative	Yes
FLY	0.31285	Neg	Falklands negative	Yes
KYLIE	0.31745	Neg	Falklands negative	Yes
MAXI	0.12875	Neg	Falklands negative	Yes
JACK	0.3129	Neg	Falklands negative	Yes
JESSIE	0.44425	Pos	Falklands negative	No
MEG	0.70135	Pos	Falklands negative	No
TAG	0.41765	Pos	Falklands negative	No
DOG 2 TUNISIA	0.67835	Pos	necropsy positive	Yes
DOG 3 TUNISIA	0.34955	Neg	necropsy positive	No
DOG 9 TUNISIA	0.98885	Pos	necropsy positive	Yes
DOG 11 TUNISIA	0.8722	Pos	necropsy positive	Yes
DOG 13 TUNISIA	0.35395	Neg	necropsy positive	No
DOG 15 TUNISIA	0.31725	Neg	necropsy positive	No
DOG 16 TUNISIA	0.47425	Pos	necropsy positive	Yes
DOG 17 TUNISIA	0.98805	Pos	necropsy positive	Yes
KIM	0.36735	Neg	Falklands negative	Yes
ASHA	0.48415	Pos	Falklands negative	No
GAUCHO	0.5569	Pos	Falklands negative	No
JESS	0.63515	Pos	Falklands negative	No
X50	2.2979	Pos	Spike	Yes
KK#8	0.26265	Neg	Purge, ELISA, Abbasi, seq positive	No
KK#18	0.27865	Neg	Purge, ELISA, Abbasi, seq positive	No
X100	1.4078	Pos	Spike	Yes
TAFF	0.1272	Neg	Falklands negative	Yes
TYPE	0.2845	Neg	Falklands negative	Yes
MIA	0.21435	Neg	Falklands negative	Yes
NEG	0.1699	Neg	Provided negative	Yes
NEG	0.20965	Neg	Provided negative	Yes
POS	1.0365	Pos	Provided positive	Yes
POS	1.17635	Pos	Provided positive	Yes

10.2 Chapter 6 Appendix 1

Dog owner questionnaire administered to householders in the Alay Valley, Kyrgyzstan

Questionnaire for individual dogs *(Анкета для собак)*

Date *(Дата)* _____ **Grid point/GPS location** *(Расположение)* _____

Village code: _____
Код села

Household number/№ _____
Номер дома

Name: _____
ФИО

Address: _____
Адреси

Dog data *(Данные собаки)*

1. Dog No. (in household): _____
Номер собаки

2. Dog ID (photo☐/microchip☐): _____
ИД номер собаки (фотография/микрочипа)

3. Dog name *(Кличка)*: _____

4. Dog description: _____
Описание собаки

5. Age of dog *(Возраст)*: _____yrs

Exact☐ **estimate**☐ **unknown**☐
точно *около* *неизвестно*

6. Sex: **male** ☐ **female** ☐
Пол *самец* *самка*

7. Neutered: **No** ☐ **Yes** ☐
Кастрирован *нет* *да*

8. Breed *(Порода)*: _____

9. Size of dog: small☐ **medium**☐ **large**☐
Габарит собаки: малый средний большой

10. Approximate weight of dog: _____kg
примерный вес собаки

11. If entire female is she pregnant?
если самка, беременная?
Yes *(да)*☐ **No** *(нет)*☐

12. Reproductive data (if female)
(Репродуктивные данные если собака сука):

a. Number of litters in the last year
(сколько раз у вашей суки были беременности в течение последних 12 месяцев?) _____

b. Total number of puppies born: _____
Сколько родились щенки?

c. Number survived: _____
Сколько из них выжили?

d. Number kept by household: _____
Сколько щенят оставили себе?

e. Number given away: _____
Сколько щенят отдали?

f. Number of litters produced over lifetime: _____
сколько раз может быть у суки беременности за всю жизнь?

13. Source of dog *(Происхождение собаки)*

a.Offspring of own bitch ☐
от собственной собаки

b. Sourced/traded with neighbours ☐
купил или приобрел от соседей

c. Bought/traded from outside neighbourhood ☐
купил или приобрел в базаре или другого села

14. Type of dog *(тип собаки)*: pet *(домашний)*☐ guard dog *(сторожевой)*☐ sheep dog *(отарный)*☐ hunting dog *(охотничий)*☐ other *(другое)* _____

15. If owners travel to Summer pasture *(если хозяин идет в пастбища)*

a. Did dog travel to Summer pasture last year? No ☐ Yes ☐
последний год, брали вашу собаку собой в пастбищу *нет* *да*

b. Will dog travel to Summer pasture this year? No☐ Yes ☐
возьмете вашу собаку в этом году собой в пастбищу *нет* *да*

16. Dog is fed by : People in household☐ Neighbours☐ Forage for themselves☐
кто кормит вашу собаку *члены семьи* *соседи* *собака сама находит*

B1

17. Dogs are fed: *питание*	never/very rarely *никогда/очень редко*	monthly *ежемесячно*	weekly *недельный*	almost every day *каждый день*
a. Uncooked meat *(сырое мясо и потроха)*	☐	☐	☐	☐
b. Offal *(мусор)*	☐	☐	☐	☐
c. Table scraps *(остатками со стола)*	☐	☐	☐	☐
d. Commercial feed *(специальный корм,для собак)*	☐	☐	☐	☐
e. Seen eating rodents *Питается ваша собака с грызунами?*	☐	☐	☐	☐

18. How is the dog kept? *(Как держите вашу собаку?)*
chained at night ☐ *ночью в цепи* chained during day ☐ *днем в цепи* always chained ☐ *всегда в цепи* never chained ☐ *никогда в цепи*

19a. % of time dog is indoors *(% времени собака в помещении конуре):* _____
19b. % of time dog is outside leashed *(% времени собака в поводке):* _____
19c. % of time dog is outside unleashed *(% Времени собаки без поводка):* _____

20. Persons who handle or play with dog *(кто отвествен для собаки дома или кто играет с собакой):*
Adults of HH☐ *Взрослые* **Children of HH**☐ *Дети* **Friends&neighbours**☐ *Друзья и соседи* **Strangers**☐ *чужие* **Nobody**☐ *Никто*

21. Has the dog had any worming treatment in the last 6 months? **No** ☐ *нет* **Yes** ☐ *да*
Лечили собаку последние 6 месяцев?

22. If yes, by whom? **Vet**☐ **People in HH**☐ **Government worker**☐ **Other**_____
Если да, то кто делал? ветеринара члены семьи правительство работника другое

23. What drug(s) were used? *(какой препарат (ы) был/были использованы)*_____

24. How many tablets were given? *(Сколько таблеток давали)*_____

25. Approximately when was the dog last dosed _____
Примерно, когда была последняя дегельминтизация

26. Has dog been vaccinated against *(собака когда нибудь вакцинирована против):*
Rabies☐ *Бешенство* Distemper☐ *чума собак* Canine hepatitis☐ *гепатит собак* Leptospirosis☐ *Лептоспироз*

27. If dog has been vacc against rabies, date of last vaccination and vaccine type
Если собака была привита против бешенства укажите дату вакцинации и типа вакцины:
_____

Faecal sample taken? *взята кот проба?* **No**☐ *нет* **Yes**☐ *да*
PBS ☐ **Alcohol** *(спирт)* ☐
If not collected, why not?
*если проба не взята почему*_____
Sample ID *(ид номер пробы)* _____
Given arecoline? *(давали ареколин)* **No**☐ *нет* **Yes**☐ *да*
Purged? *(понос был)* **No** *(нет)*☐ **Yes** *(да)*☐

Frozen *(заморозен)* ☐
Alcohol *(спирт)* ☐
Adverse reaction to arecoline? ☐
различные э ффекты к ареколину?
Dosed with praziquantel: No☐ **Yes**☐
давали празиквантел нет да
Date of dosing:_____**20**____
Дата дачи таблетки
Dose min 1 day after arecoline if purged
минимум 1день после ареколина если очищен

B2

10.3 Chapter 7 Appendices

10.3.1 Chapter 7 Appendix 1

LQAS table with decision rules for samples sized between 12-30 and coverage between 10%-95% (Valadez et al., 2002)

Overhead 3

LQAS Table: Decision Rules for Sample Sizes of 12-30 and Coverage Targets/Average of 10%-95%

Sample Size*	Average Coverage (Baselines) / Annual Coverage Target (Monitoring and Evaluation)																	
	10%	15%	20%	25%	30%	35%	40%	45%	50%	55%	60%	65%	70%	75%	80%	85%	90%	95%
12	N/A	N/A	1	1	2	2	3	4	5	5	6	7	7	8	8	9	10	11
13	N/A	N/A	1	1	2	3	3	4	5	6	6	7	8	8	9	10	11	11
14	N/A	N/A	1	1	2	3	4	4	5	6	7	8	8	9	10	11	11	12
15	N/A	N/A	1	2	2	3	4	5	6	6	7	8	9	10	10	11	12	13
16	N/A	N/A	1	2	2	3	4	5	6	7	8	9	9	10	11	12	13	14
17	N/A	N/A	1	2	2	3	4	5	6	7	8	9	10	11	12	13	14	15
18	N/A	N/A	1	2	2	3	5	6	7	8	9	10	11	11	12	13	14	16
19	N/A	N/A	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
20	N/A	N/A	1	2	3	4	5	6	7	8	9	11	12	13	14	15	16	17
21	N/A	N/A	1	2	3	4	5	6	8	9	10	11	12	13	14	16	17	18
22	N/A	N/A	1	2	3	4	5	7	8	9	10	12	13	14	15	16	18	19
23	N/A	N/A	1	2	3	4	6	7	8	10	11	12	13	14	16	17	18	20
24	N/A	N/A	1	2	3	4	6	7	9	10	11	13	14	15	16	18	19	21
25	N/A	1	2	2	4	5	6	8	9	10	12	13	14	16	17	18	20	21
26	N/A	1	2	3	4	5	6	8	9	11	12	14	15	16	18	19	21	22
27	N/A	1	2	3	4	5	7	8	10	11	13	14	15	17	18	20	21	23
28	N/A	1	2	3	4	5	7	8	10	12	13	15	16	18	19	21	22	24
29	N/A	1	2	3	4	5	7	9	10	12	13	15	17	18	20	21	23	25
30	N/A	1	2	3	4	5	7	9	11	12	14	16	17	19	20	22	24	26

N/A: *not applicable*, meaning LQAS can not be used in this assessment because the coverage is either too low or too high to assess an SA.

: shaded cells indicate where *alpha* or *beta* errors are ≥ 10%.

: hashed cells indicate where *alpha* or *beta* errors are > 15%.

10.3.2 Chapter 7 Appendix 2

Arecoline purged domestic dog faecal samples (n=20) used as part of ROC curve panels. Neg=Negative, Pos=Positive. Eg= E. granulosus. Em=E. multilocularis

Sample	Source	Method	Status	Worm burden estimate	Further detail
KK1	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
KK2	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
KK3A	Alay Valley	Arecoline	Pos for *Echinococcus*	Low	PCR +ve for *E. g* s.l ("Abbasi" primers)
KK3B	Alay Valley	Arecoline	Pos for *Echinococcus*	Low	PCR +ve for *E. g* s.l ("Abbasi" primers)
KK4	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
KK6	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
KK7	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
KK8	Alay Valley	Arecoline	Pos for *Echinococcus*	High	PCR +ve for *E. g* s.l ("Abbasi" primers)
KK9	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
KK10	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
KK11	Alay Valley	Arecoline	Pos for *Echinococcus*	Medium	PCR +ve for *E. g* s.l ("Abbasi" primers)
KK13	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
KK14	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
KK15	Alay Valley	Arecoline	Pos for *Echinococcus*	Low	PCR +ve for *E. g* s.l ("Abbasi" primers)
KK16	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
KK18	Alay Valley	Arecoline	Pos for *Echinococcus*	Low	PCR +ve for *E. g* s.l ("Abbasi" primers)
KK19	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
IRISBAEV	Alay Valley	Arecoline	Neg for *Echinococcus*	n/a	n/a
NURIK	Alay Valley	Arecoline	Pos for *Echinococcus*	Low	PCR +ve for *E. g* s.l ("Abbasi" primers)
ONHOEB	Alay Valley	Arecoline	Pos for *Echinococcus*	High	PCR +ve for *E. g* s.l ("Abbasi") & *E. m* (ND1 primers)

10.3.3 Chapter 7 Appendix 3

Domestic dog faecal samples (n=38) from necropsied dogs in Hobukesar County, Xinjiang, used as part of ROC curve panels. Neg=Negative, Pos=Positive. Eg= E. granulosus.

Sample	Source	Method	Status	Worm burden estimate	Further detail
XP1	Xinjiang	Necropsy	Neg for Echinococcus	n/a	n/a
XP2	Xinjiang	Necropsy	Pos for Echinococcus	~50 worms	Sequenced as *E. granulosus* G1
XP3	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP4	Xinjiang	Necropsy	Pos for *Echinococcus*	~50 worms	Sequenced as *E. granulosus* G1
XP5	Xinjiang	Necropsy	Neg for Echinococcus	n/a	n/a
XP6	Xinjiang	Necropsy	Pos for *Echinococcus*	>5000 worms	Sequenced as *E. g* G1
XP7	Xinjiang	Necropsy	Pos for Echinococcus	~100 worms	Sequenced as *E. g* G1
XP8	Xinjiang	Necropsy	Pos for *Echinococcus*	~20 worms	Sequenced as *E. g* G1
XP9	Xinjiang	Necropsy	Pos for *Echinococcus*	~10 worms	Sequenced as *E. g* G1
XP10	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP11	Xinjiang	Necropsy	Neg for Echinococcus	n/a	n/a
XP12	Xinjiang	Necropsy	Neg for Echinococcus	n/a	n/a
XP13	Xinjiang	Necropsy	Pos for *Echinococcus*	(not recorded)	Sequenced as *E. g* G1
XP14	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP15	Xinjiang	Necropsy	Pos for *Echinococcus*	2 worms	Sequenced as *E. g* G1
XP16	Xinjiang	Necropsy	Pos for *Echinococcus*	~100 worms	Sequenced as *E. g* G1
XP17	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP18	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP19	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP20	Xinjiang	Necropsy	Pos for *Echinococcus*	~ 300 worms	Sequenced as *E. g* G1
XP21	Xinjiang	Necropsy	Pos for *Echinococcus*	~500 worms	Sequenced as *E. g* G1
XP22	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP23	Xinjiang	Necropsy	Pos for *Echinococcus*	~100 worms	Sequenced as *E. g* G1
XP24	Xinjiang	Necropsy	Pos for *Echinococcus*	3 worms	Sequenced as *E. g* G1
XP25	Xinjiang	Necropsy	Pos for *Echinococcus*	>10,000 worms	Sequenced as *E. g* G1
XP26	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP27	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP28	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP29	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP30	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP31	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP32	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP33	Xinjiang	Necropsy	Pos for *Echinococcus*	>10,000 worms	Sequenced as *E. g* G1
XP34	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP35	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP36	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP37	Xinjiang	Necropsy	Neg for *Echinococcus*	n/a	n/a
XP38	Xinjiang	Necropsy	Pos for *Echinococcus*	>10,000 worms	Sequenced as *E. g* G1

Dog ownership, dog behaviour and transmission of *Echinococcus* spp. in the Alay Valley, southern Kyrgyzstan

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SUMMARY

Echinococcosis is a re-emerging zoonotic disease in Kyrgyzstan, and the incidence of human infection has increased substantially since the collapse of the Soviet Union in 1991. Domestic dogs are hosts of *Echinococcus* spp. and play an important role in the transmission of these parasites. The demography, ecology and behaviour of dogs are therefore relevant in studying *Echinococcus* spp. transmission. Dog demographics, roles of dogs, dog movements and faecal environmental contamination were assessed in four rural communities in the Alay Valley, southern Kyrgyzstan. Arecoline purge data revealed for the first time that *E. granulosus, E. canadensis* and *E. multilocularis* were present in domestic dogs in the Alay Valley. Surveys revealed that many households had dogs and that dogs played various roles in the communities, as pets, guard dogs or sheep dogs. Almost all dogs were free to roam, and GPS data revealed that many moved outside their communities, thus being able to scavenge offal and consume rodents. Faecal environmental contamination was high, presenting a significant infection risk to the local communities.

Key words: *Echinococcus granulosus*, *Echinococcus canadensis*, *Echinococcus multilocularis*, Kyrgyzstan, domestic dogs, demography, behaviour.

INTRODUCTION

Echinococcosis is a neglected zoonotic disease (World Health Organization, 2010) caused by infection with the larval stage (metacestode) of tapeworms within the genus *Echinococcus* (Eckert and Deplazes, 2004). The most common types of echinococcosis are cystic and alveolar which are caused by *E. granulosus* and *E. multilocularis*, respectively (World Health Organization/World Organisation for Animal Health, 2001). The life-cycles of *E. granulosus* and *E. multilocularis* involve two mammalian hosts. The adult cestode inhabits the small intestine of a definitive host (usually a canid) and produces eggs which are released into the environment (Eckert and Deplazes, 2004) and may then be ingested by an intermediate host. In the case of *E. granulosus* the intermediate host is usually a sheep, but may include other herbivore species (Sweatman and Williams, 1963; World Health Organization/World Organisation for Animal Health, 2001). In the case of *E. multilocularis*, small mammals, including voles (e.g. *Arvicola* spp., Hofer *et al*. 2000), pika (*Ochotona* spp., Schantz *et al*. 2003), and Tibetan hare (*Lepus oiostolus*, Xiao *et al*. 2004) may act as intermediate hosts. If the intermediate host is consumed by a definitive host, the cycle is complete. Humans may also inadvertently ingest eggs expelled by the definitive host and develop cystic or alveolar echinococcosis (Deplazes and Eckert, 2001).

In Asia, echinococcosis is a serious public health concern in several areas including the Tibetan Plateau (Budke *et al*. 2005), central China (Craig *et al*. 1992), and Mongolia (Ebright *et al*. 2003; Ito *et al*. 2010). There is concern that echinococcosis is re-emerging in Central Asia following the collapse of the Soviet Union (Torgerson *et al*. 2006). In Kyrgyzstan, the annual human surgical incidence of cystic echinococcosis has increased dramatically, rising from about 5 cases per 100 000 to nearly 20 cases per 100 000 between 1991 and 2002 (Torgerson *et al*. 2006). Alveolar echinococcosis is also thought to be increasing in Kyrgyzstan (Torgerson *et al*. 2010; Usubalieva *et al*. 2013).

Although several species of wild canids may be hosts for *E. granulosus*, e.g. grey wolves, *Canis lupus* (Abdybekova and Torgerson, 2012) and *E. multilocularis*, e.g. red fox, *Vulpes vulpes* (Ziadinov *et al*. 2010), infected domestic dogs, *Canis familiaris*, are considered to pose the greatest risk of human infection (Budke *et al*. 2005). Domestic dogs are hosts for a

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number of zoonotic pathogens, and due to their close association with people they may be sources of human infections (Macpherson, 2005). The demography, ecology and behaviour of dogs are therefore relevant in studying diseases that may be spread by them, and describing the dog population in a community may help to assess transmission potential, zoonotic risks and optimization of intervention programmes (Butler and Bingham, 2000). This concept has been recognized in studies relating to rabies (Perry, 1993; Butler and Bingham, 2000; Kitala *et al*. 2001; Macpherson, 2005), but has to date rarely been applied to studies on echinococcosis (but see Vaniscotte *et al*. 2011). Here we aim to determine the presence of *Echinococcus* spp. in domestic dogs in four communities in southern Kyrgyzstan. We further aim to characterize the domestic dog population in these communities by describing their demographics, roles, husbandry and roaming behaviour, as well as the levels of environmental contamination with dog faeces. In doing so, we aim to understand better the role of dogs in *Echinococcus* spp. transmission in these communities.

MATERIALS AND METHODS

Study site

The Alay Valley is located in the south of Kyrgyzstan, and covers most of the Osh oblast. It is located at an altitude of approximately 3000 m a.s.l. and is bordered by the Pamir Mountains to the south (on the border with Tajikistan), and the Alay Mountains to the north (Wikipedia, 2013). Based on a cluster of human AE cases derived from hospital records reported by Usubalieva *et al*. (2013), we selected four rural communities in the Alay Valley for a study of canine echinococcosis, namely Taldy Suu (39·70°, 72·98°) Sary Mogol (39·68°, 72·89°), Kara Kavak (39·66°, 72·72°) and Kashka Suu (39·64°, 72·67°).

Household questionnaires

Detailed questionnaire-based surveys were carried out in May 2012. Questionnaires were designed using WHO guidelines (World Health Organization and World Society for the Protection of Animals, 1990). Questions were asked about the age, sex and source of dogs, as well as their reproductive status (if female). Questions were also asked about the role of the dog, i.e. pet, guard, sheep dog or other. Further questions were included about the diet of the dog, and whether it was ever tied up. On a subsequent visit in October 2012, shorter questionnaires were employed to ask households if they still owned the previously registered dogs and if any new dogs had been acquired. If the previously registered dogs were no longer present, the reason for this was asked. Not all questions were answered by all respondents, so that numbers reported are at times less than the total number of dogs registered. All questionnaires were administered in Kyrgyz by native speakers (BM and IZ).

Faecal quadrats to assess environmental contamination

ArcGIS was used to create shapefiles of the approximate boundaries of the four villages (based on imagery from the SPOT5 satellite, Google Maps, 2012). Within each of these four areas, 17 random points were generated which were used to define one corner of each quadrat. If the point fell in an inaccessible location (e.g. a house) the nearest possible point was taken. The direction of the quadrat was usually determined by the surrounding buildings, fences, etc. Where there was enough space for the 50×50 m quadrat to be done facing several directions, the second hand on a watch was used to determine the direction. Where it was not possible to measure out 50×50 m due to the presence of buildings, smaller areas were measured and the size was recorded. Quadrats were searched for faeces by slowly pacing up and down whilst looking at the ground. Canid faeces were identified by their size and shape by a researcher experienced in the identification of canid faeces (FvK). In all likelihood, faeces found in villages were from domestic dogs although some may have been from foxes and possibly wolves. As dogs, foxes and wolves are all hosts for *Echinococcus* spp. and therefore all pose an infection risk to humans, no effort was made to distinguish between these using host PCR. The faecal density was calculated as the number of faeces/100 m^2. All quadrats were performed by the same researcher, and the same 68 quadrats were searched for faeces in May and in October 2012.

During each visit, four quadrats were selected in each village using a random number generator in Microsoft Excel® (Microsoft, Redmond, WA). In all four quadrats, one third of faecal samples (or at least six, if the total number of faeces was less than 18) were selected by sequentially ordering the samples prior to using an Excel® random number generator. Since these samples were collected from the ground we cannot be sure that samples collected were from different individual dogs. Sub-samples of each of these were stored in 0·3% PBS Tween buffer with 10% formalin and 95% ethanol for coproantigen ELISA and coproPCR analysis, respectively and were shipped to the University of Salford at ambient temperature.

Coproantigen ELISA

All faecal samples collected were analysed using a genus-specific coproantigen ELISA. This allowed us to detect those samples that contained

Echinococcus spp. antigens before using coproPCR (see below) to identify whether the samples were positive for *E. multilocularis* or *E. granulosus* sensu lato. Coproantigen ELISA allows for rapid analysis of large numbers samples and can detect pre-patent infections (Fraser and Craig, 1997), and can be recommended as a primary diagnostic tool followed by PCR (e.g. Eckert and Deplazes, 2004). Samples were first stored at −80 °C for a minimum of 5 days in order to kill any infective eggs of *Echinococcus* spp. (World Health Organization/World Organisation for Animal Health, 2001). Samples were then defrosted and homogenized with wooden spatulas, shaken and centrifuged at 2500 rpm for 5 min using an Eppendorf centrifuge 5804. Supernatants were decanted into Bijoux tubes and stored at −20 °C until used for analysis. A genus-specific sandwich ELISA, using the protocol described by Allan *et al.* (1992) and Craig *et al.* (1995), was used to test for *Echinococcus* spp. coproantigen. Supernatants of two known positives (an arecoline purge-positive sample from Kara Kavak and an antigen-spiked sample, spiked 1:100 with *E. granulosus* whole worm extract) were used as positive controls throughout. Two known negatives from non-endemic and endemic areas (Manchester, UK and the Falkland Islands) were also included as negative controls.

CoproPCR

CoproDNA was extracted from 1 g of dog faeces (weighed using a balance accurate to 0·01 g) using QIAamp® DNA Stool Mini Kit following the manufacturer's instructions. The DNA retrieved was used to test for *E. granulosus* using two sets of primers. Initially, samples were tested for *E. granulosus* G1 (common sheep strain) using highly specific ND1 primers to amplify a species-specific 226 bp fragment (Boufana *et al.* 2013). However, because the strains of *E. granulosus* present in this area are unknown, another protocol was used to detect *E. granulosus* (sensu lato) by amplifying a 269 bp tandem repeat region (Abbasi *et al.* 2003) using modifications described by Boufana *et al.* (2008). DNA samples were also tested for *E. multilocularis* using PCR-specific primers (Boufana *et al.* 2013) to amplify a 207 bp fragment within the ND1 mitochondrial gene. Negative controls (PCR grade water) were included throughout. A Stratagene Robocycler (La Jolla, CA) was used for all cycling profiles and the PCR products were separated by electrophoresis using a 1·5% (w/v) agarose gel (Bioline) in 1 × Tris-Borate-EDTA buffer (Severn Biotech, Kidderminster, UK) at 110 V, stained with gel red DNA dye (Cambridge Biosciences, Cambridge, UK), and visualized using Syngene G:Box gel documentation system (Cambridge Biosciences).

Arecoline purges

Twenty dogs (16 from Kara Kavak and 4 fromTaldy Suu) were voluntarily brought in by their owners and dosed with a 0·4% solution of arecoline hydrobromide in water (7 mg arecoline/kg body weight) and were restrained safely by their owners until they purged. The purges were examined in the field using a handheld magnifying glass and scored for presence/absence of *Echinococcus* spp. and *Taenia* spp. based on gross morphology by an experienced fieldworker (PSC). Subsamples of these purges were stored in 0·3% PBS Tween buffer with 10% formalin and 95% ethanol for coproantigen and coproPCR analysis, respectively and shipped to the University of Salford at ambient temperature.

Dog tracking

A total of 40 dogs (11 from Sary Mogol, 14 from Taldy Suu, 12 from Kashka Suu and three from Kara Kavak) were fitted with iGotU® GPS trackers. The iGotU® unit is a GPS tracker that can record GPS positions at programmed intervals (www.i-gotu.com). These units were attached to regular dog collars using ziplock bags and adhesive tape. The accuracy of the GPS units was validated both by leaving units in set locations (stationary recording) and by moving units along a path (dynamic recording). These activities were undertaken in both the Alay Valley and in relatively sparsely built-up areas in the United Kingdom (adjacent to South Park, Macclesfield for stationary recording and Peel Park, Salford for dynamic recording). In both cases, a Garmin® GPS60 unit was used for comparison.

Dogs were selected for collaring in the field during dog registration. Selection could not be completely random as only those dogs present and tame enough to be handled were selected. Although an effort was made to track each selected dog for 24 h this was not always possible due to field logistics and limitations in battery life. In addition, a number of GPS trackers could not be retrieved. Dogs were tracked for between 1·5 and 47 h (mean = 20 h, S.D. = 9 h), and trackers were set to record GPS positions every 5 min, with between 25 and 380 positions recorded per dog (mean = 156, S.D. = 81). Dogs were recorded for a total of 787 h, with a total of 6256 GPS points recorded. However, dogs with fewer than 50 points recorded ($n = 3$) were removed from further analysis, leaving a total of 37 dogs. Of these 37, 26 were male (SM = 7, TS = 11, KS = 5, KK = 3) and 7 were female (SM = 4, KS = 3). For four dogs the sex was not recorded.

Data analysis

Data were analysed and figures were made using R statistical software version 2.15.2 (R Development

Core Team, 2012). In order to analyse the quadrat data, the glme function in the lme4 package (Bates *et al*. 2012) was used to create a Poisson-normal generalized linear mixed effects model. This was used to model the number of faeces within each quadrat and investigate the effect of village and date of visit. The log area of the quadrat was included as an offset variable in the model and the quadrat ID was included as a random effect in order to account for overdispersion. Models were created including both village-specific and overall random effects and were compared using a likelihood ratio test. In order to assess for overdispersion in the final model, the ratio of the sum of squared Pearson residuals to the residual degrees of freedom was calculated with a value of greater than one used to suggest overdispersion.

The accuracy of the iGotU units used for monitoring dog movements was tested using both stationary and dynamic recordings. The accuracy of the stationary units was estimated by calculating the distance recorded by the units from the true location (as determined by the Garmin® unit), using the 'Hub Distance' tool in the MMQGIS add-on (http://michaelminn.com/linux/mmqgis) for Quantum GIS 1.8.0 (Quantum GIS Development Team, 2012). For the dynamic data, all points were matched to the nearest time point recorded by the Garmin® unit and the distance between these points was estimated using the 'Hub Lines' tool in the MMQGIS add-on for Quantum GIS.

Analysis of dog movements was conducted in order to characterize both the size of the 'home range' of these animals and the total distances travelled from the household. The R package 'adehabitatHR' (Calenge, 2006) was used for the estimation of home range size. For this, the characteristic hull polygon (CHP) method first developed by Downs and Horner (2009) was used due to the recognized limitations of the usual minimum convex polygon (MCP) and kernel density methods which have been used historically. The total areas of these home ranges were estimated using Quantum GIS 1.8.0 and exported to R for further analysis.

The 'Hub Lines' tool in the MMQGIS add-in for Quantum GIS was used to estimate the minimum distance between each relocation point and the start point for each animal. Violin plots were created using the 'vioplot' package' (Adler, 2005), and confidence intervals for the village-specific median distance travelled (calculated from the median distances travelled per dog) were bootstrapped from the data using the 'boot' package (Canty and Ripley, 2012) with 1000 replications. Researchers conducting household surveys carried Garmin GPS units with tracking mode enabled. These data were analysed using the MCP method in the Geoprocessing Tools of Quantum GIS in order to identify the boundaries of the villages under study. These were then used to estimate the number and proportion of dog relocations which were outside the village boundaries. Differences in home ranges and median distance moved (per dog) between male and female dogs were compared using the Wilcoxon rank sum test.

Table 1. Characteristics of the populations under investigation

	Village			
	Sary Mogol	Taldy Suu	Kashka Suu	Kara Kavak
Number of households registered	368	125	86	65
Total number of people	2173	588	518	398
Total number of dogs reported	178	119	50	46
Percentage of households with at least one dog	38%	74%	51%	52%
Total number of dogs registered	155	115	49	38

RESULTS

Dog owner questionnaire data

A total of 644 households were registered in the four communities, with a combined population of 3677 people (Table 1). Questionnaire data revealed that between 38·0 and 74·4% of households in the four communities had at least one dog, with a total estimated dog population of 393, or 1 dog for every 9·36 people (although this does not include the total dog population in Kashka Suu, where only a sample of dogs was taken).

Reported dog ages ranged from 2 weeks to 15 years, with a median age of two years. Males represented around 77% of the total dog population. Fig. 1 shows the population pyramid for all dogs sampled.

People in all four communities believed there were un-owned 'stray' dogs in their village (SM: 45·9%, TS: 8·8%, KK: 4·6%, KS: 14·0%) although few people who reported a stray dog population had any idea of the size of it. We registered 357 dogs and asked questions about their role and management. Around 75% of dogs for which this question was answered were described as pets, although many of these were also described as guard dogs, as shown in Fig. 2. Dogs were never reported as being kept as a source of meat, and consumption of dog meat is not customary in these mostly Muslim communities.

The proportion of dogs which were never restrained and free to roam at will was higher in Taldy Suu (110/114 = 96%) than in the other villages (232/265 = 88%, Chi square $P = 0{\cdot}01$). Of the remaining dogs, most were always chained (see Fig. 3).

Dogs were all fed by household members and were most often fed table scraps, although offal was also reported to be commonly fed (Fig. 4). Dogs were

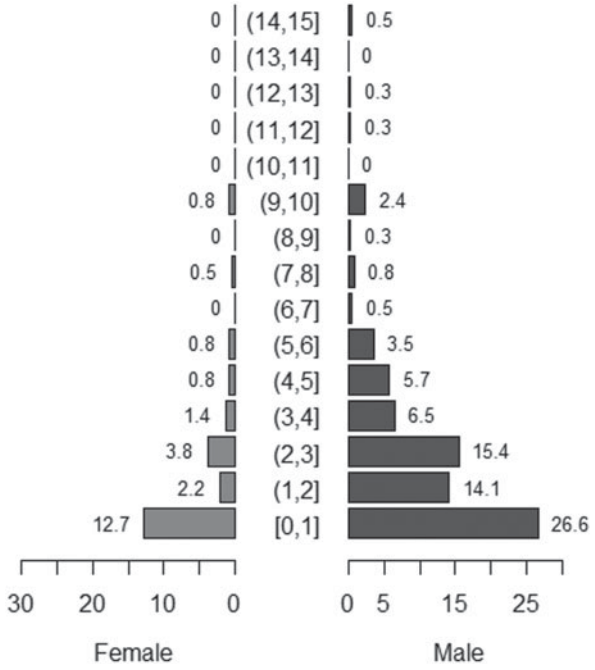

Fig. 1. Population pyramid for all dogs sampled in May 2012 ($n = 383$). Numbers represent total proportion of dogs in each age and sex group.

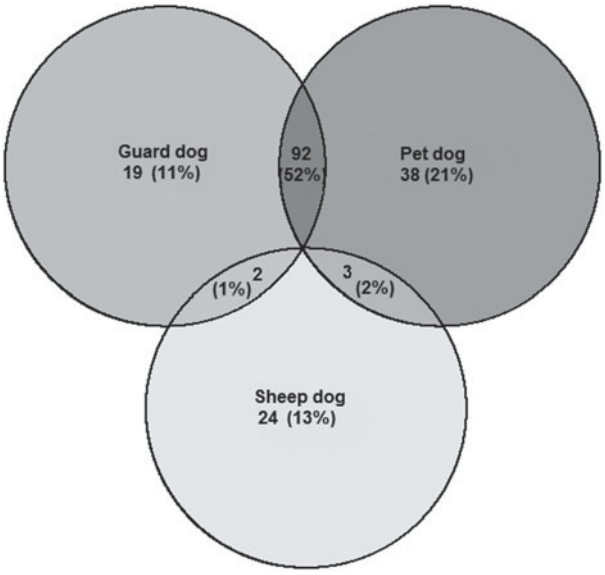

Fig. 2. Euler diagram of the reported uses of dogs registered in the study. Numbers represent total number of dogs in each category.

rarely observed eating rodents, although this was occasionally reported – especially in Kashka Suu and Taldy Suu (Fig. 4).

In May 2012, a total of 222 owned dogs were thought to be present in Sary Mogol; 141 in Taldy Suu; and 41 in Kara Kavak. Based on crude estimates of numbers of households from remote sensing data, it is assumed that around 25% of households in Kashka Suu were visited – suggesting a total owned dog population of around 200 in this village. In

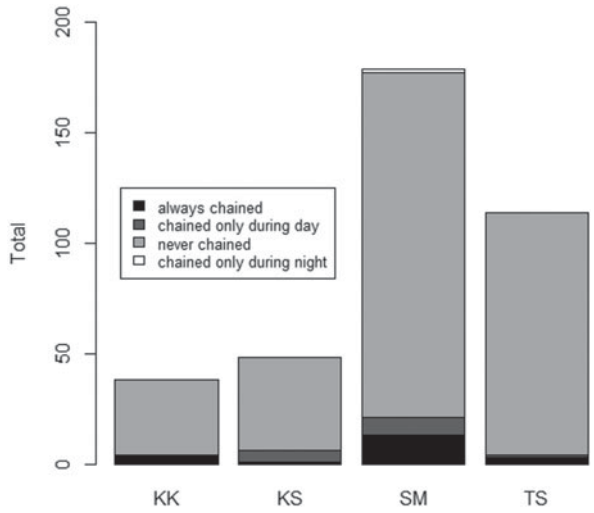

Fig. 3. Frequency of dog restraint in the study villages.

October, the owned dog population was reduced in all three of these villages – to 121 dogs in Sary Mogol; 126 in Taldy Suu; and 36 in Kara Kavak. The majority of this difference resulted from the loss of dogs (usually either as missing dogs, through accidental death or by culling), although some dogs had moved to mountain pastures. Between the two visits, a total of 52 new dogs (usually puppies) were obtained in Sary Mogol, 36 in Taldy Suu and four in Kara Kavak (although it should be noted that in Kara Kavak, only those households which previously had dogs were revisited in October 2012). Using the data from the census villages of Sary Mogol and Taldy Suu, this suggests that per owned dog present in May, the rate of removal over the 5 months between visits was around 0·7 in Sary Mogol and 0·4 in Taldy Suu, whereas the replacement rate was around 0·2 in Sary Mogol and around 0·3 in Taldy Suu. It is also important to note that the estimates for Taldy Suu were made prior to the second visit of the person responsible for dog culling, whereas those for Sary Mogol were made after this visit.

Quadrats for assessing faecal environmental contamination

In 42/68 quadrats it was not possible to measure out 50×50 m due to the presence of buildings or other structures. In these cases. smaller areas (mean = 1660·7 m^2, S.D. = 588·6 m^2) were measured and the size was recorded, with faecal densities calculated as faeces/100 m^2. Canid faecal densities ranged from a median of 0·45 faeces/100 m^2 in Kara Kavak to 1·20 faeces/100 m^2 in Kashka Suu in May; and from a median of 0·22 faeces/100 m in Sary Mogol to 0·60 faeces/100 m in Kashka Suu in October. The Poisson-Normal GLMM found no evidence that random effects were village specific, nor evidence of any interaction between village and date of visit. The final model including quadrat ID as an overall

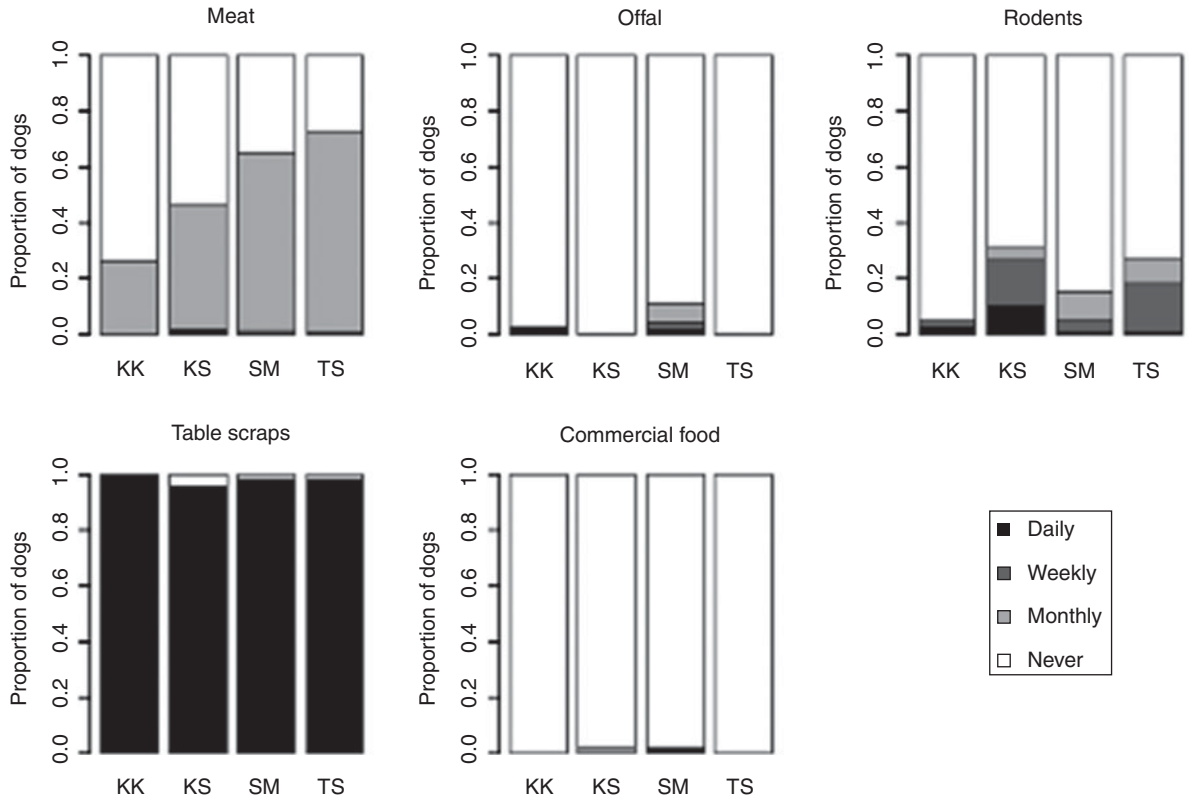

Fig. 4. Proportion of dogs fed different food types and reported frequencies of feeding.

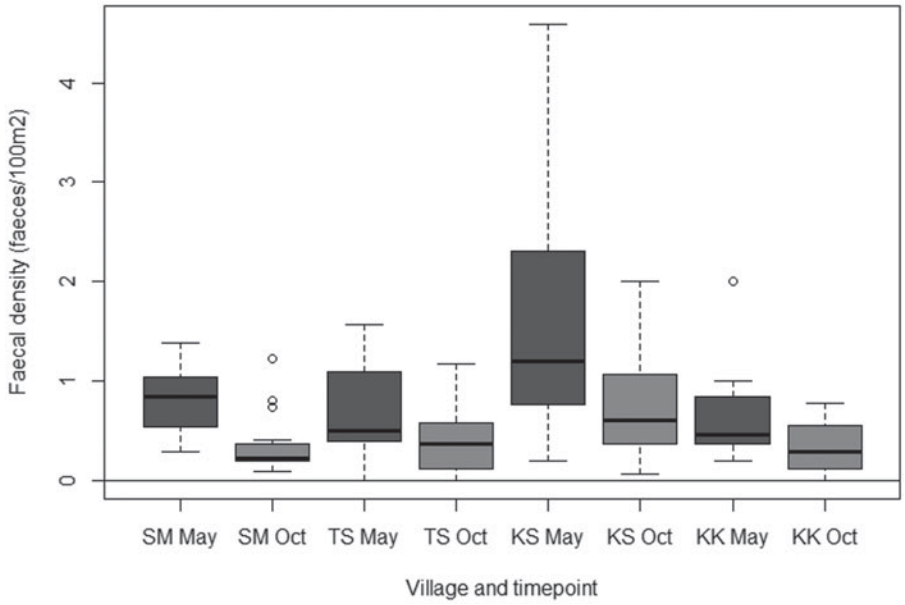

Fig. 5. Canid faecal densities amongst the different villages visited in May and October 2012 (KK = Kara Kavak, KS = Kashka Suu, SM = Sary Mogol, TS = Taldy Suu).

random effect showed no evidence of overdispersion. There was strong evidence of a significant difference between faecal contamination in Kashka Suu and all other villages (Wald $P < 0{\cdot}001$). Compared to Kashka Suu, the density of faeces in Sary Mogol was 0·46 (95% confidence interval 0·37–0·57); in Taldy Suu 0·57 (0·46–0·70); and in Kara Kavak 0·42 (0·34–0·52). Additionally, there was very strong evidence of a reduction in faecal contamination between visits (Wald $P < 0{\cdot}001$), with the density of faeces in October being 0·53 of that in May (95% CI 0·50–0·56). Fig. 5 shows the crude estimates of the faecal densities amongst the different villages over the two visits.

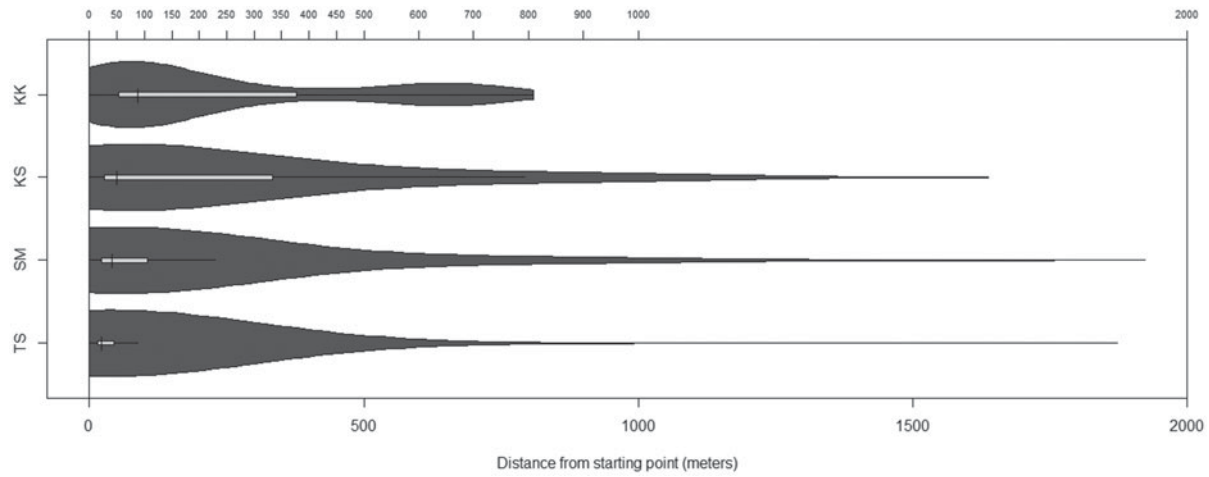

Fig. 6. Distances travelled by dogs from each village. The light bar represents the interquartile range, the horizontal black line represent the distances to the 'inner fence' (1·5 times the interquartile range), and the vertical black lines represent the median. The dark grey areas represent the probability density.

CoproELISA results of faecal quadrat samples

In May 2012, a total of 104 faecal samples were collected from the quadrats in the four villages (KK = 24, KS = 28, SM = 28, TS = 24), of which 7 (6·7%) tested positive for *Echinococcus* spp. ELISA positives ranged from 1/28 in Kashka Suu to 3/24 in Taldy Suu, with 1/24 and 2/28 ELISA positives in Kara Kavak and Sary Mogol respectively. In October 2012 a total of 100 ground faecal samples were collected (KK = 24, KS = 24, SM = 28, TS = 24) of which 18 (18%) tested positive. ELISA positives ranged from 2/24 in Kara Kavak to 8/24 in Taldy Suu, with 3/24 and 5/28 ELISA positives in Kashka Suu and Sary Mogol, respectively.

PCR results of faecal quadrat samples

All 25 ELISA-positive samples that had been collected from the faecal quadrats were analysed for *E. multilocularis, E. granulosus* G1, and *E. granulosus* (sensu lato) using coproPCR. Three samples (1 from KS, 1 from SM, 1 from TS) tested positive for *E. granulosus* (sensu lato), and four samples (1 from KK, 1 from SM and 2 from TS) tested positive for *E. multilocularis*. One of these (a sample collected from a quadrat in Sary Mogol in October) was a mixed infection, testing positive for both *E. multilocularis* and *E. granulosus* (sensu lato). All coproPCR-positive samples were collected in October; all seven ELISA positive samples collected in May were coproPCR negative. The remaining 18 ELISA positive samples that were analysed with coproPCR were PCR negative.

Arecoline purge data

Of the 20 arecoline purges, eight were scored macroscopically as *Echinococcus* spp. positive in the field. Of these eight positive samples, three also harboured *Taenia* spp. In addition, seven faecal samples were scored macroscopically as *Taenia* spp. positive but *Echinococcus* spp. negative. All 20 purges were analysed using coproELISA. Seven of the eight *Echinococcus* spp. purge-positive samples were also coproELISA positive (five from Kara Kavak and two from Taldy Suu). Additionally, one sample (from Kara Kavak) that had not been scored as *Echinococcus* spp. positive in the field (but was scored as *Taenia* spp. positive) was coproELISA positive. The remaining six *Taenia* spp. purge positives were coproELISA negative. The eight coproELISA positive samples were analysed for *Echinococcus* species using coproPCR. Three of these tested positive for *E. granulosus* G1 using the ND1 primers (Boufana *et al*. 2013), and all eight tested positive for *E. granulosus* (sensu lato) using the Abbasi primers (Abbasi *et al*. 2003; Boufana *et al*. 2008). One sample from Taldy Suu also tested PCR positive for *E. multilocularis* (Boufana *et al*. 2013), indicating a mixed infection. The eight coproPCR-positive samples were sequenced using the Abbasi primers (Beckman Coulter Genomics, Essex, UK). BLAST search gave 99% match to *E. granulosus* (accession number DQ157697) with no specification of genotype. One sample, for which there was sufficient DNA present (from Kara Kavak), was further analysed and sequenced using generic cestode primers (von Nickisch-Rosenegk *et al*. 1999). BLAST search gave 99% match to *E. canadensis* (NCBI accession number AB794685).

Dog movement data

Four stationary loggers were evaluated simultaneously over a period of 12 hours in the UK (633 recorded points). The distance recorded from the true location for the UK loggers ranged from

Table 2. Dog movements and home range sizes for the dogs monitored in this study. (Figures in brackets relate to the bootstrapped confidence interval for the median.)

Village	Village area (km^2)	Number of points	Number of dogs monitored	Median home range (m^2)	Median of median distance travelled per dog (m)
SM	3·32	1494	11	22 650	39 (31–84)
TS	1·63	2459	13	15 700	20 (20–29)
KS	1·16	1637	10	37 490	46 (28–308)
KK	0·81	666	3	29 730	62 (58–629)
Overall	–	*6256*	*37*	*22 650*	*35 (28–48)*

0 m to 206 m, with 95% of recorded locations for each logger being less than 45 m. The stationary logger left for 3.5 h in Kyrgyzstan (total 40 points), recorded a difference of 0 to 32 m, with a median of 5 m.

Of the two dynamic recordings made in Kyrgyzstan (a total of 155 points), the median difference from the true location was 16 m, with 95% of readings being within 70 m of the true value. The dynamic recordings made in the UK (35 points) showed a median difference of 31 m with 95% of readings being within 90 m of the true value.

Table 2 shows the median 95% CHP areas and distances travelled from the start location for those dogs monitored in each village. Dogs with less than 50 points recorded were excluded from analysis. A significant difference in distance travelled was found between villages (Kruskal Wallis test $P<0·001$) which was present when each individual village was compared with each other using a pairwise Wilcoxon test with Holm–Bonferroni correction ($P<0·001$ in all cases). The same overall effect was also found when individual dogs were accounted for by comparing the median distance travelled (Kruskal Wallis test $P=0·004$), although with this analysis there was only a significant difference found between Taldy Suu and Kara Kavak or Kashka Suu (Wilcoxon with Holm–Bonferroni correction $P=0·02$ and $P=0·01$, respectively). No difference was found in the size of the home ranges between villages ($P=0·13$). There was also no difference in the size of the home range between males and females, either overall (Wilcoxon rank sum, $P=0·50$) or within those villages with both sexes represented (SM $P=0·53$; KS $P=0·25$). There was also no difference in the median distance travelled (per dog) according to sex (overall Wilcoxon rank sum $P=0·85$; SM only $P=0·41$; KS only $P=0·25$). Village areas based on MCP methods are shown in Table 2. Of all 37 dogs studied, 22 (59%) left the village boundary at least once during monitoring. No difference was found in this proportion between different villages (Chi square test $P=0·73$).

DISCUSSION

Echinococcosis is a national public health concern in Kyrgyzstan (Torgerson *et al*. 2002; Usubalieva *et al*. 2013). However, no studies of canine echinococcosis have been undertaken in the Alay Valley of Osh oblast in the south-west of the country. Furthermore, no information exists in Kyrgyzstan about environmental faecal contamination and behaviour of dogs in relation to transmission of *Echinococcus* spp.

The faecal samples collected and analysed from arecoline-purged dogs confirmed that *Echinococcus* spp. are present in domestic dogs in the Alay Valley. This expands the known distribution of canine echinococcosis in southern Kyrgyzstan (Ziadinov *et al*. 2008). The ELISA-positive arecoline purges (8/20) were all tested using coproPCR and the results show that *E. granulosus* sensu lato (8/8), including *E. granulosus* G1 (3/8), as well as *E. canadensis* (1/8), and *E. multilocularis* (1/8) are present in domestic dogs in the Alay Valley. With regard to environmental contamination, three of the ELISA-positive faecal samples collected from the faecal quadrats (25/204) tested DNA positive for *E. granulosus* (sensu lato), and four samples tested DNA positive for *E. multilocularis*. For 72% of ELISA-positive samples however, the coproPCR analysis (for *E. granulosus* and *E. multilocularis*) yielded a negative result. This is likely due to the fact that many of the ground faecal samples collected from the quadrats were not fresh, and DNA in faeces is known to degrade over time unless preserved properly (e.g. Olson *et al*. 2005). Although these data confirm the presence of *Echinococcus* spp. in dogs in the Alay Valley, the data presented here are neither sufficient to infer canine infection rates with *Echinococcus* spp. nor to assess seasonality of canine echinococcosis. Further surveys are currently being undertaken by us to assess canine echinococcosis in the owned dog populations in the Alay Valley more accurately.

Between 38·0 and 74·4% of households in the four communities surveyed in the Alay Valley owned at least one dog. Male dogs were more commonly kept than females, as is often the case in rural communities (e.g. Butler and Bingham, 2000), and this may be related to males being seen as better guard/sheep dogs, or it may be due to people not wanting to deal with pups. Questionnaire analysis revealed that almost all dogs were free to roam, with very few dogs being leashed. In addition, the dog population in all four communities appeared to have a high turnover. The local municipality in Gulcha (district

administrative capital) arranges for dogs to be culled at least once a year in order to control dog population numbers (Akjol Tagaibekov, local veterinarian, personal communication), and we observed a decline in dog numbers between May and October. Although many people acquired new dogs between visits, few of these did so to replace dogs which were lost or died. As such, based on current observations, the dog population in these Kyrgyz communities appears to be quite dynamic, with changes in dog numbers and dog ownership.

Contamination with dog faeces was high in all four villages, with an overall faecal density of 77·6 and 41·3 faeces/hectare in May and October, respectively; this is higher than the faecal contamination reported in highly endemic *Echinococcus* spp. rural communities in western China (Vaniscotte *et al*. 2011). The overall density of dog faecal contamination in the communities was significantly lower in October than in May. This may be due to the dog culling that took place before and during October, or that faeces degrade faster in the warmer months between May and October than in earlier months. Based on these two sampling times, however, we cannot conclude that environmental contamination is always higher during spring than autumn. Dog culling may take place twice a year and be at different times of the year (Akjol Tagaibekov, local veterinarian, personal communication), and this will clearly affect dog faecal densities. Dog faeces present a risk to humans as *Echinococcus* spp. eggs may survive in the environment for hundreds of days (Veit *et al*. 1995). The majority of dogs in these communities were free roaming, and as a result even gardens belonging to families that did not own dogs or areas surrounding dog-free households were often contaminated with dog faeces (F. van Kesteren, personal observation). Faecal contamination was also notably higher in Kashka Suu than in the other three villages, probably due to a higher dog density in this village.

The stationary and dynamic recordings by the iGotU® units suggest that these GPS loggers can be used to monitor dog movements with reasonable accuracy. Although the battery life of the iGotU® units was limited and several units switched off prior to collection, the iGotU® units have several advantages over conventional GPS animal monitoring units. These benefits include a very small size (20 g), frequent recording capacities and affordability. As in community dogs in western China (Vaniscotte *et al*. 2011), dogs mostly stayed within a few hundred metres of their owners' homes (median 11–931 m), with median home ranges between 15 700–37 490 m^2. Dogs also roamed up to 2 km away from their owners' home and most (59%) left the village boundary. Furthermore, our estimates of dog movements are probably conservative as we could include only dogs that were present and tame enough to be handled (i.e. those dogs that accompany livestock to pasture during the day were not included and aggressive dogs were not included but may have been more active than tamer dogs). There were significant differences in the distances travelled by dogs between different villages, with dogs in Taldy Suu generally travelling shorter distances than those in other villages. Although there was no evidence of any significant difference in the sizes of their core home ranges, this may be a result of the relatively small sample size as the general trend in home range size was similar to that of median distance travelled (Table 2). In addition, although previous studies have found that male dogs generally move further than females (e.g. Vaniscotte *et al*. 2011), no evidence of a sex difference was found here. Although over 88% of people in all villages reported never feeding their dogs offal and only few people (5–39% per village) reported seeing their dogs eat rodents, the fact that dogs roamed freely and moved outside of their communities meant that people could not be sure of what their dogs were eating. Dogs were observed eating offal on several occasions (F. van Kesteren, personal observation) and are likely to consume small rodents in or around villages.

Kyrgyzstan became independent around the time of the collapse of the Soviet Union in 1991 and has since been through considerable political and economical changes (Torgerson *et al*. 2002). During Soviet times, the rearing of sheep (the primary intermediate host for *E. granulosus*) took place on large collectivized farms, slaughter was undertaken in large slaughterhouses under veterinary inspection and treatment of farm dogs with praziquantel every four months was compulsory (Torgerson *et al*. 2002), contributing to relatively low levels of human echinococcosis (Torgerson *et al*. 2002, 2006). In contrast, since independence collective farms have broken up into small farms, home slaughter has increased, and the dog population has grown (Torgerson *et al*. 2006), which has been implicated as the cause of higher rates of human echinococcosis (Torgerson *et al*. 2002, 2006). Our data show that dogs are common in rural communities in the Alay Valley in southern Kyrgyzstan. The majority of dogs roamed freely and may roam several kilometres away from their owners' home, thus being able to scavenge offal and consume rodents, putting them at risk of infection with *E. granulosus*, *E. canadensis* and *E. multilocularis*. The free-roaming dogs also defaecate wherever they are, thus putting people in the community at a potential risk of infection with *Echinococcus* spp.

We have now confirmed the first reports of *E. multilocularis*, *E. granulosus* G1 and *E. canadensis* in dogs in the Alay Valley, Osh oblast, Kyrgyzstan. Attempts to control and even eliminate echinococcosis have been carried out in several different locations, with differing degrees of success (Gemmell *et al*. 1986; Craig and Larrieu, 2006). The World

Bank has recently proposed an *Echinococcus* control programme for Kyrgyzstan, which includes providing anthelminthics for dogs (World Bank, 2011), and this programme was already underway in October 2012 (Akjol Tagaibekov, local veterinarian, personal communication). However, hydatid control programmes will benefit from being informed by an understanding of dog population size, basic dog ecology and dog behaviour. Collecting data such as that presented here can improve the efficacy of intervention programmes. Further studies to gain knowledge on dog population turnover and infection and re-infection rates will be beneficial, especially to determine optimal cost-benefits of dog dosing schedules.

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Independent evaluation of a canine Echinococcosis Control Programme in Hobukesar County, Xinjiang, China

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ABSTRACT

The Xinjiang Uyghur Autonomous Region in northwest China is one of the world's most important foci for cystic echinococcosis. Domestic dogs are the main source for human infection, and previous studies in Xinjiang have found a canine *Echinococcus* spp. coproELISA prevalence of between 36% and 41%. In 2010 the Chinese National Echinococcosis Control Programme was implemented in Xinjiang, and includes regular dosing of domestic dogs with praziquantel. Six communities in Hobukesar County, northwest Xinjiang were assessed in relation to the impact of this control programme through dog necropsies, dog *Echinococcus* spp. coproantigen surveys based on Lot Quality Assurance Sampling (LQAS) and dog owner questionnaires. We found that 42.1% of necropsied dogs were infected with *Echinococcus granulosus*, and coproELISA prevalences were between 15% and 70% in the communities. Although approximately half of all dog owners reported dosing their dogs within the 12 months prior to sampling, coproELISA prevalence remained high. Regular praziquantel dosing of owned dogs in remote and semi-nomadic communities such as those in Hobukesar County is logistically very difficult and additional measures should be considered to reduce canine echinococcosis.

1. Introduction

The Xinjiang Uyghur Autonomous Region (Xinjiang) is an autonomous region of the People's Republic of China, located in the northwest of the country (Bart et al., 2006). Xinjiang is a multi-ethnic province, with ethnic groups including Uyghur, Han Kazakh, Hui and Mongol (Wang et al., 2001). Many people in north-western Xinjiang live in pastoral areas and have traditional (semi) nomadic lifestyles (Wang et al., 2001).

Xinjiang is one of the most important foci of human cystic echinococcosis in China and the world (Bart et al., 2006), and surveys in Hobukesar Mongol Autonomous County (Hobukesar County) in north-west Xinjiang found a human CE prevalence by ultrasound of 2.7% (Wang et al., 2005). Previous dog surveys in Hobukesar County have found necropsy and coproELISA prevalences of 36% (Wang et al., 2005), and a study conducted in Fuhai and Emin counties in north Xinjiang found that 41.2% of dogs were coproELISA positive for *Echinococcus* spp. (Wei et al., 2005). In 2006, the Chinese government implemented the National Echinococcosis Control Programme in Sichuan Province, and in 2010 this programme was expanded to include other provinces in China, including Xinjiang (WHO, 2011).

The Echinococcosis Control Programme aimed to achieve monthly praziquantel dosing of domestic dogs (Chinese Ministry of Health, 2007), as well as identifying human cases through ultrasound screening and subsequent medical treatment of patients (WHO, 2010). Specific methods proposed for reducing canine echinococcosis included registering all owned dogs in endemic areas, and deworming dogs using praziquantel (0.2 g/tablet), with 1–2 tablets administered to dogs weighing more than 15 kg. A de-worming frequency of once a month was aimed for, involving supervised dosing with praziquantel in baits. Workers dosing

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dogs should confirm the tablets were swallowed and the date of de-worming recorded on the dog registration card. The Control Programme also aimed to collect dog faeces 5 days after de-worming and bury or burn these to prevent environmental contamination, as well as taking measures to control dog numbers such as culling stray dogs (Chinese Ministry of Health, 2007). In April 2013 we visited six rural communities in Hobukesar County in north-west Xinjiang to assess the impact of the Control Programme in this County.

2. Materials and methods

2.1. Communities

Six communities in Hobukesar County were included: Narenhebuke (46.47°, 85.30°), Budengjian (46.65°, 85.31°), Changan Kul (46.48°, 85.57°), Chahete (46.06°, 86.30°), Bayenoma (46.51°, 86.09°) and Tiebukenwusan (46.48°, 85.23°). These communities included ethnic Mongolians, Kazakhs, and Han Chinese, and were based around livestock husbandry, although Chahete was established in 2010 as an agricultural community and consisted mostly of ethnic Han people that were relocated from Gansu and Sichuan provinces.

2.2. Dog necropsies

Thirty-eight unwanted or stray dogs were provided by a local dog catcher, including from Bayenoma ($n = 3$), Narenhebuke ($n = 4$), Changan Kul ($n = 16$), and three other County villages called Yikewutubulage ($n = 9$), Mogete ($n = 2$) and Busitinge ($n = 1$), with the locations of three dogs not recorded. All dogs were adults (estimated to be at least 1 year old). Twenty-one were male, and 17 were female. Dogs were captured alive and euthanised by a qualified animal technician (JT) using intravenous ketamine. The small intestine of each dog was removed post-mortem and inspected in the field by experienced researchers (PSC&JT) using a magnifying glass. Dogs were scored as *Echinococcus* spp. and *Taenia* spp. present/absent, with worm burdens estimated for *Echinococcus* spp. and counted for *Taenia* spp. Tapeworms were washed in water and stored in 70% ethanol for DNA analysis. Faecal samples were collected per rectum post-mortem and stored in 0.3% PBS Tween with 10% formalin for coproELISA testing. All samples were transported to Salford University, UK, at room temperature.

2.3. Lot Quality Assurance Sampling (LQAS)

LQAS is a form of stratified sampling which requires a relatively low number of samples whilst retaining a statistical basis (the small sample size required sometimes leads to misunderstanding of the statistical basis of LQAS, as described by Pagano and Valadez (2010). Although originally developed for quality evaluation in the manufacturing industry (Dodge and Romig, 1929), LQAS has more recently been applied to studies on disease and healthcare (for a review see Robertson and Valadez, 2006). The central concept of LQAS is the classification of 'supervision areas' (e.g. villages) in a dichotomous fashion – according to whether a target has been achieved – rather than attempting to present prevalence estimates for each area. For the purposes of the current study, a simplified form of LQAS was used, which requires only one input; the minimum 'threshold' prevalence of the outcome of interest which could be considered a 'success' or 'failure'. The binomial distribution can then be used to estimate the cumulative probability distribution of the expected number of positive outcomes for a small sample size (often set at 19), given that the prevalence is at this stated threshold. From this, the minimum number of expected positive outcomes which gives a cumulative probability of greater than 0.1 can be estimated – known as the 'decision rule'. If the number of positive individuals in a sample is lower than the decision rule, it can be stated that there is some statistical evidence that the threshold has not been reached.

As echinococcosis is commonly a disease of remote, marginalised communities (Craig et al., 2007), surveillance is often hindered by logistical difficulties, and relatively quick and efficient methods are desirable. As such, we used LQAS to evaluate coproELISA prevalence, praziquantel dosing, and local knowledge about echinococcosis in the six communities studied.

2.4. Faecal sample collection

A minimum of 19 dogs were sampled in each community (a sample size of 19 minimises the risk of type A and B errors, Valadez et al., 2002), with additional dogs sampled where possible (Bayenoma = 19, Budengjian = 20, Changan Kul = 27, Chahete = 20, Narenhebuke = 21, Tiebukenwusan = 19). Dogs were selected by starting from each community's health centre and walking in a randomly chosen direction (determined by the second hand on a watch) and enquiring about dogs in alternate houses. If dogs were present, these were included in sampling, with ground faecal samples collected from around their owners' houses. If midway through the sampling day it appeared that a minimum of 19 dogs would not be reached by the end of the day, we asked local villagers who served as translators/facilitators to direct us to areas where they knew dogs were present, thus moving away from our chosen random direction. In these areas alternate houses were targeted. The age and sex of each dog was recorded, and dog owners were asked when the dog was most recently dosed with praziquantel. Nine dogs were sampled without their owners present; these dogs were chained and faecal samples were collected from the ground. The sex of these dogs was recorded but no questionnaires were administered. In four communities (Bayenoma, Budengjian, Changan Kul Tiebukenwusan) owners were asked to describe echinococcosis to assess their knowledge about this disease. Questionnaires were administered in Mandarin Chinese, Mongolian or Kazakh depending on the dog owner's native language. Subsamples of faecal samples were stored in 70% ethanol and 0.3% PBS Tween with 10% formalin respectively, and shipped to Salford University at room temperature.

2.5. CoproELISA

Faecal samples were extracted by homogenizing, shaking and centrifuging at 2500 r.p.m. (1125 g) for 5 min and collecting the supernatant. Faecal samples were analysed for *Echinococcus* spp. coproantigen with a genus-specific sandwich ELISA using the protocol originally described by Allan et al. (1992) with a modification in that the capture and conjugate antibodies were derived from different rabbit antisera. The conjugate antibody was prepared from hyperimmune rabbit IgG raised against a surface extract from adult *Echinococcus granulosus* worms (Elayoubi and Craig, 2004), and the capture antibody was anti-*E. granulosus* whole worm somatic (Allan et al., 1992). Faecal supernatants of two known positives (an arecoline *Echinococcus* spp. purge positive sample from Kyrgyzstan, and a sample spiked with *E. granulosus* whole worm extract at a 1:100 concentration) were used as positive controls throughout. Two known negative faecal samples from a low endemic area (Falkland Islands) were included as negative controls.

Because Gaussian approaches for calculating ELISA cut-off values (e.g. Allan et al., 1992), are usually based only on a panel of known negatives (often from a non-endemic area) and do not consider the true distribution of both negatives and positives from the population being studied (Gardner and Greiner, 2006), we calculated our cut-off using Receiver Operating Characteristic (ROC)

curves (Gardner and Greiner, 2006). All faecal samples collected from necropsied dogs were analysed by coproELISA, and this was treated as a panel of known positives ($n = 16$, with estimated *Echinococcus* spp. worm burdens between 2 and >10,000) and *Echinococcus* spp. negatives, $n = 22$). Using this panel, a coproELISA cut-off of 0.11685 was determined, giving a sensitivity of 94%, a specificity of 77% and an overall accuracy of 84%.

2.6. DNA extraction, PCR and sequencing

DNA was extracted from *Taenia* spp. and *Echinococcus* spp. worms using a Qiagen® DNEasy Blood & Tissue kit following the manufacturer's instructions. DNA was extracted from faecal samples using a QIAamp® DNA Stool kit, following the manufacturer's instructions, but using 1 g of faeces. Extracted tissue samples were analysed by PCR using generic cestode primers (von Nickisch-Rosenegk et al., 1999). For the faecal samples it was found that these primers were not suitable, as they cross reacted with non-target DNA (personal observation). Therefore faecal samples were analysed for *Echinococcus multilocularis* (Boufana et al., 2013), and *E. granulosus* (Abbasi et al., 2003; with modifications described by Boufana et al., 2008) using published primers and following described protocols. Positive controls (sequenced DNA from adult *E. multilocularis*/*E. granulosus*/*Taenia. hydatigena*) were used as appropriate for each protocol. Negative controls (PCR grade water) were included in all PCRs. A Stratagene Robocycler (La Jolla, CA) was used for all cycling profiles and PCR products were separated by electrophoresis at 110 V on 1.5% (w/v) agarose gels in Tris–Borate–EDTA buffer (Severn Biotech, UK), stained with GelRed (Cambridge Biosciences, UK). Gels were visualised using Syngene G:Box gel imaging system (Cambridge Biosciences). Tissue samples that were successfully extracted and amplified were sequenced by Beckman Coulter (Essex, UK) and resulting sequences analysed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.7. Data analysis

The population pyramid for the dog population in Hobukesar and the bar chart of praziquantel dosing were made using 'package sciplot' (Morales, 2013) and 'package pyramid' (Nakazawa, 2013) in R statistical software, version 2.15.0 (R Development Core Team).

To use LQAS methodology thresholds and corresponding decision rule values must be selected (Valadez et al., 2002). Setting a threshold can be done in several ways, for example a target can be selected (e.g. target for proportion of people vaccinated, etc.), and decision numbers chosen to test whether or not this target has been met. In this case we wanted to assess whether or not the control scheme had led to a reduction in coproELISA prevalence from pre-intervention prevalences. The pre-intervention prevalence was estimated from dog surveys conducted in Hobukesar County prior to the start of the control programme (Wang et al., 2001, 2005). For these surveys, 139 dogs were sampled in Narenhebuke using rectal loops, with the samples tested at Salford University using a similar sandwich coproELISA, for which 50 dogs (36%) were found to be coproELISA positive (Wang et al., 2001, 2005). As the simplified LQAS methodology using tables allowed for setting thresholds to the nearest 5% (Valadez et al., 2002), we conservatively set the upper threshold for coproELISA positive dogs at 35%, to identify communities where the coproantigen prevalence had decreased from this 'baseline' value, as would be expected 3 years after the implementation of a dog dosing control programme (WHO, 2011). Decision rule values based on this threshold were estimated (using tables provided in Valadez et al., 2002) as follows: four for Bayenoma ($n = 19$), Budengjian ($n = 20$), Chahete ($n = 20$), Narenhebuke ($n = 21$) and Tiebukenwusan ($n = 19$), and five for Changan Kul ($n = 27$).

To assess whether or not the Echinococcosis Control Programme was reaching households in the local communities, we determined the proportion of dog owners who had dosed their dogs at least once in the 12 months prior to our data collection. We set the threshold at 90%, assuming conservatively that a successful dosing campaign should reach almost all owned dogs at least once a year. Nine dogs sampled without their owners present were excluded from this analysis. The decision rule values were set at 12 for Chahete ($n = 15$), 14 for Bayenoma ($n = 17$), 15 for Narenhebuke and Tiebukenwusan ($n = 19$ each), 16 for Budengjian ($n = 20$), and 21 for Changan Kul ($n = 27$).

In four communities, householders were asked to describe echinococcosis in order to assess people's knowledge of the disease. Studies relating to echinococcosis have been carried out in Hobukesar County previously (Wang et al., 2005) and the National Echinococcosis Control Programme has been carried out in Xinjiang since 2010 (WHO, 2011). We therefore set the knowledge threshold at 65%, i.e. we expected at least 65% of people to be able to describe echinococcosis. As such the decision rule value was set at 7 for Bayenoma ($n = 13$), 8 for Tiebukenwusan ($n = 15$) and 10 for Budengjiang and Changal Kul (both $n = 19$).

3. Results

3.1. Necropsy panel

Of the 38 dogs necropsied, 20 (52.6%) had *Taenia* spp. and 16 (42.1%) had *Echinococcus* spp. on visual inspection, and 13 dogs (34.2%) were infected with both parasites (Table 1). Only 14 dogs (36.8%) had neither parasite.

A total of 18 *Taenia* spp. tapeworms were collected, but one sample was lost in transport. From the remaining samples, DNA was successfully extracted, analysed by PCR and sequenced, and all 17 were identified as *T. hydatigena* (≥99% match, accession number GQ228819.1). 16 samples of *Echinococcus* spp. were collected, but one sample was lost in transport. For the remaining samples DNA was successfully extracted and amplified, and all 15 were successfully sequenced as *E. granulosus* G1 (≥99% match, accession number DQ408422.1).

3.2. Dog demographics and praziquantel dosing

A total of 126 owned dogs were sampled in the six communities, with questionnaires administered to 117 owners. The majority of dogs were male (78.6%), and most (72.2%) were 4 years old or younger (Fig. 1).

Of the 117 owners questioned, 43 (36.8%) reported never dosing their dogs with praziquantel, and 16 (13.7%) owners did not know when the dog had last been dosed, if ever. Twenty-six dogs (22.2%) were reportedly dosed within the 6 weeks prior to sampling, with others dosed at various times between 6 weeks and 2 years prior to sampling ($n = 32$, 27.4%, Fig. 2; for dosing details per village see Table 2).

In Bayenoma, 13 people were asked to describe echinococcosis and 5 (38.5%) could accurately do so. In Budengjiang 14 of 19 people asked (73.7%) could accurately describe the disease. In Changal Kul and Tiebukenwusan 19 and 15 people were asked about

Table 1
Necropsy results ($n = 38$ dogs).

	Taenia spp.	*Echinococcus* spp.	*Taenia* spp. and *Echinococcus* spp.
Positive	18 (47.4%)	16 (42.1%)	13 (34.2%)
Negative	20 (52.6%)	22 (57.9%)	25 (65.8%)
Total	38 (100%)	38 (100%)	38 (100%)

Table 2
Most recent reported dog dosing with praziquantel in each of the six communities sampled.

Community	No. of dogs sampled	No. of questionnaires administered	No. of dogs reportedly never dosed	No. of dogs with unknown latest dosing	No. of dogs dosed in 6 weeks prior to sampling	No. of dogs dosed >6 weeks to <2 years prior to sampling
Bayenoma	19	18	9 (50.0%)	2 (11.1%)	2 (11.1%)	5 (27.8%)
Budengjian	20	20	1 (5.0%)	5 (25.0%)	6 (30.0%)	8 (40.0%)
Changan Kul	27	27	4 (14.8%)	0 (0%)	12 (44.4%)	11 (40.7%)
Chahete	20	15	14 (93.3%)	1 (6.7%)	0 (0%)	0 (0%)
Narenhebuke	21	19	4 (21.1%)	6 (31.6%)	2 (10.5%)	7 (36.8%)
Tiebukenwusan	19	18	11 (61.1%)	2 (11.1%)	4 (22.2%)	1 (5.6%)
Total	126	117	43 (36.8%)	16 (13.7%)	26 (22.2%)	32 (27.4%)

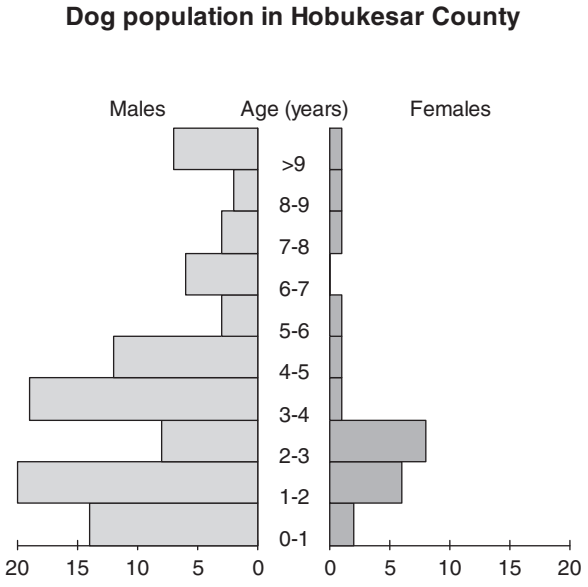

Fig. 1. Dog demographics in the six communities sampled in Hobukesar County (n = 117 dogs).

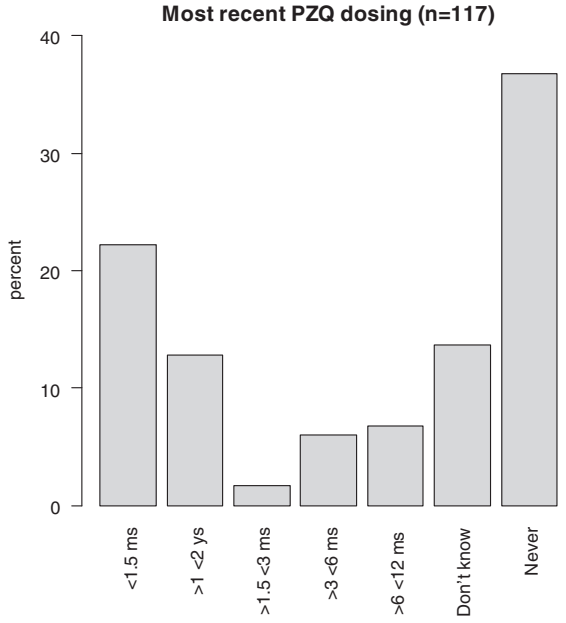

Fig. 2. Most recent praziquantel dosing of the dogs sampled in the six communities in Hobukesar County (ms = months, ys = years).

echinococcosis, respectively, with 18 (94.7%) and 4 (26.7%) respondents being able to accurately describe the disease.

3.3. Canine echinococcosis in six communities in Hobukesar County

All 126 dog faecal samples were analysed by coproELISA. CoproELISA prevalences ranged from 15.0% in Chahete to 70.0% in Budengjian, with an overall coproELISA prevalence of 41.3% (Table 3).

All coproELISA positive ground faecal samples (n = 52) were analysed by coproPCR. In total 26 samples (50%) tested positive for *E. granulosus* DNA. All samples were negative for *E. multilocularis* DNA. Twenty-six samples (50%) were coproELISA positive but coproPCR negative. As these samples were collected from the ground in a relatively dry and warm environment, any DNA in the samples may have been degraded (e.g. Olson et al., 2005), and the presence of PCR inhibitory substances may lead to false negatives (e.g. Mathis and Deplazes, 2006).

3.4. Using LQAS to evaluate canine coproELISA prevalence, PZQ dosing and knowledge of echinococcosis in Hobukesar County

The LQAS decision rule for coproELISA positives was met in five of the six communities, with only Chahete having fewer than four coproELISA positive dogs. This provides evidence that the true coproELISA prevalence in Chahete was lower than the 35% threshold. There is no evidence that the true coproantigen prevalence in the other five communities (Bayenoma, Budengjian, Changan Kul Narenhebuke and Tiebukenwusan) was below the 35% threshold.

The decision rule for reported praziquantel dosing scheme coverage over the previous year was only met in Changan Kul where 23 dogs were reportedly dosed in the last year. Therefore, this provides evidence that the praziquantel coverage was lower than 90% in Bayenoma, Budengjian, Chahete, Narenhebuke and Tiebukenwusan.

The decision rule for knowledge of echinococcosis was only reached in Budengjiang and Changal Kul providing some evidence that the level of echinococcosis knowledge was lower than 65% in Bayenoma and Tiebukenwusan.

4. Discussion

Cystic echinococcosis is a neglected zoonotic disease that is very difficult to control or eliminate (WHO/OIE, 2001) and to date, only Iceland, New Zealand and Tasmania have declared elimination status for *Echinococcus* spp. (Craig and Larrieu, 2006). Control programmes may include education campaigns, praziquantel dosing of dogs, controlled slaughter (Gemmell et al., 1986), and vaccination of sheep, the intermediate host for *E. granulosus* (Barnes et al., 2012). Echinococcosis Control Programmes are more likely to succeed on islands, where border control is possible and the area targeted is finite and clearly defined (Craig and Larrieu, 2006).

Table 3
CoproELISA positives in each of the six communities sampled. Baye, Bayenoma; Bude, Budengjian; Chan, Changan Kul; Chah, Chahete; Nare, Narenhebuke; Tieb, Tiebukenwusan.

Village	BAYE	BUDE	CHAN	CHAH	NARE	TIEB	Total
Positive	6(31.6%)	14(70.0%)	13(48.2%)	3(15.0%)	8(38.1%)	8(42.1%)	52(41.3%)
Negative	13(68.4%)	6(30.0%)	14(51.8%)	17(85.0%)	13(61.9%)	11(57.9%)	74(58.7%)
Total	19(100%)	20(100%)	27(100%)	20(100%)	21(100%)	19(100%)	126(100%)

However, continental areas present greater challenges for control of echinococcosis, especially regions that are relatively remote and where people are nomadic or semi-nomadic (e.g. Schantz et al., 2003). In these cases frequent praziquantel dosing of domestic dogs (recommended dosing every 6 weeks) may not be practically feasible (Gemmell et al., 1986; Lembo et al., 2013).

In 2006 the Chinese government implemented a National Echinococcosis Control Programme in western China, starting in Sichuan and expanding to other areas including Xinjiang in 2010 (WHO, 2011). It is important to evaluate Echinococcosis Control Programmes and assess how well these are meeting their targets (Craig and Larrieu, 2006; Craig et al., in press). Such assessments are likely to suffer from some of the same challenges as the control programme itself, such as remoteness of communities, logistical challenges and limited time and budgets. Practical assessment tools are therefore highly desirable. We undertook a dog focused assessment of the application and impact of the National Control Programme in Hobukesar County, including dog necropsies, and an LQAS approach to coproELISA tests, and dog owner questionnaires. Whilst the LQAS methodology provides a relatively quick and low-cost assessment tool, it is important to remember that it is not appropriate for estimating prevalences at the village level (i.e. any estimates would be expected to have wide confidence intervals, with the exception of villages where the total number of dogs was comparable to the number of dogs sampled).

We found that of 38 necropsied dogs, 20 (52.6%) had *T. hydatigena*, 16 (42.1%) had *E. granulosus*, and 13 (34.2%) dogs had both parasites. Only 14 dogs (36.8%) had neither parasite. Presence of either *Echinococcus* or *Taenia* tapeworms suggests that the dog had not been dosed recently, and had access to livestock offal (Gemmell et al., 1977). The dogs were provided by a local dog catcher, who recorded the location the dogs were sourced, but the exact origin and circumstances of the dogs was not known. Therefore it is important to bear in mind that these dogs are not necessarily representative of the owned dog population, as they were all either stray or unwanted. As praziquantel dosing schemes such as the current one will generally only include owned dogs, stray dogs will not benefit from dosing, and dosing compliance may be lower for unwanted dogs. Furthermore, stray/unwanted dogs may receive less or no food from people, and may be less likely to be restrained and therefore be more likely to scavenge. Stray or unwanted dogs may therefore have higher prevalences of *Echinococcus* and/or *Taenia* spp. infections. Nevertheless, the current findings suggest that active transmission of *E. granulosus* occurs in our study communities, with a high prevalence of canine echinococcosis and taeniasis in the study area.

We used LQAS methodology to investigate three factors related to the success of the control programme: coproELISA prevalence, reported praziquantel dosing, and knowledge of echinococcosis. It is important to note that the coproELISA prevalence is likely to differ from the true prevalence due to limitations in the test sensitivity and specificity. However, as we were only attempting to assess whether the coproELISA prevalence differed from a previous coproELISA estimate, no attempt was made to adjust for this. One challenge associated with LQAS is selecting the thresholds used. In this case, we used data collected from a dog survey in Narenhebuke prior to the start of the control programme (Wang et al., 2001, 2005). This was the only pre-intervention data available from this study area, and we therefore made the assumption that the dogs surveyed in Narenhebuke prior to the control scheme were representative of the dogs in other communities in Hobukesar County. Other surveys in nearby Fuhai and Emin Counties in Xinjiang found that 54/131 dogs surveyed (41.2%) were coproELISA positive (Wei et al., 2005). However, sensitivity analysis found that changing the threshold from 35% to 40% did not affect our results or conclusions (i.e. the same communities would meet or fail to meet the decision number). It should also be considered that the aim of the current study is not necessarily to identify villages which *individually* have experienced a particular reduction in coproantigen prevalence from their own pre-control status, but to identify those villages which currently have a lower coproantigen prevalence than the county-wide 'average' pre-control coproantigen prevalence (as individual villages may have had different individual pre-control prevalences). The current approach rather identifies all villages which may be in need of further attention, regardless of the reasons for this. Of the six communities studied, only one (Chahete) showed evidence of having a coproELISA positive prevalence below 35%. Although LQAS identified this village as being different from the other five in meeting the decision number, in this particular case we cannot speak of a reduction in coproELISA prevalence as this community was newly established and would not have existed at the time that Wang et al. ([Wang et al., 2001]2001, [Wang et al., 2005]2005) conducted their surveys. Furthermore, none of the dog owners interviewed in this community reported having dosed their dogs in the previous 2 years (Table 2). This suggests that the relatively low prevalence recorded in this community was unlikely to be due to successful intervention. Chahete was unique in being newly established and based largely on agriculture rather than livestock, which may explain the lower coproELISA prevalence (livestock ownership has been identified as a significant risk factor for human echinococcosis, e.g. Craig and Larrieu, 2006).

In Budengjian and Changan Kul there was no evidence that knowledge of echinococcosis was lower than 65%, and in Changan Kul there was no evidence that the praziquantel dosing rates over the previous year was lower than 90%. However, in both of these villages there was no evidence of a reduction in coproantigen prevalence from the previous estimate (35%). This may be due to infrequent dosing; it is generally suggested that, in order to impact on coproELISA prevalence, praziquantel dosing must be conducted at least four times per year (Lembo et al., 2013).

We found that even modest praziquantel dosing targets (at least 90% of dogs dosed in the previous 12 months) were not met in five communities (Bayenoma, Budengjian, Chahete, Narenhebuke and Tiebukenwusan), and in only one community (Chahete) was there evidence of a reduction in *Echinococcus* spp. coproELISA prevalence to less than the previously recorded 35%. This suggests that the echinococcosis control campaign has had little or no positive impact in these communities.

Although the aims of the Echinococcosis Control Programme, including monthly supervised dosing (Chinese Ministry of Health, 2007), were recommended, it appears that they were over-ambitious in locations such as Hobukesar County, given the associated challenges of the semi-nomadic lifestyles of local people and logistical challenges associated with remote communities. From our data, it appears that sufficiently frequent praziquantel dosing is not being achieved in the communities evaluated.

Praziquantel dosing, although highly effective against canine echinococcosis, is often impractical because of the frequent dosing and high proportion of dog coverage required. Although praziquantel rids the dosed dog of worms, it provides no protection against reinfection. Indeed in our samples we found that of the 26 dogs whose owners reported having dosed them no more than 6 weeks prior to sampling, 15 (57.7%) were coproELISA positive. Furthermore, there are other challenges associated with praziquantel dosing, including the fact that dogs dislike the taste and smell of tablets, so that ensuring that the whole dose has been consumed is difficult, as well as difficulties with dosing, as dog weights are usually estimated in the field, and dogs may be under-treated (Larrieu and Zanini, 2012). Therefore, other measures to reduce echinococcosis should be considered.

Dog dosing frequencies of every 6 weeks (eight times a year) are often suggested during a control programme (e.g. Gemmell et al., 1986; Lembo et al., 2013), with the aim of preventing *Echinococcus* spp. from reaching patency even in the case of immediate reinfection (Thompson and McManus 2001), and therefore preventing the release of any eggs from dogs. If this is carried on for a sufficient time period to allow for previously infected intermediate hosts such as sheep to be removed from the population, the transmission cycle of *Echinococcus* can be suspended (see also Larrieu and Zanini, 2012; Torgerson, 2003). However, these dosing frequencies are often not achieved in *Echinococcus* endemic areas (Craig and Larrieu, 2006; Larrieu and Zanini, 2012). As such, it may be better to set more realistic goals; even if it is not feasible to eliminate echinococcosis from a certain area, reductions in transmissions to humans can be achieved with more modest dosing frequencies. For example, mathematical models have suggested that dosing frequencies can be reduced to once every 3 months and still reduce prevalence rates in dogs and livestock to less than 1% within 10–15 years (Torgerson, 2003). It may also be advisable to ensure that supervised dosing of dogs is conducted by trained operatives, rather than relying on dog owners to administer the tablets, as this has been a feature of most successful control campaigns to date, and can help ensure compliance (Craig and Larrieu, 2006).

Previous studies have found that education campaigns could present a practical way of reducing echinococcosis (e.g. Huang et al., 2011). Inclusion of health education has the potential to reduce echinococcosis through increased compliance with dog dosing, a reduction in offal being fed to dogs and/or through improved hygiene, although education alone is unlikely to achieve the desired dosing frequency and decrease in coproELISA prevalence (Craig and Larrieu, 2006; Lembo et al., 2013). Another possible avenue of echinococcosis control is the vaccination of the intermediate host. A safe and effective vaccine against echinococcosis is available for sheep (Heath et al., 2003). Mathematical models suggest that a combination of dog dosing and sheep vaccination is the most effective strategy for echinococcosis control (Torgerson, 2003; Torgerson and Heath, 2003) and vaccination has been successfully trialled in endemic areas (Larrieu et al., 2013). However, there are challenges associated with the vaccine, including the fact that lambs need two doses of the vaccine, and a booster vaccine when they are 1–1.5 years of age (Heath et al., 2003; Larrieu et al., 2013), and the fact that sheep populations are usually much larger than dog populations (Larrieu et al., 2013). This can increase the challenges associated with logistics, although vaccination could be incorporated into other veterinary measures targeting sheep (Heath et al., 2003).

5. Conclusions

Our results suggest that the Echinococcosis Control Programme in Hobukesar County in north-west China is still facing several challenges. Although half (50.4%) of all people asked reported dosing their dogs in the last 12 months, the coproELISA prevalence amongst owned dogs remained high in most communities, suggesting little or no reduction has been achieved by the control programme. It is likely that even quarterly praziquantel dosing in these communities is very difficult; they are small rural and remote communities, and many people have semi-nomadic lifestyles that make regular dosing by authorities difficult. The logistical challenges associated with frequent praziquantel dosing and the high coproELISA prevalences found here suggest that additional methods, such as health education and livestock vaccination should be considered to improve compliance levels and the effectiveness of the Echinococcosis Control Programme in Hobukesar County and similar areas. Although many authors agree that elimination of *Echinococcus* spp. from continental areas is often infeasible, attempts to reduce *Echinococcus* spp. transmission should be undertaken in endemic areas where echinococcosis is a public health concern. Instead of aiming to dose dogs every month, which is likely to be overambitious in remote areas, government workers could aim to dose dogs two to four times a year. Public health education could also help reduce transmission to humans, and avenues to integrate sheep vaccination into existing veterinary practices could be explored.

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Ethical approval

Ethical approval for this study was provided by the Ethical Review Committee at the First Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang, China.

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Appendix: Manuscript in prep. for submission to the Journal of Helminthology

Investigation of risk factors for *Echinococcus* coproantigen positivity in dogs in the Alay valley, Kyrgyzstan

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Abstract

Echinococcosis caused by the zoonotic cestodes *Echinococcus granulosus* (sensu lato) and *Echinococcus multilocularis* is highly endemic in the Central Asian Republic of Kyrgyzstan, and is increasingly being identified as public health problem especially amongst pastoral communities. As domestic dogs are considered to be the main source of human infection in these communities, the identification of potential transmission pathways can be of use when considering implementing a control scheme for echinococcosis. The current report describes the results of an analytic study of canine echinococcosis (based on the results of coproantigen ELISA testing) in the Alay valley of southern Kyrgyzstan prior to the commencement of a praziquantel dosing scheme amongst dogs. A logistic regression model using a form of Bayes modal estimation was used to identify possible risk factors for coproantigen positivity, and the output was interpreted in a Bayesian context (posterior distributions of the coefficients of interest). The study found that sheepdogs had lower odds of coproantigen positivity, as did households with donkeys, some knowledge of echinococcosis, and which did not engage in home slaughtering. There was no evidence of an association between free roaming or previous praziquantel dosing and coproantigen positivity, as has been found in previous studies. Possible reasons for these findings are discussed and suggestions made for further work.

Introduction

Human echinococcosis, caused by infection with the metacestode stage of cestodes of the genus *Echinococcus*, is an important public health concern in various parts of the world. Due to the parasite's complex lifecycle and long period between infection and clinical signs in human hosts, accurate investigation of risk factors for human infection can be challenging. However, in cases where domestic dogs act as a definitive host (most areas of *E. granulosus* endemicity, and some areas of *E.multilocularis* endemicity (Craig *et al.*, 1992)), identification of risk factors for canine infection can provide useful information on potential human risk. Knowledge of risk factors for canine infection can also be invaluable for designing and monitoring *Echinococcus* control schemes based on treatment of infection in dogs. Although infection in definitive hosts is generally asymptomatic, a number of diagnostic tools are available for diagnosis of infection which can give a good indication of current infection status. Detection of coproantigens, which are excretory/secretory products released by adult worms, in faeces is of particular use, and has been advised as the mainstay of surveillance of echinococcosis in endemic areas by the WHO, OIE and PAHO (Eckert *et al.*, 2001; Morel *et al.*, 2013).

As the transmission cycle of *Echinococcus* spp. would be expected to vary between different locations, it is useful to conduct studies in the particular area of interest in order to identify risk factors specific to that particular transmission system. However, it is also useful to evaluate commonly identified risk factors from the wide range of studies that have been conducted worldwide (see review in (Otero-Abad & Torgerson, 2013)). Due to difficulties in obtaining good quality parasitological data (such as purge or necropsy samples), coproantigen test results are often used to approximate canine infection status. Interpretation of coproantigen data is generally in a dichotomous fashion – classifying samples as coproantigen 'negative' or 'positive', according to their OD value in relation to a defined cut-off value. This will naturally lead to some misclassification, which has been addressed in some studies by combining the results of purgation and coproPCR

(Ziadinov *et al.*, 2008). Due to the variation in transmission systems and in classification of positive individuals, a variety of different risk factors have been identified, making it difficult to state any overarching conclusions on risk factors of importance.

One useful approach is to categorise potential risk factors according to a number of general transmission processes: risk factors associated with access to infected material (whether this be infected offal, in the case of *E.granulosus*, or rodent intermediate hosts in the case of *E.multilocularis*); factors associated with variability in infection given ingestion of infectious material, and factors associated with removal of infection (such as history of anthelmintic treatment). As expected, the most commonly identified risk factors are those relating to access to infected material, and may be further subdivided into those risk factors associated with purposeful feeding of potentially infectious material (generally in relation to *E. granulosus*) (Moro *et al.*, 1999; Buishi *et al.*, 2006; Antolová *et al.*, 2009); use of dogs for hunting (in the case of *E. multilocularis*) (Ziadinov *et al.*, 2008; Antolová *et al.*, 2009); lack of canine restraint (for both *E. granulosus* and *E. multilocularis*) (Parada *et al.*, 1995; Budke *et al.*, 2005; Buishi, Walters, *et al.*, 2005; Buishi *et al.*, 2006; Guzel *et al.*, 2008; Huang *et al.*, 2008; Ziadinov *et al.*, 2008; Antolová *et al.*, 2009; Mastin *et al.*, 2011), or close proximity to potentially infectious material (Parada *et al.*, 1995; Wang *et al.*, 2001; Shaikenov *et al.*, 2003; Buishi, Njoroge, *et al.*, 2005; Acosta-Jamett *et al.*, 2010). Spatial factors are also relatively commonly identified as associated with the probability of infection (especially with *E. multilocularis*) (Bchir *et al.*, 1987; Wang *et al.*, 2007, 2010; Dyachenko *et al.*, 2008; Mastin *et al.*, 2011; Reyes *et al.*, 2012), which likely also represent proximity to infectious material.

Risk factors may also be identified at the dog level: in particular, dog type and dog age have both been found to be associated with canine infection. Working dogs such as sheepdogs and farm dogs (Moro *et al.*, 1999; Shaikenov *et al.*, 2003; Buishi, Njoroge, *et al.*, 2005) have been repeatedly found to have higher odds of infection with *E. granulosus*, which likely relates to a combination of the risk factors described above (i.e. increased availability of potentially infectious material and increased

access to this). Younger dogs have also been repeatedly found to have higher odds of infection than older dogs (Sharifi & Zia-Ali, 1996; Buishi, Njoroge, *et al.*, 2005; Buishi *et al.*, 2006; Acosta-Jamett *et al.*, 2010; Inangolet *et al.*, 2010). Whilst this could result from behavioural differences between younger and older dogs which impact upon access to infected material, modelling approaches have suggested that this could represent acquired immunity in the face of high infection pressure (possibly more associated with *E.granulosus* than *E.multilocularis*) (Torgerson, Shaikenov, *et al.*, 2003; Torgerson, 2006)), and as such could be classified as a factor describing variability in the probability of infection given ingestion of infectious material. Finally, a lack of recent anthelmintic dosing has been commonly identified as a risk factor for canid infection (Parada *et al.*, 1995; Buishi, Njoroge, *et al.*, 2005; Huang *et al.*, 2008; Acosta-Jamett *et al.*, 2010).

It should be noted here that studies of risk factors for infection of domestic dogs (rather than foxes) with *E. multilocularis* are relatively uncommon, as domestic dog infection with *E. multilocularis* is most commonly identified in particular pastoral communities (often in association with *E. granulosus*), such as Tibetan communities in China or Kyrgyz communities in Kyrgyzstan (Budke *et al.*, 2005; Wang *et al.*, 2007, 2010; Ziadinov *et al.*, 2008). However, recent work has also identified infection in domestic dogs in central and eastern Europe (Dyachenko *et al.*, 2008; Antolová *et al.*, 2009), and so evaluation of risk factors for *E. multilocularis* in domestic dogs may become increasingly common.

Identification of risk factors is most commonly based on multivariable statistical methods – in particular, logistic regression modelling, usually adopting a frequentist framework. Frequentist methods assume that the available data represent just one possible iteration of a theoretically infinite number of repeat samplings from a particular population. This population is considered static and can be defined by a specific set of precise parameters (i.e. with no variability in the parameter coefficients). The available data is considered a best estimate of these parameters, but the sampling process introduces variability and uncertainty, which is commonly quantified by the use of

confidence intervals, which indicate a range of values within which the population-level parameters can be considered to lie. As these confidence intervals are a representation of a dichotomous process (i.e. they can either contain the 'true' parameter, or not), they only take a probabilistic interpretation with reference to the theoretical repeated sampling mentioned above.

Bayesian theory considers the population parameters as unknown to some degree – meaning that a single value could never accurately describe them. Instead, the population parameters are modelled using probabilistic distributions, and are considered revisable in the light of additional data. A central component of Bayesian methodology is that through Bayes' theorem, data can be combined with a 'prior' estimate of the population parameter in order to improve our understanding of this parameter. Therefore, the output of Bayesian analysis (the 'posterior') is a probabilistic description of our knowledge of the true value of any parameters in question. One method of summarising the posterior is through the use of a 'highest density interval', which describes the smallest range of values which both captures a particular proportion (often 0.95) of the total posterior distribution estimates, and contains no values with a frequency density lower than any point outside the range.

Both frequentist and Bayesian approaches have their advantages and disadvantages, and selection of the most appropriate strategy will largely depend upon the question being asked and the available data. However, one beneficial aspect of Bayesian strategies is that they can be used (with caveats) with small and sparse datasets. A common problem with frequentist logistic regression is that of separation – where the outcome of interest is perfectly predicted by certain predictor variables under investigation. When a frequentist approach is used, this results in unstable coefficient estimates and very wide estimated standard errors, making interpretation difficult. A common approach to separation in these cases is to remove those predictor variables causing the problem. This is far from ideal, since these variables (by definition) appear to be describing the outcome well, and as such are likely to be strong predictors of the outcome (Zorn, 2005). An alternate strategy is to adopt a Bayesian approach by incorporating a 'prior' distribution (Gelman *et*

al., 2008), which will result in stabilisation of the parameter estimates. The main challenge in these cases is the definition of suitable 'prior' distributions, since they can have a considerable impact upon the resultant coefficient estimates.

The "bayesglm" procedure in the "arm" package (Gelman & Su, 2014) for R (R Development Core Team, 2014) was used in the current study in order to develop a Bayesian-based logistic regression model. The basic computational strategy and output from this procedure is derived largely from the "glm" procedure in the "stats" package of R (R Development Core Team, 2014), and as such incorporates features of both frequentist and Bayesian approaches. Further details on the precise model structure are available elsewhere (Gelman *et al.*, 2008; Gelman & Su, 2014), but to briefly summarise, the procedure has been designed to be applicable to most datasets, without requiring adjustment of the prior distributions of the coefficient estimates. All input variables are first standardised to a mean of zero and Cauchy distributions are used to model the priors (with all coefficients except the intercept modelled with a scale parameter of 2.5 for binary predictors or 2.5/(2*standard deviation) for other predictors; and the intercept modelled with a scale parameter of 10, as shown in figure 1).

These Bayesian priors are incorporated into the iterative likelihood-maximising approach used for parameter estimation and model fitting in glm. This iterative procedure is based upon using an estimate of the parameter of interest in order to make model predictions, which are then compared with the actual data using weighted least squares: allowing an updated estimate of the parameter to be made and the process to continue (Nelder & Wedderburn, 1972; Fox, 2008). At each stage, the derivative of the log likelihood is evaluated, and model convergence is suggested as this approaches zero. In bayesglm, this procedure is adapted using data augmention techniques (Cole *et al.*, 2012) in order to incorporate the prior and the data when maximising the likelihood. The log posterior density ($log\ p(\beta, \sigma|y)$) is maximised using an iterative process combining the weighted least squares approach described above and an expectation-maximisation (EM) algorithm (Dempster,

Laird & Rubin, 1977). In line with the output of the glm procedure, the bayesglm output provides point estimates of the coefficients and their standard errors (based upon the estimated parameter value when the augmented likelihood is maximised) – i.e. a largely frequentist interpretation. However, posterior simulation based upon these parameters can be used to approximate a traditional Bayesian posterior distribution.

The current study describes an investigation of risk factors for canine *Echinococcus* spp. coproantigen positivity, using faecal samples collected prior to the commencement of a praziquantel dosing scheme in the Alay valley in the Osh oblast of Kyrgyzstan – an area of known high endemicity of human alveolar echinococcosis (Usubalieva *et al.*, 2013). A combination of Bayesian and frequentist strategies were utilised in order to identify and describe these risk factors.

Materials and Methods

Site

In May 2012, four communities in the Alay valley of southern Kyrgyzstan were visited (Sary-Mogol [39.68°, 72.89°], Taldu-Suu [39.70°, 72.98°], Kashka'Suu [39.64°, 72.67°], and Kara-Kabak [39.66°, 72.72°]), prior to the commencement of a praziquantel-based pilot intervention for canine echinococcosis. A more detailed description of the study site can be found elsewhere (van Kesteren *et al.*, 2013). All occupied households in Sary-Mogol, Taldu-Suu and Kara-Kabak, and a random selection of households (approximately 25%) in Kashka'Suu were visited. For each household visited, a questionnaire was administered relating to details such as general demographics (age, sex, occupation of interviewee), dog ownership (number of dogs currently owned, management of these dogs), dog demographics (dog age, dog sex, dog weight), and perception of echinococcosis (recent administration of praziquantel to dogs, understanding of source of human echinococcosis). However, not all questions were answered by all interviewees. Of 692 households registered, a total

of 329 individuals reported owning dogs, and a total of 388 dogs in total were registered. All questionnaire data were entered into Microsoft Access.

Samples

Wherever dogs were owned and available (i.e. not in mountain pasture), faecal samples were collected (rectally where possible, otherwise from the ground near the household), with an attempt made to match individual samples to individual dogs wherever possible. Each sample collected was divided and each part stored in either saline buffer (PBS with Tween) or ethanol before being transported to the University of Salford, England, where they were stored at -80°c for a minimum of five days prior to testing (Eckert *et al.*, 2001). A total of 318 collected dog faecal samples were available which included a subsample stored in saline buffer and which could be matched to a household. These samples were tested using a well-known sandwich ELISA for coproantigen detection after (Allan *et al.*, 1992), with modifications in that the capture and conjugate antibodies were raised from two different hyperimmune rabbit sera. All samples tested by coproantigen ELISA were tested using the same reagents in the same batch period (no more than four days), with each sample tested in duplicate (in adjacent wells).

Data analysis

Initial data processing was conducted using Microsoft Access 2010, and all further data processing and analysis was conducted using R version 3.1.1 (R Development Core Team, 2014). The difference in coproantigen ELISA OD between the two duplicates for each sample was calculated and the Studentized residuals of an intercept-only linear regression were inspected for outliers. A Bonferroni-corrected t-test p-value of 0.05 or less (using the “outlierTest” function in the “car” package for R (Fox & Weisberg, 2011)) was used to indicate a possible failure of replication, and these results were removed from the dataset and the samples retested. Of the 318 samples, 23 could not be matched to an individual questionnaire (due to illegible or damaged sample labels), but

were retained in the model as the village was known. Receiver-operator characteristic (ROC) curve analysis (Zweig & Campbell, 1993; Greiner, Pfeiffer & Smith, 2000) was used on a panel of parasitologically defined dog faecal samples taken from Xinjiang province in China during an evaluation of a control scheme (van Kesteren *et al.*, 2015) The Youden index approach (i.e. maximisation of both test sensitivity and specificity) (Youden, 1950) was used to determine the optimal cut-off point. The resultant cut-off point (OD 0.07635) gave an estimated test sensitivity of 96% and specificity of 83%, based upon the panel evaluated.

Risk model

Prior to analysis, the number of variables with missing data was assessed. All variables with more than 250 missing data points were removed from further analysis, as were those categorical variables with fewer than five outcomes in any single category. This process left a total of 41 variables to be investigated, as shown in table 1.

Initial analysis utilised simple nonparametric univariable methods (Fisher's exact test, Chi-square test or Mann-Whitney U test) to identify those variables with some evidence of association with coproantigen status (using a p-value of less than 0.3 to suggest some association). Any collinear parameters identified at this stage were reduced to one parameter based on p-value obtained. Twelve variables were selected for inclusion in the preliminary regression model. A number of different models were developed, as detailed in table 3.

All variables identified in the previous stage were added to a Bayesian logistic regression model, using the "bayesglm" function described above (Gelman & Su, 2014). Model evaluation and selection was based largely on frequentist interpretation of the model output and null hypothesis testing. A manual stepwise process was used to remove individual variables based upon their Wald test p-values, and a likelihood ratio test was used to identify possible contribution to the model (with a p-value of 0.1 or less initially used to suggest some contribution). Possible confounding was assessed

by monitoring coefficients of other variables before and after variable removal, with a change of 30% or more suggestive of some confounding effect (with the exception of coefficients less than 0.001 in magnitude, where an absolute change in the magnitude of the coefficient of 0.001 or more was suggestive of a confounding effect). Following this process, all plausible interactions between the remaining variables, and any quadratic and cubic trends in any continuous variables, were assessed using a likelihood ratio test with a p-value of 0.05 or less suggestive of a significant effect. Model diagnostics were conducted using residual plots and influence plots, and observations removed as appropriate. Variables were then sequentially removed from the final model, using a likelihood ratio test of 0.05 or less to suggest model contribution. The fit of the final model was assessed using a likelihood ratio goodness of fit test.

The final model summary was interpreted in both a frequentist and a Bayesian fashion, with the latter expressed using the modes (estimated from the kernel density, according to (Parzen, 1962), using the "modeest" package in R (Poncet, 2012)) and the highest density intervals (HDI) (using the "HPDinterval" procedure in the "coda" package (Plummer *et al.*, 2006)) of the posterior distributions of the coefficient estimates.

Results

A total of 318 canine faecal samples were tested from the four communities (see table 2), of which 78 were classified as coproantigen positive (25%) using the cut-off as calculated from ROC curves. The distribution of OD values from these samples showed a clear right skew, as shown in figure 2. Of the variables in table 2, 10 categorical variables were found to be associated with coproantigen status at the end of the first stage of analysis (p-value <0.3). None of the continuous variables were found to be associated. Of the categorical variables, two variables relating to donkey ownership were identified: one based upon a dichotomous classification of whether or not donkeys were owned, and one where donkey ownership was split according to whether only one donkey was

owned, or if the number of donkeys was greater than this. As the latter variable was found to have a higher p-value (chi-square p=0.07) than the former, this was removed from further analysis. The variables found to be associated with coproantigen status in the first stage of analysis are shown in table 3.

At the end of the second stage of analysis, the home slaughter, knowledge of hydatid source in dog, sheepdog and donkey ownership variables were found to be associated with coproantigen status, however there was no evidence of interaction between these variables. In the final stage of model selection, likelihood ratio tests were used to assess variable contribution to the model, which found all four remaining variables significant at $p<0.05$. The final model estimates are shown in table 4. The likelihood ratio goodness of fit test gave a p-value of 0.27, suggesting a reasonable model fit.

The exponents of the individual simulated posterior estimates (which describe the change in the log odds of infection associated with each variable) were calculated and the resultant distribution described in order to estimate the odds ratios of the effect of the different variables on coproantigen positivity, as shown in table 5.

Discussion

The aim of the current study was not to estimate the prevalence of canine echinococcosis amongst owned dogs in the Alay valley of Kyrgyzstan, but to identify possible risk factors for infection amongst these dogs. Due to the limitations in the coproantigen test, especially in the case of low worm burdens (Allan & Craig, 2006), the true prevalence is likely to differ from the coproantigen prevalence. Four risk factors were found to be associated with reduced odds of coproantigen positivity: ownership of donkeys; description of the dog as a sheepdog; knowledge that dogs are a source of human echinococcosis; and a lack of home slaughtering in the household. The overall copro-prevalence in the communities studied was estimated as 25%.

The logistic regression modelling framework used for the risk factor study utilised a combination of frequentist and Bayesian methodologies: a Bayesian prior was incorporated into the model in order to ensure model identifiability even when data were sparse (as was the case with the home slaughter and owner knowledge of risk factors for human echinococcosis). Model selection was based on frequentist interpretation of coefficient estimates, but the model likelihoods being compared using the likelihood ratio test incorporated prior information, and so could be considered partly Bayesian. Initial interpretation of the final model was based upon frequentist point estimates and simulated Bayesian posterior distributions, with final conclusions being Bayesian in nature. The distinction between these approaches is of importance in terms of the communication of model selection and the final conclusions. The use of well-known frequentist strategies such as likelihood ratio testing ensures that the model selection process can be understood by people not familiar with Bayesian methods, but the final interpretation of the model output in a Bayesian setting makes the model output conceptually easier to understand.

Donkey ownership was found to be associated with reduced odds of coproantigen positivity in owned dogs. This is an unexpected finding, and has not been reported in any previous studies. Unlike most other livestock (which were commonly moved for grazing to pastures and foothills surrounding the village during the day), donkeys generally remained close to households until the summer months, when they were moved permanently to pasture. Donkeys appeared to be most commonly owned by poorer families, and were usually used as draught animals (for example, to carry water from the local river). Being haram, donkeys were not used as food animals, and generally received very little veterinary care. The cause of the association between donkey ownership and dog coproantigen positivity is unclear, but it is unlikely to represent any direct mechanism relating to pathogen transmission. One possibility is that donkey ownership reflects a socioeconomic factor (as described above, people with donkeys were generally poorer), which could affect the risk of canine infection (for example, home slaughter may be less commonly performed due to the costs of livestock). Another possible reason of the association between donkey ownership and coproantigen

status of dogs may be due to their use in water transportation. Households with donkeys may be more likely to be located further from a water source than those without donkeys, meaning that if proximity to a water source was associated with an increased infection risk for dogs, then households with donkeys could be less likely to be exposed to this factor. Water sources have been reported as a potential source of human infection with *Echinococcus* spp., due to egg survival in water (Dowling, Abo-Shehada & Torgerson, 2000; Torgerson, Karaeva, *et al.*, 2003; Wang *et al.*, 2006), but it has rarely been reported as a risk factor for canine infection. One study of *Echinococcus granulosus* infection of dogs living around eight abattoirs in Lima, Peru, found that dogs from abattoirs located close to the river were more likely to be infected (Reyes *et al.*, 2012). Although this finding may represent spatial or other unrecorded differences between different abattoirs not directly related to proximity to water, it was suggested that this association may be due to infected offal being discarded into the river. Similar practices were not observed in Kyrgyzstan, and therefore further work would be required in order to better understand the possible relationship identified here between donkey ownership and coproantigen positivity.

Sheepdogs were found to have lower odds of coproantigen positivity than other dogs. This is unexpected, and contrary to previous studies, which have routinely identified sheepdogs as having a higher probability of coproantigen positivity or infection than non-sheepdogs (Moro *et al.*, 1999; Buishi, Njoroge, *et al.*, 2005). Similarly, farm dogs have also been identified as having a higher probability of infection than village dogs in Kazakhstan (Shaikenov *et al.*, 2003). It is interesting to note that most dogs were described as both pet and guard dogs, whereas dogs described as sheepdogs were rarely also described as other dog types (van Kesteren *et al.*, 2013). In the current analysis, of 44 sheepdogs, only 9 (20%) were also described as either guard dogs or pet dogs, whereas 46% (116/251) of the non-sheepdogs were described as both pet and guard dogs (94/251, or 37%, were described as pet dogs). This suggests a clear distinction between sheepdogs and non-sheepdogs (which were most commonly described as pets), which likely relates to the function of the dog. It would be expected that sheepdogs would be involved with herding and guarding

livestock, whereas pet and guard dogs are probably more involved with guarding of possessions and companionship. In studies where sheepdogs were found to have higher odds of infection, this is usually considered to be due to access to infected offal. Most dogs in the current study site were free ranging and therefore would be expected to have similar access to infected offal regardless of their role, which could explain a lack of association between dog type and odds of coproantigen positivity. However, reasons for lower odds of coproantigen positivity in sheepdogs are unclear. The major possibilities are variation in dog management (for example, sheepdogs may be more highly valued by their owners than pets and therefore offered higher quality food and given appropriate veterinary care), or in dog behaviour (for example, sheepdogs may wander around the village less than other dogs, in case they are required to work). Regardless of the cause, this finding could be of particular importance as it represents the only identified dog-level risk factor for (possible) infection in the current study, with possible implications for implementation of monitoring of a praziquantel dosing scheme

Owner knowledge that dogs were a risk factor for human echinococcosis was associated with a reduced probability of canine infection, and this variable was found to be contributing to the model when assessed using the likelihood ratio test. It is important to note that very few people reported knowledge of dogs as a source of human infection (none of whom owned dogs which tested coproantigen positive). Although the resultant coefficient instability is largely mitigated by the inclusion of the Bayesian prior (as described above), the confidence intervals of the unadjusted model coefficients cross the threshold of zero. The frequentist confidence interval described here is based on a Gaussian approximation of the distribution, which is not ideal for the sparse distribution of positive responses for this variable. Due to the strong skew in the data, the highest density interval shown in table 5 (which is parameterised differently from a confidence or credible interval) does not cross the threshold of 1.0. Combined with the presence of a low likelihood test p-value, this is suggestive that the variable is contributing to the model. Knowledge of cystic echinococcosis has been identified to be a significant risk factor for canine coproantigen status in previous studies

(Buishi, Njoroge, *et al.*, 2005; Huang *et al.*, 2008), and it is likely that people with knowledge of echinococcosis are less likely to engage in practices which could facilitate transmission to dogs (such as feeding of infected offal), and may be more likely to dose their dogs with praziquantel (although there was no evidence that this was the case according to the available data). This finding demonstrates some of the potential benefits of education campaigns as an adjunct to echinococcosis control schemes.

The variable indicating whether or not home slaughter was performed in the household was also found to be contributing to the risk model. However, the final model parameters were less clear, since the 95% HDI estimate of the estimated odds ratio extends below 1 (which would suggest no effect, or even a negative association between home slaughter and coproantigen positivity). Whilst this would suggest that the current data is insufficient to confidently state that there is a positive effect of this variable on coproantigen positivity, a similar problem of variable sparsity to that described above was found here. Only a few people did not report home slaughtering (in fact, it is likely that almost all household slaughtered animals at some point), and amongst these only one owned a dog which tested coproantigen positive. As before, model contribution was ultimately assessed using the likelihood ratio test, which was less than 0.05 for this variable. Home slaughter has also been found in previous studies to have a positive association with coproantigen positivity (although in some cases this was only apparent in univariable analysis) (Buishi, Walters, *et al.*, 2005; Acosta-Jamett *et al.*, 2010, 2014). It is likely that home slaughtering is a particular risk factor for *E. granulosus* infection, rather than *E. multilocularis*, with transmission to dogs taking place when unwanted infected offal is offered to dogs during slaughter.

Interestingly, there was no apparent association between the odds of coproantigen positivity in owned dogs and recent praziquantel dosing. Although it would be logical to assume a relationship between praziquantel dosing and echinococcosis, as has been found in a number of studies (Parada *et al.*, 1995; Buishi, Njoroge, *et al.*, 2005; Huang *et al.*, 2008; Acosta-Jamett *et al.*, 2010). Some

studies have found no evidence of an association (Buishi, Njoroge, *et al.*, 2005; Buishi *et al.*, 2006; Mastin *et al.*, 2011), and some have even found a negative association between coproantigen prevalence and anthelmintic use (Acosta-Jamett *et al.*, 2014). The lack of any identified association between reported dosing history and current status in the current study may result from the fact that dosing history over a relatively long time period (six months) was enquired about. Since praziquantel has no residual effect after administration, dosed dogs can therefore become reinfected immediately after dosing, if exposed. Recall bias amongst owners is also likely to be present (i.e. people who have not dosed their dogs recently may report they have, or people who have dosed recently may report that they haven't), which would tend to reduce any coefficient estimates towards zero.

Another interesting lack of association is that of free roaming, which is probably the most commonly identified risk factor for echinococcosis in dogs (Parada *et al.*, 1995; Budke *et al.*, 2005; Buishi, Walters, *et al.*, 2005; Buishi *et al.*, 2006; Guzel *et al.*, 2008; Huang *et al.*, 2008; Ziadinov *et al.*, 2008; Antolová *et al.*, 2009; Mastin *et al.*, 2011). In the Alay valley, most dogs were free to roam throughout the village, with only 28/288 dogs (10%) were reported to be chained at all – of which, 16 (6%) were reported to be chained all of the time. No evidence was found of any association between roaming status and coproantigen status during initial univariable analysis (Fisher's exact test p=0.72). This would suggest that even chained dogs are gaining access to infected material. Whether this is through purposeful feeding (especially in the case of infected offal) or opportunistic scavenging is unclear.

One issue with any risk factor study based on identification of 'significant' risk factors from a large number of possible variables is that as the number of variables considered is increased, the probability of type I errors (i.e. finding a 'significant' association when this is not truly the case) also increases. In total, 41 variables were assessed in the current study, meaning that with an alpha error of 0.05, approximately two associations would be expected to be identified as 'significant' due to

random variation alone. This effect is therefore another possible reason for some of the associations identified. There are three other major considerations which are particular to this study, and which should be considered when interpreting the conclusions. These are the limitations in coproantigen test sensitivity and specificity; the lack of any differentiation between *E. granulosus* (sensu lato) and *E. multilocularis;* and the fact that relatively few faecal samples were conclusively matched to individual dogs.

Most studies based upon coproantigen data classify all samples as 'negative' or 'positive' based upon a single ELISA optical density (OD) cut-off. This strategy will generally result in some misclassification: in particular, in the case of animals with low *Echinococcus* burdens (i.e. imperfect sensitivity), and animals infected with other taeniid cestodes (i.e. imperfect specificity). Therefore, estimates of the coproantigen prevalence are likely to differ from the true prevalence of infection. Whilst this is a particular problem when attempting to estimate the true prevalence of infection, it may be less of an issue in the case of analytic studies, where the intention is to identify risk factors for infection. If it is considered that high ELISA OD values amongst *Echinococcus*-negative animals is most likely to represent coinfections with other taeniid species, then classification of these individuals as positive (i.e. a low test specificity) may still allow the identification of risk factors for infection with *Echinococcus* (since these would be expected to be similar to some degree: given that the main other taeniid species in the area would be expected to be *Taenia hydatigena* (acquired through ingestion of livestock offal). On the other hand, if the OD classification has a low sensitivity, then since there is a well-reported association between OD and *Echinococcus* worm burden, it would be expected that risk factors for heavier infections (which will be of main relevance to the transmission cycle) would be predominantly identified.

A major limitation in the current study is the lack of *Echinococcus* species discrimination. Previous work has shown that *E. granulosus* (both *E. granulosus* sensu stricto and *E. canadensis* G6) and *E. multilocularis* are present in dogs in the Alay valley (van Kesteren *et al.*, 2013), although interestingly

the public health problem appears to be mainly due to *E. multilocularis* to date (Usubalieva *et al.*, 2013). Further work will be undertaken to investigate risk factors for infection as identified by PCR, and it is hoped that methods of combining results obtained from these different testing methodologies (as has been achieved in other studies (Ziadinov *et al.*, 2008; Hartnack *et al.*, 2013)) will be developed in due course. It is also hoped that coproantigen ELISA tests will one day be developed which are specific for a variety of different species and strains of *Echinococcus* –allowing species discrimination in a surveillance setting (Eckert *et al.*, 2001).

Finally, despite efforts to sample dogs per rectum whenever possible, most of the samples collected were done so from the ground around the household, and therefore cannot be definitively matched to individual dogs (or even individual households, due to the free roaming behaviour of the dogs). This problem of identifying samples from individual dogs is an important when conducting *Echinococcus* surveillance in many countries (indeed, it would be expected in most cases where ground samples are collected from free roaming dogs), and investigation of methods of accounting for this in a surveillance context is currently underway.

Conclusions

The current study used a logistic regression model to identify two previously identified and two novel potential risk factors for canine *Echinococcus* coproantigen positivity in a pastoral community in southern Kyrgyzstan, prior to the instigation of a praziquantel dosing scheme in the area. Sheepdogs and dogs from households which owned donkeys appeared to have lower odds of coproantigen positivity, although the reasons for these associations are currently unclear. A lack of owner knowledge of echinococcosis was found to be associated with higher odds of coproantigen positivity, as was home slaughter. Although *Echinococcus granulosus* and *Echinococcus multilocularis* are coendemic in the study area, these risk factors have previously only been found to be associated with *E. granulosus* infection. The modelling approach described here adopts features of both

frequentist and Bayesian methodologies, and was selected due to the distribution of the data and the need for easily interpretable output. Further work will concentrate on identifying the species or *Echinococcus* present and evaluating risk factors for these different species, with a view towards informing future control measures. It is also hoped that the results of this and other studies will assist in the development of a comprehensive surveillance strategy including aspects of sampling, coproantigen testing and coproPCR testing, which facilitate the evaluation of echinococcosis control schemes in Kyrgyzstan and similar areas.

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Table 1. Variables considered in the risk factor modelling process.

Variable type	Variables
Location	Village
Animal ownership	Number of dogs owned in last 10 years Sheep owned (and number) Goats owned (and number) Cattle owned (and number) Horses owned (and number) Yaks owned (and number) Donkeys owned (and number)
Dog demographics	Dog age Dog size (small/medium/large) Dog weight Dog sex Hunting dog Guard dog Pet dog Sheep dog
Dog behaviour	Dog wormed in last six months Percentage of time spent free roaming Dog known to eat rodents Dog fed meat Dog fed offal Dog chained at all Dogs handled by adults in the household Dogs handled by children in the household Dogs handled by friends of the family Dogs not handled Dog visited pasture previous year Dog will visit pasture this year
Animal slaughter	Animals slaughtered at home (own and others) Organs from slaughtered animals thrown away Organs from slaughtered animals given to dogs Organs from slaughtered animals buried
Knowledge about human echinococcosis	Dogs perceived source of hydatid disease Cats perceived source of hydatid disease Livestock perceived source of hydatid disease Source of hydatid disease not known

Table 2. Numbers of faecal samples analysed from the four study villages in the Alay valley

Village	Number of samples	Proportion of total samples
Sary-Mogol	155	0.49
Taldu-Suu	86	0.27
Kara'Kabak	42	0.13
Kashka-Suu	35	0.11

Table 3. Variables identified as associated with coproantigen status (chi-square p<0.3) at the end of the first stage of study

Variable	Coproprevalence amongst negative respondents	Coproprevalence amongst positive respondents	χ^2 test p-value
Hunting dog	59/227 = 26%	12/68 = 18%	0.21
Home slaughter practiced	1/14 = 7%	70/281 = 25%	0.23
Organs thrown away	44/200 = 22%	27/95 = 28%	0.29
Dogs perceived source of human hydatid disease	71/283 = 25%	0/12 = 0%	0.10
Cats perceived source of human hydatid disease	71/288 = 24%	0/7 = 0%	0.29
Sheepdog	66/251 = 26%	5/44 = 11%	0.05
Dog handled by adults in HH	29/139 = 21%	42/156 = 27%	0.28
Owns donkeys	48/165 = 29%	23/130 = 18%	0.03

Table 4. Comparison of coefficient estimates using frequentist and Bayesian interpretations of the model output

Variable	Frequentist interpretation		Bayesian interpretation	
	Coefficient estimate	95% confidence interval	Modal estimate of coefficient	95% highest density interval
Home slaughter practiced	1.45	-3.81– -0.32	1.43	-0.30 – 3.19
Dogs perceived source of human hydatid disease	-2.33	-5.06 – 0.39	-2.12	-5.02 – 0.43
Sheepdog	-1.07	-2.02 – -0.11	-1.05	-2.00 – -0.13
Owns donkeys	-0.76	-1.33 – -0.19	-0.75	-1.32 – -0.20

Table 5. Odds ratios of the variables included in the final regression model

Variable	Odds ratio (mode)	95% highest density interval
Home slaughter practiced	2.04	0.18 – 18.46
Dogs perceived source of human hydatid disease	0.03	0.0005 – 0.95
Sheepdog	0.27	0.09 – 0.77
Owns donkeys	0.46	0.24 – 0.77

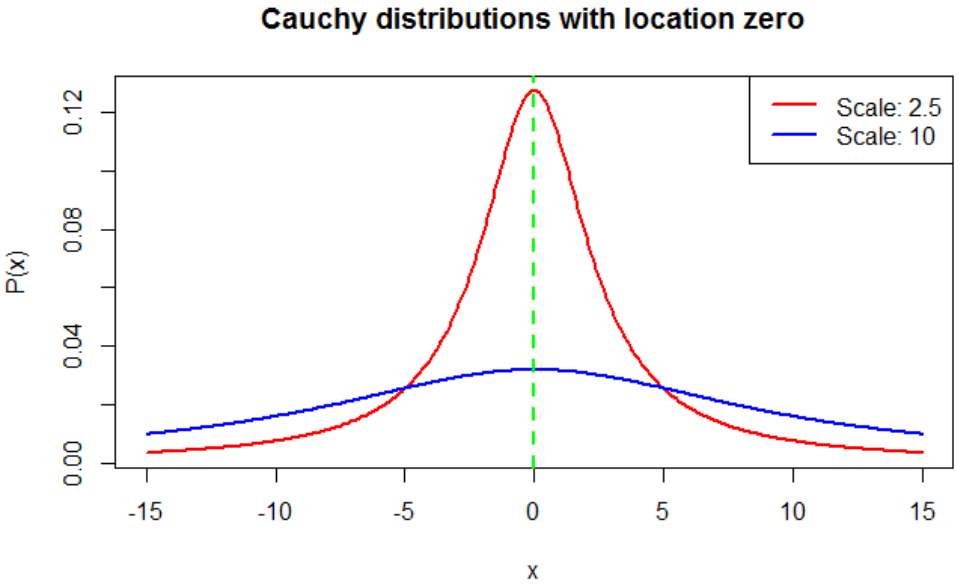

Figure 1. Cauchy distributions with a location parameter of zero and scale parameters of 2.5 and 10

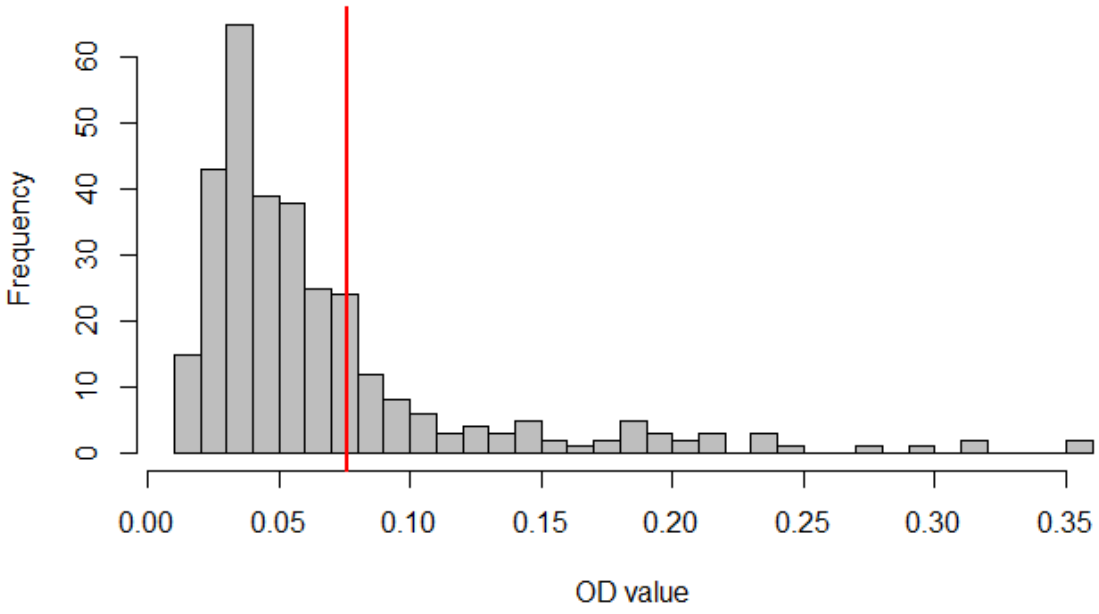

Figure 2. Distribution of coproELISA OD values for all dog faecal samples tested (n=318). The red line indicates the cut-off for positivity (OD=0.07635).