Prevalence and Molecular Detection of Gastrointestinal Pathogens in Sheep, Goats and Fish from Papua New Guinea

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Abstract

Gastrointestinal parasites of livestock cause diseases of important socio-economic concern worldwide. The two main types of intestinal parasites are helminths and protozoa. They can cause high mortality, reduce production and lead to significant overall economic losses. In tropical and subtropical areas, parasitic nematodes are recognised as a limiting factor to the expansion and improvement of smallholder production of small ruminants. In addition to their veterinary importance, some parasites of domesticated livestock can also be transmitted to companion animals and wildlife, and are a threat to public health.

Little is known of the prevalence of internal pathogens in domesticated animals or wildlife in Papua New Guinea (PNG). There have been various reports on gastrointestinal helminth and *Eimeria* infections in sheep and goats in PNG. However, those surveys have been restricted in their geographical range and have only been performed on animals raised in government or institutional (either university or agriculture research institution) farms. There is insufficient information on the epidemiology of gastrointestinal parasites infecting sheep and goats in smallholder farms. Fish, both cultured and wild caught, are an important food source in PNG, but there have been few previous surveys of parasite infection in fish. Therefore, the aim of this study was to investigate the prevalence of various pathogens including gastrointestinal nematodes, *Eimeria*, *Cryptosporidium*, *Giardia* and *Chlamydia* in sheep and goats; and *Cryptosporidium*, *Giardia* and anisakids in fish in PNG. A total of 504 faecal samples were collected from sheep (*n* = 276) and goats (*n* = 228) in smallholder, government and institutional farms, and a total of 614 fish (25 species) (cultured freshwater *n* = 132, wild freshwater *n* = 206 and wild marine *n* = 276) were sampled from February to August 2011. The study sites for sheep and goats covered a wide geographical area, representing the major sheep and goat farming regions of PNG. Samples were screened using microscopy and molecular techniques.

The overall prevalence of gastrointestinal parasites in sheep and goats in 165 small ruminants (110 sheep and 55 goats) using a modified McMaster technique was 77.6 % (128/165), with 72 % (79/110) of sheep and 89 % (49/55) of goats having one or
more species of gastrointestinal parasite. The gastrointestinal parasites found and their prevalences in sheep (S) and in goats (G) were: trichostrongylids 67.3 % (S), 85.5 % (G); *Eimeria* 17.3 % (S), 16.4 % (G); *Strongyloides* 8.2 % (S), 23.6 % (G); *Fasciola* 5.5 % (S), 18.2 % (G); *Trichuris* 1.8 % (S), 3.6 % (G); and *Nematodirus* 1.8 % (S), 3.6 % (G). Two additional genera were found in goats: *Moniezia* (9.1 %) and *Dictyocaulus* (3.6 %). To identify the pathogenic trichostrongylids, further analysis using a species-specific qPCR was performed on genomic DNA from faeces of 263 sheep and 228 goats. The prevalence of each nematode in sheep (S) and goats (G) were: *Haemonchus contortus* 41.1 % (S) and 41.7 % (G), *Teladorsagia circumcincta* 21.3 % (S) and 24.1 % (G) and *Trichostrongylus* spp. 14.8 % (S) and 14 % (G). In addition, a total of 94 samples (mostly *Eimeria*-positives by microscopy), which included 57 faecal samples from sheep and 37 faecal samples from goats, were screened by qPCR. The prevalence of *Eimeria* was 64.9 % (37/57) for sheep and 91.9 % (34/37) for goats. This is the first study to quantitatively examine the prevalence of gastrointestinal parasites in goats in PNG. The high rates of parasitism observed in the present study are likely to be associated with poor farming management practices including lack of pasture recovery time, lack of parasite control measures and poor quality feed.

Using molecular tools (nested PCRs), genomic DNA samples from sheep ($n = 276$), goats ($n = 228$) and fish ($n = 614$) were screened for *Cryptosporidium* spp. and positives were genotyped at the 18S rRNA, 60 kDa glycoprotein (*gp60*) and actin loci. The overall prevalence for *Cryptosporidium* at the 18S rRNA locus was 2.2 % (6/276) in sheep, 4.4 % (10/228) in goats and 1.14 % (7/614) in fish. In sheep, *C. parvum* (subtypes IIaA15G2R1 and IIaA19G4R1), *C. andersoni* and *C. scrofarum* were identified. In goats, *C. hominis* (subtype IdA15G1), *C. parvum*, *C. xiaoii* and rat genotype II were identified. In fish, *C. hominis* (subtype IdA15G1), *C. parvum* (IIaA14G2R1, IIaA15G2R1 and IIaA19G4R1) and a novel genotype were identified in cultured freshwater ($n = 2$), wild freshwater ($n = 1$), and wild marine ($n = 4$) fish hosts. *Cryptosporidium* was found in four different fish species; Nile tilapia (*Oreochromis niloticus*), silver barb (*Puntius gonionotus*), mackerel scad (*Decapterus macarellus*) and oblong silver biddy (*Gerres oblongus*). A novel piscine genotype in fish (piscine genotype 8) was identified in two marine oblong silver biddies and it exhibited a 4.3 %
genetic distance from piscine genotype 3 (its closest relative) at the 18S locus. The three subtypes of *C. parvum* identified in sheep and fish (IIaA14G2R1, IlaA15G2R1 and IlaA19G4R1) were all zoonotic. This is the first report of *Cryptosporidium* spp. in sheep, goats and fish in PNG. Identification of *Cryptosporidium* in livestock warrants better care of farm animals to avoid contamination and illness in vulnerable populations. The detection of zoonotic *Cryptosporidium* in livestock suggests these animals may serve as reservoirs for human infection. Zoonotic *Cryptosporidium* were identified in fish samples from all three groups (cultured and wild freshwater fish and wild marine fish). The identification of zoonotic *Cryptosporidium* genotypes in fish is important to public health in PNG and should be further investigated. In particular, detection of *Cryptosporidium* among fingerlings from aquaculture farms warrants further research to gain a better understanding of the epidemiology of *Cryptosporidium* infection in cultured fish.

*Giardia duodenalis* was screened in sheep (*n* = 276), goats (*n* = 228) and fish (*n* = 272) using qPCR targeting the glutamate dehydrogenase (*gdh*) gene. The overall prevalence of *G. duodenalis* was 9.1 % (25/256) in sheep, 12.3 % (28/228) in goats and 7 % (19/272) in fish. Although the present study found a low prevalence for *G. duodenalis* in adult small ruminants and fish, these animals can be considered as sources of contamination for susceptible hosts in PNG, because low numbers of *Giardia* cysts can cause infection. This is the first study to identify *Giardia* spp. in sheep, goats and fish in PNG. It demonstrates that *G. duodenalis* is prevalent in small ruminants and fish in PNG. *Giardia duodenalis* assemblage E (livestock genotype) was more prevalent in sheep and goats than zoonotic assemblages A and B. Further research into the characterisation of *G. duodenalis* assemblages in livestock, fish and humans in PNG is required to better understand its zoonotic potential.

The prevalence of anisakid nematode larvae in marine fish collected on the coastal shelves off the townships of Madang and Rabaul in PNG was investigated using microscopy and molecular techniques. The third-stage larvae of several genera of anisakid nematodes are important etiological agents for zoonotic human anisakidosis. Nematodes were found in seven fish species including *Decapterus macarellus, Gerres*
oblongus, Pinjalo lewisi, Pinjalo pinjalo, Seler crumenophthalmus, Scomberomorus maculatus and Thunnus albacares. They were identified by both light and scanning electron microscopy as Anisakis Type I larvae. Sequencing and phylogenetic analysis of the internal transcribed spacers (ITS) and the mitochondrial cytochrome C oxidase subunit II (cox2) gene identified all nematodes as Anisakis typica. This study represents the first in-depth characterization of Anisakis larvae from seven new fish hosts in PNG. The overall prevalence of larvae was low (7.6 %) and no recognised zoonotic Anisakis species were identified, suggesting a low threat of anisakidosis in PNG.

Finally, the prevalence of Chlamydia abortus and C. pecorum in sheep (n = 221) and goats (n = 128) were investigated using two species-specific qPCR assays, targeting the outer membrane protein cell surface antigen gene (ompA). The overall prevalence of C. abortus was 12.2 % (27/221) in sheep and 8.6 % (11/128) in goats, while for C. pecorum, it was 7.7 % (17/221) in sheep and 8.6 % (11/128) in goats. This is the first study to identify C. abortus and C. pecorum in sheep and goats in PNG. It also demonstrates that C. abortus and C. pecorum are prevalent in sheep and goats in PNG, highlighting a need for further research to quantify the production impacts of these bacteria. Furthermore, the results of the present study indicate that C. abortus could pose a risk of transmission to humans, especially, in PNG, where small ruminants in villages are cared for by women on a daily basis.

In summary, the present study represents the most detailed investigation of gastrointestinal parasites using molecular tools in sheep, goats and fish in PNG to date. A variety of gastrointestinal pathogens were identified. This provides an important update on internal pathogens of sheep and goats from previous studies (Quartermain, 2004b). Future investigations should include longitudinal studies and larger cohorts to further assess parasite epidemiology in the diverse agro-climatic zones in the country.
Declaration

I declare this thesis is my own account of my research and contains as its main content, work which has not been previously submitted for any degree and is not currently being submitted for any other degree or qualification. I declare that I have conducted the research described except where otherwise acknowledged.

Melanie Koinari

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<td>18S rRNA</td>
<td>18S ribosomal RNA</td>
</tr>
<tr>
<td>bg</td>
<td>beta (β) giardin</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
</tr>
<tr>
<td>CFT</td>
<td>complement fixation test</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>Ct</td>
<td>threshold cycle</td>
</tr>
<tr>
<td>cox2</td>
<td>cytochrome c oxidase subunit II</td>
</tr>
<tr>
<td>DAL</td>
<td>Department of Agriculture and Livestock</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>EB</td>
<td>elementary body</td>
</tr>
<tr>
<td>EPG</td>
<td>eggs per gram</td>
</tr>
<tr>
<td>EHP</td>
<td>Eastern Highlands Province</td>
</tr>
<tr>
<td>EAE</td>
<td>enzootic abortion of ewes</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>FECRT</td>
<td>faecal egg count reduction test</td>
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<td>FET</td>
<td>Fisher’s exact test</td>
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<td>gdh</td>
<td>glutamate dehydrogenase</td>
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<td>GI</td>
<td>gastrointestinal parasites</td>
</tr>
<tr>
<td>gp60</td>
<td>60 kDA glycoprotein</td>
</tr>
<tr>
<td>IAC</td>
<td>internal amplification control</td>
</tr>
<tr>
<td>IFA</td>
<td>immunofluorescent assay</td>
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<tr>
<td>ITS</td>
<td>internal transcribed spacer</td>
</tr>
<tr>
<td>ML</td>
<td>maximum likelihood</td>
</tr>
<tr>
<td>MP</td>
<td>maximum parsimony</td>
</tr>
<tr>
<td>mt-DNA</td>
<td>mitochondrial DNA</td>
</tr>
<tr>
<td>NARI</td>
<td>National Agriculture Research Institute</td>
</tr>
<tr>
<td>NJ</td>
<td>neighbour joining</td>
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<td>OEA</td>
<td>ovine enzootic abortion</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PNG</td>
<td>Papua New Guinea</td>
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<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
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<td>RB</td>
<td>reticulate body</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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<tr>
<td>SSU rRNA</td>
<td>small subunit of nuclear ribosomal RNA</td>
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<tr>
<td>Syn.</td>
<td>synonym</td>
</tr>
<tr>
<td>tpi</td>
<td>triose phosphate isomerase</td>
</tr>
<tr>
<td>WHP</td>
<td>Western Highlands Province</td>
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<td>W Ayalew: 5 %</td>
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<td>M Koinari: 80 %</td>
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<td>A Lymbery: 10 %</td>
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<td></td>
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<td></td>
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<td>R Yang: 5 %</td>
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<td></td>
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<td>R Elliot: 5 %</td>
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<td>Prevalence of <em>Chlamydia</em> from sheep and goats from PNG.</td>
<td>M Koinari: 85 %</td>
</tr>
<tr>
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</table>
Chapter 1

General Introduction
1.1 Background

1.1.1 Papua New Guinea: sheep, goats and fish.

Papua New Guinea (PNG) lies north of Australia between latitudes 1° and 12° south and longitudes 140° and 160° east. It consists of a mainland and a number of islands of varying sizes. The centre of the mainland consists of a series of mountain ranges with thickly frosted mountains in the centre, lowlands along the coast and swampy alluvial plains in the southwest. The climate in the coastal areas is generally hot and humid, with average temperatures ranging from 25 - 35 °C, while the valleys of the central highlands are warm in the daytime but cooler in the night (18 - 20 °C) and at the higher altitudes, frost occurs during certain times of the year. The rainfall varies from 1000 mm to over 4000 mm (Bourke, 2010).

PNG’s population in 2012 was approximately 6.3 million with an annual growth rate of 2.7 % (2011 census) and the majority (87 %) live in rural areas (CIA, 2012). As in other developing countries, agriculture dominates the economy in rural villages. About 50 % of the agricultural output comes from production of food items, of which livestock contributes about 13 % (Quartermain, 2004a). Livestock production has not increased in the recent past, but meat consumption is increasing steadily and is predicted to increase at 5 % per annum commensurate with the growth of the population (Quartermain, 2004a). The shortfall in local supply is met with importation of meat, especially, sheep (100 %) and beef (80 %), with the cost of imported livestock products exceeding US$50 million (Quartermain, 2004a). In 1998, imports of sheep meat and beef reached 41,800 tons (shipped weight), involving an outlay of around PGK130 million (Igua, 2001). Thus, sheep account for 38 % of meat consumption, poultry meat 39 % and beef 17 % (Igua, 2001). In order to reduce its reliance on imported sheep meat, the PNG government has attempted to increase production of small ruminants and also to promote self-sufficiency (Vincent and Low, 2000; Igua, 2001).

The country is self-sufficient with poultry and pig products, which are produced in either commercial or subsistence systems (Quartermain, 2004a). There are, however, few commercial cattle farms and no commercial sheep and goats farms.
Sheep and goats are raised in government institutions (semi-commercial) or more commonly in subsistence systems, where animals are kept primarily for household consumption, but any animals not used for household consumption are sold for cash or exchanged for other livestock or crops produced. In subsistence production systems, the animals graze or scavenge unattended and are housed at night.

There are an estimated 20,000 goats and 10,000 sheep in PNG (Quartermain, 2004b). Initially, tropical sheep from Southeast Asia and a variety of dairy goats were introduced by colonial administrators and post-colonial settlers in the 1800s (Quartermain, 2004a). Many of the tropical sheep introduced were lost during the Second World War, but a flock was gathered by the government livestock officers at Erap in the 1970s for the purpose of breeding and distribution to smallholder farmers. Those sheep then became known as Priangan, although they do not resemble the Indonesian Priangan sheep, but instead resemble the Javanese thin-tailed sheep (Quartermain, 2004a). In addition, the government attempted to farm temperate sheep in the highlands (at Aiyura and Nondugal), but high mortality and low fertility made that venture unsuccessful. In the 1980s, a research and breeding station for sheep was established in Menifo in the Eastern Highlands Province (EHP) by the PNG Department of Agriculture and Livestock (DAL), with technical assistance from New Zealand (Quartermain, 2004a). The aims of the station were to develop suitable sheep breeds that would perform well in the highlands of PNG, to increase sheep numbers rapidly and to supply other provincial sheep centres for distribution to smallholder farmers, especially, in the highlands provinces. The temperate sheep (Corriedale and Perendale) were imported from New Zealand and crossed with PNG Priangan sheep, which resulted in a new breed called Highland Halfbred (Quartermain, 2004b). The Highlands Halfbred now dominates the cooler highlands areas while the PNG Priangan sheep are raised on warm lowland areas.

Of the various dairy goats introduced during the early colonial period, few survived in the villages. From those surviving goats, a flock was gathered at the PNG University of Technology and on DAL stations at Erap and Benabena in 1975; those goats are now referred to as PNG genotype goats (Quartermain, 2004a). Since then,
there were small introductions of goats derived from Australian and New Zealand feral populations to reinforce the highland flocks (Quartermain, 2004a).

Apart from livestock, fish is one of the major economic sources in PNG (Smith, 2007). Many rural villagers living along the coast and major rivers earn their income by selling fish at local markets. Because of the nutritional value of the fish, recent efforts of national and international collaborators have encouraged the development of aquaculture in PNG (Smith, 2007). Freshwater fish farming began in the 1960s with the introduction of carp (Cyprinus carpio) and rainbow trout (Oncorhynchus mykiss) and later in 2002, a genetically improved farmed strain of tilapia (Nile tilapia; Oreochromis niloticus) was introduced (Smith, 2007). Several major hatcheries were set up, including the Highlands Aquaculture Development Centre (HAQDEC) and Erap Aquaculture Centre (EAC) to distribute fingerlings to smallholder farmers throughout the country (Smith, 2007). HAQDEC produces common carp, Nile tilapia and to a lesser extent, Java carp (Puntius gonionotus). EAC produces tilapia fingerlings. Trout farmers obtain their stock from Lake Pindi hatchery or from other smallholder hatcheries and wild caught trout. There are over 300 smallholder inland fish farms in the country (Smith, 2007). There is very little known about diseases or pathogens of fish in PNG.

1.1.2 The impact of gastrointestinal parasites on sheep and goats

Parasites are commonly found in sheep and goats and they cause diseases of major socio-economic importance worldwide. Infections can result in irreversible damage or even death to the infected sheep and goats (Hepworth et al., 2010). In addition, animals which are overburdened with parasites can be hindered in their reproductive performance, experience reduced growth rates and become less productive overall whether their purpose is meat, fibre or milk (Hepworth et al., 2010). Furthermore, parasites can cause considerable financial loss. For example, the annual cost associated with parasitic diseases in sheep and cattle in Australia has been estimated at more than 1 billion dollars (McLeod, 1995; Sackett and Holmes, 2006). In tropical and subtropical areas, parasitic nematodes are recognised as a limiting factor to the expansion and improvement of smallholder production of small ruminants (Al-Quaisy et al., 1987). In PNG, limiting factors associated with production of sheep and
goats are generally attributed to lack of management skills and parasitic diseases, however, studies assessing the economic cost of endemic diseases on profitability of cattle, sheep or goat producers are lacking.

1.1.3 Gastrointestinal pathogens of sheep and goats

Most species of nematodes and protozoa cause parasitic gastritis and enteritis in sheep and goats. The most pathogenic (disease causing) nematodes are the stomach worms (*Haemonchus contortus*, *Teladorsagia* (*Ostertagia*) *circumcincta*, and *Trichostrongylus axei*) and intestinal worms (*Trichostrongylus colubriformis*, *Trichostrongylus vitrinus*, *Nematodirus* sp., *Bunostomum trigonocephalum*, and *Oesophagostomum columbianum*) (Table 1-1) (Lichtenfels and Hoberg, 1993; Aiello et al., 2012b). *Cooperia curticei*, *Strongyloides papillosus*, *Trichuris ovis*, and *Charbertia ovina* also may be pathogenic in sheep (Table 1-1) (Lichtenfels and Hoberg, 1993; Aiello et al., 2012b). In addition, lung worms (*Dictyocaulus* spp. or *Muellerius capillaries*), liver flukes (*Fasciola hepatica*) and cestodes (*Moniezia expansia*) are also pathogenic to small ruminants (Villarroel, 2013).

Typical signs of gastrointestinal helminth infections include weight loss, diarrhoea, rough hair coat, depression, weakness, fever, anaemia, and bottle jaw (Villarroel, 2013). Parasitic nematodes of livestock are controlled mainly through anthelmintic treatment. In some areas, optimally-timed (strategic) treatments, are used, however, this type of control is expensive and in most cases only partially effective (Gasser et al., 2008). Resistance to anthelmintics has emerged as a major economic problem (Woodgate and Besier, 2010), despite the development of new drugs (Kaminsky et al., 2008).

Infection with *Eimeria* is one of the most economically important diseases of sheep and goats due to disease (diarrhoea) but also because of subclinical infections (especially, poor weight gain) (Chartier and Paraud, 2012). The disease caused by *Eimeria* is called coccidiosis, which is usually due to acute invasion and destruction of intestinal mucosa (Aiello et al., 2012a). *Eimeria* infections can be treated with coccidiostats (Villarroel, 2013).
<table>
<thead>
<tr>
<th>Parasite</th>
<th>Superfamily</th>
<th>Distribution</th>
<th>Organ</th>
<th>Damage</th>
<th>Clinical signs</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Haemonchus contortus</em></td>
<td>Trichostrongyloidea</td>
<td>Tropical, subtropical and summer rainfall.</td>
<td>Abomasum</td>
<td>Invade tissue, suck blood</td>
<td>Submandibular oedema (bottle jaw), anaemia, poor condition</td>
</tr>
<tr>
<td><em>Teladorsagia circumcincta</em></td>
<td>Trichostrongyloidea</td>
<td>Subtropical and winter rainfall</td>
<td>Abomasum</td>
<td>Hypertrophy of gastric mucosa</td>
<td>Submandibular oedema, diarrhoea, emaciation</td>
</tr>
<tr>
<td><em>Trichostrongylus spp.</em></td>
<td>Trichostrongyloidea</td>
<td>Tropical, subtropical and winter rainfall.</td>
<td>Abomasum, small intestine</td>
<td>Localised necrosis, erode mucosa, minor haemorrhages</td>
<td>Anorexia, diarrhoea, weight loss, depressed growth</td>
</tr>
<tr>
<td><em>Nematodirus spp.</em></td>
<td>Trichostrongyloidea</td>
<td>Worldwide</td>
<td>Small intestine</td>
<td>Disrupts mucus membrane</td>
<td>Unthriftiness, diarrhoea, dehydration</td>
</tr>
<tr>
<td><em>Bunostomum trigoecephalum</em></td>
<td>Ancylostomatidae</td>
<td>Worldwide</td>
<td>Small intestine</td>
<td>Suck blood, penetrate skin</td>
<td>Dermatitis, local lesions, diarrhoea, anaemia</td>
</tr>
<tr>
<td><em>Cooperia curticei</em></td>
<td>Trichostrongyloidea</td>
<td>Worldwide</td>
<td>Small intestine</td>
<td>Disrupts mucus membrane</td>
<td>Diarrhoea, anorexia, emaciation</td>
</tr>
<tr>
<td><em>Strongyloides papillosus</em></td>
<td>Strongyloidea</td>
<td>Worldwide</td>
<td>Small intestine</td>
<td>Suck blood, penetrate skin</td>
<td>Dermatitis, local lesions, diarrhoea, anaemia</td>
</tr>
<tr>
<td><em>Trichuris ovis</em></td>
<td>Trichuridae</td>
<td>Worldwide</td>
<td>Caecum, colon</td>
<td>Disrupts mucus membrane</td>
<td>Caecal mucosa oedema, diarrhoea, anaemia</td>
</tr>
<tr>
<td><em>Oesophagostomum columbianum</em></td>
<td>Strongyloidea</td>
<td>Worldwide</td>
<td>Caecum, colon</td>
<td>Nodules with small abscesses</td>
<td>Diarrhoea, weakness, loss weight</td>
</tr>
<tr>
<td><em>Charbertia ovina</em></td>
<td>Strongyloidea</td>
<td>Worldwide</td>
<td>Caecum, colon</td>
<td>Severe damage to mucosa</td>
<td>Unthrift, soft faeces with streaks of blood</td>
</tr>
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</table>
Cryptosporidium and Giardia are important intestinal protozoa, which cause acute or chronic enteric disease in young or immunosuppressed animals. The clinical symptoms of Cryptosporidium infections in lambs and goat kids are diarrhoea, weight loss and mortality (de Graaf et al., 1999), while Giardia infections can result in diarrhoea and impaired weight gain (Aloisio et al., 2006). For both cryptosporidiosis and giardiasis, the disease is usually mild and self-limited, and supportive care, primarily hydration, is important (Foreyt, 1990). Only one drug, nitazonaxide, can be used to treat diarrhoea caused by Cryptosporidium in humans (White, 2004; Fox and Saravolatz, 2005) and several drugs (metronidazole, tinidazole and nitazoxanide) can be used to treat Giardia infection in humans (Escobedo and Cimerman, 2007). In livestock, the following drugs have been used: halofuginone and paromomycin for treatment of cryptosporidiosis (Fayer and Ellis, 1993; Johnson et al., 2000), and benzimidazoles (fenbendazole, mebendazole and albendazole) for treatment of giardiasis (O'Handley et al., 1997; Geurden et al., 2006a; Geurden et al., 2006b; Geurden et al., 2010a). Control involves strict sanitation and quarantining of infected animals. In addition to their veterinary importance, Cryptosporidium and Giardia are well known as zoonotic pathogens and small ruminants have long been considered as reservoirs for human infections (Geurden et al., 2008b).

Members of the genus Chlamydia cause disease in humans and animals and most are zoonotic (Everett et al., 1999; Vlahovic et al., 2006). Two species of Chlamydia have been reported to cause infections in sheep, Chlamydia abortus and Chlamydia pecorum (Berri et al., 2009; Lenzko et al., 2011). Both are known to cause abortions in sheep with C. pecorum also known to cause enteritis in sheep (Berri et al., 2009). Chlamydia abortus, which is the causative agent of enzootic abortion of ewes (EAE), is also potentially zoonotic (Longbottom and Coulter, 2003; Baud et al., 2008; Rodolakis and Yousef Mohamad, 2010).

In PNG, previous studies have found a variety of gastrointestinal parasites including cestodes, nematodes and Eimeria species in sheep and goats (Table 1-2) (Anderson, 1960; Egerton and Rothwell, 1964; Varghese and Yayabu, 1985; Asiba, 1987; Owen, 1988, 1989, 1998; Quartermain, 2004b). In the lowlands, H. contortus, Trichostrongylus spp., Strongyloides, Oesophagostomum and Cooperia were found to
be dominant, while in the highlands, *H. contortus* and *T. colubriformis* were dominant (Quartermain, 2004b).

### Table 1-2: Previously reported gastrointestinal parasites in sheep and goats from PNG (Quartermain, 2004b).

<table>
<thead>
<tr>
<th>Gastrointestinal Parasite</th>
<th>Sheep</th>
<th>Goat</th>
<th>References</th>
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</thead>
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<td>Cestoda</td>
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</tr>
<tr>
<td><em>Moniezia expansia</em></td>
<td>*</td>
<td>*</td>
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</tr>
<tr>
<td>Nematoda</td>
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<tr>
<td><em>Bunostomum trigonocephalum</em></td>
<td>*</td>
<td>*</td>
<td>Anderson, 1960; Egerton and Rothwell, 1964.</td>
</tr>
<tr>
<td><em>Cooperia curticei</em></td>
<td>*</td>
<td></td>
<td>Owen, 1988; Owen, 1998.</td>
</tr>
<tr>
<td><em>Haemonchus contortus</em></td>
<td>*</td>
<td>*</td>
<td>Anderson, 1960; Egerton and Rothwell, 1964.</td>
</tr>
<tr>
<td><em>Nematodirus sp.</em></td>
<td>*</td>
<td>*</td>
<td>Anderson, 1960; Egerton and Rothwell, 1964.</td>
</tr>
<tr>
<td><em>Nematodirus spathiger</em></td>
<td></td>
<td>*</td>
<td>Anderson, 1960; Egerton and Rothwell, 1964.</td>
</tr>
<tr>
<td><em>Oesphagostomum venulosum</em></td>
<td>*</td>
<td></td>
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<td><em>Oesphagostomum columbianum</em></td>
<td>*</td>
<td>*</td>
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<td>*</td>
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<td>*</td>
<td>Anderson, 1960; Egerton and Rothwell, 1964.</td>
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<td><em>Trichuris globulosa</em></td>
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<td>*</td>
<td>Anderson, 1960; Egerton and Rothwell, 1964.</td>
</tr>
<tr>
<td><em>Trichuris ovis</em></td>
<td>*</td>
<td>*</td>
<td>Owen, 1988; Owen, 1998.</td>
</tr>
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<td>Coccidia</td>
<td></td>
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</tr>
<tr>
<td><em>Eimeria ahsata</em></td>
<td>*</td>
<td></td>
<td>Varghese and Yayabu, 1985.</td>
</tr>
<tr>
<td><em>Eimeria crandallis</em></td>
<td>*</td>
<td></td>
<td>Varghese and Yayabu, 1985.</td>
</tr>
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<td><em>Eimeria faurei</em></td>
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<td></td>
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<td><em>Eimeria granulosa</em></td>
<td>*</td>
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<td>Varghese and Yayabu, 1985.</td>
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#### 1.1.4 Gastrointestinal parasites of fish

All of the major groups of animal parasites are found in fish and healthy, wild fish often carry heavy parasite burdens. Protozoa such as internal flagellates and coccidia cause anorexia, weight loss and mortality; cestodes cause sterility; acanthocephalids cause enteritis and mortality and nematodes such as *Capillaria,*
Camillanus, Philometra, Eustrongylides cause weight loss and pot belly (Aiello et al., 2012c). Worms are often visualised protruding from the anus of the infected fish or within the viscera of the fish without causing any symptoms in fish (Aiello et al., 2012c). In farmed fish, poor health management practices, such as high stocking density and inadequate sanitation has led to a high incidence of parasitic diseases (Seng et al., 2006). Mass mortalities of juvenile fish due to protozoan and metazoan parasites are frequently reported (Burkholder et al., 1995; Scholz, 1999). The farm production loss in 1990 to fish diseases in a variety of fish in both freshwater pond culture and marine cage culture in developing Asian countries has been estimated to cost US$1.36 million (ADB/NACA, 1991). In PNG, there is a lack of information on the effect of disease on fish in cultured systems.

While numerous parasites have been reported in fish, only few are capable of infecting people. The most important are anisakid nematodes, cestodes and trematodes, and parasitic infections are associated with the consumption of raw or undercooked seafood (Chai et al., 2005). Species of the anisakids, particularly, Anisakis simplex and Pseudoterranova decipiens cause anisakidosis in humans, which is characterised by abdominal pain, nausea, vomiting and allergy (Bouree et al., 1995; Hochberg and Hamer, 2010). There has been a marked increase in the prevalence of anisakidosis throughout the world in the last thirty years, due in part to the growing consumption of raw or lightly cooked seafood (Lymbery and Cheah, 2007). In addition, Cryptosporidium and Giardia which are known to cause diarrhoea in humans have also been identified in fish (further details are provided in subsections 1.4.1 and 1.5.1).

1.2 Gastrointestinal parasitic nematodes of sheep and goats

1.2.1 Distribution and taxonomy

The members of the superfamily Trichostrongyloidea are principal parasites of ruminants. In tropical and subtropical regions, the most economically important parasitic gastrointestinal nematode of sheep and goats is the abomasal worm Haemonchus contortus (Waller and Chandrawathani, 2005). Of significance are also
Teladorsagia (Ostertagia) circumcincta and Trichostrongylus spp. (O’Connor et al., 2006).

Species belonging to the genus Haemonchus (barber’s pole worm) are the major abomasal pathogens of ruminants and they have a global distribution (O’Connor et al., 2006). Haemonchus contortus was first described in 1803 by Karl Rudolphi (Soulsby, 1982). Twelve species are described within the genus Haemonchus, of which H. contortus, H. placei and H. similis are found in the abomasum of domesticated ruminants (Hochberg and Hamer, 2010). Haemonchus contortus is mainly a parasite of sheep and goats (Love and Hutchinson, 2003). Its principal pathogenic features are its fourth-stage larvae and adults that feed on host blood and high parasite numbers can cause fatal anaemia (Love and Hutchinson, 2003).

Species belonging to the genus Teladorsagia (Ostertagia) (small brown stomach worm) are found in the abomasum of ruminants throughout the world. There are six described species in this genus of which two, Teladorsagia (Ostertagia) circumcincta and T. trifurcata, are found in sheep and goats (Lichtenfels et al., 1997). Heavy infections, if accompanied by Trichostrongylus spp., can cause profuse scouring, ill thrift and possibly deaths in sheep and goats (Love and Hutchinson, 2003).

Species of the genus Trichostrongylus (black scour worms) are parasites of birds, ruminants and rodents and are distributed worldwide (Anderson, 2000). There are 43 known species in this genus of which three species Trichostrongylus axei, T. colubriformis, and T. vitrinus are commonly found in sheep and goats (Lichtenfels et al., 1997; Love and Hutchinson, 2003; Rossin et al., 2006). Trichostrongylus axei infects the abomasum, while T. colubriformis and T. vitrinus infect the intestines and occur in mixed infections with Ostertagia and produce similar clinical signs (inappetence, weight loss and scouring) (Love and Hutchinson, 2003).

The overall prevalence rates of gastrointestinal trichostrongylid infections reported in sheep and goats from tropical and sub-tropical areas are usually very high (> 20 %) (Dorny et al., 1995; Cheah and Rajamanickam, 1997; Faizal and Rajapakse, 1995).
2001; Maichomo et al., 2004; Regassa et al., 2006; Tariq et al., 2008; Gadahi et al., 2009; Abebe et al., 2011; Dagnachew et al., 2011).

1.2.2 Life cycle and morphology

Most trichostrongyles have a direct life cycle, which is composed of preparasitic (free living) and parasitic stages (Fig. 1-1.) (Gasser et al., 2008). Female worms inside their host produce relatively large numbers of eggs, which are excreted in the faeces into the external environment. The first-stage larva (L1) develops inside the egg, which hatches (within 1-2 days, depending on environmental conditions) and develops into second-stage larva (L2). Both L1 and L2 larvae feed on bacteria and other microorganisms in the external environment (faeces). L2 in turn molts into the ensheathed third-stage larva (L3). Sheep and goats become infected when they graze and eat grasses containing infective L3 larvae. After L3 is ingested by the animal and passes through the stomach (s), it exsheaths (xL3) and (after a tissue phase) develops through to fourth-stage larva (L4) and subsequently into the adult at the predilection site in the alimentary tract (Gasser et al., 2008). The maturation of the worms takes place on the surface of the mucous membrane. The male and female adults mate and live in the abomasum or small intestine. Worms can be found attached to the mucosa or free in the lumen.

The length of time that infective larvae can survive on the pasture is important for control, especially pasture management programs (Zajac, 2006). The length of time required for development to the L3 stage varies with temperature and moisture. The minimum length of time required for the development of H. contortus larvae is about 3 to 4 days (Zajac, 2006). At its early life cycle stages (egg to L3), H. contortus is highly susceptible to cold and desiccation, high mortality occurs below 10 °C and L3 larvae require warmth and moisture for transmission (O’Connor et al., 2006). The free living stages of Trichostrongylus colubriformis however, have intermediate susceptibility to cold and desiccation with high mortality occurring below 5 °C; survival of L3 larvae require cool or warm moist weather (O’Connor et al., 2006). In addition, the free living stages of Teladorsagia circumcincta have low susceptibility to cold and desiccation, hatching below 5 °C; L3 stages require cool, moist weather and sub-freezing winters.
The free living stages of *Trichostrongylus* *spp.* can develop at lower temperatures and the larvae may remain dormant until conditions become favourable for transmission (Aiello et al., 2012b).

The L4 larvae of *Haemonchus* and *Teladorsagia* may undergo a period of arrested development (hypobiosis) in the host, which can be influenced by conditions in the environment which are unfavourable for development and the survival of eggs and larvae or immune status of the host (Eysker, 1997).

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**Figure 1-1: Generalised life cycle of parasitic nematodes (order Strongylida) of the alimentary tract of livestock.** (Image from Gasser et al., 2008). Adult female nematodes within the gastrointestinal tract of host animals produce eggs, which are passed out in the faeces. The larvae hatch from the eggs and develop through larval stages L1, L2 and L3 of development. Sheep and goats become infected when they graze and eat grasses containing infective L3 larvae. After the L3 is ingested by the animal and passes through the stomach (s), it exsheaths (xL3) and (after a tissue phase) develops through to fourth-stage (L4) and subsequently into the adult. Specific features of the development of such strongylid nematodes include embryogenesis (E), sexual differentiation (S), sexual reproduction (R), microbial feeding phase (F1), feeding phase inside the alimentary tract (F2), rapid growth phases (G) and the potential to undergo conditional arrested development (hypobiosis) (L4*).
Female *H. contortus* worms are 18 - 30 mm long and are easily recognised by the ‘barbers pole’ appearance of the white ovaries and uteri twisting for the length of the worm around a red blood-filled intestine, while the males are 10 - 20 mm long and uniformly reddish-brown (Love and Hutchinson, 2003). *Teladorsagia* are small brownish worms, of which adult females measure 8 - 12 mm and males 7 - 9 mm (Love and Hutchinson, 2003). *Trichostrongylus axei* are very small, slender, hair-like and reddish-brown worms (females are 5 - 8 mm long and males 4 - 7 mm long) (Love and Hutchinson, 2003). Intestinal *Trichostrongylus* spp. (*T. columbriformis* and *T. vitrines*) are small, hair-like reddish brown worms (females 6 - 8 mm and males 6 - 7 mm long) (Love and Hutchinson, 2003).

### 1.2.3 Pathogenicity and clinical signs

*Haemonchus contortus* has a tooth or lancet in its poorly developed oral cavity to perforate the gastric mucosa and suck blood (Angulo-Cubillan et al., 2007). Both the L4 larvae and adult *H. contortus* cause punctiform haemorrhages at feeding sites on the abomasal mucosa, which may be oedematous (Love and Hutchinson, 2003). Clinical signs of haemonchosis include anaemia, digestion-absorption syndromes and weight loss but no diarrhoea (Aiello et al., 2012b).

There are two types of *Teladorsagia* (*Ostertagia*) infections in ruminants. In type I ostertagiosis, which results from recent infection, most worms present are adults. As the larvae develop and emerge from gastric glands, hyperplasia of gastric epithelium may cause enlargement and coalescing of nodules, thus mucosal congestion and oedema is evident with thickening of abomasal folds (Love and Hutchinson, 2003). Clinical signs include inappetence, scours and rapid weight loss (Love and Hutchinson, 2003). Type II ostertagiosis, consists of adult worms arising from simultaneous maturation of many inhibited early L4 larvae, with glandular hyperplasia, loss of gastric structure, abomasitis, impairment of protein digestion, and leakage of plasma proteins, especially albumin, into the gut lumen (Love and Hutchinson, 2003). The mucosa appears thickened and oedematous.
In *Trichostrongylus axei* infections, the mucosa of the abomasum may show congestion and superficial erosions, which are sometimes covered with a fibrinonecrotic exudate. Intestinal *Trichostrongylus* spp. (*T. columbriformis* and *T. vitrines*) larvae burrowing in the crypts of intestinal mucosa result in damage of the mucosa and villus atrophy. The clinical signs of intestinal *Trichostrongylus* spp. are similar to those of abomasal *Teladorsagia*, which include anorexia, persistent diarrhoea, weight loss, impaired digestion and malabsorption, and protein loss (Love and Hutchinson, 2003).

### 1.2.4 Diagnosis

Techniques presently used for the diagnosis of strongylid nematodes infecting livestock can be presumptive or based on laboratory aids including parasitological, serological/immunological and DNA-based approaches. Presumptive diagnosis based on clinical signs including diarrhoea, anaemia, mortality or decreased fertility, reduced body weight, meat, milk and/or wool production in the host alone is unreliable, can be complicated in cases of mixed species infections and does not specifically detect or identify the causative agent (Gasser et al., 2008).

A diagnostic tool called FAffa MAlan CHArt (FAMACHA) method is sometimes used, in which only certain sheep or goats in the flock are selected for treatment against *H. contortus* (van Wyk and Bath, 2002). FAMACHA is a standard colour chart that is used for assessing or scoring the level of anaemia by comparison of the colour of the inner lower eyelid and then deciding which individuals in a flock should be selectively treated for haemonchosis (van Wyk and Bath, 2002).

Serological and immunological (e.g., coproantigen detection) approaches have been assessed for the specific diagnosis of nematode infections, however cross-reactivity using antigens of related parasites can occur, reducing the specificity of these methods as a diagnostic test (Johnson et al., 1996; Larsen et al., 1997; Johnson et al., 2004).

Parasitological approaches involve the identification of strongylid nematodes (at any developmental stage) on the basis of morphological characters. This approach
includes direct faecal smears, faecal flotations and post-mortem. A direct faecal smear can be examined to indicate the presence of parasite eggs. However, faecal flotations techniques are the primary laboratory test used for detection of nematode eggs and for determination of the parasite load of an animal by quantification of the worm egg per gram (EPG) of faeces. Faecal flotation techniques involve separation and concentration of eggs from faecal samples and identification based on egg morphology using light microscope (Dryden et al., 2005). The drawback of faecal worm egg counts is that EPG does not represent an accurate indication of the number of adult worms present and specific identification of strongylid nematode eggs is impractical due to close similarities in the morphology of ova (Gasser et al., 2008). EPG counts can be negative or low in the presence of large numbers of immature and adults worms, which are suppressed by host immune reactions or recent anthelmintic treatment (Aiello et al., 2012b). Also, variations in the egg-producing capability of different worms (significantly lower for Teladorsagia and Trichostrongylus than Haemonchus) may misrepresent the true worm burden (Aiello et al., 2012b). To achieve genus-level identification, L3 larvae can be produced from eggs in the faeces using the method of larval culture. For these, faecal samples are processed and incubated to hatch eggs and the resulting larva identified (Corner and Bagust, 1993). The disadvantage of larval cultures is that the yields are never 100 % (Eysker and Ploeger, 2000); it is unreliable for establishing the relative abundance of eggs from different species present in the faecal sample as their survival rates and development can vary, depending on culture conditions (Dobson et al., 1992). Also, it is time-consuming, (requiring 1-2 weeks for eggs to hatch and larvae to develop to L3 stage, depending on conditions) and requires an experienced microscopist to identify and distinguish L3 larvae (Gasser et al., 2008). Routine post-mortem examinations can provide valuable data about the status of the rest of the herd or flock (Aiello et al., 2012b). It can be performed on “traces” (parasite-naïve fully susceptible animals that graze with the herd/flock for a short period) or selecting sentinel animals from the herd/flock (Eysker and Ploeger, 2000). The faecal egg count reduction test (FECRT) is used to assess the efficacy of anthelmintic against gastrointestinal strongylids, but it is not suitable for confirmation of clinical diagnosis (Eysker and Ploeger, 2000).
A wide range of molecular approaches, based on polymerase chain reaction (PCR) techniques, are used for the specific diagnosis of nematodes including restriction fragment length polymorphism (RFLP), mutation scanning, PCR and DNA sequencing (Gasser et al., 2008). More recently, molecular methods including quantitative PCR (qPCR) for the genus- or species-specific diagnosis of strongylid infections in livestock have been developed (Zarlenga et al., 2001; Siedek et al., 2006; Harmon et al., 2007a; Harmon et al., 2007b; Bott et al., 2009; Learmount et al., 2009; von Samson-Himmelstjerna et al., 2009; Roeber et al., 2011; Sweeny et al., 2011a; Burgess et al., 2012; Roeber et al., 2012a; Roeber et al., 2012b; Demeler et al., 2013; McNally et al., 2013). Some of these studies have used copro-culture of larvae or eggs purified from faecal samples and then used PCR analysis to determine the presence/absence of selected strongylid species (Zarlenga et al., 2001; von Samson-Himmelstjerna et al., 2002; Harmon et al., 2006; Siedek et al., 2006; Harmon et al., 2007b; Bott et al., 2009; Burgess et al., 2012). However, the time and labour required for egg or larvae purification is time-consuming and is subject to the limitations described above.

1.2.5 Treatment and control

Clinically affected animals due to infections with parasitic worms can be treated with drugs (chemotherapy agents), commonly known as anthelmintics. Anthelmintics either kill egg-laying adults, or kill larvae before they become adults and become capable of laying eggs. Three families of anthelmintic drugs; (i) benzimidazoles (fenbendazole and albendazole), (ii) nicotinic agonist consisting of imidazothiaoles and tetrahydropyrimidines (levamisole, pyrantel and morantel), and (iii) macrocylic lactones comprising avermectins (ivermectin, doramectin and eprinomectin) and milbemycins (moxidectin) are available for deworming small ruminants (Schoenian, 2010).

The main goal in attempting to control gastrointestinal parasites is to break the life cycle, which can be done in a variety of ways including the use of anthelmintics, animal management and pasture management. Anthelmintics are predominantly used to control internal parasitic worms and they can be used to reduce pasture contamination. It is recommended that after treating animals for parasites, they
should be left in the original pasture for a couple of days before rotating or keep them on a dry lot for 12 - 24 h after deworming, to ensure the eggs and larvae that survived the anthelmintics are not deposited on safe pasture (Hepworth et al., 2010). In some areas, optimally-timed (strategic) treatments, are used, however, this type of control is expensive and in most cases are partially effective (Gasser et al., 2008). Anthelmintic drugs have to be used sparingly to control parasites in sheep and goats as frequent use may enable the worms to become resistant to drugs and also they are costly. Resistance to anthelmintics has emerged as a major economic problem in many parts of the world (Wolstenholme et al., 2004; Coles, 2006; Woodgate and Besier, 2010).

Pasture management is a non-chemical way to control nematodes. Pastures are allowed enough time to rest so that lower numbers of larvae are infective and will not be a problem to the grazing animal (Hepworth et al., 2010). However, good pasture management cannot entirely eliminate the parasite problem and therefore good rotational grazing techniques combined with an anthelmintic program are required; the success of which will depend on the level of anthelmintic resistance in a particular flock (Hepworth et al., 2010).

1.3 Eimeria

1.3.1 Distribution and taxonomy

Apicomplexan parasites that belong to the genus Eimeria, infect a wide range of vertebrate hosts, worldwide (McDonald and Shirley, 2009). The taxonomy of species in this genus is controversial due to phenotypic characters traditionally used for the classification of eimeriid coccidia (including the morphology of available parasite stages and host specificity) (Tenter et al., 2002). Eimeria spp. are thought to be host-specific and are not transmitted from sheep to goats (McDougald, 1979). In temperate areas, twelve species have been reported in sheep; E. ovinoidalis and E. weybrigensis (syn. E. crandallis) are most prevalent, followed by, E. ahsata, E. bakuensis, E. faurei, E. granulosa, E. intricata, E. marsica, E. parva and E. pallida (Reeg et al., 2005; Dittmar et al. 2010). Nine species are commonly reported in goats with E. ninakohlyakimovae, E. arloingi, and E. alijevi being the most frequent species, followed by E. caprina, E.
christenseni, E. jolchijevi, E. caprovina, E. hirci and E. apsheronica (Norton, 1986; Koudela and Bokova, 1998; Balicka-Ramisz, 1999; Harper and Penzhorn, 1999; Ruiz et al., 2006; Chartier and Paraud, 2012). *Eimeria* have been found in sheep and/or goats in tropical and subtropical areas including PNG (Varghese and Yayabu, 1985), Senegal (Vercruysse, 1982), Ghana (Nuvor et al., 1998), Nigeria (Woji et al., 1994), Kenya (Kanyari, 1993; Maingi and Munyua, 1994), Zimbabwe (Chhabra and Pandey, 1991), Sri Lanka (Faizal and Rajapakse, 2001), Turkey (Abdurrahman, 2007) and Brazil (Cavalcante et al., 2011). The prevalence of *Eimeria* can reach up to > 85 % in both sheep and goats (Wang et al., 2010a).

Of all the *Eimeria* species, the most pathogenic in sheep are *E. ovinoidalis* and *E. crandallis* (usually in 1-6 month old lambs) with *E. ovina* being less pathogenic (Aiello et al., 2012c). In goats, *E. arloingi*, *E. christenseni* and *E. ovinoidalis* are highly pathogenic, especially, in kids (Aiello et al., 2012c).

### 1.3.2 Life cycle and morphology

*Eimeria* has a complex life cycle (which includes three stages, sporogony, schizogony/merogony and gamogony) and requires only one host (Fig. 1-2). Sporogony is the maturation of the oocyst, which occurs outside the host, while schizogony (the asexual proliferation) and gamogony (sexual multiplication) are parasitic endogenous phases that occur within the host (Soulsby, 1982). The infected host excretes unsporulated oocysts (not infective) into the environment with the faeces. After 2 - 7 days, depending on the species of *Eimeria* and the environmental conditions (such as moisture, oxygen and temperature), the oocysts sporulate (sporogony) (Chartier and Paraud, 2012).

Once the sporulated oocyst is ingested by a suitable host, the oocyst excyst and releases sporozoites in the intestine. Each sporozoite then penetrates an epithelial cell of the small intestine, where it transforms into a schizont (Chartier and Paraud, 2012). The schizont undergoes schizogony to produce merozoites. The merozoites released from the schizont can either re-initiate asexual reproduction or develop into sexual stages called micro (male) and macro (female) gametocytes (Chartier and Paraud, 2012).
Figure 1-2: Life cycle of *Eimeria* species. Top (1): Schematic representation of the life cycle of *Eimeria* in an infected mammal. Once oocysts are ingested by a host (a), they release sporozoites (b), which invade the epithelial cells lining the intestine (c-e). This invasion may occur from 1 - 6 h after the ingestion occurs. After invasion, the sporozoites undergo replication, which leads to a rapid increase in another stage of the parasite called merozoites (f). The merozoites invade more cells of the gut, multiplying once again (g-i). The effects of coccidiosis are generally associated with the lysing of host epithelial cells by merozoites. Some merozoites develop into the sexual stages called micro (male) and macro (female) gametocytes (j). These develop into micro- and macrogametes, which fuse to form a zygote (o-r). The zygote develops into an oocyst that is eventually released in the faeces (t). The oocysts first undergo further development (sporulation) in the litter to become infectious (u-x). Bottom (2): Illustration of a fully-formed (sporulated) oocyst (a, c) and sporocyst (b, d). (Adapted from Entzeroth et al., 1998; Lindsay and Todd Jnr., 1993; Sam-Yellowe, 1996).
A zygote is formed when micro and macro gametes fuse together (gamogony) and develops into an oocyst (non-sporulated), which is eventually released in the faeces. The oocysts first undergo further development (sporulation) in the litter and are resistant to environmental extremes. Once sporulation is completed, the sporozoites within the oocysts are immediately infective to the next suitable host animals that may ingest them.

For pathogenic species, such as *E. ninakohlyakimovae* and *E. ovinoidalis*, the first schizogony takes place in the ileum (10 days post infection), the second in the crypt cells of the caecum (12 days post infection) and the colon, and eventually gamogony occurs in the large intestine (13 days post infection), with a prepatent period of 15 days (Chartier and Paraud, 2012).

The oocysts of *Eimeria* from sheep and goats are generally ovoid to ellipsoid in shape, their length ranges from 13 - 51 µm and width from 11 - 37 µm, and may contain specialized structures, such as polar caps, micropyles, residual and crystalline bodies (Vercruysse, 1982).

1.3.3 Pathogenicity and clinical signs

In sheep, *Eimeria* mostly affects the ileum, cecum and upper colon, which may be thickened, oedematous and inflamed; sometimes, there is mucosal haemorrhage (Aiello et al., 2012a). Clinical signs include diarrhoea (sometimes, containing blood or mucus), dehydration, fever, inappetence, weight loss, anaemia, wool breaking and death (Aiello et al., 2012a). In goats, stages and lesions are usually confined to the small intestine, which may appear congested, haemorrhagic or ulcerated, and have pale, yellow to white macroscopic plaques in the mucosa (Aiello et al., 2012a).

1.3.4 Diagnosis

Coccidiosis is suspected when there are digestive troubles in young animals bred under poor hygienic conditions, intensive breeding or sudden mortality. In epidemiological settings, a poor growth rate can suggest a diagnosis of subclinical coccidiosis (Chartier and Paraud, 2012). Traditional diagnosis of *Eimeria* species is based on oocyst morphology, host specificity, and pathology or examination of the gut
after necropsy (Tenter et al., 2002; Chartier and Paraud, 2012). Coproscopical examination also includes quantitation of oocysts using sodium chloride (NaCl) or magnesium sulphate (MgSO\(_4\)) flotation (Chartier and Paraud, 2012). In recent years, there have been numerous studies, which have used molecular data based on 18S ribosomal RNA (18S rRNA) sequences to discriminate the species of *Eimeria* (Power et al., 2009; Gibson-Kueh et al., 2011).

### 1.3.5 Treatment and control

The control of coccidiosis relies on preventative measures such as good hygienic conditions, reduction of stressors, an adequate nutrition and the use of anticoccidial drugs (Chartier and Paraud, 2012). Anticoccidial products belong to a number of chemical families with different modes of action on the endogenous phase of life cycle and these include sulphonamides, amprolium, ionophores, decoquinate, toltrazuril and diclazuril (Chartier and Paraud, 2012).

The greatest risk for transmission of oocysts for young lambs and goat kids (1 - 6 months old) include lambing pens, intensive grazing areas, feedlots, ration change, crowding stress, severe weather, and contamination of the environment with oocysts from infected ewes and does (Aiello et al., 2012a). Under management systems where coccidiosis is predictable, it is recommended to administer anticoccidials prophylactically for 28 consecutive days beginning a few days after the lambs or goat kids are introduced into the area (Aiello et al., 2012a).

### 1.4 Cryptosporidium

#### 1.4.1 Distribution and taxonomy

*Cryptosporidium* is an apicomplexan protozoan parasite, which invades the gastrointestinal and respiratory epithelium of a wide range of vertebrate species, and may cause considerable economic losses in livestock (Sreter and Varga, 2000; Fayer, 2008). Infections in immunocompetent individuals are usually self-limiting, but they can be life-threatening in immunocompromised individuals (O'Donoghue, 1995).

*Cryptosporidium* developmental stages were first discovered in the gastric glands of laboratory mice by Ernest Edward Tyzzer (Tyzzer, 1907, 1910). The species,
which was given the name *Cryptosporidium muris*, was later found to infect the gastric glands of several other mammals, but not humans. Tyzzer identified a second species (*C. parvum*) in 1912, which was found to infect the small intestine of laboratory mice (Tyzzer, 1912). The impact of *Cryptosporidium* on animal health was not recognised until its association with diarrhoea in 1950s in young turkeys due to *C. meleagrisidis* and in the 1970s in calves due to *C. parvum* (Slavin, 1955; Panciera et al., 1971). *Cryptosporidium* infection has been implicated in numerous waterborne and foodborne outbreaks associated with human and livestock diseases (Smith and Rose, 1998; Smith et al., 2007; Budu-Amoako et al., 2011). Since the first description of *C. muris*, numerous species of the genus *Cryptosporidium* were described based on the animal hosts from which they were isolated, however, subsequent morphological, cross-transmission and genetic studies have invalidated many species (Egyed et al., 2003; Xiao et al., 2004). The taxonomic status and naming of this genus is constantly changing as new information based on molecular data is published. At present, there are at least 27 valid species of the genus *Cryptosporidium* and over 40 different isolates or genotypes (Fayer et al., 2008; Robertson, 2009; Xiao, 2010; Ryan and Xiao, 2013).

In sheep, eight species and several genotypes have been identified including *C. parvum*, *C. ubiquitum*, *C. xiaoi*, *C. andersoni*, *C. hominis*, *C. fayeri*, *C. suis* and *C. scrofarum* with *C. xiaoi*, *C. ubiquitum* and *C. parvum* most prevalent (Ryan et al., 2005; Goma et al., 2007; Karanis et al., 2007; Santin et al., 2007; Soltane et al., 2007; Fayer et al., 2008; Geurden et al., 2008b; Mueller-Doblies et al., 2008; Quilez et al., 2008; Fayer and Santin, 2009; Giles et al., 2009; Paoletti et al., 2009; Yang et al., 2009; Diaz et al., 2010a; Robertson et al., 2010; Wang et al., 2010b; Xiao, 2010; Fiuza et al., 2011; Shen et al., 2011; Sweeney et al., 2011b; Caccio et al., 2013; Connelly et al., 2013; Imre et al., 2013; Yang et al., 2013). Three of these species (*C. parvum*, *C. hominis* and *C. xiaoi*) have also been identified in goats (Giles et al., 2009; Diaz et al., 2010b; Fayer et al., 2010; Robertson et al., 2010).

*Cryptosporidium* has a global distribution. *Cryptosporidium* infections in sheep have been reported in numerous studies in Europe (Belgium, Czech Republic, Italy,
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Gastrointestinal pathogens in sheep, goats and fish

Poland, Portugal, Spain and UK), Americas (Brazil, Mexico, Trinidad, Tobago, USA and Canada), Australia, Asia (Iraq, Iran, Oman, Sri Lanka and Taiwan) and Africa (Egypt and Zambia) with cross-sectional prevalence range from < 5 % to > 70 % (mean ~30 %, n = 20) (Robertson, 2009; Fiuza et al., 2011). Although, fewer epidemiological studies have examined Cryptosporidium spp. in goats, it appears that prevalence is similarly variable, with values of < 10 % to > 40 % reported (Robertson, 2009). Cryptosporidium infections in goats have been reported in Europe (Belgium, France, Spain and UK), the Americas (Brazil, Trinidad, and Tobago), Asia (Iraq, Sri Lanka and Taiwan) and Africa (Zambia) (Giles et al., 2009; Robertson, 2009). It has been diagnosed in outbreaks of diarrhoea and often deaths in goat kids and lambs (< 6 months old) in Australia and several European countries (de Graaf et al., 1999).

Cryptosporidium has been described in more than 17 species of both freshwater and marine fish, with prevalence rates of < 1 % to 100 % (Alvarez-Pellitero and Sitja-Bobadilla, 2002; Alvarez-Pellitero et al., 2004; Ryan et al., 2004; Murphy et al., 2009; Reid et al., 2010; Zanguee et al., 2010; Morine et al., 2012). Parasitic stages have been reported to be located deep within and on the surface of the stomach or intestinal epithelium (Alvarez-Pellitero and Sitja-Bobadilla, 2002; Alvarez-Pellitero et al., 2004; Ryan et al., 2004; Murphy et al., 2009; Reid et al., 2010; Zanguee et al., 2010; Morine et al., 2012). Currently, the only recognised species infecting fish is Cryptosporidium molnari, which was identified in gilthead sea bream (Sparus aurata) and European sea bass (Dicentrarchus labrax) (Alvarez-Pellitero and Sitja-Bobadilla, 2002) and was characterised genetically in 2010 (Palenzuela et al., 2010). Cryptosporidium molnari primarily infects the epithelium of the stomach and seldom the intestine (Alvarez-Pellitero and Sitja-Bobadilla 2002). In 2004, C. scophthalmi was described in turbot (Psetta maxima. sny. Scophthalmus maximus) (Alvarez-Pellitero and Sitja-Bobadilla, 2002; Alvarez-Pellitero et al., 2004). However, no genetic sequences are available for C. scophthalmi and it can thus not be considered a valid species due to the high genetic heterogeneity and morphological similarity among Cryptosporidium species in fish. In addition to C. molnari, a total of 3 species and 10 genotypes have been characterised genetically in fish (Table 1-3).
Table 1-3: Species/genotypes of *Cryptosporidium* reported in fish in previous studies. N = the number of specimens in which each species/genotype of *Cryptosporidium* was identified. Fish host scientific name in italics and common name in brackets.

<table>
<thead>
<tr>
<th>Species/genotype</th>
<th>N</th>
<th>Fish host</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. molnari</td>
<td>&gt; 100</td>
<td><em>Sparus aurata</em> (Gilt head sea bream) and <em>Dicentrarchus labrax</em> (European seabass)</td>
<td>Alvarez-Pellitero and Sitja-Bobadilla, 2002; Palenzuela et al., 2010</td>
</tr>
<tr>
<td>C. scophthalmi</td>
<td>49</td>
<td><em>Scopthalmus maximus</em> (Turbot)</td>
<td>Alvarez-Pellitero and Sitja-Bobadilla, 2002; Alvarez-Pellitero et al., 2004</td>
</tr>
<tr>
<td>C. molnari -like</td>
<td>7</td>
<td><em>Monodactylus argenteus</em> (Butter bream), <em>Pseudanthias dispar</em> (madder seaperch), <em>Ctenochaetus tominiensis</em> (bristle tooth tang), <em>Synodontis nigriventris</em> (upside-down catfish), <em>Paracanthurus hepatus</em> (wedgetailed blue tang), <em>Chromis viridis</em> (green chromis) and <em>Gyrinocheilus aymonieri</em> (golden algae eater).</td>
<td>Zanguee et al., 2010</td>
</tr>
<tr>
<td>Piscine genotype 1</td>
<td>2</td>
<td><em>Poecilia reticulate</em> (Guppy) and <em>Paracheirodon innesi</em> (neon tetra)</td>
<td>Ryan et al., 2004; Zanguee et al., 2010</td>
</tr>
<tr>
<td>Piscine genotype 2</td>
<td>&gt; 5</td>
<td><em>Pterophyllum scalare</em> (Angelfish), <em>Paracheirodon innesi</em> (neon tetra) and <em>Astronatus ocellatis</em> (Oscar fish).</td>
<td>Murphy et al., 2009; Zanguee et al., 2010</td>
</tr>
<tr>
<td>Piscine genotype 3</td>
<td>2</td>
<td><em>Mugil cephalus</em> (Sea mullet)</td>
<td>Reid et al., 2010</td>
</tr>
<tr>
<td>Piscine genotype 4</td>
<td>4</td>
<td><em>Aymonieri</em> (Golden algae eater), <em>Chrysiptera hemicyanes</em> (kupang damsel), <em>Astronatus ocellatis</em> (Oscar fish) and <em>Paracheirodon innesi</em> (neon tetra)</td>
<td>Zanguee et al., 2010; Morine et al., 2012</td>
</tr>
<tr>
<td>Piscine genotype 5</td>
<td>3</td>
<td><em>Pterophyllum scalare</em> (Angelfish), <em>Monodactylus argenteus</em> (butter bream) and <em>Gyrinocheilus aymonieri</em> (golden algae eater).</td>
<td>Zanguee et al., 2010</td>
</tr>
<tr>
<td>Piscine genotype 6</td>
<td>1</td>
<td><em>Poecilia reticulate</em> (Guppy)</td>
<td>Zanguee et al., 2010</td>
</tr>
<tr>
<td>Piscine genotype 6-like</td>
<td>1</td>
<td><em>Trichogaster trichopterus</em> (Gold gourami)</td>
<td>Morine et al., 2012</td>
</tr>
<tr>
<td>Piscine genotype 7</td>
<td>3</td>
<td><em>Moenkhausia sanctaefilomenae</em> (Red-eye tetra)</td>
<td>Morine et al., 2012</td>
</tr>
<tr>
<td>Rat genotype 3-like</td>
<td>1</td>
<td><em>Carassius auratus</em> (Goldfish)</td>
<td>Morine et al., 2012</td>
</tr>
<tr>
<td>C. scrofarum</td>
<td>2</td>
<td><em>Sillago vittata</em> (School whiting)</td>
<td>Reid et al., 2010</td>
</tr>
<tr>
<td>C. parvum</td>
<td>1</td>
<td><em>Sillago vittata</em> (School whiting)</td>
<td>Reid et al., 2010</td>
</tr>
<tr>
<td>C. xiaoi</td>
<td>1</td>
<td><em>Sillago vittata</em> (School whiting)</td>
<td>Reid et al., 2010</td>
</tr>
</tbody>
</table>
Approximately 15 species of *Cryptosporidium* have been identified in humans, but 5 species including *C. hominis, C. parvum, C. meleagridis, C. felis* and *C. canis* are responsible for most human cryptosporidiosis cases (Xiao and Feng, 2008). Prevalence rates of *Cryptosporidium* infections in humans ranged as low as 1 % in Europe and North America to 30 % in other countries and pooled data from developed countries showed an average of 2.1 %, while in developing countries, the average prevalence was 6.1 % (Franzen and Muller, 1999). Recently, *Cryptosporidium* was identified as one of the main causes of serious diarrhoea in children in developing countries (Kotloff et al., 2013).

### 1.4.2 Life cycle and morphology

Transmission occurs via an environmentally robust oocyst (Fig. 1-3), which is excreted in the faeces of the infected host (Franzen and Muller, 1999). *Cryptosporidium* is capable of completing all stages of its life cycle within an individual host (Fig. 1-4). Once ingested, the oocyst excysts in the gastrointestinal tract and releases infective sporozoites, which subsequently attach to the epithelial cells of the small intestine and they become enclosed within parasitophorous vacuoles (O'Donoghue, 1995). The sporozoites develop into trophozoites, which then undergo asexual proliferation by merogony into two types of meronts (type I and type II meronts). Type I meronts form 8 merozoites, which are liberated from the parasitophorous vacuole when mature and type II meronts form 4 merozoites, which produce sexual reproductive stages (called gamonts) (O'Donoghue, 1995). Type I merozoites can cause autoinfection by attaching to epithelial cells or developing into Type II merozoites, which initiate sexual multiplication (Chen et al., 2002). Type II merozoites attach to the epithelial cells, where they become either macrogamonts or microgamonts (Chen et al., 2002). Microgamonts develop into microgametocytes, which produce up to 16 non-flagellated microgametes (O'Donoghue, 1995). Macrogamonts develop into uninucleate macrogametocytes, which are fertilized by mature microgametes (sexual reproduction) (O'Donoghue, 1995). The resultant zygotes undergo further asexual development (sporogony) leading to the production of sporulated oocysts (O'Donoghue, 1995). While most oocysts are thick-walled and are excreted in the faecal material, some are thin-walled and have been reported to
encyst within the same host animal. These auto-infective oocysts and recycling type I meronts are believed to be the means by which persistent chronic infections may develop in hosts without further exposure to exogenous oocysts (O'Donoghue, 1995). The entire life cycle may be completed in 2 days in many hosts and infections may be short-lived or may persist for several months (O'Donoghue, 1995). The prepatent period, which is, the time between infection and oocysts excretion, ranges from 2 - 14 days.

The oocysts are spherical to ovoid and measure 3 - 8 µm in diameter (Fig. 1-3) (World Organisation for Animal Health-OIE, 2008; Ryan, 2010). Each sporulated oocyst contains four, flat, thin, motile, crescent-shaped sporozoites and a residuum composed of numerous small granules and a spherical or ovoid membrane-bound globule (Fayer and Ungar, 1986). Trophozoites are round or oval intracellular forms which are found within a parasitophorous vacuole surrounded by host cell membrane (Fayer and Ungar, 1986).

Figure 1-3: A scanning electron micrograph of Cryptosporidium parasites lining the intestinal tract (Photo courtesy U.S. Department of Agriculture) (Gardiner et al., 1988).
Figure 1-4: Life cycle of *Cryptosporidium parvum* and *C. hominis* (Image from CDC [http://www.cdc.gov/](http://www.cdc.gov/) Retrieved 26th October 2013). Sporulated oocysts with 4 sporozoites each are excreted by infected host (either human or animal) through faeces (1). *Cryptosporidium* oocyst is generally transmitted through contact with contaminated drinking or recreational water (2). After the parasite is ingested (3), excystation occurs and the sporozoites are released from oocysts (a, b). The sporozoites infect epithelial cells of the gastrointestinal tract. In these cells, the parasite undergoes asexual multiplication (schizogony or merogony) (d, e, f) and then sexual multiplication, or gametogony, producing microgamonts (males) (g) and macrogamonts (h). When the macrogamont is fertilized by the microgametes (i), oocysts (j, k) develop that sporulate in the infected hosts. Two different types of oocysts are produced, the thick-walled, which is commonly excreted from the host (j), and the thin-walled oocyst (k), which is primarily involved in autoinfection.
1.4.3 Pathogenicity and clinical signs

*Cryptosporidium* spp. attach intimately to the microvillus membrane of surface epithelia of the intestinal tract and cause a range of histologic abnormalities in crypt and villous structure, including villous atrophy and crypt hyperplasia, which are usually accompanied by inflammatory responses (Chen et al., 2002).

The main clinical sign of cryptosporidiosis is diarrhoea accompanied by the shedding of a large number of oocysts (de Graaf et al., 1999). High morbidity and mortality have been reported in lambs and goat kids, especially, the neonates (Fayer and Ungar, 1986; de Graaf et al., 1999). Clinical signs of ovine cryptosporidiosis include diarrhoea, which can last from 2 - 12 days and is sometimes accompanied by anorexia, poor growth, stiffness, hyperpnoea, slow gait, limb muscle fasciculations and depression (Fayer and Ungar, 1986). In fish, *Cryptosporidium* can cause high morbidity with clinical signs including variable levels of emaciation, poor growth rates, swollen coelomic cavities, anorexia, listlessness and increased mortality (Murphy et al., 2009). In humans, although infections can be asymptomatic, most patients have profuse watery diarrhoea and the duration and severity of clinical symptoms depend largely on the immune status of infected person (Chen et al., 2002). It can result in life-threatening diseases in immunodeficient people, especially, AIDS patients (Current et al., 1983).

1.4.4 Diagnosis

A variety of laboratory tests have been developed for the differential diagnosis of cryptosporidiosis. These include microscopic, immunologic and molecular detection of the parasite in the faeces and histological examination of autopsy or biopsy material. Endogenous developmental stages of *Cryptosporidium* can be diagnosed from autopsy or biopsy material after staining with haematoxylin and eosin or Giemsa and examined under light and/or scanning electron microscopes (O'Donoghue, 1995). In addition, histological techniques may be used to investigate pathological and cytological changes accompanying infections, however, these are not recommended for routine diagnosis (O'Donoghue, 1995).
Oocysts in faecal material can be detected by examining stained faecal smears. There are numerous staining methods available including direct, differential, acid-fast, fluorochrome and negative staining techniques, which have been described to stain the walls and/or contents of mature oocysts (O'Donoghue, 1995). Acid-fast staining is the simplest and a widely used method for detecting oocysts in faecal material, however, like other conventional microscopic examination methods, the drawback is that oocysts can be difficult to recover and identify because of their small size and frequent similarities of their morphological features (i.e. size, shape and internal structures) (O'Donoghue, 1995; Chen et al., 2002). The sensitivity and specificity of microscopic examination methods can be improved with sedimentation and flotation techniques prior to microscopy, and immunological-based techniques such as immunofluorescent antibody tests and antigen-capture ELISAs (Chen et al., 2002). However, antibody techniques suffer from lack of specificity and in the last two decades, numerous molecular techniques have been developed for detection and differentiation of Cryptosporidium at species/genotype and subtype level.

Molecular tools are widely used in epidemiological studies of cryptosporidiosis in endemic and epidemic areas, in which they have improved the understanding of transmission of cryptosporidiosis in humans and animals (Xiao, 2010). Different types of molecular tools have been used for the differentiation of Cryptosporidium species/genotypes or subtypes. 18S rRNA-based tools are extensively used in genotyping Cryptosporidium in humans, animals and water samples (Xiao, 2010). The widespread use of the 18S rRNA gene in genotyping Cryptosporidium is largely due to the multi-copy nature of the gene and the presence of semi-conserved and hyper-variable regions, which facilitate the design of genus-specific primers (Xiao, 2010). Other genes are also used in combination with 18S rRNA-based tools including oocysts the actin gene, the heat shock protein 70 (Hsp70) gene, and the 60 kDA glycoprotein gene (gp60) (Table 1-4) (Caccio et al., 2005).

The typing and subtyping methods used for veterinary samples are important for veterinary and public health applications. Currently, subtyping tools are only applicable to C. parvum, C. hominis, C. cuniculus, C. ubiquitum, C. tyzzeri and C. fayeri
and have been used extensively in studies of the transmission of *C. hominis* in humans and *C. parvum* in humans and ruminants (Xiao, 2010; Li et al., 2014). One gene that is popular for subtyping Cryptosporidium is *gp60*. The *gp60* gene has variations in the number of trinucleotide repeats and extensive sequence differences in the non-repeat regions, which categorize *C. parvum* and *C. hominis* to several subtype families (Xiao, 2010). It is similar to a microsatellite sequence by having tandem repeats of the serine-coding trinucleotide TCA, TCG or TCT at the 5’ end of the gene (Xiao, 2010).

**Table 1-4: Molecular techniques for characterising Cryptosporidium.** RFLP = restriction fragment length polymorphism; Hsp70 = heat shock protein 70; COWP = Cryptosporidium oocyst wall protein gene; *gp60* = 60 kDa glycoprotein gene. (Adapted from Caccio et al., 2005).

<table>
<thead>
<tr>
<th>Amplification target</th>
<th>Assay type</th>
<th>Identification for</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rDNA</td>
<td>PCR, nested PCR, sequencing, PCR-RFLP, qPCR, microarray</td>
<td>Species &amp; genotype</td>
</tr>
<tr>
<td>Hsp70</td>
<td>PCR, nested PCR, sequencing, PCR-RFLP, microarray</td>
<td>Species &amp; genotype</td>
</tr>
<tr>
<td>COWP</td>
<td>PCR, nested PCR, sequencing, PCR-RFLP, microarray</td>
<td>Species &amp; genotype</td>
</tr>
<tr>
<td>Actin</td>
<td>PCR, nested PCR, sequencing</td>
<td>Species &amp; genotype</td>
</tr>
<tr>
<td>β-Tubulin</td>
<td>PCR, nested PCR, sequencing, PCR-RFLP</td>
<td>Species &amp; genotype</td>
</tr>
<tr>
<td><em>gp60</em></td>
<td>PCR, nested PCR, sequencing</td>
<td>Subtype</td>
</tr>
<tr>
<td>Microsatellites</td>
<td>PCR, nested PCR, sequencing, fragment typing</td>
<td>Subtype</td>
</tr>
<tr>
<td>Minisatellites</td>
<td>PCR, nested PCR, sequencing, fragment typing</td>
<td>Subtype</td>
</tr>
<tr>
<td>Extra-chromosomal double stranded RNA</td>
<td>Reverse transcriptase PCR, sequencing, heteroduplex mobility assays</td>
<td>Subtype</td>
</tr>
</tbody>
</table>

**1.4.5 Treatment and control**

There is no effective specific treatment for cryptosporidiosis in sheep and goats, however, because the disease is usually self-limiting, supportive therapy, such as rehydration and maintenance of energy requirements, is usually sufficient. Preliminary results obtained in calves, lambs and goat kids infected by *Cryptosporidium* sp. have indicated a partial prophylactic efficacy of halofuginone lactate when administered at 100 μg/kg body weight (BW) (De Waele et al., 2010). Recently, an efficacy demonstration study showed that halofuginone lactate, which when given as a prophylactic treatment at 100 μg/kg BW, reduced oocyst shedding, diarrhoea and mortality in goat kid cryptosporidiosis (Petermann et al., 2014).
1.5 Giardia

1.5.1 Distribution and taxonomy

*Giardia* is a flagellated protozoan parasite and is one of the most common nonviral causes of diarrhoea in humans and livestock worldwide (Thompson et al., 2000). The parasite was first observed by Van Leeuwenhoek in 1681 (Dobell, 1920) and more fully described by Lamb in 1859 (Lamb, 1859). There are six recognised species in the genus *Giardia* (1952-2007); *Giardia duodenalis* (syn. *G. intestinalis, G. lamblia*) in mammals, *G. agilis* in amphibians, *G. muris* and *G. microti* in rodents, and *G. ardeae* and *G. psittaci* in birds (Monis et al., 2009). *Giardia duodenalis* is frequently found in livestock, companion animals and humans (Xiao and Fayer, 2008). Within this species, there are eight morphologically identical but genetically distinct genotypes or assemblages designated A – H (Thompson, 2004; Lasek-Nesselquist et al., 2010) with assemblages A and B known to infect a wide range of hosts including humans, livestock, cats, dogs and other mammals (Thompson, 2004; Xiao and Fayer, 2008). Assemblages C and D infect dogs, assemblage E infects livestock (cattle, sheep, pigs), assemblage F infects cat, assemblage G infects domestic rats and assemblage H infects marine vertebrates (Thompson, 2004; Lasek-Nesselquist et al., 2010).

In small ruminants, especially, lambs and goat kids, giardiasis can cause significant economic losses (O’Handley et al., 2001; Aloisio et al., 2006). The prevalence of *Giardia* ranged from 2.7 - 68.8 % in sheep and 4.0 - 42.2 % in goats (Robertson, 2009). Based on genotyping analyses in the last decade, *G. duodenalis* assemblage E was found to be the most prevalent genotype in sheep and goats, with assemblage A and B less prevalent in Australia (Ryan et al., 2005; Yang et al., 2009; Sweeny et al., 2011b), USA (Santin et al., 2007), Belgium (Geurden et al., 2008b), Italy (Giangaspero et al., 2005; Aloisio et al., 2006), The Netherlands (van der Giessen et al., 2006) and Spain (Castro-Hermida et al., 2006; Ruiz et al., 2008; Gomez-Munoz et al., 2009).

Although *Giardia* has been reported in seals, sea lions, a shark and clams, very little is known about the prevalence, genetic diversity and effect of *Giardia* species in marine or freshwater systems and the role that aquatic animals play in transmission of these parasites to human (Yang et al., 2010). In fish, prevalences of 3.8 % and 3.3 %
have been reported and *G. duodenalis* assemblages A, B, E and *G. microti* have been found (Yang et al., 2010; Ghoneim et al., 2011).

In humans, giardiasis is a well-recognised waterborne disease in both developed and undeveloped countries (Levine et al., 1990; Thompson, 2000; Leclerc et al., 2002; Dawson, 2005; Baldursson and Karanis, 2011; Budu-Amoako et al., 2011). The reported prevalence of *G. duodenalis* assemblages A and B ranged from 20 - 100 % in at least 14 countries including Italy, Wales, England, The Netherlands, USA, Canada, Australia, China, Korea, Laos, India, Peru, Uganda and Turkey (Caccio et al., 2005). Although, little epidemiological evidence exist that strongly supports the importance of zoonotic transmission of giardiasis to humans, the occurrence of zoonotic *G. duodenalis* assemblages A and B in sheep and goats suggest that these animals may act as reservoirs for human infection (Xiao and Fayer, 2008).

### 1.5.2 Life cycle and morphology

The life cycle of *Giardia* is composed of two major stages: cysts and trophozoites (Fig. 1-5). Cysts are environmentally stable and responsible for transmission. Infection is initiated when the cyst is ingested with contaminated water or, less commonly, food or through direct faecal-oral contact (Adam, 2001). Following ingestion and exposure to the acidic environment of the stomach, cysts excyst and develop into trophozoites in the proximal small intestine (Adam, 2001; Ankarklev et al., 2010). The trophozoites then attach to the surface of the intestinal epithelium in the crypts of the duodenum and upper jejunum and reproduce asexually by binary fusion, causing intestinal tissue damage (characterised by diarrhoea and malabsorption) (Ortega and Adam, 1997). After exposure to host specific-factors such as high levels of bile, low levels of cholesterol and a basic pH, some trophozoites form cysts in the jejunum and are passed in the faeces, allowing completion of the life cycle by infecting a new host (Lauwaet et al., 2007).

*Giardia* cysts are either round or oval and measure 11 - 14 x 7 - 10 µm (Fig. 1-6) (Ortega and Adam, 1997). Each cyst has four nuclei and contains axonemes and median bodies (Ortega and Adam, 1997). The cysts are metabolically inactive and have
a hard wall (consisting of 60% carbohydrate and 40% protein) that protect them from hypotonic lysis in the environment ( Ankarklev et al., 2010).

Figure 1-5: Life cycle of *Giardia duodenalis* (Image from Ankarklev et al., 2010). *Giardia* cysts are exposed to gastric acid during their passage through the host’s stomach, triggering excystation. This causes differentiation of the cyst into the vegetative trophozoite, which attaches to the intestinal epithelium via its adhesive disc and replicates. After exposure to bile salts, some trophozoites form non-motile, infective cysts (a process known as encystation) in the lower intestine and are passed in the faeces.

Figure 1-6: Electron photomicrograph of *Giardia duodenalis* cysts (A) and trophozoites (B). (Bar = 10 μm) (Image from Ortega and Adam, 1997).
Giardia trophozoites measure 10 - 20 µm in length and 5 - 15 µm in width (Fig. 1-6) (Ortega and Adam, 1997). The shape of the trophozoites varies among species: *G. duodenalis* is pear shaped and has one or two transverse, claw-shaped median bodies; *G. agilis* is long and slender and has a teardrop-shaped median body, and *G. muris* trophozoites are shorter and rounder and have a small, rounded median body (Adam, 2001). *Giardia duodenalis* trophozoites are bilaterally symmetrical, measure 12 - 15 µm x 6 - 8 µm, and have a convex dorsal surface and a large unique adhesive or ‘sucking disc’ on the ventral surface (Thompson, 2004). They have two nuclei, four pairs of flagellae and a pair of distinctive median bodies with a simple vesicular secretory system, but no mitochondria or peroxisomes (Marti et al., 2003; Ankarklev et al., 2010). The flagellae and adhesive disc are used for adhesion to the upper intestinal walls, while the role of median body is unknown (Ankarklev et al., 2010).

### 1.5.3 Pathogenicity and clinical signs

The pathogenesis of *Giardia* is not clearly understood, however, several mechanisms have been proposed based on studies on human epithelial cell lines, on laboratory animals, goat kids and calves that indicate that pathogenesis of giardiasis is a combination of both parasite and host factors (Ankarklev et al., 2010; Geurden et al., 2010b). The main virulence factors include the trophozoite’s adhesive disc and the four flagellae, which when they interact with host cells can induce apoptosis, loss of epithelial-barrier function, diffuse shortening of microvillus (which leads to malabsorption of electrolytes, solutes and water), mucosal inflammation (after infiltration of lymphocytes and mast cells) and interference with bile salts metabolism (Ankarklev et al., 2010).

The clinical signs of giardiasis include acute and chronic diarrhoea, dehydration, abdominal pain and weight loss. *Giardia duodenalis* infections have been reported in lambs in which abnormal faeces, impairment in feed efficiency, a decreased rate of weight gain and severe weight loss have been observed (Olson et al., 1995; Aloisio et al., 2006). In histological sections, mild to severe infiltrative enteritis with eosinophils, lymphocytes and plasma cells have been reported (Aloisio et al., 2006). In humans, symptoms include watery diarrhoea, epigastric pain, nausea, vomiting and weight loss,
which appear 6 - 15 days after the infection (Ankarklev et al., 2010). The clinical impact is more severe in young children and undernourished or immunodeficient individuals.

1.5.4 Diagnosis

As the symptoms of giardiasis are similar to other gastrointestinal diseases, clinical diagnosis in production animals is mainly based on clinical history, data on farm management and the exclusion of other infectious disease, such as coccidiosis, and confirmed by detection of the parasite in a faecal sample by microscopic examination, antigen detection and PCR-based tools (Geurden et al., 2010b).

Traditional microscopic methods include the application of faecal concentration techniques, especially, zinc sulphate flotation and centrifugation (Zajac et al., 2002) and followed by stains such as iodine or trichrome (Geurden et al., 2010b). It is cheaper to perform a microscopic examination but it has relatively low sensitivity and requires a skilled and experienced person to discriminate the parasite (Xiao and Herd, 1993; Amar et al., 2002; Geurden et al., 2010b).

For detection of parasite antigen, immunofluorescence assays (IFAs) (Xiao and Herd, 1993), enzyme-linked immunosorbent assays (ELISAs) (Boone et al., 1999) and rapid solid-phase qualitative immunochromatography assays (Garcia et al., 2003) are available. IFAs and ELISA use monoclonal antibodies against cyst wall proteins. Immunochromatographic techniques also target cyst wall proteins as well as trophozoite proteins and unlike, IFA and ELISA; they allow on-site and quick diagnosis of infection (Garcia et al., 2003). Antigen detection tests are more sensitive and specific than microscopic examination for diagnosis of infection, however, they too require trained personnel and are expensive (Geurden et al., 2010b). These tests have been developed and evaluated using human stool samples, and used in calves (Geurden et al., 2004), however, they have not been used in sheep (Geurden et al., 2010b).

Molecular tools are mainly used for differentiating *Giardia* at species and genotype level for taxonomical and epidemiological research, but can be used for diagnosis. Most studies have relied on the analysis of the well conserved eukaryotic
genes such as the 18S rRNA, glutamate dehydrogenase (gdh), triose phosphate isomerase (tpi), elongation factor 1-alpha gene (ef-1α) or genes uniquely associated with the parasite such as the β-giardin gene (Table 1-5) (Ryan and Caccio, 2013). Other genes used for characterising Giardia include G. lamblia open reading frame (GLORF)-C4 and the inter-genomic rRNA spacer region (IGS) (Caccio et al., 2005; Lee et al., 2006) (Table 1-5).

**Table 1-5: Molecular techniques for characterising Giardia** (Adapted from Caccio et al. 2005). gdh = glutamate dehydrogenase, tpi = triose phosphate isomerase, EF-1α = elongation factor 1-alpha gene, GLORF-C4 = G.doudenalis open reading frame C4, IGS = inter-genomic rRNA spacer region.

<table>
<thead>
<tr>
<th>Amplification target</th>
<th>Assay type</th>
<th>Main application</th>
</tr>
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<td>Species and genotype identification</td>
</tr>
<tr>
<td>gdh</td>
<td>PCR, nested PCR, sequencing, PCR-RFLP</td>
<td>Species and genotype identification</td>
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<td>tpi</td>
<td>PCR, nested PCR, sequencing, qPCR, microarray</td>
<td>Species and genotype identification</td>
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<tr>
<td>β-giardin</td>
<td>PCR, nested PCR, sequencing, PCR-RFLP, q PCR, microarray</td>
<td>Genotype identification</td>
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<tr>
<td>EF-1α</td>
<td>PCR, nested PCR, sequencing</td>
<td>Species and genotype identification</td>
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<td>GLORF-C4</td>
<td>PCR, sequencing, PCR-RFLP</td>
<td>Genotype identification</td>
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<tr>
<td>IGS</td>
<td>PCR, sequencing</td>
<td>Genotype identification</td>
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### 1.5.5 Treatment and control

Several compounds, specifically, benzimidazoles and paromomycin, have a known efficacy against *Giardia* both *in vitro* and in laboratory animals, yet there are no drugs registered for the treatment of giardiasis in sheep and goats (Geurden et al., 2010b). Several benzimidazole compounds and paromycin were shown to be efficacious in experimental conditions, especially, in calves (O'Handley et al., 1997; O’Handley et al., 2000; O’Handley et al., 2001; Geurden et al., 2006b; Geurden et al., 2010a). Fenbendazole has been shown to effectively clear *Giardia* infection in natural infections in lambs (Aloisio et al., 2006). In humans, nitroimidazoles and benzimidazoles are used for treating giardiasis (Thompson, 2004).

To control *Giardia* infection in sheep and goats, a combined approach consisting of chemotherapy and hygienic measures could be taken (Geurden et al.,
Preventive measures should include low stocking rate, isolation of infected lambs from animal housing, regular cleaning and disinfection of the housing facilities (Geurden et al., 2010b).

1.6 Anisakids

1.6.1 Distribution and taxonomy

Anisakid nematodes are economically significant in fish farming and are medically important to humans. The presence of anisakid larvae on and in the viscera and flesh or free in the body cavity of many economically important cephalopod and fish species is an aesthetic problem, which leads to notable adverse effects on marketing (Abollo et al., 2001). In addition, removing parasites adds appreciably to the costs of packaging, reducing the value of the product and representing a financial loss in marketing value (Abollo et al., 2001).

Anisakidosis refers to infection of people with larval stages of anisakid nematodes belonging to the family Anisakidae or Rhaphidascarididae and its clinical symptoms include acute abdominal pain, nausea, vomiting and allergic responses (Chai et al., 2005; Audicana and Kennedy, 2008). The first case of human infection by an anisakid was reported in the Netherlands by Van Thiel (1960), who described the presence of a marine nematode in a patient suffering from acute abdominal pain (Van Thiel et al., 1960). Anisakidosis occurs throughout the world. Approximately 20,000 cases of human anisakidosis are reported each year with majority of the cases (>90) from Japan, followed by Europe and then the rest of the world (the Americas, South-east Asia, Egypt and New Zealand) (Lymbery and Cheah, 2007; Hochberg and Hamer, 2010).

Anisakids are ascarid nematodes, which utilise aquatic vertebrate (fish, reptiles, piscivorous birds, or mammals) as definitive hosts and whose transmission depends on water and usually involves aquatic invertebrates and fish as intermediate or paratenic hosts (Lymbery and Cheah, 2007). The nematode superfamily Ascaridoidea contains >50 genera. There are controversies in taxonomic classification in this superfamily; few classification schemes exist due to inconsistencies in the key morphological structures.
or life history features (Mattiucci et al., 2003; Mattiucci and Nascetti, 2008). Recent reported phylogenetic relationships based on molecular data have not fully resolved taxonomic uncertainty within this superfamily, however, they tend to support the classification proposed by Fagerholm (1991) (Lymbey and Cheah, 2007). The two genera most often associated with anisakidosis are Anisakis and Pseudoterranova.

The genus Anisakis consists of nine described species in two clades. The first clade includes 6 species; Anisakis simplex complex (which comprised A. simplex sensu stricto (s.s.), A. pegreffii and A. simplex C), A. typica and the sister species A. nascettii and A. ziphidarum, while the second clade consists 3 species; A. brevispiculata, A. paggiae and A. physeteris (Mattiucci and Nascetti, 2006; Mattiucci et al., 2009). Currently, only A. simplex s.s., A. pegreffii and A. physeteris have been shown to cause infection in humans (Asato et al., 1991; Hochberg and Hamer, 2010; Mattiucci et al., 2011; Arizono et al., 2012). The distribution patterns of Anisakis species are likely to be a consequence of dispersal through the faeces of infested oceanic delphinids (Kuhn et al., 2011). Anisakis simplex s.s. is found in the northern hemisphere in Atlantic and Pacific Oceans from 20 °N to 80 °N (the Artic polar circle) (Mattiucci and Nascetti, 2006; Kuhn et al., 2011). Anisakis pegreffii is distributed in southern oceans from Mediterranean waters through to East Atlantic Ocean and down to the Antarctic Peninsula and also in Japanese and Chinese waters (Mattiucci and Nascetti, 2006; Kuhn et al., 2011). Anisakis physeteris is distributed throughout the central Atlantic Ocean (Mattiucci and Nascetti, 2006; Kuhn et al., 2011). In the tropics and subtropics, A. typica is the most dominant species between 45 °N and 25 °N (Mattiucci and Nascetti, 2006; Kuhn et al., 2011). Anisakis typica is a common parasite of various dolphin species of warmer temperate waters, such as Tucuxi (Sotalia fluviatilis), the Common Bottlenose Dolphin (Tursiops truncates) and the Pantropical Spotted Dolphin (Stenella attenuate) (Kuhn et al., 2011).

The genus Pseudoterranova consists of three species: Pseudoterranova kogiae, P. cetiola and P. decipiens. To date, only P. decipiens (seal worms) have been reported to cause infection in humans (Hochberg and Hamer, 2010). Based on genetic analysis, P. decipiens represents a species complex comprising 6 different species, namely,
P. azarasi, P. bulbosa, P. cattani, P. decipiens sensu stricto (s.s.), P. decipiens E and P. krabbei (Mattiucci and Nascetti, 2008). Pseudoterranova decipiens s.s. has a wider distribution including the North East Atlantic (NEA), the North West Atlantic (NWA), arctic and sub-arctic areas (Mattiucci and Nascetti, 2008). The main geographic distribution of P. krabbei is NEA, P. bulbosa is NWA and North West Pacific (NWP), P. azarasi is NWP and waters of Japan, P. decipiens E is Antartica and P. cattani is South-East Pacific (Mattiucci and Nascetti, 2008).

1.6.2 Life cycle and morphology

Anisakid nematodes have a complex life cycle involving a free-living stage and multiple host species (Fig. 1-7). The definitive hosts for Anisakis species are cetaceans (dolphins, porpoises and whales) and for Pseudoterranova are pinnipeds (seals, walruses, and sea lions) (Hochberg and Hamer, 2010). Adult nematodes living in the gut of marine mammals produce unembryonated eggs, which are shed with the faeces of their hosts. The egg embryonate in the sea and develops into first stage larva (L1) and then free-living L2 or L3 larvae (Lymbery and Cheah, 2007; Hochberg and Hamer, 2010). Free-living larvae are ingested by small crustaceans (e.g. krills, first intermediate host), in which L2 can develop into L3 for some anisakid nematodes (Lymbery and Cheah, 2007; Hochberg and Hamer, 2010). Infected crustaceans are eaten by fish and squid (second intermediate hosts), in which the L3 migrates into the viscera and peritoneal cavity (Hochberg and Hamer, 2010). L3 larvae are often transferred from fish to fish along the food chain, as a result, large numbers of larvae may accumulate in piscivorous fish (Hochberg and Hamer, 2010). Upon ingestion by a definitive host, L3 develop into L4 then adult. Humans can only be considered accidental hosts in this life cycle, and have no influence on the transmission of these parasites (since L3 larvae do not develop any further and the cycle cannot be completed).

Like other nematodes, anisakid are cylindrical (or round in cross section) and non-segmented. They have both an oral and anal opening and a complete digestive tract, which includes an oesophagus, a ventriculus and an intestine (Berland, 1961). In addition, the mouth of third stage larvae of Anisakis is surrounded by three relatively small, inconspicuous lips with a prominent boring tooth, an excretory pore and a
mucron (Cannon, 1977). *Anisakis* larvae are white, usually coil in a characteristic watch-spring shape and can grow up to 40 mm in length and 0.6 mm in width (Cannon, 1977; Hurst, 1984).

**Figure 1-7: Complex life cycle of anisakid nematodes.** Image from CDC, (http://www.cdc.gov/parasites/anisakiasis/biology.html), retrieved 30\(^{th}\) October 2013. Adult nematodes living in the stomach of marine mammals produce unembryonated eggs, which are shed by their hosts (1). Eggs become embryonated, hatched and develop into free-living third stage larvae (L3) (2a, b). The free-living L3 are ingested by planktonic crustaceans (e.g. euphausiid oceanic krill and copepods) (intermediate/paratenic hosts) (3). Infected crustaceans are eaten by fish and cephalods (e.g. squid) and through predation the larvae are transferred from fish to fish (4, 5). When fish or squid containing L3 larvae are ingested by marine mammals, the larvae molt twice and develop into adult worms (6). Adult worms produce eggs to continue the life cycle. Humans become infected through eating L3-infected raw or undercooked fish or cephalods. Humans act as accidental hosts, since L3 usually do not develop any further and the cycle cannot be completed (7).
The L3 larvae of *Pseudoterranova* spp. have three well-defined lips, a boring tooth, an excretory pore ventral between ventral lips, no ventricular appendages, a well-developed intestinal caecum and a bluntly rounded tail with little mucron (Hurst, 1984). They are dark red in colour and measure 25 - 36 mm in length and up to 1.2 mm in width (Hurst, 1984).

### 1.6.3 Pathogenicity and clinical signs

Anisakid larvae found inside the fish are usually tightly coiled and found on the mesenteries, liver, and gonads and in the musculature of the body wall of infected fish. Studies have demonstrated that the presence of larvae in fish induces various pathophysiological changes including host cell necrosis in liver and muscle, reduced cell size, organ compression, gastrointestinal eosinophilic granulomas, proliferation of host cellular immune responses during hours of invasion and chronic inflammatory responses near the site of infected liver and muscles (Hauck and May, 1977; Elarifi, 1982; Heckmann, 1985).

In marine mammals, anisakids cause gastric lesions, ulceration, connective tissue proliferation, granulation, peritonitis and erode the gastric lining, which results in auto digestion of the stomach wall by the host (Spraker et al., 2003).

Human anisakidosis can take several forms depending on the location and histopathological lesions caused by the larva. In non-invasive infections, larvae remain in the alimentary canal without penetrating the mucosa wall and are only discovered when the worms are expelled by coughing, vomiting or defecating (Lymbery and Cheah, 2007). This form of infection is usually asymptomatic but occasionally produces a “tingling throat” syndrome when worms migrate back up the oesophagus into the oropharynx (Sakanari and McKerrow, 1989). Non-invasive infections are often due to *Pseudoterranova* sp. (Lymbery and Cheah, 2007).

In the invasive form of anisakidosis, larvae penetrate the alimentary tract and associated organs in infected people. Penetration of the buccal mucosa and pharyngeal mucosa occurs only rarely, by the larvae of both *Anisakis* and *Pseudoterranova* (Amin et al., 2000; Lymbery and Cheah, 2007). Symptoms of
oropharyngeal anisakidosis include slight pain, feelings of discomfort, and difficulty in swallowing (Amin et al., 2000). Penetration of the gastric or intestinal mucosa is the most common form of anisakidosis. It is associated with four major clinical signs including gastric anisakidosis, intestinal anisakidosis, ectopic (or extra-gastrointestinal) anisakidosis, and allergic diseases in infected people. Infections with *Anisakis* can result in both gastric and intestinal anisakidosis, while *Pseudoterranova* causes gastric invasions (Bouree et al., 1995). The symptoms of gastric anisakidosis occur 1 - 12 h after ingestion of infected fish and is associated with severe epigastric pain, nausea, vomiting, low grade fever and occasionally, a rash (Lymbery and Cheah, 2007; Hochberg and Hamer, 2010). Blood is often observed in gastric juices and stool (Sakanari and McKerrow, 1989). Acute gastric anisakidosis is often misdiagnosed and can become a chronic disease with clinical features, which are similar to peptic ulcers, gastric tumours, acute gastritis, and cholecystitis (Lymbery and Cheah, 2007). Intestinal anisakiasis usually manifests as an acute disease, occurring 5 - 7 days after ingestion of the anisakid larvae and is characterised by intermittent or constant abdominal pain, nausea, vomiting, fever, diarrhoea with blood (Lymbery and Cheah, 2007; Hochberg and Hamer, 2010). Ectopic (extra-alimentary) anisakidosis is less common and results from larval penetration of the gastrointestinal tract and migration in the body cavity where they usually lodge in the peritoneum or subcutaneous tissues, forming tumour-like, eosinophilic granulomas or abscesses (Lymbery and Cheah, 2007; Hochberg and Hamer, 2010). Associated symptoms include abdominal pain, vomiting and bloody stools (Lymbery and Cheah, 2007). Anisakidosis is often associated with strong allergic responses, with clinical symptoms ranging from isolated swellings to urticarial and life-threatening anaphylactic shock (Audicana and Kennedy, 2008; Alonso et al., 1999; Hochberg and Hamer, 2010).

### 1.6.4 Diagnosis

As symptoms (acute epigastric or abdominal pain) of gastrointestinal anisakidosis are nonspecific, clinical diagnosis requires careful examination of clinical symptoms and patient history of recent consumption of raw or incompletely cooked fish. Gastric anisakidosis is often misdiagnosed as peptic ulcer or stomach tumour,
while intestinal anisakidosis as appendicitis or peritonitis (Sakanari and McKerrow, 1989). Diagnosis of gastric anisakidosis includes endoscopy, which allows larvae to be removed and identified morphologically or genetically, and radiology, which can detect morphological changes in the host, such as thickened, narrowed and obstructed areas (Hochberg and Hamer, 2010). Intestinal anisakidosis is more complicated to diagnose and differential diagnosis is recommended (Hochberg and Hamer, 2010).

Worms isolated from fish samples can be identified on the basis of gross morphological features, which may be observed microscopically in live or preserved specimens. For examination, the use of clearing agents such as xylene may be necessary to make the nematode cuticle more transparent and a slide mount can be prepared. The morphological characters are used to distinguish adult worms include: (i) structures of labiae and ventriculus, (ii) the shape of a tail, (iii) the shape of lobes (Berland, 1961; Cannon, 1977; Olson et al., 1983).

Molecular tools such as PCR, PCR-based RFLP and single-strand conformation polymorphism are widely used for characterisation of anisakid species. The two most common targets are the rDNA ITS regions (Zhu et al., 1998; D’Amelio et al., 2000; Nadler et al., 2000; Pontes et al., 2005; Abe et al., 2006; Umehara et al., 2006; Zhu et al., 2007; Umehara et al., 2008; Kijewska et al., 2009b;) and the mt-DNA cytochrome oxidase subunit II (cox2) gene (Valentini et al., 2006; Mattiucci et al., 2009; Cavallero et al., 2011; D’Amelio et al., 2011; Murphy et al., 2010; Setyobudi et al., 2011). The rDNA ITS region is a more conserved target and provides a useful approach for the identification of both distantly and closely related ascaroid species as these spacers show interspecific sequence differences in the presence of low-level intraspecific variation (Zhu et al., 1998). On the other hand, mitochondrial DNA evolve at a faster rate than the rDNA ITS and therefore, provide useful information for phylogenetic reconstruction of closely related species of nematodes or for identification of cryptic species (Blouin, 1998, 2002).

1.6.5 Treatment and control

*Anisakis* and *Pseudoterranova* cannot survive in humans and will eventually die (Nakaji, 2009). Endoscopic treatment is the definitive treatment for gastric anisakidosis
(Hochberg and Hamer, 2010). Endoscopy allows the physician to remove the larva with the biopsy forceps and identify the worm morphologically. Early removal of worm may prevent the formation of eosinophilic granulomas caused by allergic reaction to the degenerating worm in chronic infections (Sakanari and McKerrow, 1989). For intestinal anisakiasis, recommended treatments include anthelmintics, isotonic glucose solutions and removal of tissue by surgery (Moschella et al., 2005, Pacios et al., 2005). Control measures for anisakidosis should focus on postharvest handling, storage and cooking procedures for fish (Lymbery and Cheah, 2007) and educating the public about the dangers of eating raw or inadequately cooked, marinated, or salted marine fish or squid (Hochberg and Hamer, 2010).

1.7 Chlamydia

1.7.1 Distribution and taxonomy

Members of the genus *Chlamydia* are gram-negative obligate intracellular bacteria, which are distributed globally and cause a variety of diseases including pneumonia, gastroenteritis, encephalomyelitis, conjunctivitis, arthritis, sexually transmitted diseases and abortion in man, other mammals and birds (Longbottom and Coulter, 2003). The first descriptions of chlamydial organisms were provided by Halberstaedter and von Prowazek in 1907, who described the transmission of trachoma from man to orangutans by inoculating their eyes with conjunctival scrapings (Halberstädter and von Prowazek, 1907a, b). It was thought that those organisms were protozoa and they were named ‘chlamydozoa’ after the Greek word ‘chlamys’ for mantle, because the reddish elementary bodies of the bacterium appeared to be embedded in a blue matrix or mantle (Longbottom and Coulter, 2003). Chlamydial infections in domestic mammals was first reported in 1936 following abortions in sheep in Scotland (Grieg, 1936). The aetiological agent was identified in 1950 by Stamp et al., and subsequently, the disease was termed enzootic abortion of ewes (EAE, synonym, ovine enzootic abortion or OEA) (Stamp et al., 1950; Stamp et al., 1952).

The taxonomy of *Chlamydiaceae* has recently undergone several reclassifications based on sequence analysis of the 16S and 23S ribosomal RNA and was divided into two genera, *Chlamydia* and *Chlamydophila* (Everett et al., 1999).
However, the use of the two-genus classification system, while initially helpful in distinguishing animal species in the genus *Chlamydia*, has not been widely adopted and indeed caused considerable confusion. Efforts are underway to confirm the "reunification" of the genus *Chlamydia* and in 2010, the subcommittee on the taxonomy of the Chlamydiae proposed the return to the combined genus *Chlamydia* (Greub, 2010). There are nine species in the genus *Chlamydia; Chlamydia trachomatis, C. muridarum, C. suis, C. pecorum, C. abortus* (formerly known as *Chlamydia psittaci* serotype 1), *C. pneumonia, C. psittaci, C. caviae* and *C. felis* (Everett et al., 1999). Of these, two species, *C. abortus* and *C. pecorum*, infect sheep and goats (Berri et al., 2009; Rodolakis and Yousef Mohamad, 2010; Lenzko et al., 2011;). Both *C. abortus* and *C. pecorum* are known to cause abortion in small ruminants with *C. pecorum* also known to cause enteric diseases in these animals (Berri et al., 2009; Rodolakis and Yousef Mohamad, 2010).

*Chlamydia abortus*, in particular, efficiently colonizes the placenta, leading to abortion or premature births in sheep and goats and financial losses in the affected flocks (Longbottom and Coulter, 2003). It is a most common cause of reproductive loss in sheep and goats worldwide due to abortion, especially, in Northern Europe (Stuen and Longbottom, 2011). In the United Kingdom, OEA is responsible for approximately 45 % of all diagnosed infectious cause of abortion (Stuen and Longbottom, 2011). Globally, the prevalence (mostly seroprevalence) of *C. abortus* in sheep and goats can vary drastically from < 5 % to > 50 % (Wang et al., 2001; Borel et al., 2004; Szeređi et al., 2006; Bagdonas et al., 2007; Cislakova et al., 2007; Masala et al., 2007; Czopowicz et al., 2010; Pinheiro Junior et al., 2010; Lenzko et al., 2011; Runge et al., 2012).

In addition to its effect on sheep and goat production, *C. abortus* is hazardous for pregnant women due to its zoonotic potential (Rodolakis and Yousef Mohamad, 2010). Zoonotic abortion due to *C. abortus* infection has been confirmed by genetic analysis of isolates from a woman who worked with sheep (Herring et al., 1987). Confirmed cases of abortions in people have also been reported in several countries including France (Villemonteix et al., 1990), the US (Jorgensen, 1997), the Netherlands
(Kampinga et al., 2000), Switzerland (Pospischil et al., 2002) and Italy (Walder et al., 2005).

*Chlamydia pecorum* was first associated with disease (sporadic bovine encephalomyelitis) in cattle in 1940 (McNutt, 1940). The name *Chlamydia pecorum* was given by Fukushi and Hirai in 1992 on the basis of genetic data when they isolated the organism from diseased cattle and sheep with clinical signs including sporadic encephalitis, infectious polyarthritis, pneumonia and diarrhoea (Fukushi and Hirai, 1992). *Chlamydia pecorum* infects a wide range of mammals including sheep, goats, cattle, pigs and koalas and causes a wide range of diseases such as enteritis, polyarthritis, conjunctivitis, pneumonia, encephalomyelitis, metritis, salpingitis, mastitis and infertility (Mohamad and Rodolakis, 2010). Its involvement in small ruminant abortion cases have been reported in France and in North African countries (Rodolakis and Souriau, 1989; Berri et al., 2009). Several studies have reported the prevalence of *C. pecorum*; in Germany, it was 47 % in sheep (Lenzko et al., 2011), while in Egypt, it was 6.7 % in sheep and 50 % in goats (Osman et al., 2011). The zoonotic potential of *C. pecorum* is unknown (Longbottom and Coulter, 2003; Robertson et al., 2010).

### 1.7.2 Life cycle and morphology

Species of *Chlamydia* have a unique life cycle, with alteration between two morphological forms, an elementary body (EB) and a reticulate body (RB) (Fig. 1-8) (Longbottom and Coulter, 2003). The EBs are small (approximately, 0.3 µm in diameter), round, electron dense, non-replicating, extracellular infectious forms of the organism (Matsumoto, 1973; Eb et al., 1976). The RBs are larger (0.5 - 1.6 µm in diameter) and their cytoplasm appears granular with diffuse, fibrillar nucleic acids (Plaunt and Hatch, 1988). RBs are non-infectious and they are bounded by an inner and outer-membrane resembling other gram-negative bacteria. Their surfaces are covered with projections and rosettes that extend from the bacterial surface through the inclusion membrane, similar to those on EBs but at a higher density (Matsumoto, 1982; Abdelrahman and Belland, 2005).
In pregnant animals, elementary bodies (EBs) enter the placental cells by endocytosis, inside a vacuole or inclusion body, which is formed out of the placental cell wall. EBs transformed into metabolically active reticulate bodies (RBs) that replicate by binary fission inside the inclusion body. When the inclusion body occupies most of the cell volume, the RBs transformed back to EBs, which are then released upon cell lysis and infect other cells.

The life cycle is initiated with the endocytosis of EBs by eukaryotic cells. On entering the host cell, RBs develop from EBs via numerous morphologically intermediate stages so that by 8 - 12 h after infection, almost pure RB populations are seen (Moulder, 1991). The mature RBs undergo multiplication by binary fission. The phagocytic membrane surrounding the ingested EB becomes the inclusion membrane, which now encloses the micrology of dividing RBs (Moulder, 1991). After division (12 - 24 h depending on the species), RBs revert back to metabolically inactive, infectious EBs, which are released by the cell through exocytosis (Longbottom and Coulter, 2003).

Species of *Chlamydia* are shed in faeces, urine, respiratory secretions, birth fluids or placentas of infected animals (Rodolakis and Yousef Mohamad, 2010).
Transmission of *C. abortus* in ruminants can occur from ingestion of placenta or grass contaminated by vaginal discharge and placenta (Rodolakis and Yousef Mohamad, 2010). The transmission of *C. abortus* to people generally occurs via inhalation of infectious dust and aerosols inducing infection of the mucosal epithelial cells and macrophages of the respiratory tract and eventually spread through blood stream to various organs (Rodolakis and Yousef Mohamad, 2010).

### 1.7.3 Pathogenicity and clinical signs

Chlamydial abortions in small ruminants are primarily caused by *C. abortus* and sometimes by *C. pecorum*, in combination with other co-factors such as nutrition, parasites or animal breed (Mohamad and Rodolakis, 2010). After *C. abortus* infects its host, it enters a latent phase where it causes no clinical signs and is undetectable (Jones, 1995). The animal host produces cytokines in response to chlamydial infection, specifically, interferon-gamma, which restricts the growth of the bacteria in host cells in a dose dependant manner (Entrican et al., 2001). During latency, it is thought that bacteria reside in the tonsils, from which they are spread by blood or lymph to other lymphoid tissues (Jones and Anderson, 1988). During pregnancy, it is thought that immune modulation releases the organisms from latency, allowing them to multiply, which in turn initiates placental infection (Longbottom and Coulter, 2003). Placental infection occurs at around day 60 of gestation, although pathological changes are not seen until after day 90 of gestation (Buxton et al., 1990). The chlamydial invasion of the placentome leads to aggressive inflammatory response, thrombotic vasculitis and necrotic damage to the cotyledons and intercotyledonary membranes of the placentome (Buxton et al., 1990; Longbottom and Coulter, 2003). Pathological changes in the foetus consist of focal necrosis of the liver, lung, spleen and less frequently, brain and lymph nodes (Buxton et al., 1990). The destruction of chorionic epithelium, associated lesions and impairment to the placentome are enough to compromise the maternal-foetal exchange of oxygen and nutrients, leading to foetal death (Longbottom and Coulter, 2003). In addition, placental chlamydial infection induces significant changes in hormone concentration, which can also contribute to premature labour (Longbottom and Coulter, 2003). The abortion of the foetus occurs around 2 - 3
weeks prior to the expected term (Longbottom and Coulter, 2003). Following abortion, the aborting females develop protective immunity that prevents them from aborting due to chlamydial organism in subsequent pregnancies (Kerr et al., 2005).

Clinical signs associated with *C. abortus* infection in ewes and does include abortion, stillbirth and retained placenta, which may lead to metritis (Rodolakis and Yousef Mohamad, 2010). Infected ewes and does show no clinical signs prior to abortion, although vaginal discharge is sometimes observed (Rodolakis and Yousef Mohamad, 2010). Lambs and kids born live from infected females may have arthritis, conjunctivitis and pneumonia (Rodolakis and Yousef Mohamad, 2010). In male animals, *C. abortus* can reach the seminal vesicles and cause epididymitis, while in non-pregnant female animals, infections generally develop into an asymptomatic form (Waldhalm et al., 1971). In people, clinical signs of *C. abortus* infection include malaise, influenza-like illness, mild dry cough and dyspnoea (Rodolakis and Yousef Mohamad, 2010). Pregnant women may experience lower abdominal pain, followed by abortion and sometimes severe complications such as acute renal failure, disseminated intravascular coagulation, or respiratory distress, necessitating mechanical ventilation (Rodolakis and Yousef Mohamad, 2010).

*Chlamydia pecorum* is commonly found in the digestive tract of healthy ruminants (Clarkson and Philips, 1997), however, some have been isolated from ovine and caprine abortion cases in Morocco, Egypt and France (Rekiki et al., 2004; Osman et al., 2011). The common clinical signs of *C. pecorum* are diarrhoea, conjunctivitis, arthritis, encephalitis, orchitis, and pneumonia in small ruminants (Fukushi and Hirai, 1992; Berri et al., 2009; Mohamad and Rodolakis, 2010). Also, *C. pecorum* can be spread from the intestines through blood circulation, as a result of unknown physiopathological changes, and may reach the placenta, where they induce abortion (Osman et al., 2011).

### 1.7.4 Diagnosis

There are two main approaches for diagnosing *Chlamydia* species in mammals: (i) by identification of the agent using smears, antigen detection, DNA, tissue sections
and isolation of the agent by cell culture, and (ii) by serological tests such as complement fixation test and ELISA. The choice of the test depends on the type of sample received (blood, placental membranes, foetal tissues, swabs), organism viability, presumptive diagnosis and clinical history (Sachse et al., 2009). A presumptive diagnosis of infection can be made if the abortion occurs in the last 2 to 3 weeks of gestation and by the presence of gross pathology of both the intercotyledonary membranes and the cotyledons upon examination of fresh placenta (Stuen and Longbottom, 2011). This can be confirmed by microscopic examination of smears made from infected cotyledons prepared using a modified Ziehl-Neelsen, Giemsa, Gimenez, or Machiavello procedure (Longbottom and Coulter, 2003; Sachse et al., 2009). Smears can also be made from vaginal swabs of females, which have aborted within the previous 24 h, or from the moist fleece of a freshly aborted or stillborn lamb (Longbottom and Coulter, 2003). *Chlamydia abortus* and rickettsia (*Coxiella burnettii*) have similar morphology and staining characteristics and therefore, further tests such as fluorescent antibody tests using a specific antiserum or monoclonal antibody are useful for identification of *C. abortus* in smears (Longbottom and Coulter, 2003). Antigen detection methods also include immunohistochemical staining of tissue sections using specific monoclonal antibodies against dominant chlamydial surface antigens (Stuen and Longbottom, 2011).

Serological tests based on complement fixation tests (CFT) may be used to detect a rise in antibody titre to *C. abortus* after abortion or stillbirth (Longbottom and Coulter, 2003). For this, paired blood samples taken at the time of abortion and then at least 3 weeks later is used for detection. However, because *C. abortus* shares common antigens with *C. pecorum* and some Gram-negative bacteria, detection using CFT can be problematic due to antigenic cross-reactivity (Longbottom and Coulter, 2003).

DNA-based techniques provide an alternative approach for verifying the presence of chlamydial organism. Over recent years, a variety of molecular tools including conventional PCR, qPCRs and DNA microarray technology, have been developed and used for specific detection of chlamydial organisms (Sachse et al., 2009). qPCR assays have been the preferred method in diagnostic laboratories because
of their rapidity, high throughput and ease of standardisation (Sachse et al., 2009). The
targets of these tools include 16S rRNA, 16-23S intergenic spacer region, 23S rRNA,
and the outer membrane protein gene (ompA) (Sachse et al., 2009).

1.7.5 Treatment and control

Chlamydiosis is usually treated with tetracyline. If ovine enzootic abortion is
suspected to be present in a flock of pregnant ewes, long-acting oxytetracycline (20
mg/kg body weight, intramuscularly) may be given to reduce severity of infection and
losses resulting from abortion (Longbottom and Coulter, 2003; Stuen and Longbottom,
2011). Treatment should be given soon after day 95 - 100 of gestation, when
pathological changes start to occur in the placenta, with subsequent treatment given
at 2 week intervals until the time of lambing (Longbottom and Coulter, 2003; Stuen
and Longbottom, 2011). Tetracyline does not effectively eliminate the infection nor
reverse any pathological damage in the animals, but reduces the disease in the flock
(Longbottom and Coulter, 2003).

In people with chlamydial infections, treatment includes supportive therapy
(fluids, oxygen), measures to combat toxic shock and administration of tetracyclines,
erthromycin and clarithromycin (orally or parenterally) depending on clinical severity
(Stuen and Longbottom, 2011).

For control, good hygiene such as hand washing is essential before tending to
other animals. *Chlamydia* is shed in faeces, urine, respiratory secretions, birth fluids,
and placentas of infected symptomatic and asymptomatic animals and as EBs do not
survive very long, they require close contact for transmission (Rodolakis and Yousef
Mohamad, 2010). After abortion, the infected ewes or does should be immediately
isolated, all dead newborns, placentals and bedding safely disposed of and lambing
pens cleaned and disinfected to limit the risk of spreading contamination (Longbottom
and Coulter, 2003). Pregnant women should avoid contact with pregnant or aborting
ruminants and, if possible, sheep or goats if OEA is suspected in a flock.
1.8 Aims of the present study

Given the lack of knowledge of parasitic infections in sheep, goats and fish in PNG, the main objectives of this thesis were to:

(i) Investigate the prevalence of gastrointestinal parasites in faecal samples from sheep and goats by light microscopy;
(ii) Detect *Haemonchus contortus*, *Teladorsagia circumcincta*, and *Trichostrongylus* spp. in faecal samples of sheep and goats using molecular tools;
(iii) Detect *Eimeria* spp. in faecal samples of sheep and goats using molecular tools;
(iv) Investigate the prevalence and perform molecular characterisation of *Cryptosporidium* spp. and *Giardia* spp. in sheep, goats and fish;
(v) Investigate the prevalence and characterise the species of anisakid nematodes in fish using morphological and molecular tools; and
(vi) Investigate the prevalence of *Chlamydia abortus* and *C. pecorum* in faecal samples of sheep and goats using molecular tools.
Chapter 2

Materials and Methods
2.1 General background of the study

The samples for the present study were collected in various locations in PNG (Fig. 2-1). Most of the laboratory components of this study were conducted at Murdoch University, but some analyses were carried out at Divine Word University (DWU) in Madang, PNG and at the University of Western Australia, Perth, Australia. Specifically, coprological analyses of faecal samples from sheep and goats were performed at DWU in April 2011 and scanning electron microscopy of anisakid larvae was conducted at the Centre for Microscopy Characterisation and Analysis (CMCA) at the University of Western Australia in May 2012. Light microscopy analysis of anisakid larvae and histological analysis of fish tissues were conducted at the School of Veterinary and Life Sciences at Murdoch University. All molecular work was conducted in the State Agriculture and Biotechnology Centre (SABC) laboratory at Murdoch University between September 2011 and October 2013.

2.2 Human and animal ethics approval

A questionnaire survey for sheep and goat farmers was approved by Murdoch University Human Research Ethics committee (Permit/Project no: 2010/174). Faecal sample collection from sheep and goats and collection of fish was approved by Murdoch University Animal Ethics Committee (Permit no: R2368/10).

2.3 Sampling

2.3.1 Study locations for sheep and goats

PNG has extremely variable agro-ecological conditions, although, it is located in the tropics. It has at least three broad climatic zones, namely, wet lowland (< 1, 200 m), dry lowland (< 1, 200 m) and moderately wet highland (> 1, 200 m) areas (Quartermain, 2004b). The wet lowland areas receive from 2,000 - 5,000 mm of rainfall annually, while dry lowland areas receive less than 2,000 mm of rainfall and are characterized by pronounced dry periods of up to six months of the year (Quartermain, 2004b). The highland areas exhibit temperate climate with occasional frosts above 1,800 m and rainfall between 2,000 and 2,300 mm with high seasonality (Quartermain, 2004b).
The study locations for sheep and goats were chosen because they represented the largest flocks in the country (Fig. 2-1). As there was no available demographic information on the small ruminant farms, the farms were chosen based upon consultation with agricultural scientists at the National Agricultural Research Institute (NARI).

**Figure 2-1: Study locations for sheep, goats and fish in the present study.** The dots on the map represent sites where sheep and goats were collected. Research institutional farms were in Labu (insert 1) and Tambul (insert 6). The public institutional farm was in Baisu (insert 6). The government farm was in Menifo (insert 5). Smallholder farms were in Chuave (insert 4); Daulo, Goroka, Benabena and Korefeigu (insert 5). The stars represent sites where fish were collected. Cultured freshwater fish were obtained from ponds in Bathem (insert 2), Kundiewa (insert 4), Asaro (insert 5) and Mumeng (insert 1). Wild freshwater fish were collected in Ramu River near Sausi (insert 3) and Sepik River near Pagwi (insert 2). Wild marine fish were collected in Bilbil and Madang (insert 3), and Pilapila and Tavana (insert 7).
The chosen study locations were located in two broad agro-climatic regions:

- the warm and wet lowland area. Specific study site is Labu in Morobe Province.
- the cool and moderately wet highlands region. Specific study sites are Korefeigu, Benabena, Menifo, Goroka and Daulo in Eastern Highlands Province (EHP); Chuave in Simbu Province; and Baisu and Tambul in Western Highlands Province (WHP).

The study locations were divided into 5 groups based on the farm management systems (Table 2-1). These include:

(i) Lowland research institutional farm (Labu),  
(ii) Highland research institutional farm (Tambul),  
(iii) Public institutional farm (Baisu),  
(iv) Government station farm (Menifo),  
(v) Smallholder farms (Benabena, Korefeigu, Goroka, Daulo and Chuave).

Table 2-1: Sampling locations and farm information for the chosen sheep and goat flocks in the present study. The combined numbers of sheep and goats at the time of sampling in Labu, Baisu, Tambul and Menifo were 125, 70, 143 and 55, respectively. Smallholder farmers kept 3 - 19 animals per herd. The asterisk (*) refers to flocks that graze free range over a wide area near road-sides, gardens, hills etc. References for climate data: Quartermain, 2004b and Bourke, 2010. S = sheep, G = goats, O = others (such as pigs, dogs, chickens, or cattle).

<table>
<thead>
<tr>
<th>Study locations</th>
<th>Mean altitude (m)</th>
<th>Mean annual temperature (°C)</th>
<th>Mean annual rainfall (mm)</th>
<th>Farm ownership</th>
<th>Farm size (ha)</th>
<th>Animals on property</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labu</td>
<td>0</td>
<td>26</td>
<td>&gt; 4000</td>
<td>Research</td>
<td>20 - 60</td>
<td>S, G, O</td>
</tr>
<tr>
<td>Tambul</td>
<td>2320</td>
<td>13.8</td>
<td>3000</td>
<td>Research</td>
<td>20 - 60</td>
<td>S, G</td>
</tr>
<tr>
<td>Menifo</td>
<td>1608</td>
<td>20.1</td>
<td>1000 - 1500</td>
<td>Government</td>
<td>20 - 60</td>
<td>S, G, O</td>
</tr>
<tr>
<td>Baisu</td>
<td>1730</td>
<td>18.3</td>
<td>3000</td>
<td>Public</td>
<td>20 - 60</td>
<td>S, G</td>
</tr>
<tr>
<td>Chuave</td>
<td>1530</td>
<td>20.4</td>
<td>2000</td>
<td>Smallholder</td>
<td>Varies*</td>
<td>G, O</td>
</tr>
<tr>
<td>Goroka</td>
<td>1600</td>
<td>20.1</td>
<td>1500</td>
<td>Smallholder</td>
<td>Varies*</td>
<td>S, G, O</td>
</tr>
<tr>
<td>Korefeigu</td>
<td>1600</td>
<td>20.1</td>
<td>1500</td>
<td>Smallholder</td>
<td>Varies*</td>
<td>S, G, O</td>
</tr>
<tr>
<td>Benabena</td>
<td>1600</td>
<td>20.1</td>
<td>1500</td>
<td>Smallholder</td>
<td>Varies*</td>
<td>S, G, O</td>
</tr>
</tbody>
</table>

2.3.2 Study locations for fish

Fish were collected from four cultured freshwater ponds, two large river systems and marine environments in various locations throughout PNG (Fig. 2-1). Both
of the river systems are widely used by people for transportation and for sourcing fish. For marine fish, waters near the township of Madang (Madang province) and Rabaul (East New Britain province) were chosen, as they are centrally located and relatively easily accessible.

Specifically, these locations included:

- Mumeng in Morobe Province - a cultured freshwater pond and hatchery that actively distributes fingerlings to local customers,
- Kundiawa in Simbu Province - a cultured freshwater fish-pond, which was built in the centre of the town,
- Asaro in EHP - a cultured freshwater fish-pond, which was set up in a forested area just outside the village,
- Betham in East Sepik Province - a cultured freshwater fish-pond, which was built in the grassland area near the village,
- Sepik River near Pagwi in East Sepik Province for wild freshwater fish,
- Ramu River near Sausi in Madang Province for wild freshwater fish,
- Madang-coastal areas near Madang town, Madang Province for wild marine fish,
- Bilbil, Madang Province for wild marine fish,
- Pilapila, East New Britain (ENB) Province in for wild marine fish, and
- Tavana, ENB Province for wild marine fish.

2.4 Survey questionnaire

The sheep and goat owners/managers completed questionnaires regarding farm management and animal health programs for their flocks (sample questionnaire form is attached in Appendix A).

Farm management

The flocks from the three institutional farms (Labu, Baisu and Tambul) and the government farm (Menifo) grazed pasture in fenced areas (20 - 60 ha) at daytime. At night-time, the flocks were kept in houses with wooden, slatted floors in institutional
farms and on the ground in the government farm. At the time of sample collection, the combined numbers of sheep and goats in Labu, Tambul, Baisu and Menifo were 125, 143, 70 and 55, respectively. The subsistence farmers kept few animals, usually less than 20, which grazed free range or were tethered and housed at night on slatted floors or on the ground underneath the farmer’s house (Fig. 2-2).

Feeding system

Most animals grazed on native grasses and shrubs. Smallholder farmers also fed their animals with starchy vegetables, mostly sweet potatoes. The interviewed farmers also indicated that there were feed shortages due to shortage of pasture for grazing, fires or drought. The animals drank from troughs, sourced from main water supplies or rainwater tanks, rainwater run-off or ponds.

Herd health programs

Hygiene and management levels for animal housing were sub-optimal. For example, the floors of the houses where animals were kept at night-time were unclean. The animals were penned on dirty floors, bare ground or on bare concrete floors. The smallholder farmers did not shear their sheep as they did not have the resources for it. Most farm managers reported that the most common signs of illness in their animals were diarrhoea and coughing, followed by itching and hair loss. Most smallholder farmers did not know about causes of diseases in their sheep and goats or know about or use anthelmintic drugs for controlling gastrointestinal parasite infections. For instance, a farmer in Benabena reported the death of his entire flock of sheep ($n = 25$) and noticed nematode worms in the gut of a dead sheep. The three large institutional flocks were drenched with benzimidazole (Panacur) nominally at bimonthly intervals. At the time of sampling animals had been drenched two months previously in Labu, four months previously in Baisu and Tambul and six months previously in Menifo.
Figure 2-2: Pictures of sheep and goat farming in PNG. A. Staffs of National Agriculture Research Institute (NARI) in Tambul. B. Lambing pens at NARI, Tambul. C. Sheep tethered and housed under the owner’s house in Goroka. D. A subsistence farmer tethering her goats in Daulo. E. A wooden slatted floored night house for sheep and goats in Korefeigu. F. Sheep and goats housed together in a smallholder farm in Benabena.
Farm types

The flocks at Labu and Tambul are managed by the NARI. Both had relatively high numbers of animals (Labu = 125, Tambul = 143). Labu is in Morobe Province, which is located in a humid lowland area with very high rainfall. The flocks in Labu are made up of PNG Priangan sheep and PNG goat genotype, which possibly came from the PNG University of Technology in Lae and Erap in Markham, which were established in 1970-80s. Tambul’s climate is wet and cool with high rainfall per annum (2500 mm). The Highlands Halfbred sheep flock in Tambul was thought to have been established from flocks from Aiyura and Menifo and PNG genotype goats from Simbu Province.

The flocks at Baisu are managed by a correctional service (a government institution). The foundation flock for Baisu was provided from Tambul. There were approximately 70 sheep and goats available during sample collection.

Menifo is a government research and breeding station for sheep. It was established in 1975. It had 55 sheep and 15 goats at the time of sampling.

The flocks in Benabena, Goroka, Korefeigu, Daulo and Chuave are owned by smallholder farmers. These flocks were thought to have been established from flocks in Menifo and Aiyura government breeding stations. Smallholder flocks were established much earlier than those in research institutional farms (Labu and Tambul) and consisted of Highlands Halfbred sheep and PNG genotype goats.

2.5 Animals

2.5.1 Sheep and goats

For the present study, a total of 504 faecal samples were collected directly from the rectum of sheep (n = 276) and goats (n = 228) and stored in 25 mL screw top plastic containers (Table 2-2). Most animals sampled were adults. No lambs or goat kids were sampled as only a few were present during the sampling period and it was difficult to obtain faecal samples from them. During sampling, the following factors were recorded for each animal; faecal moisture and consistency and clinical symptoms such as active versus lethargic, bright pink versus pale mucous membranes, coughing,
chest congestion and diarrhea. Almost all the faeces collected were hard, dry pellets or soft moist pellets. On a few occasions, liquid/fluid faeces were encountered but they were either too small in quantity or too watery and difficult to collect.

Table 2-2: Number (N) of sheep and goat samples collected in the present study.

<table>
<thead>
<tr>
<th>Type of farm</th>
<th>Sheep (N)</th>
<th>Goat (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Research Institute (Labu)</td>
<td>40</td>
<td>43</td>
</tr>
<tr>
<td>Research Institute (Tambul)</td>
<td>115</td>
<td>35</td>
</tr>
<tr>
<td>Public Institution (Baisu)</td>
<td>27</td>
<td>39</td>
</tr>
<tr>
<td>Government Station (Menifo)</td>
<td>40</td>
<td>6</td>
</tr>
<tr>
<td>Smallholder</td>
<td>54</td>
<td>105</td>
</tr>
<tr>
<td>total</td>
<td>276</td>
<td>228</td>
</tr>
</tbody>
</table>

Handling of faecal samples

All faecal samples were processed in a NARI laboratory in Labu, Morobe Province. Samples that were collected from the flocks in Labu were taken directly to the laboratory and processed within a day. In contrast, samples collected from the rest of the study sites were kept in ice-filled Coleman Coolers (for up to 10 days), until transported to the laboratory for processing. At Labu, each faecal sample from a 25 mL screw top container was placed into a 2 mL Eppendorf tubes and preserved in 70 % ethanol for molecular screening. Excess samples were left in the 25 ml container for morphological analyses by a faecal flotation technique (Whitlock, 1948). All samples were kept at 4 °C until shipment or analysis.

2.5.2 Fish

A total of 614 fish from cultured freshwater, wild freshwater and wild marine environments were collected (Fig. 2-3). Cultured fish (n = 133) included three species, which were collected from four smallholder cultured freshwater fish-ponds in Mumeng, Asaro, Kundiawa and Bathem (Table 2-3).
Figure 2-3: Pictures of fishing activities in PNG. A. A fish-pond in Bathem village. B. A fish farmer in Sausi. C. Freshwater fish market in Pagwi near the Sepik River. D. The field team processing fish near a river in Bathem. E & F, Toilet huts over the sea and the estuary, respectively, in Madang.
Wild freshwater fish \((n = 205)\) included six species and were collected from Ramu and Sepik Rivers, while 276 fresh marine fish, consisting of 16 species were bought from local fishermen in Bilbil, Madang, Pilapila and Tavana (Table 2-3).

**Table 2-3: Cultured freshwater, wild freshwater and marine fish species collected in the present study.**

<table>
<thead>
<tr>
<th>Cultured freshwater fish</th>
<th>Locations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kundiawa</td>
</tr>
<tr>
<td>Nile tilapia ((Oreochromis niloticus))</td>
<td>36</td>
</tr>
<tr>
<td>Common carp ((Cyprinus carpio))</td>
<td>-</td>
</tr>
<tr>
<td>Mozambique tilapia ((Oreochromis mossambicus))</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>36</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Wild freshwater fish</th>
<th>Locations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ramu River</td>
</tr>
<tr>
<td>Silver barb ((Puntius gonionotus))</td>
<td>13</td>
</tr>
<tr>
<td>Highfin catfish ((Neoarius berneyi))</td>
<td>-</td>
</tr>
<tr>
<td>Mozambique tilapia ((Oreochromis mossambicus))</td>
<td>-</td>
</tr>
<tr>
<td>Pacu ((Colossoma bidens))</td>
<td>-</td>
</tr>
<tr>
<td>Indo-Pacific tarpon ((Megalops cyprinoids))</td>
<td>-</td>
</tr>
<tr>
<td>Redtail catfish ((Phractocephalus hemioliopterus))</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>13</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Wild marine fish</th>
<th>Bilbil</th>
<th>Madang</th>
<th>Pilapila</th>
<th>Tavana</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bigeye scad ((Selar crumenophthalmus))</td>
<td>46</td>
<td>-</td>
<td>60</td>
<td>-</td>
<td>106</td>
</tr>
<tr>
<td>Bigeye trevally ((Caranx sexfasciatus))</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>Blackfin barracuda ((Sphyraena qenie))</td>
<td>9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9</td>
</tr>
<tr>
<td>Coachwhip trevally ((Carangoides oblongus))</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Indo-Pacific tarpon ((Megalops cyprinoids))</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mackerel scad ((Decapterus macarellus))</td>
<td>5</td>
<td>-</td>
<td>24</td>
<td>-</td>
<td>29</td>
</tr>
<tr>
<td>Oblong silver biddy ((Gerres oblongus))</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>54</td>
<td>55</td>
</tr>
<tr>
<td>Oriental bonito ((Sarda orientalis))</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>Pinjalo snapper ((Pinjalo pinjalo))</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Rainbow runner ((Elagatis bipinnulatus))</td>
<td>-</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>Reef needlefish ((Strongylura incisa))</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>Slender pinjalo ((Pinjalo lewisi))</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Spanish mackerel ((Scomberomorus maculatus))</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Talang queenfish ((Scomberoides commersonnianus))</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Wahoo ((Acanthocybium solandri))</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Yellow fin tuna ((Thunnus albacares))</td>
<td>-</td>
<td>34</td>
<td>-</td>
<td>-</td>
<td>34</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>69</strong></td>
<td><strong>51</strong></td>
<td><strong>87</strong></td>
<td><strong>69</strong></td>
<td><strong>276</strong></td>
</tr>
</tbody>
</table>
On average, the time when fish were at the stalls before being sold and the time lag between purchasing and processing the fish was approximately 4 h. The only live fish collected were from the aquaculture farms. These were euthanized by ice slurry. Length and weight of each fish were measured. Fish were necropsied, checked for worms under white light and further dissected. The stomach and intestinal walls were removed using a scalpel blade. Parts of the gut (stomach and intestinal walls) tissues were placed into 2 mL Eppendorf tubes and preserved in 70 % ethanol for molecular screening. Remaining stomach and intestinal tissues were fixed in 10 % buffered formalin for histological analysis. The stomach and intestinal walls were collected because protozoan parasites (Cryptosporidium and Giardia) attach to the surface epithelia of these regions of the gut and cause a range of histological abnormalities (Chen et al., 2012). All samples were stored at 4 °C in PNG until sample collection was completed.

2.6 Shipment of biological samples

Preserved samples were packaged according to the International Air Transport Association (IATA) standard of shipment of biological samples and transported by air to Perth, Western Australia. Appropriate permits were obtained in PNG for export and in Australia for import.

2.7 Parasitology

2.7.1 Faecal worm egg count

All faecal worm egg counts were performed in Madang, PNG, using a modified McMaster technique (Whitlock, 1948). For this method, approximately, 2 g of faeces were weighed and placed in a 60 mL mixing jar and 2.5 mL of tap water was added to soften the faecal pellets. The faeces were mashed up using a pair of forceps and allowed to soak for more than 1 h. A flotation salt solution (saturated sodium chloride solution with specific gravity 1.20 – 1.25) was added to the sample to make a final volume of 50 mL solution. The faecal suspension was mixed vigorously using a tongue depressor until a homogenous solution was obtained. Using a pipette, 0.6 mL of solution were drawn out from the center of the mixed faecal suspension and added to
the counting chamber of a Whitlock Paracytometer slide. The eggs were allowed 10 minutes (maximum time) to float up under the glass before counting at 40x magnification using a light microscope (Zeiss Axio Scope). All visible eggs within the double line boundaries of the Whitlock Paracytometer counting chamber were identified and recorded.

Parasites were identified morphologically to genus, or (in the case of pathogenic trichostrongyles) to superfamily level (superfamily = Trichostrongyloidea) using published keys (Soulsby, 1965) (Fig. 2-4). Parasite egg load was expressed as eggs per gram faeces (EPG). The volume of the flotation fluid used in the examinations was 50 mL and the fluid volume examined in the counting chamber was 0.3 mL. Since EPG is calculated using Equation [1], the minimum EPG count in our analyses was 83 eggs per gram and occurred when only one egg was observed in the counting chamber.

\[ EPG = \frac{\text{eggs counted} \times \text{total volume [mL]}}{(\text{examined volume [mL]} \times \text{weight of faeces [g]})} \]  

[1]

Figure 2-4: Trichostrongylid egg (left) and Eimeria oocyst (right) at 40x magnification.

2.7.2 Morphological analysis of anisakid nematodes

Whole nematodes were cleared in lactophenol for more than 48 h and individually mounted onto microscope slides. The body lengths of the nematodes were directly measured. Images were taken with an Olympus BX50 light microscope equipped with Olympus DP70 Camera at 40x and 100x magnification. The following features were measured: body width, oesophagus length, ventriculus length and mucron length. Morphological identification was conducted according to keys previously reported (Berland, 1961; Cannon, 1977; Shamsi et al., 2009a, b).

Scanning electron micrographs (SEMs) were taken for representative specimens to study further morphological details. SEMs were obtained on a Phillips
XL30 scanning electron microscope at the Centre for Microscopy Characterisation and Analysis (CMCA) at the University of Western Australia. For SEM, parasite samples were fixed in 2 % glutaraldehyde and 1 % paraformaldehyde in phosphate buffer saline (PBS) for 60 minutes at 4 °C and washed twice with PBS (pH = 7.4) in 1.5 mL Eppendorf tubes. Samples were dehydrated using a PELCO Biowave microwave processor (TedPella Inc., Redding, CA, USA) by passage through increasing ethanol concentrations in water (33 %, 50 %, 66 % and 100 %), followed by two washes in dry acetone. Samples were then dried in a critical point dryer (Emitech 850, Quorum Technologies, Ashford, UK), attached to aluminium sample holders and coated with a 5 nm thick platinum coating to enable surface electrical conduction.

2.8 DNA isolation

2.8.1 Isolation of genomic DNA from faeces

Genomic DNA was extracted directly from faeces using a PowerSoil® DNA isolation kit (MO BIO laboratories, Carlsbad, California, USA). The kit is designed to remove faecal PCR inhibitors, which potentially increases the DNA quality. Approximately, 0.25 grams of each faecal sample were placed in a Power bead tube and then mixed by vortexing. To ensure complete lysis of any Cryptosporidium oocysts that may have been present, five freeze thaw cycles were conducted, which consisted of dipping the sample into liquid nitrogen for approximately 10 seconds until frozen and then incubating at 95 °C until thawed. Sixty microliters of solution C1 were then added to the Power bead tubes containing the sample and the mixture was again mixed by vortexing. The Power bead tube was secured horizontally onto a flat-bed vortex pad with tape and vortexed at maximum speed for 10 minutes and then centrifuged at 10, 000 x g for 30 seconds at room temperature. The supernatant was then transferred to a clean 2 mL collection tube. To this, 250 µL of solution C2 were added and the contents were mixed by vortexing for 5 seconds and incubated at 4 °C for 5 minutes. After incubation, the tube was centrifuged at room temperature for 1 minute at 10, 000 x g and approximately 600 µL of supernatant was transferred to a clean 2 mL collection tube and 200 µL of solution C3 were added to it. The tubes were vortexed briefly and incubated at 4 °C for 5 minutes. Following incubation, the tubes
were centrifuged at room temperature for 1 minute at 10,000 x g. Up to 750 µL of supernatant were collected in a clean 2 mL collection tube and 1,200 µL of solution C4 were added to the supernatant. The solutions were mixed by vortexing for 5 seconds. Approximately, 675 µL of the supernatant mixture were loaded onto a spin filter, which captures the DNA. The tube was centrifuged at 10,000 x g for 1 minute at room temperature. The flow-through was discarded. An additional 675 µL of supernatant mixture were loaded onto the spin filter and the process was repeated until all the remaining supernatant were loaded onto the spin filter and centrifuged. This was followed by an addition of 500 µL of solution C5 (ethanol wash solution) to rinse the captured DNA by centrifugation at room temperature for 30 seconds at 10,000 x g. The flow-through was discarded. The spin filter tubes were centrifuged again and the spin filter was placed in a clean 2 mL collection. To concentrate the DNA, 50 µL of solution C6 (sterile DNA elution buffer) (instead of the recommended 100 µL) was then added to the centre of the white filter membrane of the spin filter, and the tube was centrifuged at room temperature for 30 seconds at 10,000 x g to elute the DNA. The spin filter was discarded and the tube containing the DNA was stored at -20 °C.

2.8.2 Isolation of genomic DNA from anisakid larvae

A Qiagen DNeasy® blood and tissue kit (Catalogue number: 69504, Qiagen, Hilden, Germany) was used to isolate DNA from the anisakid nematodes analysed in the present study. The kit contains InhibitEX® tablets that remove PCR inhibitors from the DNA. Also, the kit includes a tissue lysis step using the enzyme, Proteinase K, to digest protein and remove contamination from preparations of DNA. The protocol (spin-column protocol) for the purification of total DNA from animal tissues on page 28 of the DNeasy® blood and tissue handbook was followed with minor modifications.

Prior to DNA extraction, each nematode larva was cut into small pieces to enable more efficient lysis. Approximately, 25 mg of cut sample was placed in a 1.5 mL microcentrifuge tube and 180 µL Buffer ATL and 20 µL Proteinase K were added. The sample was mixed thoroughly by vortexing and incubated at 56 °C for 3 h for complete tissue lysis. Several times during incubation, the tube was vortexed briefly to disperse the sample. After incubation, the tube was vortexed for 15 seconds followed by
addition of 200 μL Buffer AL and thorough mixing by vortexing. The mixture was pipetted into the DNeasy® Mini spin-column within a 2 mL collection tube. The tube was centrifuged at 10,000 x g for 1 minute. The flow-through and collection tube was discarded. The DNeasy® Mini spin-column was placed in a clean 2 mL collection tube. Five hundred μL of buffer AW1 were loaded onto the DNeasy® Mini spin-column and centrifuged at 10,000 x g for 1 minute. Again, the flow-through and the collection tube were discarded. The DNeasy® Mini spin-column was placed in another clean 2 mL collection tube and 500 μL of buffer AW2 were loaded onto it. The column was centrifuged at 20,000 x g for 3 minutes. Again the flow-through and the collection tube were discarded. The DNeasy® Mini spin-column was placed in a clean 1.5 mL collection tube and 50 μL (instead of the recommended 200 μL) of buffer AE were loaded directly onto the DNeasy® Mini spin-column membrane. The tube was incubated at room temperature for 1 minute, and then centrifuged at 10,000 x g for 1 minute to elute the DNA. All extracted samples were stored at -20 °C until screening.

2.8.3 Isolation of genomic DNA from fish gut scrapings

Preserved intestines and stomachs were washed 5 times with water to remove the ethanol and scraped using a scalpel blade. DNA was isolated from 0.25 grams of the tissue scrapings using a PowerSoil® DNA isolation kit (MO BIO laboratories, Carlsbad, California, USA) as described in Section 2.8.1. DNA was eluted in 50 μL of elution buffer. All extracted samples were stored at -20 °C until screening.

2.9 DNA amplification

As a precaution and to avoid contamination with PCR product, the PCR reactions were prepared in a designated pre-PCR area (separated from amplification areas). PCR contamination controls were used including negative controls (no DNA). Quantitative PCRs were performed on a Rotor-Gene 6000 thermocycler (Corbett Research, Victoria, Australia) for detection of trichostrongyles, *Giardia* and *Chlamydia* and on a Roche LightCycler® 480 thermocycler (Roche, Castle Hill, NSW, Australia) for *Eimeria*. All single step and nested PCRs were performed in a Perkin Elmer Gene Amp PCR 2400 thermocycler (Perkin Elmer, Foster City, CA, USA). The magnesium chloride (MgCl₂) and deoxynucleotide triphosphates (dNTPs) were ordered from (Fisher
Biotech, Wembley, WA, Australia). All probes were synthesised by Biosearch Technologies (Novato, CA, USA) and primers were ordered from Invitrogen™ (http://www.lifetechnologies.com/au/en/home/brands/invitrogen.html).

2.9.1 Molecular detection of trichostrongylid nematodes by qPCR

A previously developed multiplex quantitative PCR (qPCR) assay was used (Yang et al., 2014e). Primers and probe for *Haemonchus* were modified from Harmon et al. (2007a). A fragment (92 bp) of the *Haemonchus* rRNA ITS1 region was amplified using the forward primer HC-ITS F1 (5'-CATATACATGCAACGTGATGTATGAA-3'), the reverse primer HC-ITS R1 (5'-GCTCAGGTGCATTACAAATGATAAA-3') and the probe (5'-FAM-ATGGCGACGATGTTC-BHQ1-3'). The *Haemonchus* probe was prepared by substitution of C-5 propynyl-dC (pdC) for dC and C-5 propynyl-dU (pdU) for dT to enhance base pairing and duplex stability. A fragment (143 bp) of the *Teladorsagia* ITS2 region was amplified using the primers TC-ITS2 F1 (5'-TCTGGTTCAGGGTTAATGAAACTA-3') and TC-ITS2R1 (5'-CCGTCGTACGTCATGTTGCAT-3') and the probe (5'-CAL Fluor Orange 560–TGTGGCTAACATATAACACTGTTTGTCGA-BHQ1-3'). A fragment of *Trichostrongylus* ITS1 region (114 bp) was amplified using TS ITS1 F1 (5'-AGTGCGCCTGTTGATTGAAACTA-3') and TS ITS1 R1 (5'-TGCGTACTCAACCACCACCA-3') and the probe (5'-CAL Fluor Red 610–TGCGAAGTTCCCATCTATGATGGTGA-BHQ2-3').

An internal amplification control (IAC) was included in this qPCR assay to monitor faecal inhibition. An IAC is a non-target DNA sequence present in the same sample reaction tube which is co-amplified simultaneously with the target sequence (Hoorfar et al., 2003). Inhibition can be due to malfunction of the thermal cycler, incorrect PCR mixture, poor polymerase activity and the presence of inhibitory substances in the sample matrix (Hoorfar et al., 2003). Thus, in a PCR with an IAC, a control signal will always be produced even when there is no target sequence present and when neither IAC signal nor target signal is produced, the PCR has failed.

In this PCR assay, an IAC consisting of a fragment of a coding region from Jembrana Disease Virus (JDV) cloned into a pGEM-T vector (Promega, Madison, WI, USA) was used, as previously described (Yang et al., 2013). The IAC primers were JDVF (5'-GGTAGTGCTGAAAGACATT-3') and JDVR (5'-ATGAGCTTGACCGGAAGT-3') and the
probe was 5'-Cy5-TGCCGCTGCGTACGAGG -BHQ2-3' (Yang et al., 2013). Inhibition in faecal samples was measured using the IAC, as the IAC was added to all faecal DNA samples to detect any PCR inhibitors present in the extracted DNA. If any inhibition is present in a sample, the IAC will not produce a signal.

The total volume of this assay was 15 μL and contained 3 mM MgCl₂, 1 μL of 2.5 nM dNTPs, 1x PCR buffer, 1.0 U Kapa DNA polymerase (Kapa Biosystems, Cape Town, South Africa), 0.2 μM each of forward and reverse primers, 0.1 mM probe, 0.2 μM each of forward and reverse IAC primers, 50 nM of the probe, 50 nM of IAC probe, 10 copies of IAC template and 1 μL of sample DNA. The PCR cycling conditions consisted of a pre-melt at 95 °C for 3 minutes and then 45 cycles of 95 °C for 30 seconds, and a combined annealing and extension step of 60 °C for 45 seconds.

2.9.2 Molecular detection of *Eimeria* spp. by qPCR and nested PCR.

For molecular detection of *Eimeria*, a subset of samples from sheep and goats were initially screened at the 18S rRNA locus by qPCR and then genotyped using a nested PCR.

**qPCR for *Eimeria***

A qPCR assay was used to screen for *Eimeria* species in faecal samples of sheep and goats at the 18S rRNA locus as previously described (Yang et al., 2014d). The assay consisted of a forward primer, Eim F1 (5’-CGACTTGCTATTAAAACCACTATAGTT-3’), a reverse primer, Eim R1 (5’-CGCATGTAAGCCATAGATAACCA-3’) and a probe (5’-Joe-ATGTCTCTTCTACATGGA-BHQ1-3’), which produced an 85 bp product (Yang et al., 2014d). An internal amplification control (IAC) was included to check if any inhibition was occurring in the sample (Yang et al., 2013) (see Section 2.9.1).

The total reaction volume in this assay was 15 μL. The reaction mixture contained 5 mM MgCl₂, 1 mM dNTP, 1x PCR buffer, 1.0 U Kapa DNA polymerase (Kapa Biosystems, Cape Town, South Africa). 0.2 μM each of forward and reverse primers, 0.2 μM each of forward and reverse IAC primers, 50 nM of the probe, 50 nM of IAC probe, 10 copies of IAC template and 1 μL of sample DNA (Yang et al., 2014d). The PCR cycling conditions consisted of a pre-melt at 95 °C for 3 minutes and then 45 cycles of
95 °C for 30 seconds, and a combined annealing and extension step of 60 °C for 45 seconds.

**Eimeria 18S rDNA PCR-Sequencing**

A nested PCR technique was used to amplify a subset of samples, which were positive for *Eimeria* by qPCR at the 18S rRNA locus. The primers used for the amplification of the 18S rRNA were those developed by Relman et al. (1996). For the primary PCR, the forward primer was CYCF1E (5’-GGAATTCCTACCCAAATGAAACAGTTT-3’) and reverse primer was CYCR2B (5’-CGGGATCCAGGAGAAGCCAAGGTAGG-3’) (Relman et al., 1996). For the secondary PCR, the forward primer was CYCF3E (5’-GGAATTCCTCGCGCTTCGCTGCGT-3’) and the reverse primer was CYCR4B (5’-CGGGATCCGTCTTCAAAACCCCTACTG-3’) (Relman et al., 1996).

The total reaction volume of the primary PCR assay was 25 µL, which contained 1 µL of genomic DNA, 2 mM MgCl₂, 200 µM of each dNTP, 12.5 pmol each of forward and reverse primers, 1x Kapa Taq buffer and 0.05 U/µL of Kapa Taq DNA polymerase (Kapa Biosystems, Cape Town, South Africa). PCR was performed as previously described (Pieniazek et al., 1996), with an initial denaturation of 95 °C for 4 minutes, followed by 45 cycles of 95 °C for 30 seconds (denaturation), 55 °C for 30 seconds (annealing), 72 °C for 90 seconds (extension) and a final extension at 72 °C for 10 minutes.

In the secondary PCR, a product of approximately 497 - 498 bp in length was amplified using 1 µL of primary PCR product and nested forward CYCF3E and reverse CYCR4B primers. The conditions were the same as for the primary PCR, except that the annealing temperature was 60 °C.

**2.9.3 Molecular detection of Cryptosporidium spp. using nested PCR**

In the present study, nested PCRs were performed to screen for *Cryptosporidium* species in faecal samples from sheep and goats and in guts of fish at the 18 rRNA, actin and gp60 loci. A positive control (*C. parvum*) was included in all sets of reactions.
**Cryptosporidium 18S rRNA PCR**

For the primary PCR, the forward primer, 18SiCF2 (5’-GACATATCATTCAAGTTTCTGACC-3’) and the reverse primer, 18SiCR2 (5’-CTGAGGAGTAAGGAAACC-3’) were used to produce a product with an approximate length of 763 bp as previously described (Ryan et al., 2003). The total reaction volume for this assay was 25 µL, which contained 1 µL of genomic DNA, 1.5 mM MgCl₂, 200 µM of each dNTP, 12.5 pmol each of forward and reverse primers, 1x Kapa Taq buffer and 0.05 U/µL of Kapa Taq DNA polymerase (Kapa Biosystems, Cape Town, South Africa). The cycling conditions consisted of an initial hot start (94 °C for 5 minutes), followed by 48 cycles of 95 °C for 40 seconds (denaturation), 58 °C for 30 seconds (annealing) and 72 °C for 45 seconds (extension), and a final extension at 72 °C for 7 minutes.

In the secondary PCR, 1 µL of primary PCR product were included in the reaction mixture containing forward primer 18SiCF1 (5’-CCTATCAGCTTTAGACGGTAGG-3’) and reverse primer 18SiCR1 (5’-TCTAAGAATTTCACTCTCGACTG-3’) to produce a product of approximately 587 bp in length. The conditions of the secondary PCR were identical to those in the primary PCR.

For the samples that failed to amplify using the nested PCR described above, a smaller fragment (298 bp) was amplified using the forward primer, 18SiF (5’-AGTGAAGAAATAACATACAGG-3’) and reverse primer, 18SiR (5’-CCTGCTTAAAGCACTCTAATTTTC-3’) as previously described (Morgan et al., 1997). PCR amplification was performed in 25 µL volumes with 1 µL of genomic DNA, 10 pmol each of forward and reverse primers, 200 µM concentration of each dNTP, 2 mM MgCl₂, 1x Kapa Taq buffer 0.05 U/µL of Kapa Taq DNA polymerase (Kapa Biosystems, Cape Town, South Africa). The cycling conditions consisted of an initial hot start (94 °C for 5 minutes), followed by 48 cycles of 95 °C for 30 seconds (denaturation), 60 °C for 30 seconds (annealing) and 72 °C for 40 seconds (extension) and a final extension at 72 °C for 7 minutes.

**Cryptosporidium actin PCR**
Attempts were made to screen Cryptosporidium-positive samples from piscine hosts at the actin locus using a previously described assay (Ng et al., 2006), however, amplifications were unsuccessful.

**Piscine-derived Cryptosporidium actin PCR**

A new set of semi-nested actin primers were designed using the software, Primer 3 (http://frodo.wi.mit.edu/), based on actin gene sequences of piscine-derived Cryptosporidium. Those primers were used in a semi-nested PCR protocol to amplify samples from fish, which were Cryptosporidium-positive at the 18S rRNA locus.

For the primary PCR, the forward primer, Actinall F1 (5’-GTAAAATACAGGCAGTT-3’) and the reverse primer, Actinall R1 (5’-GGTTGGAAACATGCTTC-3’) were used to produce a product with an approximate length of 392 bp. The total reaction volume for this assay was 25 µL, which contained 1 µL of genomic DNA, 2.0 mM MgCl₂, 200 µM of each dNTP, 12.5 pmol each of forward and reverse primers, 1x Kapa Taq buffer and 0.05 U/µL of Kapa Taq DNA polymerase (Kapa Biosystems, Cape Town, South Africa). The cycling conditions consisted of an initial denaturation step at 95 °C for 4 minutes followed by 45 PCR cycles, 95 °C for 30 seconds (extension), 46 °C for 30 seconds (annealing), and 72 °C for 30 seconds (extension), with a final extension at 72 °C for 7 minutes.

In the secondary PCR, a product of approximately 278 bp in length was amplified using 1 µL of primary PCR product and forward primer, Actinall F2 (5’-CCTCATGCTATAATGAG-3’) and the Actinall R1 reverse primer described above. The conditions for the secondary PCR were identical to those used in the primary PCR.

**Cryptosporidium gp60 PCR**

*Cryptosporidium parvum and C. hominis* positive samples were further subtyped at the 60kDa glycoprotein (*gp60*) locus using a nested PCR assay as previously described (Sulaiman et al., 2005). In the primary PCR, a fragment of the *gp60* gene (approximately 800 bp in length) was amplified using the forward AL3531
(5’-ATAGTCTCCGCTGTATTC-3’) and reverse AL3533 (5’-GAGATATATCTTGGTGCG-3’) primers (Peng et al., 2001; Sulaiman et al., 2005).

The total reaction volume for this assay was 25 µL, which contained 1 µL of genomic DNA, 3.0 mM MgCl₂, 200 µM of each dNTP, 12.5 pmol each of forward and reverse primers, 1x Kapa Taq buffer and 0.05 U/µL of Kapa Taq DNA polymerase (Kapa Biosystems, Cape Town, South Africa). PCR was performed under the following conditions: 95 °C for 4 minutes followed by 40 PCR cycles, 95 °C for 48 seconds (extension), 50 °C for 45 seconds (annealing), and 72 °C for 30 seconds (extension), with a final extension at 72 °C for 10 minutes.

In the secondary PCR, a product of approximately 400 bp in length was amplified using forward primer AL3532 (5’-TCCGCTGTATTCTCAGCC-3’), reverse primer LX0029 (5’-CGAACCACATTACAAATGAAGT-3’) and 1 µL of the primary PCR product (Sulaiman et al., 2005). The conditions of the secondary PCR assay were identical to those in the primary PCR assay except that the annealing time was reduced to 45 seconds.

2.9.4 Molecular detection of Giardia spp. using qPCR and nested PCR

Samples from sheep, goats and fish were initially screened at the glutamate dehydrogenase (gdh) locus using a qPCR assay, followed by attempts to genotype Giardia by PCR and sequencing at the gdh, triphosphate isomerase gene (tpi) and beta (β) giardin (bg) loci. However, amplifications using nested PCRs were unsuccessful. Positive control DNA samples of G. duodenalis assemblage A, B and/or E were included in all sets of reaction runs.

qPCR at the Giardia glutamate dehydrogenase (gdh) locus

For the qPCR assay, the forward primer, gdhF1 (5’-GGGCAAGTCCGACAACGA-3’), the reverse primer gdhR1 (5’-GCACATCTCCTCCAGGAAGTAGAC-3’) and the probe (5’-Quasar 670-TCATGCCTCCTGCAGAAGTAGAC-3’) were used to produce a product of approximately 261 bp in length as previously described (Yang et al., 2014b). IAC was included to check if any inhibition was occurring in the sample (Yang et al., 2013) (see Section 2.9.1).
The total volume of the PCR mixture was 15 μL, which contained 5 mM MgCl₂, 1 mM dNTPs, 1x PCR buffer, 1.0 U Kapa Taq DNA polymerase (Kapa Biosystems, Cape Town, South Africa), 0.2 μM each of forward and reverse primers, 0.2 μM each of forward and reverse IAC primers, 50 nM of the probe, 50 nM of IAC probe, 10 copies of IAC template and 1 μL of sample DNA. The PCR cycling conditions consisted of a pre-melt at 95 °C for 3 minutes and then 45 cycles of 95 °C for 30 seconds, and a combined annealing and extension step at 60 °C for 45 seconds.

**Semi-nested PCR at the *Giardia* gdh locus**

Attempts were made to amplify *Giardia*-positive samples at the *gdh* locus as previously described (Read et al., 2004). The semi-nested PCR assay involved an external forward primer, GDHeF (5′-TCAACGTAAACAGCGGYYTTCCGT-3′), an internal forward primer, GDHiF (5′-CAGTAAACTYCTTCCG-3′) and a reverse primer, GDHiR (5′-GTTTCCCTGCACATCTCC-3′), which produced a product of approximately 432 bp in length (Read et al., 2004).

In the primary PCR, the primers GDHeF and GDHiR were used. The total reaction volume for this assay was 25 μL, which contained 1 μL of genomic DNA, 1.5 mM MgCl₂, 200 μM of each dNTP, 12.5 pmol each of forward and reverse primers, 1x Kapa Taq buffer and 0.05 U/μL of Kapa Taq DNA polymerase (Kapa Biosystems, Cape Town, South Africa). The cycling conditions consisted of an initial hot start at 95 °C for 4 minutes, followed by 55 PCR cycles, 95 °C for 30 seconds (denaturation), 56 °C for 20 seconds (annealing), and 72 °C for 45 seconds (extension) and then a final extension at 72 °C for 7 minutes.

In the secondary PCR, 1 μL of primary PCR product and nested forward GDHiF and reverse GDHiR primers were included to produce a product of 432 bp in length (Read et al., 2004). The conditions for the secondary PCR were identical to the primary PCR. However, the amplifications were not successful, even with variations to the initial assay conditions. Those variations included:

1. the addition of dimethyl sulfoxide (DMSO) at a final concentration of 5 %,
2. the use of fresh aliquots of dNTPs, primers or Kapa Taq DNA polymerase,
[3] Kapa Taq DNA polymerase was replaced with Tth Plus DNA Polymerase (Fisher Biotech Catalogue number: TP-1), and


**Nested PCR at the* Giardia tpi locus**

A nested PCR was used to amplify the triphosphate isomerase (*tpi*) gene of *Giardia* positive isolates using primers complementary to the conserved sequences of various *Giardia* parasites in the primary PCR and assemblage-specific primers in the secondary PCR. Positive controls for *G. duodenalis* assemblages A, B and E were included in all reaction runs.

In the primary PCR, primers AL3543 (5’-AAATIATGCCTGCTCGTCG-3’) and AL3546 (5’-CAACCTTITCCGCAAACC-3’) were used to amplify a product of approximately 605 bp in length as previously described (Sulaiman et al., 2003). The total reaction volume for this assay was 25 µL, which contained 1 µL of genomic DNA, 3 mM MgCl₂, 200 µM of each dNTP, 12.5 pmol each of forward and reverse primers, 5 % DMSO, 1x Kapa Taq buffer and 0.05 U/µL of Kapa Taq DNA polymerase (Kapa Biosystems, Cape Town, South Africa). The cycling conditions were 95 °C for 4 minutes, followed by 45 cycles of 95 °C for 45 seconds (denaturation), 50 °C for 45 seconds (annealing) and 72 °C for 60 seconds (extension). The cycle was followed by a final extension at 72 °C for 10 minutes.

In the secondary PCR, assemblage-specific primers and 1 µL of the primary PCR product were used to screen for *G. duodenalis* assemblages A, B and E as previously described (Geurden et al., 2008a; 2009). Listed below are the primers and their annealing temperatures:

- For the amplification of the *tpi* gene of assemblage A, primers Af 5’-CGCCGTACACCTGTCA 3’ and Ar (5’-AGCAATGACAACCTCCTT-3’) were used to amplify a product of approximately 332 bp in length. The annealing temperature for this primer set was 64 °C (Geurden et al., 2008a).
- For assemblage B, primers Bf (5’-GTTGTTGTTGCTCCTTCTT-3’) and Br (5’-CCGGCTCATAGGCAATTACA-3’) were used to amplify a product of...
approximately 400 bp in length. The annealing temperature for this primer set was 62 °C (Geurden et al., 2009).

- For assemblage E, primers Ef (5’-CCCCTTCTGCGGTACATTAT-3’) and Er (5’-GGCTCGTAAGCAATAACGACTT-3’) were used to amplify a PCR product of approximately 388 bp in length. The annealing temperature for this primer set was 67 °C (Geurden et al., 2008a).

The total reaction volume for each assemblage-specific PCR assay was 25 µL, which contained 1 µL of genomic DNA, 3 mM MgCl₂, 200 µM of each dNTP, 12.5 pmol each of forward and reverse primers, 1x Kapa Taq buffer and 0.05 U/µL of Kapa Taq DNA polymerase (Kapa Biosystems, Cape Town, South Africa). The cycling conditions were 95 °C for 5 minutes, followed by 45 cycles of 95 °C for 30 seconds (denaturation), 64 °C for 30 seconds (annealing) for the assemblage A-specific primer or 62 °C for assemblage B-specific primer or 64 °C for assemblage E-specific primer and 72 °C for 45 seconds (extension). The final extension was at 72 °C for 7 minutes.

**Nested PCR at the Giardia β-giardin locus**

Attempts were made to amplify the β-giardin gene using different nested PCR protocols. Initially primers designed by Caccio et al. (2002) and Lalle et al. (2005b) were used. Specifically, forward primer, G7 (5’-AAGCCCGACGACCTACCCGAGTGC-3’) and reverse primer, G759 (5’-GAGGCCGCCCTGGATCTTGACGAC-3’) were used in the primary PCR (Caccio et al., 2002). For the secondary PCR, forward primer, βGiarF (5’-GAACGAACGAGATCGAGGTCCG-3’) and the reverse primer, βGiarR (5’-CTCGACGAGGCTCTGTT-3’) were used (Lalle et al., 2005a).

In the primary PCR reaction, primers G7 and G759 were used to produce a product of approximately 753 bp in length as previously described (Caccio et al., 2002). The total reaction volume of this assay was 25 µL, which contained 1 µL of genomic DNA 1.5 mM MgCl₂, 200 µM of each dNTP, 12.5 pmol each of forward and reverse primers, 1x Kapa Taq buffer and 0.05 U/µL of Kapa Taq DNA polymerase (Kapa Biosystems, Cape Town, South Africa). The cycling condition consisted of an initial hot start (95 °C for 4 minutes), followed by 45 cycles of 95 °C for 30 seconds
(denaturation), 65 °C for 30 seconds (annealing) and 72 °C for 60 seconds (extension),
and a final extension at 72 °C for 7 minutes. In the secondary PCR, a product of
approximately 511 bp in length was produced using 1 µL of primary PCR product and
nested primers βGiarF and βGiarR (Lalle et al., 2005b). The conditions of the secondary
PCR were identical to those used in primary PCR except that the annealing
temperature was 55 °C.

Amplification at the β-giardin was also attempted using primers designed by Dr.
Rongchang Yang, Murdoch University in a semi-nested PCR. Primers BGexF (5’-
CCGACGACCTCACCACGAGT-3’) and BGRev (5’-GCTCGGCTTCTCGCGGTG-3’) were
used in the primary PCR assay to produce a product of 682 bp in length. Primers BGinF
(5’-CCTTGCGGAGATGGGCGACACA-3’) and BGRev were used in the secondary PCR
assay to amplify a product of approximately 380 bp in length.

The total reaction volume for this PCR assay was 25 µL, which contained 1 µL of
genomic DNA in the primary PCR (1 µL of primary PCR product in the secondary PCR), 3
mM MgCl₂, 200 µM of each dNTP, 5 % DMSO, 12.5 pmol each of forward and reverse
primers, 1x Kapa Taq buffer and 0.05 U/µL of Kapa Taq DNA polymerase (Kapa
Biosystems, Cape Town, South Africa). The cycling conditions for both primary and
secondary PCR assays consisted of an initial denaturation at 95 °C for 3 minutes,
followed by 45 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds and 72°C for 60
seconds and a final extension at 72 °C for 7 minutes.

2.9.5 Molecular detection of anisakid nematodes using PCR

To determine the species of anisakid nematodes isolated from fish, two single
step PCR assays were performed. The first assay involved the amplification of the
nuclear ribosomal DNA (rDNA) region spanning the first internal transcribed spacer,
the 5.8S gene and the second internal transcribed spacer (ITS-1, 5.8S, ITS-2). The
second assay involved the amplification of the mitochondrial cytochrome oxidase
subunit 2 gene (mt-DNA cox2). Positive (genomic DNA from L3 Anisakis typica larvae)
controls were included in all PCR reactions.
**Anisakid ITS region PCR**

The ITS rDNA region of the anisakid nematodes were amplified using the forward primer NC5 (5’-GTAGGTGAACCTGCGGAAGGATCAT-3’) and the reverse primer NC2 (5’-TTAGTTTCTTTTCCCTCGCT-3’) by Zhu et al. (1998) in a single step PCR assay. The total reaction volume for this assay was 25 µL, which contained 1 µL of genomic DNA, 1.5 mM MgCl₂, 200 µM of each dNTP, 12.5 pmol each of forward and reverse primers, 1x Kapa Taq buffer and 0.05 U/µL of Kapa Taq DNA polymerase (Kapa Biosystems, Cape Town, South Africa). The cycling conditions were an initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of 95 °C for 30 seconds (denaturation), 60 °C for 30 seconds (annealing) and 72 °C for 45 seconds (extension), then a final extension at 72 °C for 10 minutes (Kijewska et al., 2009b).

**Anisakid mitochondrial cytochrome oxidase II (cox2) PCR**

A 629 bp fragment of the mitochondrial DNA cytochrome oxidase II (cox2) gene was amplified using primers 210 (5’-CACCAACTCTTAAAAATTATC-3’) and 211 (5’-TTTTCTAGTTATAGATTGRTTYAT-3’) (Nadler and Hudspeth, 2000). The total reaction volume for this assay was 25 µL, which contained 1 µL of genomic DNA, 2.5 mM MgCl₂, 200 µM of each dNTP, 12.5 pmol each of forward and reverse primers, 1x Kapa Taq buffer and 0.05 U/µL of Kapa Taq DNA polymerase (Kapa Biosystems, Cape Town, South Africa). The conditions were an initial denaturation at 95 °C for 4 minutes, followed by 34 cycles of 95 °C for 30 seconds (denaturation), 46 °C for 60 seconds (annealing) and 72 °C for 90 seconds (extension), then a final extension at 72 °C for 10 minutes (Valentini et al., 2006).

**2.9.6 Molecular detection of Chlamydia spp. by qPCR**

A multiplex qPCR assay was used to detect *Chlamydia abortus* and *Chlamydia pecorum* in faecal samples from sheep and goats as previously described (Yang et al., 2014c). This assay targets the outer membrane protein cell surface antigen gene (ompA) using species-specific primers, which were originally designed as singleplex qPCRs by Pantchev et al. (2009, 2010). IAC was included to check if any inhibition was occurring in the sample (Yang et al., 2013) (see Section 2.9.1).
A *C. abortus* species-specific qPCR, which produces an 86 bp product, was amplified using the forward primer CpaOMP1-F (5’-GCAACTGACACTAAGTCGGCTACA-3’), the reverse primer CpaOMP1-R (5’-ACAAGCATGTTCAATCGATAAGAGA-3’) and the probe CpaOMP1-Sb (5’-dTFAATATACCACGAATGGCAAGTTGGTTAGCG-BHQ-1-3’) as previously described (Pantchev et al., 2009). A species-specific 76 bp product was amplified from *C. pecorum* using the forward primer CpecOMP1 F (5’-CCATGTGATCCTTGCGCTACT-3’), the reverse primer CpecOMP1 R (5’-TGTCGAAAACATAATCTCCGTAAAAT-3’) and the probe CpecOMP1-S (5’-CAL-Fluor Orange -560-TGCGACGCGATTAGCTTACGCGTAG-TAMARA-3’) as previously described (Pantchev et al., 2010).

The total volume of this assay was 15 μL and contained 4 mM MgCl₂, 1 mM dNTPs, 1x PCR Buffer, 1.0 U Kapa DNA polymerase (Kapa Biosystems, Cape Town, South Africa) 0.2 μM each of forward and reverse primers, 0.2 μM each of forward and reverse IAC primers, 50 nM of the probe, 50 nM of IAC probe, 10 copies of IAC template and 1 μL of sample DNA (Yang et al., 2014c). The reaction was performed under the following conditions: a pre-melt at 95 °C for 3 minutes, followed by 45 cycles of 95 °C for 20 seconds (denaturation), and a combined annealing and extension step of 60 °C for 45 seconds.

### 2.10 Agarose gel electrophoresis

PCR products were allowed to run on 1 % agarose (Fisher Biotech cat. No. 901b) dissolved in 1x TAE buffer (40 mM Tris-HCl, 20 mM acetate, 2 mM EDTA, pH adjusted to 8.0 with water) and stained with 0.025 μL/mL SYBR® safe DNA gel stain (Invitrogen, Carlsbad, USA). A 1kb Plus DNA ladder (Promega, Madison, WI, USA) was added on a single lane along with PCR products to determine the size of PCR products.

Post electrophoresis products were visualized by UV transillumination using a BIO Rad Gel Doc 1000 transilluminator (Bio-Rad laboratories, Gladesville, NSW, Australia). Bands with correct lengths were cut out from the gel using a scalpel blade. Each cut-out band was placed in a separate 1.5 mL Eppendorf tube with appropriate labels.
2.11 PCR product extraction from gel

Two methods were used to extract the DNA from the gel after electrophoresis. A commercial, Ultra Clean® DNA purification kit (MolBio, West Carlsbad, CA, USA), was used to purify all PCR products generated for anisakid nematodes in *Chapter 6* according to the manufacturer’s instructions. For products described in *chapters 3 and 4*, a simple tip elution method was used to purify DNA amplicons from the sliced gels (Yang et al., 2013). Briefly, positive bands were cut from the gel and the gel fragment were transferred to a 100 µL filter tip (with the end cut off) (Axygen, Fisher Biotech, Wembley, WA, Australia) and then placed in a 1.5 mL Eppendorf tube and centrifuged at 20,000 x g for 1 minute. The filter tip was then discarded and the eluent was retained and used for sequencing without any further purification.

2.12 DNA sequencing PCR with dye terminator kit

The purified PCR products were subjected to sequencing PCR using an ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instruction, with the exception that the annealing temperatures varied (depending on the annealing temperature of individual primers). Briefly, the reaction volume for sequencing PCR was 10 µL, which contained 1.5 µL of 5x sequencing buffer (Applied Biosystems), 1 µL BigDye® Terminator (Applied Biosystems), 3.2 pmol primer (either forward or reverse primer), 5 - 20 ng PCR product and deionised water. Both forward and reverse primers were used; each primer was used in a separate reaction. The reactions were performed on a Perkin Elmer Gene Amp PCR 2400 thermal cycler (Perkin Elmer, Foster City, CA, USA) under the following conditions: 96 °C for 2 minutes (initial denaturation) followed by 25 cycles of 96 °C for 10 seconds (denaturation), 50 - 60 °C for 5 seconds (annealing) and 60 °C for 4 minutes (extension).

2.13 Post reaction purification

After thermal cycling, the reaction products were purified to remove salts and unincorporated dye terminators. For purification, the products were transferred from their original 0.2 mL tubes to 0.6 mL tubes and ethanol precipitation was performed as described below. Briefly, DNA was precipitated by adding 1 µL of 125 mM EDTA, 1 µL
of 3 M sodium acetate (pH = 5.2) and 25 μL of 100 % ethanol (Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia). The solution was mixed gently by using a pipette and incubated at room temperature for 20 minutes. After incubation, the DNA precipitate was centrifuged for 30 minutes at 20,000 x g. The supernatant was discarded and 125 μL of 70 % ethanol was added to the remaining precipitate to rinse the DNA pellet. This was followed by further centrifugation for 5 minutes at 20,000 x g. The resulting supernatant was discarded and the purified DNA pellet was dried using a Savant SpeedVac® Concentrator (Thermo Fisher Scientific, Waltham, MA, USA) for 10 minutes and submitted for sequencing.

2. 14 DNA Sequencing

DNA sequencing was performed on an Applied Biosystems 3730 DNA Analyser Instrument (Applied Biosystems, Foster City, California, USA) by a specialist (Frances Brigg) in the State Agricultural Biotechnology Centre (SABC) at Murdoch University, WA (http://www.sabc.murdoch.edu.au/).

2.15 Phylogenetic analysis

The nucleotide sequences obtained were analysed using FinchTV 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com) and compared with published sequences for identification using the for Biotechnology Information Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov) for species identification, developed by the National Institute of Health’s National Centre.

Sequences were aligned with known reference sequences using both the Clustal Omega alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalo/) and MUSCLE alignment (Edgar, 2004), in the MEGA5 software (Tamura et al., 2011). The resultant alignments were adjusted manually using the MEGA5 software.

Phylogenetic trees were constructed using additional sequences retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Distance estimation was conducted using MEGA5 (Tamura et al., 2011) based on evolutionary distances calculated using p-distance method (Nei and Kumar, 2000), the Kimura two-parameter model (Kimura, 1980) or the Tamura-Nei model (Tamura and Nei, 1993) and grouped
using neighbour-joining (NJ) (Saitou and Nei, 1987). Parsimony and maximum likelihood (ML) analyses were also conducted using MEGA5 (Tamura et al., 2011). More details of the individual phylogenetic analyses conducted are provided in the specific chapters of this thesis. Reliabilities for the trees were tested using 1000 bootstrap replications (Felsenstein, 1985) and bootstrap values exceeding 70 were considered well supported (Hills and Bull, 1993).

2.16 Statistics

Prevalences were expressed as percentage of positive samples with 95% confidence intervals calculated assuming a binomial distribution using the software Quantitative Parasitology 3.0 (Rozsa et al., 2000). Differences in prevalence among groups were compared by Fisher’s Exact Test (for two groups) or Chi-square analysis (for three or more groups). $P$ values < 0.05 were considered significant. Greater details of the individual statistical analyses conducted are provided in specific chapters. Statistical analyses were performed using GraphPad Prism GraphPad Prism version 4 for Windows, GraphPad Software, San Diego, California, USA (www.graphpad.com).
Chapter 3

Prevalence and infection levels of helminth and coccidian parasites in sheep and goat in PNG
The contents of this chapter, specifically, the microscopy analyses have been published in the Journal of Helminthology. The article is listed below and can be found in Appendix B.


This chapter describes the analyses of faecal samples from sheep and goats for helminths and coccidian parasites. Two methods were used: (1) faecal flotation and microscopic analysis (results of which have been published in the Journal of Helminthology) and (2) qPCR detection for trichostrongylid nematodes and *Eimeria* sp.

**Research highlights:**

- The overall parasite prevalence by microscopy was 72 % (79/110) in sheep and 89 % (49/55) in goats. (one or more species of gastrointestinal parasite detected) (Note this does not include *Cryptosporidium* and *Giardia*. *Cryptosporidium* requires specific stains. Also, microscopic examinations have a relatively low sensitivity for these parasites due to their small size were only screened for by PCR).
- The gastrointestinal parasites found by microscopy in both sheep and goats were trichostrongylid nematodes, *Eimeria*, *Strongyloides*, *Fasciola*, *Trichuris* and *Nematodirus*. (Note: trichostrongylids/trichostrongyles refers to the superfamily *Trichostrongyloidea* and not the genus *Trichostrongylus*).
- Two additional genera were found in goats: *Moniezia* and *Dictyocaulus*.
- The prevalence of each trichostrongyles by qPCR in sheep (S) and goats (G) were; *Haemonchus contortus* 41.1 % (S) and 41.7 % (G); *Teladorsagia circumcincta* 21.3 % (S) and 24.1 % (G) and *Trichostrongylus* spp. 14.8 % (S) and 14 % (G).
- The prevalence of *Eimeria* spp. by qPCR was 64.9 % (37/57) for sheep and 91.9 % (34/37) for goats.
- This is the first study to report *Eimeria* in goats and *Dictyocaulus* in small ruminants in PNG.
3.1 Introduction

Gastrointestinal parasites are recognised as a major threat to the production of sheep and goats in both small and large scale-farms as described in Chapter 1.

In PNG, the distribution and productivity of small ruminants are hindered mainly by poor health, nutrition and management (Quartermain, 2004a). There have been various reports on the infections of sheep and goats with gastrointestinal parasites including nematodes, cestodes and Coccidia (listed in Table 1-2, Chapter 1), (Varghese & Yayabu, 1985; Owen, 1988a; 1989b; 1998c and reviewed by Quartermain, 2004b). However, those surveys have been performed on animals raised in government or institutional farms and there is a lack of information on the epidemiology of gastrointestinal parasites infecting sheep and goats in smallholder farms. Such information is essential for understanding the economic impact these parasites can have on farmers and to support decision-making regarding the treatment and prevention of parasitic diseases in these animals.

The aims of the present study were to obtain data on the prevalence, infection levels and species of gastrointestinal parasites in different farm management types over a wide geographic area. Specifically, the parasites were identified in fresh faecal samples based on the morphology of their eggs or oocysts using the McMaster technique (Whitlock, 1948) and light microscopy. Furthermore, molecular tools were used to identify trichostrongyles (H. contortus, T. circumcincta and Trichostrongylus spp.) and Eimeria spp.

3.2 Methods

This section summarises only the most important methods used to obtain the results described in this chapter (Fig. 3-1). A detailed description of all the methods can be found in Chapter 2 of this thesis.

3.2.1 Sample collection

Sampling was conducted from February to April 2011 in two broad agro-climatic zones, the highlands (all study sites, except Labu) and lowlands (Labu) in the central region of mainland PNG (Fig. 2-1 in Chapter 2). These sites are located in a wet tropic
environment and receive high annual rainfall predominantly during the wet season, which lasts from October to May (Quartermain, 2004b). Specific details of the study sites including the climate data are presented in Chapter 2, Section 2.3.1 and Table 2-1.

Figure 3-1: Flow chart of methods used for screening gastrointestinal parasites from faecal samples of sheep and goats. After sample collection, a subset of faecal samples was analysed using parasitological methods involving faecal flotation and light microscopy in PNG. The rest of the samples was preserved in 70 % ethanol and transported to Murdoch University, WA, for molecular screening. DNA was extracted using a commercial kit and qPCR assays were performed using established methods.

For the comparison of prevalence of gastrointestinal parasites among study sites, the study sites were divided into farm types:

- Research institutions: Tambul in high altitude and Labu in lowland
- Public institutions: Baisu
- Government farms: Menifo
- Smallholder farms: Korefeigu, Benabena, Goroka, Daulo and Chuave

Furthermore, for comparison of farm management systems, data from the institutional farms (Labu, Tambul and Baisu) were pooled because in institutional farms, anthelmintics were given to animals regularly to control gastrointestinal parasites,
whereas no anthelmintics were administered to the smallholder flocks. Also, there were high stocking rates at institutional farms but not in the smallholder farms.

Fresh faecal samples were obtained directly from the rectum of sheep and goats and put into 25 mL (screw top) containers in ice-filled Coleman coolers until processing at a NARI laboratory in Labu. Each faecal sample from the 25 mL container was placed into 2 mL Eppendorf tubes and preserved in 70 % ethanol for molecular screening. Excess samples were left in the 25 ml tubes for morphological analyses by a faecal flotation technique (the time lag between sample collection and microscopic examination was 15 - 20 days). All samples were stored at 4 °C. Samples for molecular analyses were transported to Murdoch University, WA.

3.2.2 Parasitology

A subset of faecal samples consisting of 110 from sheep and 55 from goats were used for microscopic examinations. Approximately two grams of each faecal sample were examined using a modified McMaster technique (Whitlock, 1948). The detailed procedure is described in Chapter 2, Section 2.7.1 ‘Faecal worm egg count’.

3.2.3 DNA isolation

Total DNA was extracted from 250 mg of faeces using a PowerSoil® DNA isolation kit (MO BIO laboratories, Carlsbad, California, USA) as described in Chapter 2, Section 2.8.1. All extracted samples were stored at -20 °C until required for screening.

3.2.4 Screening of trichostrongyles and Eimeria species by qPCR

A species-specific qPCR assay was performed to detect H. contortus, T. circumcincta and Trichostrongylus spp. in faecal samples of sheep (n = 263) and goats (n = 228) as described in Chapter 2, Section 2.9.1.

A qPCR was also used to screen for Eimeria in faecal samples of sheep (n = 57) and goats (n = 37) at the small subunit rRNA locus (Yang et al., 2014d), while a nested PCR was used to amplify samples, which were positive for Eimeria by qPCR for genotyping (Pieniazek et al., 1996) as described in Chapter 2, Section 2.9.2. Only two
Eimeria-positive samples from goats were successfully sequenced at the 18S rRNA locus.

### 3.2.5 Data analysis

Prevalence was calculated as the percentage of positive samples in the total number of samples examined. Apart from the overall prevalence (i.e. infection with gastrointestinal parasites) in each flock, prevalence was also calculated for each parasite type, stratified by animal species (Table 3-1); sheep breed, agro-ecological zone and farm management type (Table 3-2) and different farm types (Table 3-3). For comparison of agro-ecology and farm management systems, data for sheep and goats were combined (Table 3-2). Differences in prevalence among groups were compared by Fisher’s exact test (FET) (for two groups) (specifically, Tables 3-1 and 3-2) or chi-square analysis (for more than two groups) (Table 3-3). FET were used where sample sizes were relatively small (n ranged from 27 to 127) as FET is more accurate for smaller sample sizes. Where multiple pairwise comparisons were made among groups, a Bonferroni correction was applied to maintain an experiment-wide Type I error rate of 5 %.

EPG counts were transformed to their decadic logarithms to obtain a normal distribution of values prior to statistical testing for significant differences between animal species, sheep breed, agro-ecological zones and farm types. Differences between two groups were compared by t-tests (Table 3-1 and Table 3-2) and among more than two groups by one-way analysis of variance (1-way ANOVA); followed by a post-hoc, Tukey’s HSD, test (Table 3-3).

For qPCR amplification, the threshold cycle (Ct) values obtained from the amplification plots were used to determine whether the sample was positive for each of the trichostrongylid nematodes (Fig. 3-2). Differences in prevalence between farm types (Table 3-5 to Table 3-7) were compared by Fisher’s exact test (FET) and a Bonferroni correction was applied as described above.
Figure 3-2: qPCR amplification plot for *H. contortus* as an example at the ITS-1 locus. Data was acquired at FAM channel for *H. contortus*, at JOE channel for *T. circumcineta* and at ROX channel for *Trichostrongylus* species. A threshold (red horizontal line) was adjusted manually in the exponential phase of PCR. The threshold cycle (Ct) is the cycle number at which the fluorescent signal of the reaction crosses the threshold (arrow) and was used to determine the presence of each gastrointestinal parasite in the sample. For example, where the yellow line crosses the threshold (arrow), the corresponding Ct value is 18. Ct threshold values were automatically set by the real time PCR software (Rotor-Gene 6.1.93, Corbett Research, Mortlake, NSW, Australia).

3.3 Results

3.3.1 Infection levels of gastrointestinal parasites by microscopy

From a total of 165 faecal samples from small ruminants (110 from sheep and 55 from goats) examined by microscopy, gastrointestinal parasites were found in 128 (77.6 %) animals, with 71.8 % (79/110) of sheep and 89.1 % (49/55) of goats having one or more species of gastrointestinal parasites. The overall prevalence of gastrointestinal parasites in goats was significantly higher than in sheep (FET: $p = 0.017$). Of the 128 positives, 39 % (50/128) had two or more types of gastrointestinal parasites; 32.9 % (26/79) of sheep and 49 % (24/49) of goats (not significantly different by FET: $p = 0.09$).

The gastrointestinal parasites found and their prevalences in sheep (S) and in goats (G) were as follows: trichostrongylids 67.3 % (S), 85.5 % (G); *Eimeria* 17.3 % (S), 16.4 % (G); *Strongyloides*, 8.2 % (S), 23.6 % (G); *Fasciola*, 5.5 % (S), 18.2 % (G); *Trichuris*, 1.8 % (S), 3.6 % (G); and *Nematodirus*, 1.8 % (S), 3.6 % (G). Two additional genera were
found in goats: *Moniezia* (9.1%) and *Dictyocaulus* (3.6%). The prevalence of trichostrongylids (FET: $p = 0.0002$), *Strongyloides* (FET: $p = 0.013$) and *Fasciola* (FET: $p = 0.013$) were significantly higher in goats than in sheep. In addition, the mean EPG counts for trichostrongylids were significantly higher in goats than in sheep (t-test: $p = 0.014$). Prevalences and mean EPG counts in all the sampling sites, stratified by the type of host animal (sheep or goat) and the type of parasite are listed in Table 3-1.

**Table 3-1: Gastrointestinal parasite prevalence and mean ± SD EPG counts stratified by gastrointestinal parasite type for sheep and goats.** $N$ = the total number of faecal samples. $P$ = the number of animals, which were positive for one or more gastrointestinal parasites. Given in parentheses are percent prevalences and 95% confidence intervals (CI). EPG counts are given as means ± SD. Where no SD value is given on the mean EPG count, there was only a single or few positive observations with the same values so that SD could not be calculated. * = values are significantly different between sheep and goats by either FET or t-test.

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Goat (N = 55)</th>
<th>Sheep (N = 110)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P (%), 95% CI</td>
<td>EPG ± SD</td>
</tr>
<tr>
<td>Trichostrongylids</td>
<td>20* (36.7, 24.0 - 50.5)</td>
<td>745 ± 622*</td>
</tr>
<tr>
<td><em>Strongyloides sp.</em></td>
<td>13 (23.6, 13.7 - 37.3)</td>
<td>277 ± 437</td>
</tr>
<tr>
<td><em>Eimeria sp.</em></td>
<td>9 (16.4, 8.2-2.9)</td>
<td>203± 103</td>
</tr>
<tr>
<td><em>Fasciola sp.</em></td>
<td>10 (18.2, 9.5 - 31.4)</td>
<td>257± 275</td>
</tr>
<tr>
<td><em>Trichuris sp.</em></td>
<td>2 (3.6, 0.6 -1 3.6)</td>
<td>125 ± 59</td>
</tr>
<tr>
<td><em>Moniezia sp.</em></td>
<td>5 (9.1, 3.4 - 20.7)</td>
<td>116 ± 46</td>
</tr>
<tr>
<td><em>Dictyocaulus sp.</em></td>
<td>2 (3.6, 0.6 - 13.6)</td>
<td>83 ± 0</td>
</tr>
<tr>
<td><em>Nematodirus sp.</em></td>
<td>2 (3.6, 0.6 - 13.6)</td>
<td>249 ± 117</td>
</tr>
<tr>
<td>Total</td>
<td>49/55 (89.1)*</td>
<td>927 ± 821*</td>
</tr>
</tbody>
</table>

Prevalence and mean EPG counts for gastrointestinal parasites stratified by agro-ecology, farm management systems (institutional vs smallholder) and sheep breed are given in Table 3-2. The prevalence of gastrointestinal parasites in the two agro-ecological zones ranged from 2.4% (*Moniezia* and *Trichuris*) to 75% (trichostrongyles) in the highlands, while it ranged from 4.9% (*Moniezia* and *Trichuris*) to 68.3% (trichostrongyles) in the lowlands (Table 3-2). For the farm management systems, the prevalence of gastrointestinal parasites ranged from 1.7% (*Trichuris*) to 74.8% (trichostrongyles) in institutional farms and 2% (*Moniezia*) to 70% (trichostrongyles) in smallholder farms (Table 3-2). Similarly, in sheep breeds, the
prevalence ranged from 3.7% (Strongyloides) to 66.7% (trichostrongyles) for Priangan sheep and from 3.6% (Fasciola, Trichuris, Nematodirus,) to 67.5% (trichostrongyles) for Highlands Halfbred sheep (Table 3-2).

No statistically significant differences were found in the prevalence of each gastrointestinal parasite type between the agro-ecological zones (FET: \( p > 0.05 \)), the farm management systems (FET: \( p > 0.05 \)), or the sheep breeds (FET: \( p > 0.05 \)), with one exception. That is, the prevalence of Fasciola was higher in the lowland than the highland (FET: \( p = 0.028 \)) agro-ecological zone. Similarly, there were no differences in EPG for most of the parasite types between the specific groups with the exception of Eimeria. The mean EPG for Eimeria was higher in the highlands than in the lowlands (Labu) (t-test: \( p = 0.045 \)). In addition, the mean EPG for Eimeria was higher in the smallholder farms than in institutional flocks (t-test: \( p = 0.004 \)) and the mean EPG for Eimeria in the Highlands Halfbred sheep was higher than in the Priangan sheep (t-test: \( p = 0.045 \)).

The prevalence and the mean EPG counts for each parasite in different farm types (research and public institutions, government and smallholder) are given in Table 3-3. Trichostrongylid nematodes, Strongyloides and Eimeria were prevalent in both sheep and goats in all 5 farm types except where no data were obtained (as for goats in the government farm). There were no statistically significant differences in the prevalence, but there were several significant differences in EPG counts for different gastrointestinal parasite types across the different farms types. The mean EPG counts for goats infected with Strongyloides sp. in Tambul (highland research institution) were significantly higher than the ones in Labu (lowland research institution) (1-way ANOVA: \( F = 3.91, p = 0.05 \), Tukey’s test: \( p < 0.05 \)). For sheep, the mean EPG count for Eimeria in Labu was significantly lower than in Menifo (1-way ANOVA: \( F = 3.41, p = 0.045 \); Tukey’s test: \( p < 0.05 \)). No significant differences were observed for the other genera of gastrointestinal parasites. Fasciola was found in sheep in the research institutes (Labu and Tambul) and in smallholder farms and in goats from Labu, Baisu and smallholder farms (Table 3-3). Trichuris occurred in sheep from smallholder and government farms and in goats only in Labu (Table 3-3).
Table 3-2: Gastrointestinal parasite prevalence and mean EPG counts stratified for agro-ecology, farm management and sheep breed. N = the number of animals examined. Prevalence = animals infested with one or more gastrointestinal parasite type. (%) = percent prevalence. EPG counts are given as mean ± SD. Where no SD value is given on the mean EPG count, there was only a single or few positive observations with the same EPG values so that SD could not be calculated. * = values are significantly different between sheep and goats by either FET or t-test. Institution = both research (Labu and Tambul) and the public (Baisu), H/Halfbred = Highlands Halfbred sheep.

<table>
<thead>
<tr>
<th></th>
<th>Agro-ecology</th>
<th>Farm Management</th>
<th>Sheep Breed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Highland N = 124</td>
<td>Lowlands N = 41</td>
<td>Institutional N = 115</td>
</tr>
<tr>
<td>Prevalence (%) P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trichostrongyles</td>
<td>93 (75)</td>
<td>28 (68.3)</td>
<td>86 (74.8)</td>
</tr>
<tr>
<td>Strongyloides</td>
<td>18 (14.5)</td>
<td>4 (9.8)</td>
<td>14 (12.2)</td>
</tr>
<tr>
<td>Eimeria</td>
<td>18 (14.5)</td>
<td>10 (24.4)</td>
<td>15 (13.1)</td>
</tr>
<tr>
<td>Fasciola</td>
<td>8 (6.5)*</td>
<td>8 (19.5)*</td>
<td>12 (10.4)</td>
</tr>
<tr>
<td>Trichuris</td>
<td>3 (2.4)</td>
<td>2 (4.9)</td>
<td>2 (1.7)</td>
</tr>
<tr>
<td>Moniezia</td>
<td>3 (2.4)</td>
<td>2 (4.9)</td>
<td>4 (3.5)</td>
</tr>
<tr>
<td>Dictyocaulus</td>
<td>0</td>
<td>2 (4.9)</td>
<td>2 (1.7)</td>
</tr>
<tr>
<td>Nematodirus</td>
<td>5 (4)</td>
<td>0</td>
<td>5 (4.3)</td>
</tr>
<tr>
<td>EPG (mean ± SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trichostrongyles</td>
<td>818±1156</td>
<td>551±605</td>
<td>748±942</td>
</tr>
<tr>
<td>Strongyloides</td>
<td>278±375</td>
<td>374±582</td>
<td>357±491</td>
</tr>
<tr>
<td>Eimeria</td>
<td>696±1492*</td>
<td>133±80*</td>
<td>133±69*</td>
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<tr>
<td>Fasciola</td>
<td>228±283*</td>
<td>187±165</td>
<td>152±141</td>
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<tr>
<td>Trichuris</td>
<td>111±48</td>
<td>126±59</td>
<td>125±59</td>
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<tr>
<td>Moniezia</td>
<td>138±48</td>
<td>83</td>
<td>104±42</td>
</tr>
<tr>
<td>Dictyocaulus</td>
<td>0</td>
<td>83</td>
<td>83</td>
</tr>
<tr>
<td>Nematodirus</td>
<td>166±102</td>
<td>0</td>
<td>166</td>
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</table>
Table 3-3: Gastrointestinal parasite prevalence and mean ± SD EPG counts for gastrointestinal parasite type and farm type. \(N_S\) and \(N_G\) are the total number of faecal samples collected at each study site for sheep and goats, respectively. \(P\) = the number of animals infested with one or more gastrointestinal parasite type. Given in parentheses (%) is the percent prevalence. EPG counts are given as means ± SD. Where no SD value is given on the mean EPG count, there was only a single or few positive observations with the same EPG value so that SD could not be calculated. * = values are significantly different by 1-way ANOVA/Tukey’s HSD testing.

<table>
<thead>
<tr>
<th></th>
<th>Research (Labu) (N_S=27, N_G=14)</th>
<th>Research (Tambul) (N_S=29, N_G=4)</th>
<th>Public institution (N_S=19, N_G=22)</th>
<th>Government (N_S=19, N_G=0)</th>
<th>Smallholder (N_S=16, N_G=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Trichostrongylus</strong></td>
<td>18 (66.7) 406 ± 570</td>
<td>20 (69) 1216 ± 1585</td>
<td>15 (78.9) 603 ± 389</td>
<td>10 (52.6) 1087 ± 2303</td>
<td>11 (68.8) 445 ± 677</td>
</tr>
<tr>
<td><strong>Strongyloides</strong></td>
<td>1 (3.7) 1246</td>
<td>5 (17.2) 266 ± 180</td>
<td>1 (5.3) 83</td>
<td>1 (5.3) 166</td>
<td>1 (6.3) 83</td>
</tr>
<tr>
<td><strong>Eimeria</strong></td>
<td>7 (25.9) 95 ± 31*</td>
<td>2 (6.9) 125 ± 59</td>
<td>0</td>
<td>0</td>
<td>1 (6.3) 83</td>
</tr>
<tr>
<td><strong>Fasciola</strong></td>
<td>3 (11.1) 166</td>
<td>2 (6.9) 83</td>
<td>0</td>
<td>0</td>
<td>2 (12.5) 83</td>
</tr>
<tr>
<td><strong>Trichuris</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Moniezia</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Dictyocaulus</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Nematodirus</strong></td>
<td>0</td>
<td>2 (6.9) 125 ± 59</td>
<td>1 (5.3) 83</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Goat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Trichostrongylus</strong></td>
<td>10 (71.4) 813 ± 603</td>
<td>3 (75) 478 ± 212</td>
<td>20 (90.9) 706 ± 713</td>
<td>no data</td>
<td>14 (93.3) 810 ± 582</td>
</tr>
<tr>
<td><strong>Strongyloides</strong></td>
<td>3 (21.4) 831</td>
<td>2 (50) 960 ± 996*</td>
<td>2 (9.1) 83</td>
<td>6 (40) 210 ± 170</td>
<td>3 (20) 249 ± 143</td>
</tr>
<tr>
<td><strong>Eimeria</strong></td>
<td>3 (21.4) 221 ± 95</td>
<td>0</td>
<td>3 (13.6) 138 ± 48</td>
<td>3 (20) 470 ± 374</td>
<td></td>
</tr>
<tr>
<td><strong>Fasciola</strong></td>
<td>5 (35.7) 199 ± 216</td>
<td>0</td>
<td>2 (9.1) 83</td>
<td>3 (20)</td>
<td></td>
</tr>
<tr>
<td><strong>Trichuris</strong></td>
<td>2 (14.3) 125 ± 59</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Moniezia</strong></td>
<td>2 (14.3) 83</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Dictyocaulus</strong></td>
<td>2 (14.3) 83</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Nematodirus</strong></td>
<td>0</td>
<td>2 (9.1) 125 ± 59</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

\(\text{P} = \) number of animals infested with one or more gastrointestinal parasite type.
Nematodirus was found in sheep in two sites (Baisu and Tambul) and in goats only from Baisu (Table 3-3). Moniezia was only found in goats from flocks in Labu, Baisu and smallholder farms (Table 3-3). Dictyocaulus was only found in goats from Labu (Table 3-3). There was also a trend for goats to be more heavily infected than sheep in all areas. Specifically, in Labu, mean EPG for trichostrongyles (t-test: \( p = 0.021 \)) and Eimeria (t-test: \( p = 0.004 \)) were higher in goats than in sheep. Also, in smallholder farms, the prevalence of Strongyloides sp. was higher in goats than in sheep (FET: \( p = 0.037 \)) and mean EPG for trichostrongyles in goats was higher than in sheep (t-test: \( p = 0.006 \)).

3.3.2 Prevalence of trichostrongylid nematodes by qPCR

Genomic DNA from faeces of 263 sheep and 228 goats were screened using qPCR to detect H. contortus, T. circumcincta and Trichostrongylus spp. The prevalence of each nematode in sheep (S) and goats (G) were: H. contortus 41.1 % (S) and 41.7 % (G); T. circumcincta 21.3 % (S) and 24.1 % (G) and Trichostrongylus spp. 14.8 % (S) and 14 % (G) (Table 3-4). There were significant differences among H. contortus, T. circumcincta and Trichostrongylus spp. for both sheep (Chi-square = 51.4, df = 2, \( p < 0.0001 \)) and goats (Chi-square = 19.26, df = 2, \( p < 0.0001 \)).

Differences in the prevalence of H. contortus in sheep between the different types of farms were statistically significant. Statistical data for comparison of prevalences for H. contortus in sheep between two farm types are given in Table 3-5. Specifically, the prevalence of H. contortus in Tambul (research institute) was higher than Labu (research institute), Baisu (public institute) and Menifo government farm (FET: \( p \) values < 0.005) (Table 3-5). In addition, the prevalence of H. contortus in Baisu was higher than in Labu, Menifo and smallholder farms (Table 3-5). There was, however, no significant difference in the prevalence of H. contortus in goats between farm types for all pairs of farm (FET: \( p \) values > 0.005).

There were no significant differences in the prevalence of T. circumcincta in sheep or goats between farm types for all pairs of farms (FET: \( p \) values > 0.005).

There were significant differences in the prevalence of Trichostrongylus spp. in sheep among the different types of farms (Table 3-6). The prevalence of
Trichostrongylus spp. in sheep from the public institution (Baisu) was significantly higher than in Tambul, government (Menifo) and smallholder farms (Table 3-6). However, there were no significant differences in the prevalence of Trichostrongylus spp. in goats for all pairs of farms (FET: p values > 0.005).

Table 3-4: Prevalence of trichostrongylid nematodes for sheep and goats. N = the total number of animals examined in each farm type. P = the number of animals, which were positive for one of the trichostrongylides. Given in parentheses are the per cent prevalence (%) and 95 % confidence interval (CI). RI (Labu) = lowland research institute, RI (Tambul) = highland research institute, PI = Public Institute.

<table>
<thead>
<tr>
<th>Type of farm</th>
<th>N</th>
<th>P (%, 95 % CI)</th>
<th>P (%, 95 % CI)</th>
<th>P (%, 95 % CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sheep</td>
<td>H. contortus</td>
<td>T. circumcincta</td>
<td>Trichostrongylus</td>
</tr>
<tr>
<td>RI (Labu)</td>
<td>40</td>
<td>10 (25.0; 13.3-41.5)</td>
<td>4 (10.0; 3.3-24.6)</td>
<td>6 (15.0; 6.3-30.1)</td>
</tr>
<tr>
<td>RI (Tambul)</td>
<td>109</td>
<td>64 (58.7; 48.9-67.9)</td>
<td>33 (30.3; 22-39.9)</td>
<td>15 (13.8; 7.2-20.3)</td>
</tr>
<tr>
<td>PI (Baisu)</td>
<td>27</td>
<td>19 (70.4; 52.0-88.9)</td>
<td>3 (11.1; 2.9-30.3)</td>
<td>12 (44.4; 24.4-64.5)</td>
</tr>
<tr>
<td>Government</td>
<td>37</td>
<td>4 (10.8; 3.5-26.4)</td>
<td>6 (16.2; 6.8-32.7)</td>
<td>2 (5.4; 1.5-17.7)</td>
</tr>
<tr>
<td>Smallholder</td>
<td>50</td>
<td>11 (22.0; 10.1-33.9)</td>
<td>10 (20.0; 8.5-31.5)</td>
<td>4 (8.0; 0.2-15.8)</td>
</tr>
<tr>
<td>Total</td>
<td>263</td>
<td>108 (41.1; 35.1-47.1)</td>
<td>56 (21.3; 16.3-26.3)</td>
<td>39 (14.8; 10.5-19.2)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Type of farm</th>
<th>N</th>
<th>P (%, 95 % CI)</th>
<th>P (%, 95 % CI)</th>
<th>P (%, 95 % CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Goat</td>
<td>H. contortus</td>
<td>T. circumcincta</td>
<td>Trichostrongylus</td>
</tr>
<tr>
<td>RI (Labu)</td>
<td>43</td>
<td>20 (46.5; 31.0-62.0)</td>
<td>14 (32.6; 18.0-47.2)</td>
<td>5 (11.6; 1.7-21.6)</td>
</tr>
<tr>
<td>PI (Tambul)</td>
<td>35</td>
<td>19 (54.3; 37.0-71.7)</td>
<td>10 (28.6; 12.8-44.3)</td>
<td>5 (14.3; 2.1-26.5)</td>
</tr>
<tr>
<td>PI (Baisu)</td>
<td>39</td>
<td>15 (38.5; 22.5-54.4)</td>
<td>6 (15.4; 3.5-27.3)</td>
<td>4 (10.3; 0.3-20.2)</td>
</tr>
<tr>
<td>Government</td>
<td>6</td>
<td>3 (50; 14-86.1)</td>
<td>3 (50; 14-86.1)</td>
<td>1 (16.7; 0.8-63.5)</td>
</tr>
<tr>
<td>Smallholder</td>
<td>105</td>
<td>38 (36.2; 26.9-45.5)</td>
<td>22 (21.2; 13.2-29.1)</td>
<td>17 (16.2; 9.0-23.4)</td>
</tr>
<tr>
<td>Total</td>
<td>228</td>
<td>95 (41.7; 35.2-48.1)</td>
<td>55 (24.1; 18.5-29.7)</td>
<td>32 (14.0; 9.5-19.6)</td>
</tr>
</tbody>
</table>

Table 3-5: Comparisons of prevalences for H. contortus in sheep among the farm types. All pairs of farms were compared by Fisher’s exact test. A Bonferroni correction was used to determine significance at p values < 0.005. RI = Research Institute, PI = Public Institute.

<table>
<thead>
<tr>
<th>Data set 1</th>
<th>Data set 2</th>
<th>Significant</th>
<th>p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lowland RI (Labu)</td>
<td>Highland RI (Tambul)</td>
<td>Yes</td>
<td>p = 0.0004</td>
</tr>
<tr>
<td>Lowland RI (Labu)</td>
<td>PI (Baisu)</td>
<td>Yes</td>
<td>p = 0.0004</td>
</tr>
<tr>
<td>Lowland RI (Labu)</td>
<td>Government (Menifo)</td>
<td>No</td>
<td>p = 0.1429</td>
</tr>
<tr>
<td>Lowland RI (Labu)</td>
<td>Smallholder</td>
<td>No</td>
<td>p = 0.8047</td>
</tr>
<tr>
<td>Highland RI (Tambul)</td>
<td>PI (Baisu)</td>
<td>No</td>
<td>p = 0.3782</td>
</tr>
<tr>
<td>Highland RI (Tambul)</td>
<td>Government (Menifo)</td>
<td>Yes</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Highland RI (Tambul)</td>
<td>Smallholder</td>
<td>Yes</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>PI (Baisu)</td>
<td>Government (Menifo)</td>
<td>Yes</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>PI (Baisu)</td>
<td>Smallholder</td>
<td>Yes</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Government (Menifo)</td>
<td>Smallholder</td>
<td>No</td>
<td>p = 0.2521</td>
</tr>
</tbody>
</table>
Table 3-6: Comparisons of prevalences for *Trichostrongylus* spp. in sheep among the farm types. All pairs of farms were compared by Fisher’s exact test (FET). A Bonferroni correction was used to determine significance at *p* values < 0.005. RI = Research Institute, PI = Public Institute.

<table>
<thead>
<tr>
<th>Data set 1</th>
<th>Data set 2</th>
<th>Significant</th>
<th><em>p</em> values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lowland RI (Labu)</td>
<td>Highland RI (Tambul)</td>
<td>No</td>
<td><em>p</em> = 1</td>
</tr>
<tr>
<td>Lowland RI (Labu)</td>
<td>PI (Baisu)</td>
<td>No</td>
<td><em>p</em> = 0.0102</td>
</tr>
<tr>
<td>Lowland RI (Labu)</td>
<td>Government (Menifo)</td>
<td>No</td>
<td><em>p</em> = 0.2715</td>
</tr>
<tr>
<td>Lowland RI (Labu)</td>
<td>Smallholder</td>
<td>No</td>
<td><em>p</em> = 0.5034</td>
</tr>
<tr>
<td>Highland RI (Tambul)</td>
<td>PI (Baisu)</td>
<td>Yes</td>
<td><em>p</em> = 0.0009</td>
</tr>
<tr>
<td>Highland RI (Tambul)</td>
<td>Government (Menifo)</td>
<td>No</td>
<td><em>p</em> = 0.2399</td>
</tr>
<tr>
<td>Highland RI (Tambul)</td>
<td>Smallholder</td>
<td>No</td>
<td><em>p</em> = 0.4308</td>
</tr>
<tr>
<td>PI (Baisu)</td>
<td>Government (Menifo)</td>
<td>Yes</td>
<td><em>p</em> = 0.0004</td>
</tr>
<tr>
<td>PI (Baisu)</td>
<td>Smallholder</td>
<td>Yes</td>
<td><em>p</em> = 0.0003</td>
</tr>
<tr>
<td>Government (Menifo)</td>
<td>Smallholder</td>
<td>No</td>
<td><em>p</em> = 1</td>
</tr>
</tbody>
</table>

3.3.3 Prevalence of *Eimeria* in sheep and goats by qPCR

The prevalence of *Eimeria* was 64.9 % (37/57) for sheep and 91.9 % (34/37) for goats (Table 3-7). There was a significant difference in prevalence between sheep and goats (FET: *p* = 0.030).

Table 3-7: Overall prevalence of *Eimeria* in sheep and goats by qPCR. N = the total number of faecal samples. P = the number of animals, which were positive for *Eimeria*. Given in parentheses are the percent prevalence (% P) and 95 % confidence interval (CI). * = values are significantly different between sheep and goats by FET. RI = Research Institute.

<table>
<thead>
<tr>
<th>Type of farm</th>
<th>N</th>
<th>P (% P; 95 % CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sheep</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lowland RI (Labu)</td>
<td>7</td>
<td>5 (71.4; 30.3-94.9)</td>
</tr>
<tr>
<td>Highland RI (Tambul)</td>
<td>19</td>
<td>13 (68.4; 43.5-86.4)</td>
</tr>
<tr>
<td>Public Institute (Baisu)</td>
<td></td>
<td>Not analysed</td>
</tr>
<tr>
<td>Government (Menifo)</td>
<td>10</td>
<td>7 (70, 35.4-91.9)</td>
</tr>
<tr>
<td>Smallholder</td>
<td>21</td>
<td>12 (57.1; 34.4-37.4)*</td>
</tr>
<tr>
<td>Total</td>
<td>57</td>
<td>37 (64.9; 51.1-76.8)*</td>
</tr>
<tr>
<td><strong>Goats</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lowland RI (Labu)</td>
<td>8</td>
<td>8 (100; 59.8-100)</td>
</tr>
<tr>
<td>Highland RI (Tambul)</td>
<td>4</td>
<td>3 (75; 21.9-98.7)</td>
</tr>
<tr>
<td>Public Institute (Baisu)</td>
<td>3</td>
<td>3 (100; 31-100)</td>
</tr>
<tr>
<td>Government (Menifo)</td>
<td>2</td>
<td>2 (100; 19.8-100)</td>
</tr>
<tr>
<td>Smallholder</td>
<td>20</td>
<td>18 (90; 66.9-98.3)*</td>
</tr>
<tr>
<td>Total</td>
<td>37</td>
<td>34 (91.9; 77-98.3)*</td>
</tr>
</tbody>
</table>
No significant associations were found between sheep and goats in each farm types with the exception that the prevalence of *Eimeria* for goats was higher than for sheep in smallholder farms (FET: $p = 0.0325$).

**Molecular typing of *Eimeria***

Positive samples from goats were genotyped at the 18S rRNA locus and two isolates (GE62 and GE72) from smallholder farms were typed as *Eimeria zuernii*. Unfortunately, samples from sheep could not be genotyped because chromatogram sequences were unreadable.

### 3.4 Discussion

Overall, 77.6 % (128/165) of the examined farm animals in PNG were infected with one or more types of gastrointestinal parasites by microscopy. This high rate of gastrointestinal parasitism has also been observed in other tropical countries such as Ethiopia and Kenya (Maichomo et al., 2004; Regassa et al., 2006). Trichostrongyles has the highest prevalence (67.3 % in sheep and 85.5 % in goats), followed by *Eimeria* (16.4 - 17.3 %), *Strongyloides* (8.2 - 23.6 %), *Fasciola* (5.5 - 18.2 %), *Trichuris* (1.8 - 3.6 %) and *Nematodirus* (1.8 - 3.6 %) (Table 3-1). A similar trend in prevalence for these species have also been reported in other countries (Gadahi et al., 2009; Dagnachew et al., 2011). This high parasite prevalence may be attributed to poor farm management practises, such as short pasture rest times, poor nutrition and lack of anthelmintic treatment. In all the farms studied, mixed-species flocks grazed or browsed on natural forage on the same land for most of the time or were moved to other areas with only short pasture rest times. In smallholder farms, sheep and goats were allowed to graze freely or were tethered in available communal pasture during the day and housed at night on daily basis. In institutional and government farms, the flocks were also allowed to graze at daytime and housed at night but the pastures were divided into paddocks ($n = 3$ to 5). Sheep and goats were allowed to graze in one paddock for up to 2 weeks and then changed to the next one. Studies in Fiji, Tonga, Malaysia and the Philippines found that nematodes (especially, *H. contortus* and *T. colubriformis*) have short survival times (3 to 4 days) on pasture and suggested rotational grazing as an effective measure for parasite control (Banks et al., 1990; Barger et al., 1994; Cheah
and Rajamanickam, 1997; Baker and Gray, 2004). PNG has similar climate as those countries, and therefore if rotational grazing is proposed, 3.5 days would be suitable for sheep and goats to graze in one paddock, after which the animals can be moved to another paddock.

Natural pasture and shrubs in PNG are often not very nutritious and may contain anti-nutritive factors such as tannin types, which restrict rather than enhance protein availability to the ruminant (Macfarlane, 2000). Signal grass, for example, may cause hepatic dysfunctions in farm animals (Macfarlane, 2000). Low quality feed may result in subsequent malnutrition, which may negatively affect the development of acquired immunity against gastrointestinal parasites (Kyriazakis and Houdijk, 2006).

In the present study, however, significantly lower gastrointestinal parasite prevalence at study sites, which had more frequent anthelmintic treatments, was not observed. This could be due to anthelmintic resistance or treatments not being given correctly, however, further studies are needed to confirm this.

The overall infection levels of gastrointestinal parasites in goats were higher than in sheep. Goats were also infected with a wider spectrum of gastrointestinal parasites. This contradicts some previous studies, which found a lower overall prevalence of gastrointestinal parasites in goats (Kanyari et al., 2009; Khan et al., 2010; Abebe et al., 2011) but is in agreement with a number of other studies, which reported a higher parasite prevalence in goats (Regassa et al., 2006; Nwosu et al., 2007; Gadahi et al., 2009; Dagnachew et al., 2011). Goats do not develop resistance as efficiently as sheep (Hoste et al., 2008) and this may explain the findings of the present study.

Trichostrongyloid nematodes were the most abundant parasites detected in the present study by microscopy. Quantitative PCR analyses revealed a higher prevalence of \textit{H. contortus} (41.1 - 41.7 \%) compared to \textit{T. circumcincta} (21.3 - 24.1 \%) and \textit{Trichostrongylus} spp. (14 - 14.8 \%) (Table 3-4). The results of this study were similar to those found in a study in adult goats in Malaysia, which reported that \textit{H. contortus} prevalence ranged from 35 - 46 \% and \textit{Trichostrongylus} spp. from 14 - 21 \%, although in sheep, prevalence of \textit{H. contortus} (35 – 68 \%) and \textit{Trichostrongylus} (39.6 \%) were
slightly higher (Dorny et al., 1995). Another study in Ethiopia also found higher prevalences of *H. contortus* (56.3 %) and *Trichostrongylus* spp. (39.6 %) (Abebe et al., 2011). The observed differences in prevalence between the present and previous studies could be due to variations in geographical and climatic conditions. However, the trends are similar, which is *H. contortus* occurring at a higher frequency than the other trichostrongyloid nematodes.

*Haemonchus* species are highly fecund, laying up to 5000 eggs per day, where environmental factors are favourable (Gupta et al., 1987; Le Jambre, 2006). The pre-parasitic stages of *H. contortus* develop and survive better at mean monthly maximum temperatures ≥ 18.3 °C (Gupta et al., 1987). The study sites in the present study had mean maximum annual temperatures that ranged from 18.9 °C to 31.1 °C, with only little variation throughout the year due to their proximity to the equator. Additionally, most study sites were very humid and therefore, likely to sustain the survival of the free-living stages of *H. contortus*, leading to high pasture contamination. Free-living stages of *Teladorsagia* and *Trichostrongylus*, however survive better at < 10 °C (O’Connor et al., 2006). Thus, the fact that the prevalence of *H. contortus* was higher than *T. circumcincta* and *Trichostrongylus* spp. is likely due to adult nematodes releasing more eggs in their faeces and well as optimal environmental conditions in favour of *H. contortus* development.

The overall prevalence of *Eimeria* for sheep was 17.3 % (19/110) by microscopy (Table 3-1) and 64.9 % (35/57) by qPCR (Table 3-7). A previous study in PNG reported that 89 % (67/75) of sheep were positive for *Eimeria* from three locations (in Erap and Lae, Morobe Province and in Mt. Hagen, Western Highlands Province) (Varghese and Yayabu, 1985). The low prevalence of *Eimeria* observed in the present study by microscopy may be due to the different methodology used for parasite identification. In the present study, a simple flotation procedure was used, while in previous studies, a centrifugation/flotation method was used (Varghese and Yayabu, 1985). Methods involving centrifugation are more sensitive than just faecal flotation techniques for detection of oocytes in faecal samples (Dryden et al., 2005). The overall prevalence of *Eimeria* in goats was higher by qPCR (91.1 %) (Table 3-7). Previous studies in other
countries have also reported such high prevalences in goats (Kusilukaa et al., 1996; Balicka-Ramisz, 1999). Furthermore, *Eimeria zuernii* was identified in two adult goats in the present study. *Eimeria zuernii* is highly pathogenic in calves; causing diarrhoea (Bangoura et al., 2012), enteric lesions and death of infected calves (Stockdale, 1977). The smallholder goat farms where *E. zuernii* was found shared feeding grounds with cattle and other animals. Whether the presence of *E. zuernii* identified herein represents actual or mechanical infections remains to be determined, as the oocysts may have been passing through rather than infecting these goats. Notwithstanding, this is the first study to report *Eimeria* in goats in PNG. Adult sheep and goats shed eimerid oocysts in their faeces, which could lead to contamination of pasture and therefore they could be regarded as a source of infection for lambs and kids.

The EPG counts for *Eimeria* in PNG Priangan sheep (n = 27) were significantly lower than that in the Highlands Halfbred sheep (n = 83). It is difficult to ascertain the mechanisms behind the observed difference, especially as coincidentally, most PNG Priangan sheep samples were collected in Labu, where anthelminthic drug treatment was conducted more regularly. Nevertheless, previous studies have shown that some indigenous sheep breeds exhibit higher levels of resistance against gastrointestinal parasites and this might partially explain the present findings (Baker and Gray, 2004). PNG Priangan sheep are native to tropical climates, as they originated from Southeast Asia and have been exposed to gastrointestinal parasites in PNG for over a century (Quartermain, 2004a). In contrast, Highlands Halfbred sheep, which are crossbreeds of the PNG Priangan sheep and the temperate Corriedale and Perendale breeds have only a fraction of this resistance and will therefore be more susceptible to infection. More detailed studies on immunology and feeding behaviour of the different sheep breeds are required to elucidate this problem further.

The trematode, *Fasciola*, was present in sheep and goats in research institutes (Labu and Tambul), smallholder farm (Benabena), and the public institution (Baisu). A previous study in PNG found *Fasciola* in the area of Aiyura (Eastern Highlands province) and showed that the sheep there were exposed to continual (low-level) pasture contamination leading to chronic fasciolosis at all times (Owen, 1989). In PNG,
in all areas where the intermediate snail host, *Lymnaea* species, exists, acute fasciolosis can occur in the wet season especially in areas where the land is not well-drained and the grazing pressure is high (Owen, 1989).

This is the first study reporting the prevalence of the lungworm, *Dictyocaulus*, in small ruminants in PNG. Some studies have found that goats are more susceptible to *Dictyocaulus* than sheep (Sharma, 1994; Berrag and Urquhart, 1996; Alemu et al., 2006). This study found a low prevalence (3.6 %) of *Dictyocaulus* in goats from Labu (lowland) whereas, previous studies reported a high prevalence (12 – 60 %) (Alemu et al., 2006). The observed difference between the present and previous studies could be due to the life cycle stages of the parasite. In the prepatent or post-patent phases or during hypobiosis, it is impossible to detect these parasites by faecal examinations (Frasser, 1991).

### 3.5 Conclusions

In conclusion, the information collected in this study is an important update on the presence of gastrointestinal parasites in sheep and goats in PNG. Future investigations should include longitudinal studies and larger cohorts to further assess parasite epidemiology in the diverse agro-climatic zones in the country. In addition, further research is necessary to characterise the prevalence of various *Eimeria* species in young lambs, goat kids and cattle to more fully understand the transmission dynamics of *Eimeria* among ruminants in PNG.
Chapter 4

Molecular characterisation of *Cryptosporidium* in sheep, goats and fish from PNG
The contents of this chapter have been published as journal articles and they can be found in Appendix C and D.


This chapter describes molecular screening and genotyping of *Cryptosporidium* species and subtypes in sheep, goats and fish.

Research highlights:

- The overall prevalences for *Cryptosporidium* was 2.2% (6/276), 4.4% (10/228), 1.14% (7/614) in sheep, goats and fish, respectively, at the 18S rRNA locus.
- In sheep, *C. parvum* (subtypes IIaA15G2R1 and IIaA19G4R1), *C. andersoni* and *C. scrofarum* were identified.
- In goats, *C. hominis* (subtype IdA15G1), *C. parvum, C. xiaoai* and rat genotype II were identified.
- In fish, *C. hominis* (subtype IdA15G1) and *C. parvum* (IIaA14G2R1, IIaA15G2R1 and IIaA19G4R1) and a novel genotype (which we have named, *Cryptosporidium* sp. piscine genotype 8) were identified.

### 4.1 Introduction

Species of *Cryptosporidium* are globally distributed, zoonotic intestinal protozoan parasites that cause diarrheal disease in animals and are one of the main causes of serious diarrhoea in children (Kotloff et al., 2013). Clinical effects of *Cryptosporidium* infection, which include diarrhoea, weight loss and often death in lambs and goat kids, can severely impact the economy of sheep and goat farming (de Graaf et al., 1999). In fish, *Cryptosporidium* can cause high morbidity with clinical signs including variable levels of emaciation, poor growth rates, swollen coelomic cavities,
anorexia, listlessness and increased mortality (Murphy et al., 2009). Further details on Cryptosporidium are described in Chapter 1, Section 1.4.

Little is known of the prevalence of Cryptosporidium spp. in humans, domesticated animals or wildlife in PNG. Therefore, the aim of the present study was to screen sheep, goats and fish for Cryptosporidium using molecular tools.

4.2 Methods

This section summarises the most important methods used to obtain the results described in this chapter. A detailed description of all the methods is given in Chapter 2 of this thesis. A schematic representation of methods used for molecular detection is shown in Figure 4-1.

![Flow chart of the methodology for molecular detection of Cryptosporidium spp. in the present study. DNA was extracted using commercial kits. Nested PCRs were performed and the amplified products were visualized on the gel. Amplification of the 18S rRNA, gp60 and actin loci produced approximately 298 bp, 400 bp and 278 bp products in length, respectively. The PCR products were sequenced and phylogenetic analyses were conducted.](image-url)
4.2.1 Sample collection

Faecal samples from a total of 276 sheep and 228 goats were collected from February 2011 to April 2011 in a variety of agro-economic zones in PNG. The details of the study sites, sheep and goats, and type of farms are described in Chapter 2, Sections 2.3 - 2.5.1.

A total of 614 fish from three different groups of fish; cultured freshwater fingerlings (juveniles), wild freshwater and wild marine were collected between February and August 2011. The species and numbers of fish are listed in Table 2-3 (Chapter 2, Section 2.5.2). On average, the time when fish were caught and at the stalls before being sold was 2 h and the time lag between purchasing and processing the fish was up to 4 h. The fish were weighed, measured (length and weight) and dissected. Sections of intestine and stomach were cut using a fresh scalpel blade for each fish and preserved in 70 % ethanol for molecular screening. The remaining stomach and intestine were fixed in 10 % buffered formalin for histological analysis.

4.2.2 DNA isolation

Genomic DNA were extracted from 250 mg of faecal sample and fish tissue scrapings using a PowerSoil® DNA isolation kit (MO BIO laboratories, Carlsbad, CA, USA) according to manufacturer’s instructions and stored at -20 °C until screening. The methods used are described in Chapter 2, Sections 2.8.1 and 2.8.3.

4.2.3 Cryptosporidium genotyping and subtyping

Identification of Cryptosporidium species and subtypes in faecal samples from sheep and goats and from fish gut scrapings were performed using PCR and sequence analyses of the 18S rRNA region, and gp60 and actin genes (Fig. 4-1). Details of the molecular techniques used are described in Chapter 2, Sections 2.9.3. and 2.10 - 2.15.

After sequencing, the nucleotide sequences were analysed using FinchTV 1.4.0 (Geospiza, Inc., Seattle, WA, USA). Each sequence was entered into GenBank using BLAST (http://blast.ncbi.nlm.nih.gov) to search for matching known sequences. Sequences generated as part of the present study were aligned with 35 18S, 10 gp60 and 23 actin reference sequences representing selected Cryptosporidium species and
genotypes available in GenBank. Sequences were aligned using both Clustal Omega
(http://www.ebi.ac.uk/Tools/msa/clustalo/) and MUSCLE alignment (Edgar, 2004)
using MEGA5 software (Tamura et al., 2011) and the resultant alignments were
adjusted manually using the MEGA5 program.

Phylogenetic analyses were conducted in MEGA5 software (Tamura et al.,
2011) using neighbour joining (NJ), maximum parsimony (MP) and maximum likelihood
(ML) models for the 18S and actin loci only. Distance estimation was conducted using
MEGA5 (Tamura et al., 2011) based on evolutionary distances calculated using the p-
distance model (Nei and Zhang, 2006) and grouped using neighbour-joining.
Parsimony and maximum likelihood analyses were also conducted using the MEGA5
(Tamura et al., 2011). The MP trees were obtained using the close-neighbour-
interchange (CNI) algorithm (Nei and Kumar, 2000) with search level 3 in which the
initial trees were obtained with the random addition of sequences (1 replicate). Evolutionary history was inferred using maximum likelihood. Reliabilities for the trees
were tested using 1000 bootstrap replications (Felsenstein, 1985) and bootstrap values
exceeding 70 were considered well supported (Hills and Bull, 1993).

4.2.4 GenBank nucleotide accession numbers

The partial 18S and gp60 nucleotide sequences for Cryptosporidium derived
from sheep and goats were deposited in GenBank under the accession numbers
KJ584567 - KJ584584. The unique partial 18S rRNA and actin sequences of piscine
genotype 8 (from silver biddies) were deposited in GenBank under the accession
numbers KC807985 - KC807988.

4.2.5 Histological analysis

Sections of intestinal and stomach tissues of fish were fixed in 10 % formalin
and were embedded in paraffin. Histological sections were cut at 5 µm thicknesses,
stained with haematoxylin and eosin and examined with an Olympus BX50 light
microscope at 400 and 1000 fold magnifications.
4.2.6 Analysis of prevalence

Prevalences (isolates positive at 18S locus) were expressed as a percentage of positive samples with 95 % confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rozsa et al., 2000).

4.3 Results

4.3.1 Prevalence of Cryptosporidium in sheep, goats and fish.

Cryptosporidium was detected in 2.2 % (6/276; 95 % CI 2.8 % – 6.2 %) of sheep, 4.4 % (10/228; 95 % CI = 2.8 % - 6.2 %) of goats and 1.14 % (7/614; 95 % CI 0.7 % - 1.6 %) of fish at the 18S rRNA locus. Among sheep, Cryptosporidium was detected in two animals from research institutes, one from the government station and three from smallholder flocks (Table 4-1). Among goats, Cryptosporidium was identified in four animals from research institutional farms and six from smallholder farms (Table 4-1).

Of the seven positive isolates from fish, two (1.5 %, 95 % CI 0.4 % - 2.8 %) were cultured fingerlings, one (0.5 %, 95 % CI 0.0 % - 1.1 %) was a wild freshwater species and four (1.5 %, 95 % CI 0.7 % - 2.3 %) were wild marine species. The prevalence of Cryptosporidium for specific species of fish in each study location is given in Table 4-2.

Table 4-1: Prevalence of Cryptosporidium spp. in different farm types in sheep and goats in the present study. N = the total number of faecal samples for sheep and goats. P = the prevalence, which is the number of animals, which were positive for Cryptosporidium at the 18S locus. Given in parentheses are the percent prevalence and 95 % confidence interval (CI).

<table>
<thead>
<tr>
<th>Type of farms</th>
<th>N</th>
<th>P (%, 95 % CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Research Institute</td>
<td>156</td>
<td>2 (1.28, 0.22-5.03)</td>
</tr>
<tr>
<td>Public Institute</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>Government</td>
<td>40</td>
<td>1 (2.5, 0.13-14.73)</td>
</tr>
<tr>
<td>Smallholder</td>
<td>54</td>
<td>3 (5.56, 1.45-16.35)</td>
</tr>
<tr>
<td>Total</td>
<td>276</td>
<td>6 (2.2, 0.8-4.9)</td>
</tr>
<tr>
<td>Goat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Research Institute</td>
<td>78</td>
<td>4 (5.13, 1.66-13.31)</td>
</tr>
<tr>
<td>Public Institute</td>
<td>39</td>
<td>0</td>
</tr>
<tr>
<td>Government</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Smallholder</td>
<td>105</td>
<td>6 (5.71, 2.34-12.52)</td>
</tr>
<tr>
<td>Total</td>
<td>228</td>
<td>10 (2.25-8.16)</td>
</tr>
</tbody>
</table>
Table 4-2: Prevalence of Cryptosporidium spp. in fish in the present study. N = the total number of fish sampled. P = the prevalence, which is the number of animals, which were positive for Cryptosporidium at the 18S locus. Given in parentheses are the percent prevalence and 95 % confidence interval (CI). Cultured = all fish were from freshwater fish-ponds.

<table>
<thead>
<tr>
<th>Study locations</th>
<th>Host</th>
<th>Group</th>
<th>N</th>
<th>P (%)</th>
<th>95 % CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kundiawa</td>
<td>Nile tilapia</td>
<td>Cultured</td>
<td>36</td>
<td>1 (2.78, 0.15-16.21)</td>
<td></td>
</tr>
<tr>
<td>Mumeng</td>
<td>Nile tilapia</td>
<td>Cultured</td>
<td>26</td>
<td>1 (3.85, 1.2-21.59)</td>
<td></td>
</tr>
<tr>
<td>Pagwi</td>
<td>Silver barb</td>
<td>Wild freshwater</td>
<td>39</td>
<td>1 (2.56, 0.13-15.07)</td>
<td></td>
</tr>
<tr>
<td>Bilbil</td>
<td>Mackerel scads</td>
<td>Wild marine</td>
<td>5</td>
<td>1 (20, 1.05-70.12)</td>
<td></td>
</tr>
<tr>
<td>Pilapila</td>
<td>Mackerel scads</td>
<td>Wild marine</td>
<td>24</td>
<td>1 (4.17, 0.22-23.12)</td>
<td></td>
</tr>
<tr>
<td>Tavana</td>
<td>Silver biddy</td>
<td>Wild marine</td>
<td>29</td>
<td>2 (6.9, 1.2-24.22)</td>
<td></td>
</tr>
</tbody>
</table>

4.3.2 Molecular characterisation of Cryptosporidium species identified in sheep, goats and fish at the 18S, gp60 and actin locus

Based on comparison with 18S rRNA nucleotide sequences available in GenBank database, three species of Cryptosporidium were detected in sheep, namely C. parvum (n = 4), C. andersoni (n = 1) and C. scrofarum (n = 1). Four species/genotypes were detected in goats; C. hominis (n = 6), C. parvum (n = 2), C. xiaoi (n = 1) and rat genotype II (n = 1) (Table 4-3). Rat genotype II, C. xiaoi, C. scrofarum and C. andersoni isolates were detected in animals from smallholder farms. The C. hominis isolates were from smallholder (n = 4) and institutional (n = 2) farms, while C. parvum was identified in animals from three types of farms; government (n = 1), institutional (n = 3) and smallholder (n = 2). Analysis of the gp60 gene identified the presence of two C. parvum subtypes; IIaA15G2R1 (n = 3) and IIaA19G4R1 (n = 2) in sheep and goats and a C. hominis subtype (IdA15G1) (n = 1) in a goat (Table 4-3). The phylogenetic relationship between sequences obtained from sheep and goats in the present study and other Cryptosporidium species or genotypes is shown in Figure 4-2.

In fish, sequence analyses identified C. parvum (5 isolates: Nile tilapia ON36 and ON68, mackerel scad DM17 and DM18, and silver barb PG37) and a novel piscine genotype (2 isolates: silver biddy GO18 and GO55), hereafter referred to as piscine genotype 8 at the 18S rRNA locus (Table 4-4). At the gp60 locus, three subtypes
belonging to *C. parvum* (IIaA14G2R1, IIaA15G2R1, IIaA19G4R1) and one subtype belonging to *C. hominis* (IdA15G1) were identified (Table 4-4).

The two piscine genotype 8 isolates from silver biddies were genetically identical to each other, but distinct from all isolates previously characterised at the 18S rRNA locus. Analyses of the 18S rRNA nucleotide sequences revealed that piscine genotype 8 is closely related to piscine genotype 3 from a sea mullet with 4.3 % genetic difference (i.e. 95.4 % genetic similarity) (Table 4-5). Neighbour-joining, parsimony and maximum likelihood analyses produced similar results and indicated that piscine 8 genotype, clustered most closely with the piscine 3 genotype from a sea mullet, while the other five isolates clustered with *C. parvum* (85 % bootstrap support) (Fig. 4-3). Sequences were also obtained for the two piscine genotype 8 isolates at the actin locus. At this locus, sequence information was only available for *C. molnari* and piscine genotype 1 and piscine genotype 8 grouped more closely with *C. molnari* genotypes (Fig. 4-4) with 7.3 % - 8.5 % genetic differences (91.5 % to 92.7 % genetic similarities) (Table 4-5).

Table 4-3: Species and subtypes of *Cryptosporidium* identified in sheep and goats in the present study.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Host</th>
<th>Species identified at the 18S locus</th>
<th>Type of farm</th>
<th>gp60 subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>GE51</td>
<td>Goat</td>
<td><em>C. hominis</em></td>
<td>Smallholder</td>
<td>_</td>
</tr>
<tr>
<td>GE53</td>
<td>Goat</td>
<td><em>C. hominis</em></td>
<td>Smallholder</td>
<td>_</td>
</tr>
<tr>
<td>GE66</td>
<td>Goat</td>
<td><em>C. hominis</em></td>
<td>Smallholder</td>
<td>_</td>
</tr>
<tr>
<td>GE78</td>
<td>Goat</td>
<td><em>C. hominis</em></td>
<td>Smallholder</td>
<td>IdA15G1</td>
</tr>
<tr>
<td>GM14</td>
<td>Goat</td>
<td><em>C. hominis</em></td>
<td>Research Institution</td>
<td>_</td>
</tr>
<tr>
<td>GW10</td>
<td>Goat</td>
<td><em>C. hominis</em></td>
<td>Research Institution</td>
<td>_</td>
</tr>
<tr>
<td>GM35</td>
<td>Goat</td>
<td><em>C. parvum</em></td>
<td>Research Institution</td>
<td>IIaA19G4R1</td>
</tr>
<tr>
<td>GW19</td>
<td>Goat</td>
<td><em>C. parvum</em></td>
<td>Research Institution</td>
<td>IIaA15G2R1</td>
</tr>
<tr>
<td>SW29</td>
<td>Sheep</td>
<td><em>C. parvum</em></td>
<td>Research Institution</td>
<td>_</td>
</tr>
<tr>
<td>SE03</td>
<td>Sheep</td>
<td><em>C. parvum</em></td>
<td>Government</td>
<td>IIaA15G2R1</td>
</tr>
<tr>
<td>SE83</td>
<td>Sheep</td>
<td><em>C. parvum</em></td>
<td>Smallholder</td>
<td>IIaA15G2R1</td>
</tr>
<tr>
<td>SW106</td>
<td>Sheep</td>
<td><em>C. parvum</em></td>
<td>Research Institution</td>
<td>IIaA19G4R1</td>
</tr>
<tr>
<td>SE67</td>
<td>Sheep</td>
<td><em>C. scrofarum</em></td>
<td>Smallholder</td>
<td>_</td>
</tr>
<tr>
<td>SE79</td>
<td>Sheep</td>
<td><em>C. andersoni</em></td>
<td>Smallholder</td>
<td>_</td>
</tr>
<tr>
<td>GE102</td>
<td>Goat</td>
<td><em>C. xiaoi</em></td>
<td>Smallholder</td>
<td>_</td>
</tr>
<tr>
<td>GE01</td>
<td>Goat</td>
<td>Rat genotype II</td>
<td>Smallholder</td>
<td>_</td>
</tr>
</tbody>
</table>
Table 4-4: Species, genotypes and subtypes of Cryptosporidium identified in fish in the present study. Sample DM18 was typed as *C. parvum* at the 18S locus but at *gp60* locus it was typed as *C. hominis* (IdA15G1) indicating a mixture of *C. parvum/C. hominis* in this sample. Cultured = all fish were from freshwater fish-ponds.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Host species</th>
<th>Group</th>
<th>18S</th>
<th>GP60</th>
<th>Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ON36</td>
<td>Nile tilapia</td>
<td>Cultured</td>
<td><em>C. parvum</em></td>
<td></td>
<td>IlaA19G4R1</td>
</tr>
<tr>
<td>ON68</td>
<td>Nile tilapia</td>
<td>Cultured</td>
<td><em>C. parvum</em></td>
<td>IlaA14G2R1</td>
<td>-</td>
</tr>
<tr>
<td>PG37</td>
<td>Silver barb</td>
<td>Wild freshwater</td>
<td><em>C. parvum</em></td>
<td>IlaA19G4R1</td>
<td>-</td>
</tr>
<tr>
<td>DM17</td>
<td>Mackerel scad</td>
<td>Wild marine</td>
<td><em>C. parvum</em></td>
<td>IlaA15G2R1</td>
<td>-</td>
</tr>
<tr>
<td>DM18</td>
<td>Mackerel scad</td>
<td>Wild marine</td>
<td><em>C. parvum</em>, IdA15G1</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>GO18</td>
<td>Silver biddy</td>
<td>Wild marine</td>
<td>Piscine genotype 8</td>
<td></td>
<td>Piscine genotype 8</td>
</tr>
<tr>
<td>GO55</td>
<td>Silver biddy</td>
<td>Wild marine</td>
<td>Piscine genotype 8</td>
<td></td>
<td>Piscine genotype 8</td>
</tr>
</tbody>
</table>

Table 4-5: Percentage genetic differences between piscine genotype 8 and other *Cryptosporidium* species/genotypes at the 18S rRNA and actin loci. * = Actin sequences were not available for these genotypes.

<table>
<thead>
<tr>
<th>Species/subtypes</th>
<th>18S locus</th>
<th>Actin locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piscine genotype 1</td>
<td>13.0</td>
<td>17.4</td>
</tr>
<tr>
<td>Piscine genotype 2</td>
<td>5.1</td>
<td>Not analysed*</td>
</tr>
<tr>
<td>Piscine genotype 3</td>
<td>4.3</td>
<td>Not analysed*</td>
</tr>
<tr>
<td>Piscine genotype 4</td>
<td>6.3</td>
<td>Not analysed*</td>
</tr>
<tr>
<td>Piscine genotype 5</td>
<td>5.5</td>
<td>Not analysed*</td>
</tr>
<tr>
<td>Piscine genotype 6</td>
<td>13.0</td>
<td>Not analysed*</td>
</tr>
<tr>
<td>Piscine genotype 7</td>
<td>11.8</td>
<td>Not analysed*</td>
</tr>
<tr>
<td><em>C. molnari</em></td>
<td>10.8</td>
<td>7.3-8.5</td>
</tr>
<tr>
<td><em>C. parvum</em></td>
<td>15.4</td>
<td>21.4</td>
</tr>
<tr>
<td><em>C. hominis</em></td>
<td>15.4</td>
<td>21.4</td>
</tr>
<tr>
<td><em>C. bovis</em></td>
<td>17.7</td>
<td>19.0</td>
</tr>
<tr>
<td><em>C. baileyi</em></td>
<td>14.7</td>
<td>19.4</td>
</tr>
<tr>
<td><em>C. muris</em></td>
<td>15.0</td>
<td>19.4</td>
</tr>
<tr>
<td><em>C. andersoni</em></td>
<td>13.8</td>
<td>20.1</td>
</tr>
</tbody>
</table>

4.3.3 Microscopy

No parasites were observed during microscopic examination of the intestinal or stomach tissues due to substantial autolysis of tissues.
Figure 4-2: Evolutionary relationships of Cryptosporidium isolates from sheep and goats inferred by neighbour-joining analysis of $p$ distances calculated from pairwise comparison of 18S rRNA sequences. The percentage of replicate trees, in which associated taxa clustered together in the bootstrap test (1000 replicates) are shown at the internal nodes (> 50 % only) for distance, parsimony and maximum likelihood approaches (n.s. = not supported). Accession numbers are given in parentheses. Isolates from the present study are marked with an asterisk (*).
Evolutionary relationships of Cryptosporidium piscine-derived isolates inferred by neighbour-joining analysis of p distances calculated from pairwise comparison of 18S rRNA sequences. The percentage of replicate trees, in which associated taxa clustered together in the bootstrap test (1000 replicates) are shown at the internal nodes (> 50 % only) for distance, ML and parsimony (n.s. = not supported). Accession numbers are

Figure 4-3:
given in parentheses. Isolates from the present study are marked with asterisk (*).

Figure 4-4: Phylogenetic relationships of Cryptosporidium isolates inferred by neighbor-joining analysis of the actin gene based on genetic distances calculated by the p distance model. The percentage of replicate trees, in which associated taxa clustered together in the bootstrap test (1000 replicates) are shown at the internal nodes (> 50% only) for distance, ML and parsimony (n.s. = not supported). Accession numbers are given in parentheses. Isolates from the present study are marked with asterisk (*).
4.4 Discussion

Cryptosporidium in sheep and goats

This is first study to identify and molecularly characterise Cryptosporidium in sheep and goats in PNG and analysis revealed a high diversity of Cryptosporidium parasites within these animal populations. The only other previous study of Cryptosporidium in PNG identified Cryptosporidium antibodies in 24 % of young children from Goroka (Groves et al., 1994).

Although point prevalences were low for Cryptosporidium in the present study, the true prevalence may be underestimated as only single faecal samples were screened at one time point and intermittent shedding and seasonal variation are common (O'Handley et al., 1999). In addition, only adult animals were screened and prevalences are known to be much higher in younger animals (Santin et al., 2007). Most importantly, the identification of Cryptosporidium in livestock warrants better care of farm animals to avoid contamination and illness in vulnerable populations, as Cryptosporidium spp. are known to cause diarrhoea and mortality in lambs and goat kids (de Graaf et al., 1999; Quilez et al., 2008; Giles et al., 2009).

The three species (C. parvum, C. andersoni and C. scrofarum) identified in sheep from the present study have also been reported in sheep in previous studies (Ryan et al., 2005; Santin et al., 2007; Quilez et al., 2008; Giles et al., 2009). In addition, C. andersoni is frequently reported in cattle and occasionally in humans, while C. scrofarum is commonly identified in pigs (Xiao, 2010). Cryptosporidium ubiquitum is a common species found in sheep in other countries (Ryan et al., 2005; Santin et al., 2007; Yang et al., 2009; Wang et al., 2010b), however, it was not identified in the present study.

Three species, C. hominis, C. parvum and C. xiaoi, detected in goats in the present study have also been reported in goats in other studies (Goma et al., 2007; Geurden et al., 2008b; Quilez et al., 2008; Giles et al., 2009; Diaz et al., 2010b). For example, molecular analyses confirmed infections with C. hominis and C. parvum in diarrheic goat kids in the UK (Giles et al., 2009) and C. parvum in goats in Spain (Quilez et al., 2008). Cryptosporidium xiaoi is commonly reported in sheep (Fayer and Santin,
2009) and occasionally in goats (Diaz et al., 2010b). This is the first report of rat genotype II in goats. Rat genotype II has been reported in house rats in China (Lv et al., 2009) and in the Philippines (Ng-Hublin et al., 2013), brown rats in the Philippines (Ng-Hublin et al., 2013), and in wild black rats in Northern Australia (Paparini et al., 2012). The goat in which rat genotype II was identified was from a smallholder farm in Benabena. Smallholders usually keep their goats in night houses, which are built very close to their own homes in order to avoid theft. The goat could have acquired this genotype from the house rats; however, further studies are required to confirm this and to determine if the goat was actually infected or just passing oocysts from ingestion of rat faeces. Identification of species such as C. andersoni, C. scrofarum and C. xiaoii in smallholder flocks probably reflects the management system. Typically, these small ruminants are tethered and/or allowed to graze freely on shrubs and grasses along road sides, near homes and gardens, where they share the feeding grounds with other livestock, especially cattle and pigs.

Cryptosporidium hominis and C. parvum are the most common causes of cryptosporidiosis in humans worldwide (Xiao, 2010). In the present study, C. hominis (subtype IdA15G1) was found in goats and C. parvum (subtypes IlaA15G2R1 and IlaA19G4R1) was found in both sheep and goats. The C. parvum subtype, IlaA15G2R1, has been reported in sheep and goats in previous studies in Belgium, Spain, Brazil, China and Australia (Geurden et al., 2008b; Diaz et al., 2010a; Paz et al., 2014; Yang et al., 2014a; Ye et al., 2014). The C. parvum subtype, IlaA15G2R1, is a common subtype in cattle and humans (Xiao, 2010; Feng et al., 2013) in the Americas, Europe, Northern Africa and Asia (Quilez et al., 2008; Santin et al., 2008; Soba and Logar, 2008; Brook et al., 2009; Geurden et al., 2009; Amer et al., 2010; Diaz et al., 2010a; Meireles et al., 2011; Iqbal et al., 2012; Alyousefi et al., 2013; Helmy et al., 2013; Rahmouni et al., 2014; Rieux et al., 2013). It has also been found in yaks in China (Mi et al., 2013) and in buffalo in Egypt (Helmy et al., 2013). The C. parvum subtype, IlaA19G4R1, was identified in both a goat and a sheep in the present study. Previously, C. parvum subtype IlaA19G4R1 was identified in cattle in Northern Ireland (Thompson et al., 2007) and Australia (Ng et al., 2008).
**Cryptosporidium in fish**

In fish, the overall prevalence of *Cryptosporidium* sp. was low (1.14 %, 7/614). Previous studies have also reported low prevalences in similar groups of fish (0.8 %, 6/709) (Reid et al., 2010) and in ornamental fish (3.5 %, 6/171) (Morine et al., 2012), while others have reported higher prevalences (10 - 100 %) mostly among juvenile fish (Alvarez-Pellitero et al., 2004; Sitja-Bobadilla et al., 2005; Murphy et al., 2009; Zanguee et al., 2010).

The present study identified three new fish hosts for *Cryptosporidium*: silver barb (*P. gonionotus*), mackerel scad (*D. macarellus*) and oblong silver biddy (*G. oblongus*). The fourth host Nile tilapia (*O. niloticus*) could also be a new host since previous studies have detected *Cryptosporidium* in the same genus but did not identify the species (Landsberg and Paperna, 1986; Paperna and Vilenkin, 1996).

No oocysts or life cycle stages were observed in the infected fish hosts in the present study due to substantial autolysis of tissues, which has been reported as an issue for *Cryptosporidium* detection in piscine hosts (Zanguee et al., 2010). Fish are known to have a very rapid rate of tissue autolysis compared to homeotherms (Roberts, 2012) and many of the fish were dead for up to 4 hours prior to being processed, which contributed to the problem. Previous studies have provided histological and electron microscopic evidence of considerable cellular damage associated with several *Cryptosporidium* species/genotypes that infect fish; *C. molnari* in the stomach of fingerlings and juveniles of gilt-head sea bream (Alvarez-Pellitero and Sitja-Bobadilla, 2002), piscine genotype 1 in the stomach of a guppy (Ryan et al., 2004) and piscine genotype 3 in the intestine of a mullet (Reid et al., 2010). Piscine genotype 2 was associated with gastric infections in angelfish, with the greatest morbidity and mortality seen in larval and juvenile fish (Murphy et al., 2009). Whether the *C. parvum* and *C. hominis* identified in the present study represents actual or mechanical infections remains to be determined as the oocysts may have been passing through rather than infecting these fish and further research using histological analysis of rapidly preserved tissue specimen is required to confirm this.
The zoonotic Cryptosporidium genotypes identified in this study are of significance to public health. Cryptosporidium parvum subtypes IlaA14G2R1, IlaA15G2R1 and IlaA19G4R1 were found in cultured freshwater (Nile tilapia), wild freshwater (silver barb) and a marine (mackerel scad) fish. The zoonotic C. parvum Ila subtype family has predominantly been found in calves and in humans in North America, Europe and Australia (Xiao, 2010). Only one study has previously detected zoonotic C. parvum (subtype IlaA18G3R1) in a marine fish (Reid et al., 2010). The presence of Ila subtypes in the fish samples from the present study could be due to waterborne contamination with human and animal wastes. Human sewage management systems in PNG villages typically involve dugout toilets near the homes and toilets built over the rivers or seas. No cattle farms were seen in close vicinity to where the samples were collected, whereas dugout toilets, companion animals and/or domesticated poultry and pigs were seen in the environment. Fish-ponds, rivers and seas could be contaminated from rainwater runoff and from humans and/or animals bathing in them. A previous study has detected antibodies against Cryptosporidium among children from PNG (Groves et al., 1994), however, no molecular work has been done to confirm the species or genotypes present.

One marine fish (mackerel scad DM18) was identified as C. parvum at the 18S locus, while at the gp60 locus it was subtyped as C. hominis IdA15G1R1, indicating that a mixed C. parvum/C. hominis infection was present. This is the first report of C. hominis in fish. The only other marine organism, in which C. hominis has been reported was a dugong (Dugong dugon) (Morgan et al., 2000), and its presence probably reflects human sewage contamination of the water.

The novel piscine genotype 8 was identified in two marine silver biddies. At the 18S locus, piscine genotype 8 exhibited 4.3 % genetic difference with piscine genotype 3 and 13.8 % - 17.7 % genetic difference with other Cryptosporidium spp. (Table 4-5). At the actin locus, piscine genotype 8 exhibited 7.3 % genetic difference with C. molnari 2 and 19.0 % - 21.4 % with other Cryptosporidium spp. (Table 4-5). Based on the differences in the genetic sequences, piscine genotype 8 is unique and may represent a new species; however, further research is required to confirm this.
4.5 Conclusions

These findings suggest that sheep and goats may be important reservoirs of *C. hominis* and zoonotic *C. parvum* subtypes in PNG. The detection of *C. hominis* in goats presumably reflects the very close association between humans and goats. Further research is necessary to characterise the prevalence of various Cryptosporidium species and genotypes in young lambs, goats and cattle and other hosts such as humans to more fully understand the transmission dynamics of *Cryptosporidium* in PNG.

The present study identified zoonotic *C. parvum* subtypes in fish species, which are frequently eaten in PNG. Previous studies have not identified conclusive evidence for transmission of *Cryptosporidium* from fish to humans but one study reported that urban anglers are at a risk for contracting cryptosporidiosis from exposures received, while fishing and consuming caught fish (the mean probability of infection was nearly one) (Roberts et al., 2007). It is therefore essential that fish for human consumption are handled appropriately to avoid contamination. In addition, a novel *Cryptosporidium* sp. (piscine genotype 8) was identified based on molecular data but lacks histological details of infection or morphological features of the oocysts, thus future work is required to establish this genotype as a species.
Chapter 5

Prevalence of *Giardia duodenalis* in sheep, goats and fish from PNG by quantitative PCR
Chapter 5

Gastrointestinal pathogens in sheep, goats and fish in PNG

This chapter describes work conducted using a qPCR assay to screen for *Giardia duodenalis* in sheep, goats and fish from PNG.

Research highlights:

- The overall prevalence for *G. duodenalis* in sheep, goats and fish were 9.1% (25/276), 12.3% (28/228) and 7% (19/272), respectively.
- *Giardia duodenalis* was found in sheep and goats in all farm types and in fish in all three groups (cultured freshwater, wild freshwater and wild marine).

5.1 Introduction

Giardiasis is a re-emerging infectious disease of worldwide significance caused by *Giardia duodenalis* (Thompson, 2000). *Giardia*-induced diarrhoea is common in sheep and goats. It can cause significant economic loss to farmers (O'Handley et al., 2001; Aloisio et al., 2006). See further details on *Giardia* in Chapter 1, Section 1.5.

Little is known about *Giardia* infections in fish. A recent study in Australia identified a low prevalence of *Giardia* (3.8% - 27/709) in fish hosts (Yang et al., 2010) with *G. microti* and both zoonotic (A and B) and non-zoonotic (E) assemblages of *G. duodenalis* detected. Whether the fish in the latter study were truly infected with those *Giardia* species or simply served as mechanical vectors for the dissemination of waterborne *Giardia* cysts is unclear (Feng and Xiao, 2011). Another study, in Egypt, identified *G. duodenalis* (assemblage A) in farmed and wild freshwater fish host with a prevalence of 3.3% (Ghoneim et al., 2011).

*Giardia duodenalis* is common in sheep and goats, but there are no records of infection or prevalence in these animals or other animals in PNG (Owen, 2005). Infections in humans with *G. duodenalis* have been reported in several surveys in the country (Owen, 2005). However, the importance of *G. duodenalis* as the causative agent of diarrhoea in PNG has not been assessed. As little is known of the prevalence of *Giardia* spp. in humans, domesticated animals or wildlife in PNG, the aim of the present study was to screen sheep, goats and fish for *G. duodenalis* using molecular tools.
5.2 Methods

This section summarises the most important methods used to obtain the results described in this chapter. A detailed description of all the methods is described in Chapter 2 of this thesis.

5.2.1 Sample collection

Samples were collected from sheep, goats and fish in various locations in PNG from February 2011 to August 2011. Further details of sampling are described in Chapter 2, Sections 2.1 - 2.6.

5.2.2 DNA isolation and qPCR amplification

Genomic DNA were extracted from 250 mg of faecal sample and fish tissue scrapings using a PowerSoil® DNA isolation kit (MO BIO laboratories, Carlsbad, CA, USA) as described in Chapter 2, Sections 2.8.1 and 2.8.3.

All samples were screened for the presence of *G. duodenalis* at the glutamate dehydrogenase (*gdh*) locus using a qPCR assay as previously described (Yang et al., 2014b). The details of this assay are described in Chapter 2, Section 2.9.4.

5.2.3 Data analysis

Prevalences (samples which were positive for *G. duodenalis* at the *gdh* locus) were expressed as a percentage of positive samples; with 95 % confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rozsa et al., 2000). Differences in prevalence among groups were compared by Fisher’s exact test (FET) (for two groups) or chi-square analysis (for more than two groups). Where multiple pairwise comparisons were made among groups, a Bonferroni correction was applied to maintain an experiment-wide Type I error rate of 5 %.

5.3 Results

The overall prevalence of *G. duodenalis* was 9.1 % (25/256) in sheep, 12.3 % (28/228) in goats and 7 % (19/272) in fish. For each farm type, the prevalence for sheep ranged from 5 – 12.2 % and for goats from 8.6 – 66.7 % (Table 5-1). There was no significant difference in prevalence between sheep and goats (FET: $p = 0.2474$).
There were no significant differences in prevalence of *Giardia* in sheep between farm types (FET: $p > 0.005$) (Table 5-2). There were, however, differences in prevalence of *Giardia* in goats between farms (Table 5-2). Specifically, the prevalence of *G. duodenalis* in goats was higher in Menifo compared to Labu, Tambul and Baisu ($p$ values $< 0.005$).

In the three groups of fish, the prevalence was 4.1 % (5/121) in cultured freshwater fish, 7.7 % (1/13) in wild freshwater fish and 9.4 % (13/138) in wild marine fish (Table 5-3). There were no significant difference in the prevalences between the fish groups (Chi-square: $p$ values where larger than 0.05). *Giardia duodenalis* was identified in eight fish species; Nile tilapia ($n = 4$), common carp ($n = 1$), Java carp ($n = 1$), mackerel scad ($n = 2$), rainbow runner ($n = 2$), big eye scad ($n = 4$), reef needlefish ($n = 3$) and oriental bonito ($n = 4$) (Table 5-4).

**Table 5-1: Prevalence of *G. duodenalis* in sheep and goats in different farm types in the present study.** N = the total number of faecal samples for sheep and goats. P = the number of animals, which were positive for *Giardia*. Given in parentheses are percent prevalence and 95 % confidence interval (CI).

<table>
<thead>
<tr>
<th>Type of farm</th>
<th>N</th>
<th>P (%)</th>
<th>95 % CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sheep</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lowland research institute (Labu)</td>
<td>40</td>
<td>2 (5, 0.9 - 18.2)</td>
<td></td>
</tr>
<tr>
<td>Highland research institute (Tambul)</td>
<td>115</td>
<td>14 (12.2, 7.1 - 19.9)</td>
<td></td>
</tr>
<tr>
<td>Public institute (Baisu)</td>
<td>27</td>
<td>2 (7.4, 1.3 - 25.8)</td>
<td></td>
</tr>
<tr>
<td>Government (Menifo)</td>
<td>40</td>
<td>2 (5, 0.9 - 18.2)</td>
<td></td>
</tr>
<tr>
<td>Smallholder</td>
<td>54</td>
<td>5 (9.3, 3.5 - 21.1)</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>276</td>
<td>25 (9.1, 6.1 - 13.2)</td>
<td></td>
</tr>
<tr>
<td><strong>Goats</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lowland research institute (Labu)</td>
<td>43</td>
<td>4 (9.3, 3.0 - 23.1)</td>
<td></td>
</tr>
<tr>
<td>Highland research institute (Tambul)</td>
<td>35</td>
<td>3 (8.6, 2.2 - 24.2)</td>
<td></td>
</tr>
<tr>
<td>Public institute (Baisu)</td>
<td>39</td>
<td>5 (12.8, 4.8 - 28.2)</td>
<td></td>
</tr>
<tr>
<td>Government (Menifo)</td>
<td>6</td>
<td>4 (66.7, 24.1 - 94.0)</td>
<td></td>
</tr>
<tr>
<td>Smallholder</td>
<td>105</td>
<td>12 (11.4, 6.3 - 19.5)</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>228</td>
<td>28 (12.3, 8.5 - 17.4)</td>
<td></td>
</tr>
</tbody>
</table>
Table 5-2: Comparisons of prevalences for *G. duodenalis* in sheep and goats between farm types. All pairs of farms were compared by FET. A Bonferroni correction was used to determine significance at \( p \) values < 0.005. * = values are significantly different between farm types by FET. RI = research institute, PI = public institute.

<table>
<thead>
<tr>
<th>Data sets</th>
<th>Sheep ( p ) values</th>
<th>Goats ( p ) values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lowland RI (Labu) vs Highland RI (Tambul)</td>
<td>0.3619</td>
<td>1.0000</td>
</tr>
<tr>
<td>Lowland RI (Labu) vs PI (Baisu)</td>
<td>1.0000</td>
<td>0.7298</td>
</tr>
<tr>
<td>Lowland RI (Labu) vs Government (Menifo)</td>
<td>1.0000</td>
<td>0.0043*</td>
</tr>
<tr>
<td>Lowland RI (Labu) vs Smallholder</td>
<td>0.6947</td>
<td>1.0000</td>
</tr>
<tr>
<td>Highland RI (Tambul) vs PI (Baisu)</td>
<td>0.7385</td>
<td>0.7143</td>
</tr>
<tr>
<td>Highland RI (Tambul) vs Government (Menifo)</td>
<td>0.3619</td>
<td>0.0045*</td>
</tr>
<tr>
<td>Highland RI (Tambul) vs Smallholder</td>
<td>1.0000</td>
<td>0.7614</td>
</tr>
<tr>
<td>PI (Baisu) vs Government (Menifo)</td>
<td>1.0000</td>
<td>0.0103</td>
</tr>
<tr>
<td>PI (Baisu) vs Smallholder</td>
<td>1.0000</td>
<td>0.0103</td>
</tr>
<tr>
<td>Government (Menifo) vs Smallholder</td>
<td>0.6947</td>
<td>0.0038*</td>
</tr>
</tbody>
</table>

Table 5-3: Prevalence of *G. duodenalis* in fish from the three groups of aquatic environments in the present study. \( N \) = the total number of fish samples. \( P \) = the number of animals, which were positive for *Giardia*. Given in parentheses are percent prevalences and 95 % confidence intervals (CI).

<table>
<thead>
<tr>
<th>Group</th>
<th>( N )</th>
<th>( P ) (%, 95 % CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultured freshwater</td>
<td>121</td>
<td>5 (4.1, 1.5 - 9.6)</td>
</tr>
<tr>
<td>Wild freshwater</td>
<td>13</td>
<td>1 (7.7, 0.4 – 37.9)</td>
</tr>
<tr>
<td>Wild marine</td>
<td>138</td>
<td>13 (9.4, 5.3 – 15.9)</td>
</tr>
<tr>
<td>Total</td>
<td>272</td>
<td>19 (7.0, 4.4 – 10.9)</td>
</tr>
</tbody>
</table>

Table 5-4: Prevalence of *G. duodenalis* in different fish hosts in the present study. \( N \) = the total number of fish samples. \( P \) = the number of animals, which were positive for *Giardia*. Given in parentheses are percent prevalences and 95 % confidence intervals (CI). Cultured = fish from freshwater fish ponds/farms.

<table>
<thead>
<tr>
<th>Fish group</th>
<th>Scientific name</th>
<th>Common name</th>
<th>( N )</th>
<th>( P ) (%, 95 % CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultured</td>
<td><em>Oreochromis niloticus</em></td>
<td>Nile tilapia</td>
<td>80</td>
<td>4 (5, 1.3 - 13)</td>
</tr>
<tr>
<td>Cultured</td>
<td><em>Cyprinus carpio</em></td>
<td>Common carp</td>
<td>41</td>
<td>1 (2.4, 0.13 - 14.4)</td>
</tr>
<tr>
<td>Wild freshwater</td>
<td><em>Puntius gonionotus</em></td>
<td>Java carp</td>
<td>13</td>
<td>1 (7.7, 0.4 - 37.9)</td>
</tr>
<tr>
<td>Marine</td>
<td><em>Decapterus macarellus</em></td>
<td>Mackerel scad</td>
<td>27</td>
<td>2 (7.4, 1.3 - 25.8)</td>
</tr>
<tr>
<td>Marine</td>
<td><em>Elagatis bipinnulatus</em></td>
<td>Rainbow runner</td>
<td>5</td>
<td>2 (40, 7.3 - 83)</td>
</tr>
<tr>
<td>Marine</td>
<td><em>Selar crumenophtalmus</em></td>
<td>Bigeye scad</td>
<td>77</td>
<td>4 (5.2, 1.7 - 13.5)</td>
</tr>
<tr>
<td>Marine</td>
<td><em>Strongylura incisa</em></td>
<td>Reef needlefish</td>
<td>4</td>
<td>3 (75, 21.9 - 98.7)</td>
</tr>
<tr>
<td>Marine</td>
<td><em>Sarda orientalis</em></td>
<td>Oriental bonito</td>
<td>4</td>
<td>2 (50, 9.2 - 90.8)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>251</td>
<td>19 (7.6, 4.7 - 11.8)</td>
</tr>
</tbody>
</table>
5.4 Discussion

This is the first study to identify *Giardia* spp. in sheep, goats and fish from PNG. In the present study, *G. duodenalis* was found in adult sheep in all types of farming environments, giving an overall prevalence of 9.1 % (25/276). This prevalence rate is higher than a previous study in Italy, which detected a prevalence of 1.5 % (5/325) in sheep using nested PCRs targeting the *gdh* and beta-giardin gene (Giangaspero et al., 2005), but its lower than most other studies investigating *G. duodenalis* in post-weaned/adult sheep. In those studies, the prevalences were 44 % (220/500) in Australia (Ryan et al., 2005), 37.5 % (12/32) in the US (Santin et al., 2007) and 19.2 % (86/446) in Spain (Castro-Hermida et al., 2007). The first two of these studies were based on PCR analyses, while the latter study used immunofluorescent antibody tests.

The prevalence of *G. duodenalis* in adult goats in the present study was 12.3 % (28/228) by qPCR analysis of the *gdh* gene. This rate falls in the range of reported prevalences for *G. duodenalis* in adult goats in other countries. For example, in Malaysia, a low prevalence (6.8 %, 21/310) of *Giardia* was found in goats (≥ 3 years of age) using nested PCR of the 18S locus (Lim et al., 2013), while in Spain, it was 19.8 % (23/116) in healthy adult goats using immunofluorescent antibody tests (Castro-Hermida et al., 2007). However, a similar prevalence (14.3 % - 15/105) has also been reported in goat kids (< 12 months old) in Brazil (Bomfim et al., 2005). Generally, high prevalences of *G. duodenalis* have been reported in young goats (< 10 months old). For example, in Spain, 42.6 % (133/315) were positive using molecular tools targeting the *beta-giardin* and *tpi* genes (Ruiz et al., 2008) and in Belgium, 35.5 % (53/148) were positive using immunofluorescent antibody tests (Geurden et al., 2008b).

Although the present finding showed that the prevalence of *G. duodenalis* in goats was higher in Menifo as compared to the institutional farms, this may not be a very accurate estimate as the total number of goats from Menifo, which were screened for *Giardia* was small (n = 6).

The prevalence of *G. duodenalis* was low (7 %, 19/272) in fish in the present study (Table 5-3). These results complement previous findings on overall low
prevalences of *Giardia* spp. in fish in Australia (3.8 %, 27/709) and Egypt (3.3 %, 3/92) (Yang et al., 2010; Ghoneim et al., 2011). In Australia, *G. duodenalis* cysts and trophozoites were detected in tissue sections of ten fish isolates including a marine fish (sea mullet), cultured fingerlings (barramundi, black bream and mulloway) and a freshwater fish (mulloway) (Yang et al., 2010). Whether the *G. duodenalis* identified in the present study represents actual or mechanical infections remains to be determined; the cysts may have been passing through rather than infecting these fish and further research using histological analysis of rapidly preserved tissue specimens is required to confirm this.

The present study identified *G. duodenalis* in seven new fish hosts including common carp, Java carp, mackerel scad, rainbow runner, big eye scad, reef needlefish and oriental bonito. The prevalence of *G. duodenalis* in Nile tilapia was 5 % (4/80), which is similar to that reported in Egypt (4.2 %) (Ghoneim et al., 2011).

The low prevalence of *Giardia* detected in the present study could be due to low levels of *Giardia* infections at the time of sampling. The sensitivity and specificity of the qPCR assay used in this study have been evaluated by Yang et al. (2014b) and its detection limit was 1 *Giardia* cyst per μL of faecal DNA extract. PCR is sometimes hindered by inhibitors in stool specimens, including bile acids, bilirubins, haem and complex carbohydrates (Wilson, 1997). However, in the present study, internal amplification controls (IACs), as described previously (Yang et al., 2013), were included in all the qPCR reactions to monitor faecal inhibition (see Chapter 2, Section 2.9.1).

### 5.5 Conclusions

Although the present study found low prevalences for *G. duodenalis* in adult small ruminants and fish, these animals can be considered as sources of contamination for susceptible hosts in PNG. Even low numbers (as low as ten) of *Giardia* cysts may lead to an infection in humans (Rendtorff, 1979). Furthermore, *Giardia* cysts are immediately infectious upon excretion and also are resistant and able to survive for several weeks in the environment, resulting in a gradual increase in environmental infection pressure (Geurden et al., 2010b). Previous studies have shown that
G. duodenalis assemblage E (livestock genotype) is more prevalent in sheep and goats than the zoonotic assemblages A and B (Geurden et al., 2010b), whereas a high prevalence of zoonotic assemblages (A and B) have been reported in fish (Yang et al., 2010; Ghoneim et al., 2011). In the present study, despite numerous attempts, it was not possible to genotype the Giardia positives identified. Future studies are required to genotype Giardia species and assemblages (genotypes) in these hosts and other hosts including humans to fully understand the transmission dynamics of Giardia in PNG.
Chapter 6

Morphological and molecular characterisation of *Anisakis* species in fish from PNG
The contents of this chapter have been published in the journal of Veterinary Parasitology. The article is listed below and it can be found in Appendix E.


The chapter describes morphological and molecular analyses of anisakid nematodes from wild marine fish from PNG.

Research highlights:

- This study identified only one species from the genus Anisakis, *Anisakis typica*, and the overall prevalence was 7.6%.
- *Anisakis typica* was found in seven species of fish including *Decapterus macarellus*, *Gerres oblongus*, *Pinjalo lewisi*, *Pinjalo pinjalo*, *Selar crumenophthalmus*, *Scomberomorus maculatus* and *Thunnus albacares* (these are new hosts for *A. typica*).

### 6.1 Introduction

The family Anisakidae includes parasitic nematodes of marine fauna. They have a worldwide distribution and a complex life cycle, which involves invertebrates, fish, cephalopods and mammals (Chai et al., 2005). Further details on anisakids are described in *Chapter 1, Section 1-6*. Anisakid nematodes can accidentally infect humans who can suffer from several symptoms including sudden epigastric pain, nausea, vomiting, diarrhoea and allergic reaction (Sakanari and McKerrow, 1989; Audicana and Kennedy, 2008). Most cases of human infection involve anisakid species belonging to the genus *Anisakis*. There are nine described species of *Anisakis*, which are further subdivided into two types (Fig. 6-1) (Mattiucci and Nascetti, 2008; Mattiucci et al., 2009). Of these, only *A. simplex* s.s., *A. pegreffii* and *A. physeteris* have been shown to cause infection in humans (Mattiucci et al., 2011; Arizono et al., 2012).
Figure 6-1: Type 1 and Type II *Anisakis* species belonging to the genus *Anisakis*.

Anisakid nematodes can be differentiated based on their morphological characteristics and molecular data (Fig. 6-2). Larval morphological features including the absence or presence of a ventricular appendage and an intestinal caecum are useful for distinction between several anisakid genera (Shamsi and Butcher, 2011). *Anisakis* Type I or Type II larvae can be identified based on ventriculus length and the presence of a tail spine (or mucron) (Berland, 1961; Cannon, 1977). More recently, PCR-based tools have been widely used for characterisation of anisakid species at multiple loci as discussed in Chapter 1, Section 1-6.

Anisakid nematodes are a major public health concern. In the last thirty years, there has been a marked increase in the prevalence of anisakidosis throughout the world, due in part to growing consumption of raw or lightly cooked seafood (Lymbery and Cheah, 2007; Audicana and Kennedy, 2008). The highest number of cases (> 90 %) come from Japan (with up to 1,000 cases per year) due to consumption of raw fish dishes such as sushi and sashimi (Oshima, 1972; Sugimachi et al., 1985). Most of the rest of the cases are from other countries with a tradition of eating raw or marinated fish, which include the Netherlands, France, Spain, Chile and the Philippines (Chai et al., 2005; Choi et al., 2009). Anisakidosis is caused most commonly by *Anisakis simplex* and *Pseudoterranova decipiens* (Table 6-1) (Hochberg and Hamer, 2010).

Fish are important sources of food and income for the majority of people living in the mainland coastal areas as well as islands in PNG. Little is known about the prevalence of zoonotic animal parasites such as anisakids in fish or of anisakidosis in humans in PNG. A review paper mentioned *A. simplex* in skipjack tuna (*Katsuwonus*...
pelamis) in waters on the south coast of PNG, but did not provide any supporting information (Owen, 2005). The present study was aimed at investigating the distribution of anisakid species and specifically to screen for zoonotic species of anisakids obtained in fish from PNG, using both morphology and molecular analyses.

Figure 6-2: Schematic representation of diagnostic features for differentiating anisakids. *Anisakis* sp. Type I larvae: the posterior end with a micron (see arrow) (Image from Cannon, 1977).

Table 6-1: Common zoonotic anisakids-geographic distribution, hosts, common food dishes and diseases. Host species 1 = primary host species. Host species 2 = predominant second intermediate host species.

<table>
<thead>
<tr>
<th>Nematode</th>
<th>Geographic distribution</th>
<th>Host species 1</th>
<th>Host species 2</th>
<th>Common food dishes</th>
<th>Form of anisakidosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anisakis simplex</em></td>
<td>Japan, Northern Europe, US, Korea</td>
<td>Dolphins, porpoises, whales</td>
<td>Pacific salmon, herring, anchovies, scad, hake, mackerel</td>
<td>Sushi, sashimi, salted or smoked herring, gravlax, borquerones, Hawaiian lomi-lomi, ceviche</td>
<td>Gastrointestinal, ectopic, allergy</td>
</tr>
<tr>
<td><em>Pseudoterranova decipiens</em></td>
<td>US, Canada</td>
<td>Seals, walruses, sea lions</td>
<td>cod</td>
<td>Ceviche, cod</td>
<td>Oropharyngeal</td>
</tr>
</tbody>
</table>

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6.2 Methods

This section summarises the most important methods used to obtain the results described in this chapter. A detailed description of all the methods can be found in Chapter 2 of this thesis. An overview schematic representation of methods used for sample collection and screening of anisakid nematodes in this study is shown in figure 6-3.

6.2.1 Parasite collection

A total of 276 whole fresh fish were collected from markets in the coastal towns of Madang (specific study sites: Bilbil and Madang) and Rabaul (specific study sites: Pilapila and Tavana) from March to August 2011 (see Chapter 2, Sections 2.3 and 2.5). The fish were necropsied and nematodes were collected from the body cavities. The muscles of the fish were thinly sliced and investigated under white light to check for nematode larvae. Nematodes were preserved in 70% ethanol and transported to Murdoch University, Australia, for analysis.

6.2.2 Morphological analysis

Whole nematodes were cleared in lactophenol for more than 48 h, individually mounted onto microscope slides and viewed under an Olympus BX50 light microscope equipped with Olympus DP70 Camera at 40/100x magnification. The following features were measured: body width, oesophagus length, ventriculus length and mucron length. Morphological identification was conducted according to keys previously reported (Berland, 1961; Cannon, 1977; Shamsi et al., 2009a, b). Scanning electron micrographs (SEMs) were taken for representative specimens to study further morphological details using a Phillips XL30 scanning electron microscope. Specific details of specimen preparations are described in Chapter 2, Section 2.7.2.
6.2.3 Genetic characterisation and phylogenetic analysis

DNA from individual nematodes was isolated using a Qiagen DNeasy® blood and tissue kit (Qiagen, Hilden, Germany) as described in Chapter 2, Section 2.8.2.

The ITS rRNA region was amplified using primers NCS and NC2 (Zhu et al., 1998). The mitochondrial DNA cytochrome oxidase II (mt-DNA cox2) gene was
amplified using primers 210 and 211 (Nadler and Hudspeth, 2000). PCR assays were performed as previously described (Kijewska et al., 2009a; Valentini et al., 2006). The specific details of the PCR assays are described in Chapter 2, Section 2.9.5.

All PCR products were purified using an Ultra Clean® DNA purification kit (MolBio, West Carlsbad, CA, USA). Sequencing was performed using the ABI Prism BigDye® terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 3730 DNA Analyser instrument according to manufacturer’s instructions except that the annealing temperature was lowered to 46 °C for the cox2 locus. Nucleotide sequences obtained were analysed using FinchTV 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com) and compared with published sequences for identification in the GenBank database using BLAST (http://blast.ncbi.nlm.nih.gov) for species identification. (Further details of the molecular techniques used for DNA processing are described in Chapter 2, Sections 2.10 - 2.15).

Before phylogenetic analysis, sequences were aligned with 9 ITS and 9 cox2 reference sequences, representing selected *Anisakis* species obtained from GenBank. The nucleotide sequences were aligned using MUSCLE (Edgar, 2004), edited manually and tested with MEGA5 model test to find the best DNA model to infer the phylogenetic trees using the MEGA5 software (Tamura et al., 2011). Phylogenetic analyses with other known anisakid species were conducted using both neighbour-joining (NJ) and maximum-likelihood (ML) analyses for both loci. Evolutionary relationships were calculated using the Kimura two-parameter model for ITS sequences and the Tamura-Nei model for cox2 sequences with *Contracaecum osculatum* as an outgroup. Reliabilities for both NJ and ML trees were tested using 1000 bootstrap replications (Felsenstein, 1985) and bootstrap values exceeding 70 were considered well supported (Hills and Bull, 1993). The nucleotide sequences were deposited in GenBank under the accession numbers: JX648312 - JX648326.
6.3 Results

6.3.1 Prevalence of Anisakis spp.

The overall prevalence of anisakids in fish from PNG (based on ITS PCR positives) was 7.6 % (21/276, 95 % CI = 4.9 - 11.6). Anisakid larvae were found in 7 fish species, at prevalences ranging from 2.9 - 100 % (Table 6-2). The larvae were observed mostly within the body cavities of the fish and intensity ranged from 1 to 6 per infected fish host with the exception of Pinjalo pinjalo, which had an intensity of 120 larvae per fish, with larvae being found in many other body parts including muscles, pyloric region and liver.

Table 6-2: Prevalence of anisakid larvae in different fish hosts in the present study as determined by PCR. N = the number of fish sampled. P = the number of fish, which had anisakid nematodes. Given in parentheses are the percent prevalences and 95 % confidence intervals (CI). Mean intensity (MI) is the mean number of larvae in the infected fish hosts ± SD (range). Where no SD value was given, there were only one or few positive observations and SD could not be calculated.

<table>
<thead>
<tr>
<th>Fish host</th>
<th>N</th>
<th>P (% P, 95 % CI)</th>
<th>MI ± SD (min-max)</th>
<th>Specimen code</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Decapterus macarellus</strong></td>
<td>29</td>
<td>2 (6.9, 1.2 - 24.2)</td>
<td>1</td>
<td>DM23, DM24</td>
</tr>
<tr>
<td>(mackerel scad)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Gerres oblongus</strong></td>
<td>54</td>
<td>2 (3.7, 0.6 - 13.8)</td>
<td>3 ± 0.4 (2 - 4)</td>
<td>GO14, GO15</td>
</tr>
<tr>
<td>(silver biddy)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pinjalo lewisi</strong></td>
<td>14</td>
<td>7 (50, 24 - 76)</td>
<td>5 ± 0.92 (1 - 6)</td>
<td>PL1-PL5, PL8, PL9</td>
</tr>
<tr>
<td>(slender pinjalo)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pinjalo pinjalo</strong></td>
<td>1</td>
<td>1 (100, 5.5 - 100)</td>
<td>120</td>
<td>PP1</td>
</tr>
<tr>
<td>(pinjalo snapper)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Scomberomorus maculatus</strong></td>
<td>3</td>
<td>1 (33.3, 1.8 - 87.5)</td>
<td>1</td>
<td>SM3</td>
</tr>
<tr>
<td>(Spanish mackerel)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Thunnus albacares</strong></td>
<td>34</td>
<td>1 (2.9, 0.2 - 17.1)</td>
<td>3</td>
<td>TA3</td>
</tr>
<tr>
<td>(yellow-fintuna)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Selar crumenophthalmus</strong></td>
<td>10</td>
<td>7 (6.7, 2.9 - 13.6)</td>
<td>2.9 ± 0.95 (1 - 3)</td>
<td>SC76 - SC78, SC88, SC97, SC100, SC102</td>
</tr>
<tr>
<td>(big eye scad)</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

6.3.2 Morphology of Anisakis Type I larvae

Morphological analysis showed that all anisakid nematodes examined were Anisakis Type I larvae. The larvae were white and cylindrical in shape. They measured between 20 - 36 mm in length and 0.4 - 0.45 mm in width. SEM revealed that the cuticles were irregularly striated transversely at 5.5 µm intervals. The larvae had inconspicuous lips with six papillae, a prominent boring tooth and excretory pore,
which opened ventrally at the cephalic end (Fig. 6-4, panels A and E). The mouth opening led to a cylindrical striated oesophagus (length 1.6 - 2.1 mm), which was followed by a slightly wider ventriculus (length 0.98 - 1.13 mm). The junction between oesophagus and ventriculus was transverse (Fig. 6-4 panel B). The ventriculus connected obliquely with the intestine, without a ventricular appendage and intestinal caecum (Fig. 6-4 panel C). The intestine filled the remaining part of the body. The mucron was distinct and was located at the caudal end (length 17.5 - 18.0 µm) (Fig. 6-4 panels D, F and G).

**Figure 6-4: Anisakis Type I larvae from *Selar crumenophthalmus*.** These images are exemplary for all larvae found in the present study. *Light microscopy images*: **A.** Cephalic end of larva showing the boring tooth and the excretory pore; **B.** ventriculus - oesophagus junction; **C.** ventriculus - intestine junction; **D.** caudal end showing the mucron, anal opening and anal glands. *Scanning electron microscopy images*: **E.** cephalic end; **F.** rounded tail with a mucron; **G.** mucron. *ag = anal glands, ao = anal opening, bt = boring tooth, e = oesophagus, ep = excretory pore, int = intestine, l = lips, mu = mucron, ve = ventriculus.*

### 6.3.3 Phylogenetic analysis of the ITS region

Amplification of the ITS rDNA generated an approximately 900 bp product. Based on comparison with reference ITS rDNA nucleotide sequences from GenBank
database, all 21 specimens of *Anisakis* type I larvae from the present study were identified as *Anisakis typica*. The nucleotide sequences from the present study exhibited 99.1 - 100% similarities to the published sequence of *Anisakis typica* (AB432909) found in Indian mackerel (*Rastrelliger kanagurta*) in Thailand and 96.1 - 97.6% similarities to the published sequence of *Anisakis typica* (JQ798962) found in cutlassfish (*Trichiurus lepturus*) from Brazil. The sequences exhibited 82.7 - 88.7% similarities with other *Anisakis* species (Table 6-3). Both NJ and ML analyses produced trees with similar topology. Neighbour-joining analysis of the ITS nucleotide sequences from the present study with previously reported sequences from GenBank clustered all the *Anisakis* Type I larvae examined with *Anisakis typica* (Fig. 6-5).

**Table 6-3: Percentage similarity of the *Anisakis* species analysed in the present study and their closest relatives.** At the ITS locus, comparison with *A. typica*, accession numbers AB432909 and JQ798962 were presented. *Anisakis* sp.* is conspecific with *A. nascettii* (Mattiucci et al., 2009).

<table>
<thead>
<tr>
<th>Species compared</th>
<th>% similarity at:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ITS rRNA locus</td>
<td>Cox2 locus</td>
</tr>
<tr>
<td><strong>A. typica</strong></td>
<td>99.1 - 100</td>
<td>92.4 - 99.3</td>
</tr>
<tr>
<td><strong>A. ziphidarum</strong></td>
<td>87.5 - 88.7</td>
<td>82.1 - 84.3</td>
</tr>
<tr>
<td><strong>A. pegreffii</strong></td>
<td>85.4 - 86.2</td>
<td>84.7 - 87.2</td>
</tr>
<tr>
<td><strong>A. simplex s. s</strong></td>
<td>85.6 - 86.3</td>
<td>84.7 - 86.7</td>
</tr>
<tr>
<td><strong>A. simplex C</strong></td>
<td>85.8 - 86.6</td>
<td>84.2-85.8</td>
</tr>
<tr>
<td><em>Anisakis</em> sp.*</td>
<td>86.5 - 87.8</td>
<td>not analysed</td>
</tr>
<tr>
<td><strong>A. nascettii</strong></td>
<td>not analysed</td>
<td>83.9 - 86.7</td>
</tr>
<tr>
<td><strong>A. physeteris</strong></td>
<td>82.7 – 83.9</td>
<td>82.8 - 84.5</td>
</tr>
<tr>
<td><strong>A. brevispculata</strong></td>
<td>78.6 - 80.1</td>
<td>77.0 – 79.1</td>
</tr>
<tr>
<td><strong>A. paggiae</strong></td>
<td>83.8 - 84.7</td>
<td>79.3 – 82.2</td>
</tr>
</tbody>
</table>
Figure 6-5: Phylogenetic relationships between Anisakis species from the present study (*) and other Anisakis species as inferred by neighbour-joining analysis of ITS rDNA. The evolutionary distances were computed using the Kimura-2 parameter method and the rate variation among sites was modelled with a gamma distribution with Contracaecum osculatum as an outgroup. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown at the internal nodes (> 50 % only). Specimen codes are given in Table 6-2.
Figure 6-6: Phylogenetic relationships between *Anisakis* species from the present study (*) and other *Anisakis* species inferred using the neighbour-joining analysis of *cox2* genes. The evolutionary distances were computed using Tamura-Nei model and the rate variation among sites was modelled with a gamma distribution with *Contracaecum osculatum* as an outgroup. The percentage of trees in which the associated taxa clustered together in a bootstrap test (1,000 replicates) are shown next to the branches (> 50% only). Specimen codes are given in Table 6-2.

Amplification of the *cox2* gene generated an approximately 629 bp product. As with the ITS locus, NJ and ML analyses produced trees with similar topology. Neighbour-joining analysis of *cox2* nucleotide sequences showed that all isolates clustered broadly with *A. typica* (DQ116427) but revealed more variation. Two broad
groups were produced with subgroup I consisting of 5 isolates and *A. typica* reference sequence (DQ116427), and subgroup II containing 16 isolates (Fig. 6-6). Based on genetic distance analysis, subgroup I had 98.9 - 99.3 % similarity to *A. typica* (DQ116427) while subgroup II had 92.4 - 94.6 % similarity to *A. typica* (DQ116427). The *cox2* nucleotide sequences from the present study shared 77.0 - 87.2 % similarity with other known *Anisakis* species (Table 6-3).

### 6.4 Discussion

Anisakid larvae were found in 7.6 % (21/276) of the 7 fish species examined. The intensity of infection was low (*n* ranged from 1 to 6) in all fish hosts except for *Pinjalo pinjalo* (*n* = 120) (Table 6-2). Previous studies have reported wide variation in prevalence and intensity of infection of anisakids in other fish hosts (Costa et al., 2003; Farjallah et al., 2008a; Farjallah et al., 2008b; Setyobudi et al., 2011). The relatively low infection level found in the present study could be due to the fact that most of the fish hosts sampled were relatively small in size (ranged 16 - 49 cm, fork length) compared to previous studies. In general, prevalence and parasite burden tend to increase with the size and the age of the fish host (Setyobudi et al., 2011).

All nematodes in the present study were identified morphologically as *Anisakis* Type I larvae, based on an oblique connection between the ventriculus and the intestine, lack of a ventricular appendage and intestinal caecum, and the presence of a mucron (Berland, 1961; Cannon, 1977). Larvae of *A. typica* found in cutlassfish (*Trichiurus lepturus*) from Brazil shared similar morphological characteristics with the *A. typica* larvae from the present study (Borges et al., 2012).

Phylogenetic analysis of DNA sequences indicated that all examined samples were *Anisakis typica*. At the ITS locus, all isolates examined formed a single clade with *A. typica*. The comparison of the ITS nucleotide sequences from this study with sequences previously deposited in GenBank resulted in 96.1 - 97.6 % similarities to *A. typica* found in cutlassfish (accession no. JQ798962) from Brazil and 99.1 - 100 % similarities to *A. typica* (accession no. AB432909) from Indian mackerel in Thailand.
At the cox2 locus, whilst the isolates clustered broadly with the reference *A. typica* genotype, two distinct subgroups (I: 98.9 - 99.3 % similarity and II: 92.4 - 94.6 % similarity) were identified. Previously reported cox2 trees by Valentini et al. (2006) also showed similar genetic divergence within the *Anisakis typica* clade. Furthermore, the sequence difference of 5.4 - 7.6 % between the subgroup II clade and the reference *A. typica* sequence is still within the range found between conspecifics in other nematode taxa (Blouin et al., 1998).

According to Mattiucci and Nascetti (2006), *Anisakis* species form two sister clades and *A. typica* is grouped within clade I, based on phylogenetic relationships inferred from allozyme and mitochondrial gene markers. In the present study, *A. typica* clustered within clade I at the cox2 locus, consistent with previously reported phylogenetic trees (Valentini et al., 2006; Mattiucci et al., 2009; Cavallero et al., 2011; Setyobudi et al., 2011). However, at the ITS locus, *A. typica* did not cluster within clade 1 and formed a separate group to the two clades. Other studies have shown similar tree topology at the ITS locus (Kijewska et al., 2009b; Cavallero et al., 2011). *Anisakis typica* could form a distinct lineage (resulting in three clades, rather than two, for the genus *Anisakis*). It should be noted, however, that the position of *A. typica* in both the ITS tree and cox2 tree was not well supported (< 50 % bootstrap support) in our study and therefore more sampling of the species from a wider range of hosts and geographical areas is needed to resolve this discrepancy.

The present study identified seven new fish species (*Decapterus macarellus, Gerres oblongus, Pinjalo lewisi, Pinjalo pinjalo, Selar crumenophthalmus, Scomberomorus maculatus* and *Thunnus albacares*) as hosts for *A. typica*. Previous studies have identified *A. typica* in more than 15 different fish hosts, which have an epipelagic distribution in the Atlantic Ocean close to the coastlines of Brazil, Mauritius, Morocco, Portugal and Madeira (Mattiucci et al., 2002; Pontes et al., 2005; Marques et al., 2006; Farjallah et al., 2008a; Iniguez et al., 2009; Kijewska et al., 2009b). *Anisakis typica* has also been found in the Mediterranean Sea close to Tunisia, Libya, Cyprus and Crete, and in the Indian Ocean off Somalia (Mattiucci et al., 2002; Farjallah et al., 2008b) and Australia (Yann, 2006). Furthermore *A. typica* has been found in Japan,
Taiwan, China, Thailand and Indonesia (Chen et al., 2008; Palm et al., 2008; Umehara et al., 2010). Although it has been hypothesized that *A. typica* has a global distribution that extends from a 30 °S to a 35 °N latitude (Mattiucci and Nascetti, 2006), a previous distribution model for anisakid species has not included PNG (Kuhn et al., 2011).

### 6.5 Conclusions

In conclusion, all anisakids identified from PNG in the present study were *A. typica*, which has not previously been associated with human infections. Further studies are needed to extend the knowledge of anisakid species distribution in larger fish hosts and other seafood hosts in Papua New Guinean waters, but the present results suggest that the danger from zoonotic anisakid species in PNG is very low.
Chapter 7

Prevalence of *Chlamydia* in sheep and goats from PNG by quantitative PCR
This chapter describes the screening for *Chlamydia abortus* and *C. pecorum* using a species-specific multiplex qPCR assay in sheep and goats from PNG.

Research highlights:

- The overall prevalence of *C. abortus* was 12.2 % (27/221) in sheep and was 8.6 % (11/128) in goats in all farm types.
- The overall prevalence of *C. pecorum* was 7.7 % (17/221) in sheep and was 8.6 % (11/128) in goats in all farm types.

### 7.1 Introduction

Chlamydiae are obligate intracellular bacteria, which cause a variety of diseases in humans and animals, worldwide. The taxonomy of *Chlamydia* and infection with *Chlamydia abortus* and *C. pecorum* are described in Chapter 1, Section 1.7.

Little is known about *Chlamydia* in sheep and goats in PNG. Therefore, the aim of the present study was to use a species-specific multiplex qPCR assay for *C. abortus* and *C. pecorum* to investigate the prevalence and species of *Chlamydia* found in sheep and goats over a wide geographical area, representative for the major sheep and goat farming regions of PNG.

### 7.2 Methods

This section summarises the most important methods used to obtain the results described in this chapter. A detailed description of all methods is described in Chapter 2 of this thesis.

#### 7.2.1 Sample collection

Faecal samples from 221 sheep and 128 goats collected in PNG from February to April 2011 were screened for *Chlamydia*. The details of sample collection are described in Chapter 2, Sections 2.3 - 2.6.

#### 7.2.2 DNA isolation and qPCR amplification

Total DNA was extracted from 250 mg of faeces using a PowerSoil® DNA isolation kit (MO BIO laboratories, Carlsbad, California, USA) (see Chapter 2, Section 2.8.1). All samples were screened for the presence of *C. abortus* and *C. pecorum* using a multiplex qPCR assay, which targets the amplification of the outer membrane protein.
cell surface antigen gene (ompA) as previously described (Yang et al., 2014c). The details of this assay are described in Chapter 2, Section 2.9.6.

### 7.2.3 Data analysis

Prevalences (samples which were positive for *C. abortus* or *C. pecorum*) were expressed as a percentage of positive samples, with 95 % confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rozsa et al., 2000). Differences in prevalence among groups were compared by Fisher’s exact test (FET). Where multiple pairwise comparisons were made among groups, a Bonferroni correction was applied to maintain an experiment-wide Type I error rate of 5 %.

### 7.3 Results

The overall prevalence of *C. abortus* was 12.2 % (27/221) in sheep and 8.6 % (11/128) in goats. There was no significant difference in the prevalence of *C. abortus* between sheep and goats (FET: \( p = 0.3733 \)). In each farm type, the prevalence of *C. abortus* ranged from 6.0 % (smallholder farms) to 34.8 % (Baisu) for sheep and 4.6 % (Labu) to 33.3 % (Menifo) for goats (Table 7-1). There were no differences in the prevalences of *C. abortus* between farm types for sheep with one exception, that is the prevalence observed in the public institution (Baisu) was higher than in the smallholder farms (FET: \( p < 0.005 \)) (Table 7-2). There were no differences in the prevalences of *C. abortus* between farm types for goats (FET: \( p \) values > 0.005) (Table 7-2).

The overall prevalence of *C. pecorum* was 7.7 % (17/221) in sheep and 8.6 % (11/128) in goat. There was no difference in the prevalence of *C. pecorum* between sheep and goats (FET: \( p = 0.8386 \)). In each farm type, the prevalence of *C. pecorum* ranged from 6.5 % (Tambul) to 13 % (Baisu) for sheep and 1.9 % (smallholder) to 18.2 % (Labu) for goats (Table 7-1). There were no differences in the prevalences of *C. pecorum* between farm types for both sheep and goats (FET: \( p \) values > 0.0083) (Table 7-2).
Table 7-1: Prevalence of *Chlamydia* in sheep and goats in the present study. N = the total number of faecal samples for sheep and goats. P = the number of animals, which were positive for *Chlamydia abortus* or *C. pecorum*. Given in parentheses are percent prevalence and 95 % confidence interval (CI). RI = Research institute.

<table>
<thead>
<tr>
<th>Type of farm</th>
<th>Sheep</th>
<th><em>C. abortus</em></th>
<th><em>C. pecorum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lowland RI (Labu)</td>
<td>35</td>
<td>4 (11.43, 3.7 - 27.7)</td>
<td>3 (8.6, 2.2 - 24.2)</td>
</tr>
<tr>
<td>Highland RI (Tambul)</td>
<td>93</td>
<td>10 (10.8, 5.6 - 19.3)</td>
<td>6 (6.5, 2.7 - 14.1)</td>
</tr>
<tr>
<td>Public Institute (Baisu)</td>
<td>23</td>
<td>8 (34.8, 17.2 - 57.2)</td>
<td>3 (13, 3.4 - 34.7)</td>
</tr>
<tr>
<td>Government</td>
<td>3</td>
<td>1 (33.3, 1.8 - 87.5)</td>
<td>_</td>
</tr>
<tr>
<td>Smallholder</td>
<td>67</td>
<td>4 (6, 1.9 - 15.4)</td>
<td>5 (7.5, 2.8 - 17.3)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>221</strong></td>
<td><strong>27 (12.2, 8.4 - 17.5)</strong></td>
<td><strong>17 (7.7, 4.7 - 12.2)</strong></td>
</tr>
</tbody>
</table>

Table 7-2: Comparisons of prevalences for *Chlamydia* in sheep and goats between farm types. All pairs of farms were compared by FET. A Bonferroni correction was used to determine significance at Bonferroni corrected significance levels of < 0.005 for *C. abortus* and < 0.008333 for *C. pecorum*. * = values are significantly different between farms types. RI = Research Institute.

<table>
<thead>
<tr>
<th>Data sets</th>
<th>Sheep</th>
<th><em>C. abortus</em></th>
<th>Goats</th>
<th><em>C. abortus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lowland RI (Labu) vs Highland RI (Tambul)</td>
<td>1.0000</td>
<td>0.3449</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lowland RI (Labu) vs Public Institute (Baisu)</td>
<td>0.0473</td>
<td>1.0000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lowland RI (Labu) vs Menifo</td>
<td>0.3532</td>
<td>0.2300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lowland RI (Labu) vs Smallholder</td>
<td>0.4410</td>
<td>1.0000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highland RI (Tambul) vs Public Institute (Baisu)</td>
<td>0.0087</td>
<td>0.6378</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highland RI (Tambul) vs Menifo</td>
<td>0.3087</td>
<td>0.4368</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highland RI (Tambul) vs Smallholder</td>
<td>0.3984</td>
<td>0.3972</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Public Institute (Baisu) vs Menifo</td>
<td>1.0000</td>
<td>0.2633</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Public Institute (Baisu) vs Smallholder</td>
<td>0.0015*</td>
<td>1.0000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Menifo vs Smallholder</td>
<td>0.2020</td>
<td>0.2487</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Data sets</th>
<th>Sheep</th>
<th><em>C. pecorum</em></th>
<th>Goats</th>
<th><em>C. pecorum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lowland RI (Labu) vs Highland RI (Tambul)</td>
<td>0.7043</td>
<td>0.3449</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lowland RI (Labu) vs Public Institute (Baisu)</td>
<td>0.6727</td>
<td>1.0000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lowland RI (Labu) vs Smallholder</td>
<td>1.0000</td>
<td>0.0240</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highland RI (Tambul) vs Public Institute (Baisu)</td>
<td>0.3784</td>
<td>0.3803</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highland RI (Tambul) vs Smallholder</td>
<td>1.0000</td>
<td>0.4898</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Public Institute (Baisu) vs Smallholder</td>
<td>0.4162</td>
<td>0.0193</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
7.4 Discussion

This is the first study to detect *C. abortus* and *C. pecorum* in sheep and goats (clinically healthy adult animals at the time of sampling) in PNG. The prevalence observed for *C. abortus* was 12.2 % (27/221) in sheep in the present study. This rate is within the range of reported prevalences of *C. abortus* in clinically healthy adult sheep (free from diarrhoea, history of abortion or respiratory diseases), for example, 6.7 % in Egypt (Osman et al., 2011) and 50 % in Germany (Lenzko et al., 2011). The prevalence of *C. abortus* is usually higher in diseased sheep (presence of diarrhoea, history of abortion or respiratory diseases). For example, in Egypt, the prevalence of *C. abortus* was 25.7 % in diseased sheep (Osman et al., 2011).

In this study, the prevalence of *C. abortus* was low (8.6 %, 11/128) in clinically healthy goats. A previous study in Egypt reported a higher prevalence of *C. abortus* in in clinically healthy (free from diarrhoea, history of abortion or respiratory diseases) (50 %) and diseased (presence of diarrhoea, history of abortion or respiratory diseases) (16.2 %) goats (Osman et al., 2011). The higher prevalences in the previous study compared to the present study could be due to the techniques used for screening, as well as, the total number of animals sampled. In Egypt, molecular identification was not performed directly on faecal samples collected from sheep and goats, but from embryonated chicken eggs inoculated with cell cultures of faecal samples from sheep and goats (Osman et al., 2011). Furthermore, fewer sheep and goats were screened (sheep: asymptomatic *n* = 10 and diseased *n* = 59; goats: asymptomatic *n* = 12 and diseased *n* = 24) (Osman et al., 2011), thereby, giving a higher prevalence than the present study.

An obvious limitation of the present study is that vaginal swabs were not screened and therefore the true prevalence of *C. abortus* in sheep and goats in PNG may be higher. However, *C. abortus* is commonly found in faeces as well as the genital tract (Herrmann et al., 2000; Tsakos et al., 2001; Longbottom et al., 2002; Longbottom and Coulter, 2003; Jee et al., 2004; Lenzko et al., 2011; Talafha et al., 2012) and previous studies have shown that *C. abortus* is frequently detected in faecal samples by qPCR (Jee et al., 2004; Lenzko et al., 2011).
The prevalence of *C. abortus* in sheep was higher in public institutional farms (Baisu) than in smallholder farms. The lower prevalence observed in smallholder farms could be due to the fact that the stocking rates were much lower (each smallholder farms owned 3 – 20 animals), thus resulting in little contamination and spread of *C. abortus*.

The prevalence of *C. pecorum* was 7.7 % (17/221) in sheep and 8.6 % (11/128) in goats in the present study. In contrast, other studies have detected a high prevalence of *C. pecorum* in clinically healthy adult sheep in Germany (47 %) (Lenzko et al., 2011) and Australia (30.1 %, 1027/3412) (Yang et al., 2014c). The observed differences in the prevalence could be due to stocking rates and faecal contamination of pastures and resting places. The flock sizes ranged from 6 – 115 in the present study, 250 – 1,800 in Germany (Lenzko et al., 2011) and 300 – 5,500 in Australia (Yang et al., 2014c). Faecal shedding is an important mode of transmission for *Chlamydia* spp. (Shewen, 1980; Brenner J., 2001). The high prevalences observed in Germany and Australia may be due to high contamination of pasture or resting places, where animal numbers are large.

There are very few studies which have screened goats’ faecal samples for *C. pecorum*. For example, in Egypt, Osman et al. (2011) did not identify *C. pecorum* in goats, although it was present in sheep (at low prevalence). *Chlamydia pecorum* is commonly isolated from the digestive tract of ruminants, in which the infections are clinically inconspicuous, but under circumstances of stress, host animals may shed the organism in large numbers and may suffer from clinical disease (Shewen, 1980; Osman et al., 2011).

In summary, low prevalences of *C. abortus* and *C. pecorum* in sheep and goats in the present study could be due to low distribution of the bacteria in the biological material. Also, the observed lower prevalence could be related to low contamination of environment with these bacteria; however further epidemiological studies are needed. The analytical sensitivity may be a contributing factor for the observed lower prevalence. The multiplex qPCR assay used for screening the samples in the present was previously validated by Yang et al. (2004c). The detection limit for *C. abortus* and
C. pecorum was 5 and 5 organisms respectively per 1 µL of faecal DNA extract (Yang et al., 2004c). Therefore, the detection technique is unlikely be a contributing factor to the observed low prevalences.

7.5 Conclusions

In conclusion, prevalences of C. abortus and C. pecorum in sheep and goats in PNG were low in comparison to previous studies, which also used molecular methods for detection. However, the results of the present study indicate that C. abortus occurs in sheep and goats in PNG and could pose a risk of transmission to humans, especially in villages in PNG, where the small ruminants are cared for by women on a daily basis. Little is known about chlamydial abortions in women in PNG. Previous studies in PNG have focused on C. trachomatis infections, for which the reported prevalences were from 14 – 26.1 % in females (Theunissen et al., 1995; Vallely et al. 2010).

Future studies should include surveys on the history of abortion in sheep and goats, and compare clinically healthy Chlamydia-positive flocks with diseased flocks (those with clinical chlamydial infection) and flocks with reproductive disorders without chlamydial infections to determine the clinical impact of Chlamydia in PNG. Also, further isolation and characterisation of Chlamydia spp. are required to assess their possible zoonotic potential.
Chapter 8

General discussion and concluding remarks
Chapter 8  

**Gastrointestinal pathogens in sheep, goats and fish in PNG**

### 8.1 General discussion

The focus of the work conducted in this thesis was to investigate the epidemiology of gastrointestinal pathogens in sheep and goats in PNG and to detect potential zoonotic pathogens present in these animals in smallholder and institutional flocks, as well as, government breeding sites. Also, fish were sampled in aquaculture farms, rivers and marine systems to specifically identify potential zoonotic gastrointestinal parasites (*Cryptosporidium*, *Giardia* and anisakids), which may represent a source of illness for local populations. It should be noted that sample collection in rural areas in PNG is logistically difficult, especially in the remote areas where most of the sampling was done. The present study represents the most detailed investigation of gastrointestinal parasites in sheep, goats and fish in PNG to date, using molecular tools, and a variety of gastrointestinal pathogens were identified. This provides an important update on internal pathogens of sheep and goats from previous studies (Quartermain, 2004b).

A high prevalence of gastrointestinal nematodes, especially *Haemonchus contortus*, and protozoa, particularly *Eimeria* spp., were detected in both sheep and goats (*Chapter 3*). According to the survey questionnaire, most smallholder farmers did not know about parasites and the diseases they can cause in their flocks, which may be one reason for the high prevalence observed in the smallholder settings. In the research institute farms, although anthelmintics were given to the flocks to control the burden of gastrointestinal nematodes, the animals were kept at higher stocking rates compared to smallholder farms, lambing pens were dirty and floors of the resting houses were not swept. These factors may contribute to the spread of gastrointestinal parasites (Van Metre, 2013).

The main goal of employing practises to control gastrointestinal parasites in farm management is to break their life cycle, which can be done in a variety of ways including the use of anthelmintics, pasture management and animal management. Good pasture management as well as the use of anthelmintics, depends on the level of anthelmintic resistance in a particular flock (Sani et al., 2004). Parasite control can be exercised even without the use of anthelmintics through practises such as rotational...
grazing and applying basic hygienic measures, but requires considerable efforts in management and planning and the farmers continued commitment to these activities (Sani et al., 2004). Committed farmers in turn need support and clear advice from informed veterinary practitioners, animal scientists, and extension workers on relevant, scientifically sound strategies for parasite control. In low-income countries like PNG, this infrastructure is often not established.

PNG has a climate ideal for development of *H. contortus*, *Teladorsagia*, and *Trichostrongylus* spp., where the latter two genera occur especially in the cool highlands areas. Rational grazing, where sheep and goats are allowed to graze in a paddock for 3-4 days, after which they are moved to another paddock (Sani et al., 2004) may be an appropriate strategy to reduce parasite burden in sheep and goats in PNG. The potential success of such methods is due to the relatively short larval survival times on open pasture. Previous studies of trichostrongylid nematodes on open pasture in humid tropical climates in Southeast Asia found that eggs developed to infective larvae in a minimum time of 3–4 days after faecal deposition and most larvae developed within seven days. Infective larvae on open pasture survived for 5-6 weeks, and on vegetation under tree crops survived for 5-8 weeks (Cheah and Rajamanickam, 1997; Sam-Mohan, 1995; Sani, 1994). The relatively short larval survival times observed allows for the integration of grazing management with worm control. Small ruminants can safely graze for 3-4 days in an area which is ‘rested’ for 5-6 weeks (Sani et al., 2004).

In addition, low-cost and simple diagnostic tools, such FAMACHA (described in Chapter 1, Section 1.2.4) (van Wyk et al, 2002), could be used by farmers in PNG for diagnosis of anaemia, so that the affected sheep and goats can be selectively treated with anthelmintic drugs.

The protozoan parasites, *Cryptosporidium* and *Giardia* were also found in sheep, goats and fish (Chapters 4 and 5) in the present study, however the pathogenic effects of these parasites in PNG need to be further investigated. Sheep and goat producers should be informed of the clinical signs (diarrhoea) caused by these protozoa and measures should be taken if disease signs are observed in young animals.
to prevent the spread of these parasites to other animals, or to humans. Such measures include but are not limited to an improvement of hygiene (clean pens, washing of hands).

In terms of molecular characterisation, the present study did not characterise assemblages of *G. duodenalis* and future studies using techniques such as PCR and sequencing are needed to genotype *G. duodenalis* isolates to assemblage level to better understand the zoonotic potential of this parasite in these hosts. The present study used a qPCR assay to determine the presence or absence of *G. duodenalis*.

The work described in Chapter 6 was aimed at identifying zoonotic anisakid nematodes in fish, as fish are a major source of food for people living in coastal villages and towns in PNG. Papua New Guineans prepare their fish by smoking, steaming and deep-frying. More recently, raw seafood dishes, especially, sushi and sashimi, are becoming popular in hotels and restaurants in towns and cities due to an increasing international community. In addition to the consumption of raw fish dishes, the presence of live third stage larvae of anisakid nematodes, in smoked fish or deep fried fish, could cause health implications (Bier, 1976; Hauck, 1977; Gardiner, 1990; Kim et al., 2006). The present study did not find zoonotic anisakid species in a variety of fish hosts in PNG; however, further studies are necessary to confirm the absence of zoonotic anisakid in PNG waters.

In Chapter 7, a species-specific multiplex qPCR assay was used to screen for *C. abortus* and *C. pecorum* in sheep and goats from PNG. Both pathogens were prevalent in sheep and goats in all the farms types. The presence of *Chlamydia* in small ruminants in PNG has not been investigated before. A possible explanation for this is that livestock officers, veterinarians and human health professionals are not necessarily aware of the widespread occurrence and zoonotic nature of *Chlamydia*. As both species could cause abortion in sheep and goats, this study provides valuable information for researchers and veterinarians who are working to improve production of sheep and goats throughout the country. Everyone in contact with sheep and goats should be informed about the clinical signs of *Chlamydia*. It is particularly important
that pregnant women in rural areas should be made aware of the risks of *C. abortus* and how to avoid infection.

### 8.2 Future directions

There are many open questions remaining, which require scientific work beyond the scope of this thesis and research opportunities which have arisen due to the performed work. Some of the most important questions originating from this research are raised in the next few paragraphs.

1. **What diseases are prevalent in sheep and goats in PNG?**

   There is little current research documenting the consequences of internal pathogens on sheep and goats in PNG. Documentation is limited because, in the past, small ruminants in PNG have been perceived to be less important than pigs, poultry and cattle (Quartermain, 2004b). Therefore, human and financial resources available for research, directed at sheep and goats, have been limited. The farmers in the present study said they have noticed foot-rot disease, diarrhoea and coughing in their sheep and goats, yet, most were unaware of other diseases and the underlying pathogens causing the diseases in their flocks.

   Further studies into internal pathogen species/genotype epidemiology, especially in the lowland smallholder farms in areas with different levels of rainfall, are required. The present study provided insights into gastrointestinal nematodes (including trichostrongylids, *Strongyloides* and *Trichuris*), liver fluke (*Fasciola*), cestode (*Moniezia*), protozoa (*Eimeria*, *Cryptosporidium* and *Giardia*) and bacteria (*C. abortus* and *C. pecorum*), in a warm lowland site (Labu) and the highland sites (smallholder farms, Menifo, Baisu and Tambul). However, more information about other important infectious agents of small ruminants is needed. Specifically, other important pathogens known to cause diarrhoea (*Escherichia coli*, *Salmonella* sp., *Clostridium perfringins*, and rotavirus) and abortion (*Campylobacter fetus*, *Toxoplasma gondii*, *Brucella ovis*) in sheep and goats should be investigated.

   Due to the scarcity of veterinary research in PNG and the associated severe knowledge gaps, a large scale longitudinal research study is needed to:
(i) collect data on the epidemiology of a wide range of pathogens in different geographical areas and farm types at different time points, in both warm and cool agro-ecological zones, to determine the distribution of different species/genotypes of internal pathogens (parasites, bacteria and viruses) across different geographical areas, and;

(ii) to determine in detail, the impact on human and animal health as well as the economic impact, caused by these pathogens in PNG.

However, it should be noted that such studies are logistically difficult and costly to conduct in PNG for several reasons. The smallholder farms are remote and to ensure the samples are kept viable, the sampling team has to stay in a nearby town or village, where there is power for refrigeration. Roads are usually in very poor condition and only 4WD vehicles may be used. Furthermore, farmers have to be notified a day in advance, before sampling so that animals are kept in the night house on the day the field team arrives. This is often very difficult due to the lack of telecommunication.

(2) What are the longitudinal prevalence rates of pathogens and transmission dynamics of zoonotic pathogens?

This study was a cross-sectional study (faecal samples collected at one time point) and the true prevalence, especially of Cryptosporidium spp., Giardia spp., Chlamydia abortus and Chlamydia pecorum may be underestimated due to factors such as intermittent shedding, seasonal variation and imperfect detection. Therefore, their true prevalence may be considerably higher in PNG and future longitudinal studies, as described above, could give more accurate estimates. Also, as these pathogens have zoonotic potential, further studies should include a wide range of hosts such as humans, companion animals and cattle, to more fully understand their transmission dynamics in PNG.

(3) Which fish species serve as hosts for Cryptosporidium and Giardia?

Further morphological and histological analyses of Cryptosporidium and Giardia in fish hosts are required (in addition to genetic studies) to identify sites of infection
and confirm that fish hosts are actually infected and not just serving as mechanical transmitters of Cryptosporidium and Giardia oo/cysts, as this information is currently lacking. This is particularly important for zoonotic species of Cryptosporidium and Giardia. Infection studies that inoculate fish with zoonotic species of Cryptosporidium and Giardia and subsequent morphological/histological and genetic studies should be conducted to better understand the transmission dynamics of zoonotic Cryptosporidium and Giardia in fish hosts.

(4) What is the true burden of gastrointestinal nematodes in livestock?

A wider analysis of total worm counts and histopathological analysis of tissue sections of sheep and goats in a flock are critical to determine the burden of nematode infections in the gut to allow decisions about treatment of flocks with anthelmintics. In addition, although this study used molecular tools to identify species of strongylid nematodes, future studies should include larval culture (on individual faecal samples), in addition to worm egg counts for species identification of internal helminths. In PNG, where molecular tools are rare in veterinary (research) laboratories, larval cultures are critical. However, for such studies to take place, a better veterinary science infrastructure needs to be established in PNG. But the funds available to build state-of-the-art facilities for molecular research in veterinary research are scarce. To date, there are several PCR machines in all of PNG and these are located in PNG Institute of Medical Research and the Port General Hospital for use with samples from humans.

8.3 Concluding remarks

The results generated in this study have been given to the livestock research team at NARI in Labu, who can disseminate information to breeders and farmers in the country. To reduce the burden caused by gastrointestinal parasites and improve productivity of sheep and goats, veterinary scientists at NARI could assist farmers in understanding the diseases and control measures that could be applied to prevent contamination and illness in their animals.
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Bibliography


Appendices

The following appendices include journal articles published by the candidate over the course of the candidature. They are submitted as additional evidence for candidature for the degree of Doctor of Philosophy.

Appendix A: The survey questionnaire described in Chapter 2.


Appendix A

Survey Questionnaire
Appendix A  Gastrointestinal pathogens in sheep, goats and fish in PNG

Prevalence and management factors related to the prevalence of gastrointestinal parasites in sheep and goat in wet mainland of Papua New Guinea.

Conducted by: Melanie Koinari, Murdoch University, in collaboration with National Agricultural Research Institute of Papua New Guinea, Bubia, Morobe Province.

Section 1: GENERAL MANAGEMENT
1. How many animals are there in your farm/ herd?
   Sheep: __________________________  Goats: __________________________

2a) How many animals by age group?
<table>
<thead>
<tr>
<th>Number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
</tr>
<tr>
<td>Male Adults</td>
</tr>
<tr>
<td>Female Breeder</td>
</tr>
<tr>
<td>Pre-weaned (0-2 months old lamb)</td>
</tr>
<tr>
<td>Post-weaned (2-6 months old lambs)</td>
</tr>
<tr>
<td>Goats</td>
</tr>
<tr>
<td>Male Adults</td>
</tr>
<tr>
<td>Female Breeder</td>
</tr>
<tr>
<td>Pre-weaned (0-2 months old lamb)</td>
</tr>
<tr>
<td>Post-weaned (2-6 months old lambs)</td>
</tr>
</tbody>
</table>

2b) Number of lambs in your herd/farm: __________________________
   Number of kids in your herd/ farm: __________________________

3. How large is your farm? __________________________

4. How are the animals fed?
<table>
<thead>
<tr>
<th>Feed</th>
<th>Yes/No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grazing:</td>
<td></td>
</tr>
<tr>
<td>Cut and Carry:</td>
<td></td>
</tr>
<tr>
<td>Feed:</td>
<td></td>
</tr>
<tr>
<td>Mixed feeding (eg: grazing and cut and carry):</td>
<td></td>
</tr>
</tbody>
</table>

5. If the animals are let free to graze, what type of grazing system is adopted for the herd?
   ☐ No restriction (free area)
   ☐ The animals are allowed to graze at particular areas restricted by non-electric fencing.

6. Are the animals separated according to gender? Yes/No ________

7. Do the animals get any feed supplements? Yes/No ________
   If no, go to Question number 10.
   If yes, what type of feed supplement is used? (Please specify)

8. If feed supplement is used, is it specially made or purchased?
   ☐ Made
   ☐ Purchased
   ☐ Both
9. If purchased, where do you buy the feed supplement from?
   - Feed distributor
   - Others (Please specify): ______________________

10. What is the source of water for the herd?
    - Tap water
    - River
    - Groundwater
    - Others (Please specify): ______________________

11. a) Approximately how far is your sheep herd from the nearest sheep herd?
    ______________________
    b) Approximately how far is your goat herd from the nearest goat herd?
    ______________________

12. a) Approximately how far is your sheep herd from other livestock such as goat, buffalo, cattle, and pigs?
    ______________________
    b) Approximately how far is your goat herd from other livestock such as sheep, buffalo, cattle, and pigs?
    ______________________

13. When was the last time you introduced new sheep/goat to your farm?
    Sheep: ______________________
    Goat: ______________________

14. How often do you introduce new animals to your farm?
    ______________________

15. Are there any other animals on this farm? Yes/No _________
    If yes, please specify:
    ______________________________________________________

16. Do you have any specific management practices for handling animal waste? Yes/No _________

17. If no, where do the faeces or effluent go?
    ______________________________________________________

20. If yes, how do you manage the animal waste?
    ______________________________________________________

22. If treated, what type of waste treatment system do you use?
    ______________________________________________________
Section 2: EWES - LAMB MANAGEMENT

Perinatal management

1a) Where are lambs born?
- multi-ewes lambing area
- single-ewes lambing area
- born in the field

1b) Where are kids born?
- multi-does doeling area
- single-does doeling area
- born in the field

If the lambs/kids are born in pens, please answer Questions 2 to 4. If not proceed to Question 5.

2. Do you clean the lambing pens before young are born?
   - Yes
   - No

3. What is the floor of the lambing pen made of?
   - Lamb (Yes/No)
   - Kid (Yes/No)
   - Concrete
   - Earth
   - Gravel
   - Others (Please specify)

4. If earth flooring, do you sweep the floor? Yes/No ________

5. Do you separate young animal from their mothers within 12 hours after birth?
   - Yes
   - No

Section 3: HERD HEALTH PROGRAM

1. Is medication given to your herd? Yes/No ________ If no, proceed to Section 4.

2. Is medication given to newborn animals?
   - Lamb
   - Kids
   - Yes
   - No

3. If yes, what type of medication is given to the young animals?
   - Lamb: ____________________________
   - Kid: ____________________________

4. When was the most recent medication given?
   - Date: ____________ (dd/mm/yyyy)
5. What type of medication is routinely used for the herd?
The brand name: __________________________________

6. Approximately how many times do you give treatments each year? _________

**Section 4: HEALTH**

1. Do you know any disease in your herd/flock? Yes/No _______________
   If yes, please specify: _____________________________________________
   If your herd has diarrhoea, please answer questions 2 to 4.

2. Do you know what caused this diarrhoea? Yes/No _______________
   If yes, please outline: ___________________________________________

3. Do you know the clinical signs of diarrhoea? Yes/No _______________
   If yes, please describe: __________________________________________

4. Do you know how to control diarrhoea? Yes/No _______________
   If yes, please describe: __________________________________________

General comments:

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*Thank you for taking the time to complete this questionnaire.*

Melanie Koinari  
PhD student,  
Faculty of Veterinary Medicine and Biomedical Sciences,  
Murdoch University,  
Perth, Australia
Appendix B

Infection levels of gastrointestinal parasites in sheep and goats in Papua New Guinea.
Infection levels of gastrointestinal parasites in sheep and goats in Papua New Guinea

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Abstract

Gastrointestinal parasites of livestock cause diseases of important socio-economic concern worldwide. The present study investigated the prevalence of gastrointestinal parasites in sheep and goats in lowland and highland regions of Papua New Guinea (PNG). Faecal samples were collected from a total of 165 small ruminants (110 sheep and 55 goats) from February to April 2011. Analysis by a modified McMaster technique revealed that 128 animals (72% of sheep and 89% of goats) were infected with one or more species of gastrointestinal parasites. The gastrointestinal parasites found and their prevalences in sheep (S) and in goats (G) were as follows: strongyle 67.3% (S), 85.5% (G); Eimeria 17.3% (S), 16.4% (G); Strongyloides, 8.2% (S), 23.6% (G); Fasciola, 5.5% (S), 18.2% (G); Trichuris, 1.8% (S), 3.6% (G); and Nematodirus, 1.8% (S), 3.6% (G). Two additional genera were found in goats: Moniezia (9.1%) and Dictocaulus (3.6%). This is the first study to quantitatively examine the prevalence of gastrointestinal parasites in goats in PNG. The high rates of parasitism observed in the present study are likely to be associated with poor farming management practices, including lack of pasture recovery time, lack of parasite control measures and poor-quality feed.

Introduction

Parasitism is recognized as a major threat to the production of small ruminants in both small-scale and large-scale farms. Gastrointestinal (GI) parasites cause high mortality, reduce production and lead to a significant overall economic loss (Al-Quaisy et al., 1987; McLeod, 1995; Simpson, 2000). GI parasites are highly prevalent in sheep and goats in humid subtropical and tropical areas of the world (Yadav & Tandon, 1989; Banks et al., 1990; Barger et al., 1994; Dorny et al., 1995; Cheah & Rajamanickam, 1997; Regassa et al., 2006; Nwosu et al., 2007; Gadahi et al., 2009; Abebe et al., 2011; Dagnachew et al., 2011).

There are approximately 15,000 sheep and 20,000 goats in Papua New Guinea (PNG) (Quartermain, 2002). Animals are raised by institutions for breeding or for nutritional research purposes and by smallholder farmers for subsistence meat production. There are no sheep and goats native to PNG. Tropical sheep from South-East Asia were introduced by colonial administrators and missionaries in the late 19th century and this sheep population is known as PNG Priangan. In the 1980s, temperate Corriedale and Perendale sheep from New Zealand were brought to the cooler highlands of PNG. These temperate sheep breeds were crossed with the PNG Priangan sheep to produce crossesbreeds known as Highlands Halfbred (Quartermain, 2002). PNG Priangan sheep are mainly raised in areas of lower altitude, whereas Highlands Halfbred sheep are raised at higher altitudes. A variety of dairy goats, which are now referred to as PNG genotype, were introduced during the early colonial period (Quartermain, 2002).
Distribution and productivity of small ruminants in PNG are hindered mainly by poor health, nutrition and management (Quartermain, 2004). GI parasites are expected to be widespread in PNG due to its humid tropical climate. In some parts of PNG, previous surveys, which were conducted mostly in government stations, identified a diversity of internal parasite species in small ruminants (Varghese & Yayabu, 1985; Owen, 1988, 1989, 1998; also reviewed by Quartermain, 2004). However, there is insufficient information on the epidemiology of the GI parasites infecting sheep and goats in PNG. Such information is essential for understanding the economic impact these parasites can have on farmers and to support decision-making regarding the treatment and prevention of parasitic diseases in these animals. The present study, therefore, was conducted to obtain data on the prevalence and infection levels of gastrointestinal parasites in sheep and goats on several farms in PNG.

Materials and methods

Study sites

The study was conducted from February to April 2011 in two broad agro-climatic zones, the highlands (specific study sites: Tambul, Baisu, Menifo and Ungai-Bena) and lowlands (specific study site: Labu) in the central region of mainland PNG (fig. 1).

The altitudes of the study sites are 0 m for Labu, 1600–1608 m for Menifo and Ungai-Bena, 1730 m for Baisu and 2320 m for Tambul, with mean annual temperatures of 26°C, 20.1°C, 20.1°C, 18.3°C and 14.7°C, respectively (Bourke, 2010). The mean annual rainfall for Labu is above 4000 mm and between 2000 and 3500 mm for Ungai-Bena, Baisu and Tambul (Quartermain, 2004). Menifo is drier than most of the PNG highlands and receives a mean annual rainfall of 1000–1500 mm (Quartermain, 2004).

The study sites were further characterized by a questionnaire survey, in which 20 farm managers and smallholder farmers were interviewed. It consisted of questions regarding general farm management practices, feeding systems and herd health programmes.

Farm management

The flocks from the three institutional farms (Labu, Baisu and Tambul) were kept together in fenced areas (approximately 20–60 ha), grazed pasture at a high stocking rate at daytime and were kept in houses with wooden, slatted floors at night. At the time of sample collection, the total numbers of sheep and goats in Labu, Baisu and Tambul were 125, 70 and 143, respectively. The subsistence farmers kept few animals (usually fewer than 15) which grazed free range or were tethered and housed at night on slatted floors or on the ground underneath the farmer’s house.

Feeding system

Most animals grazed on native grasses and shrubs. Smallholder farmers also fed their animals with starchy vegetables (mostly sweet potatoes). The interviewed farmers also indicated that there were shortages of feed. The animals drank from troughs (sourced from water supply or rainwater tanks), rainwater run-off water or ponds.

Herd health program

The floors of the resting houses were not swept. The animals were penned on dirty floor, ground or on bare concrete floors. The smallholder farmers did not shear their sheep and explained that they did not have the resources for it. Most farm managers reported that the most common signs of illness in their animals were diarrhea and coughing, followed by itching and hair loss. Most smallholder farmers did not know about causes of diseases in their sheep and goats or the use of anthelmintic drugs for treatment of nematode infections. For instance, a man in Ungai-Bena reported the death of his entire flock (n = 25) and noticed nematode worms in the gut of a dead sheep. The three large institutional flocks were drenched with benzimidazole (Panacur) nominally at bimonthly intervals. At the time of sampling animals had been drenched 2 months previously in Labu and 4 months previously in Baisu and Tambul.

Collection and examination of faecal samples

A total of 110 faecal samples from sheep and 55 faecal samples from goats were obtained from the rectum of randomly selected animals between 07.30 and 09.00 h and kept at 4°C until taken to the laboratory for analysis. Each sample was examined visually for consistency, mucus and macroscopic parasites. Two grams of each faecal sample were examined using a modified McMaster technique (Whitlock, 1948). Parasites were identified morphologically to genus, or in the case of strongyles, to group level (Soulsby, 1965). Parasite egg load was expressed as eggs per gram faeces (EPG). The volume of the flotation fluid used in the examinations was 50 ml and the fluid volume examined in the counting chamber was 0.3 ml. Since EPG is calculated using equation 1, the
Gastrointestinal parasites in sheep and goats in Papua New Guinea

minimum EPG count in our analyses was 83 eggs/g and occurred when only one egg was observed in the counting chamber.

$$\text{EPG} = \frac{\text{eggs counted} \times \text{total volume (ml)}}{\text{examed volume (ml) \times weight of faeces (g)}} \quad (1)$$

Data analysis

Prevalence was calculated as the percentage of positive samples in the total number of samples examined. Apart from the overall prevalence (i.e. the infection with any GI parasite) in each flock, prevalence was also calculated for each parasite type, stratified by animal species and breed, study site, agro-ecological zone and farm management type. Differences in prevalence among groups were compared by Fisher’s exact test (FET) (for two groups) or chi-squared analysis (for more than two groups).

EPG counts were transformed to their decadic logarithms to obtain a normal distribution of values prior to statistical testing for significant differences between animal species and breeds, study sites, agro-ecological zones and farm management systems. Differences between/among groups were compared by t-tests or one-way analysis of variance (ANOVA); followed by a post hoc Tukey’s HSD test. Statistical analyses were performed using GraphPad Prism version 4 (GraphPad Inc., California, USA).

Results

From the total of 165 small ruminants examined, 128 (78%) were found to be infected with one or more types of GI parasites. A mixed infection with two or more types of GI parasites was found in 39% (50/128) of the infected animals: 33% (26/79) of sheep and 49% (24/49) of goats (not significantly different by FET, $P = 0.09$).

Table 1 shows prevalence and mean EPG counts over all sampling sites, stratified by the type of host animal (sheep or goat) and the type of parasite. The overall prevalence of GI parasite infection was significantly higher (FET; $P = 0.017$) in goats (89%, 49/55) than in sheep (72%, 79/110). Specifically, prevalence of strongyle (FET; $P = 0.0002$), Strongyloides (FET; $P = 0.013$) and Fasciola (FET; $P = 0.013$) were significantly higher in goats than in sheep. In addition, the mean EPG counts for strongyle were significantly higher in goats than in sheep ($t = 2.48, P = 0.014$).

Table 2 summarizes prevalence and mean EPG data grouped by study site. There were no statistically significant differences in the prevalence but there were several significant differences in the EPG counts for different GI parasite types across the study sites.

Strongyle and Strongyloides parasites were found in both sheep and goats in all study sites. The mean EPG counts for goats infected with Strongyloides in Tambul were significantly higher than the ones in Labu (ANOVA: $F = 3.91, P = 0.05$, Tukey’s test: $P < 0.05$). *Eimeria* was found in sheep from four study sites (Labu, Ungai-Bena, Tambul and Menifo) and in goats from three sites (Labu, Ungai-Bena and Baisu). In sheep, the mean EPG count for *Eimeria* in Labu was significantly lower than in Menifo (ANOVA: $F = 3.41, P = 0.045$; Tukey’s test: $P < 0.05$).

No significant differences were observed for the other genera of GI parasites. *Fasciola* was found in sheep from three study sites (Labu, Ungai-Bena and Tambul) and in goats from three study sites (Labu, Ungai-Bena and Baisu). *Trichuris* occurred in sheep from two study sites (Ungai-Bena and Menifo) and in goats only from Labu. *Nematodirus* was found in sheep from two sites (Baisu and Tambul) and in goats only from Baisu. *Moniezia* was only found in goats from three study sites (Labu, Ungai-Bena and Baisu). *Dictyocaulus* was only found in goats from Labu.

There was also a trend for goats to be more heavily infected than sheep in all areas. In Labu, mean EPG for strongyle ($t = 2.47, P = 0.021$) and *Eimeria* ($t = 3.95, P = 0.004$) were higher in goats than in sheep. In Ungai-Bena, the prevalence for Strongyloides was higher in goats than in sheep (FET: $P = 0.037$) and mean EPG for strongyle in goats was higher than in sheep ($t = 3.03; P = 0.006$).

Table 1. GI parasite prevalence and eggs/g (EPG) counts stratified by GI parasite type for the total number of faecal samples collected in the present study. $N$ is the total number of faecal samples. The total prevalence refers to the fraction of animals infected with one or more GI parasite types. EPG counts are given as means ± SD. Where no SD value is given on the mean EPG count, there was only a single or few positive observations with the same values, so that SD could not be calculated. Values with the superscript (*) are significantly different between sheep and goats by either Fisher’s exact test (FET) or t-test.

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Goat (N = 55)</th>
<th>Sheep (N = 110)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prevalence (%)</td>
<td>EPG</td>
</tr>
<tr>
<td>Strongyle</td>
<td>85.5*</td>
<td>745 ± 622*</td>
</tr>
<tr>
<td>Strongyloides</td>
<td>23.6*</td>
<td>277 ± 437</td>
</tr>
<tr>
<td>Eimeria</td>
<td>16.4</td>
<td>203 ± 103</td>
</tr>
<tr>
<td>Fasciola</td>
<td>18.2*</td>
<td>257 ± 275</td>
</tr>
<tr>
<td>Trichuris</td>
<td>3.6</td>
<td>125 ± 59</td>
</tr>
<tr>
<td>Moniezia</td>
<td>9.1</td>
<td>116 ± 46</td>
</tr>
<tr>
<td>Dictyocaulus</td>
<td>3.6</td>
<td>83</td>
</tr>
<tr>
<td>Nematodirus</td>
<td>3.6</td>
<td>249 ± 117</td>
</tr>
<tr>
<td>Total</td>
<td>89*</td>
<td>927 ± 821</td>
</tr>
</tbody>
</table>
Table 2. GI parasite prevalence and eggs/g (EPG) counts for GI parasite type and study site. \( N_G \) and \( N_S \) are the total numbers of faecal samples collected at each study site for goats and sheep, respectively. P (%) is the prevalence in per cent. EPG counts are given as means ± SD. Where no SD value is given on the mean EPG count, there was only a single or few positive observations with the same EPG values, so that SD could not be calculated.

<table>
<thead>
<tr>
<th></th>
<th>Labu (( N_G = 14, ) ( N_S = 27 ))</th>
<th>Ungai-Bena (( N_G = 15, ) ( N_S = 16 ))</th>
<th>Baisu (( N_G = 22, ) ( N_S = 19 ))</th>
<th>Tambul (( N_G = 4, ) ( N_S = 29 ))</th>
<th>Menifo (( N_G = 0, ) ( N_S = 19 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Goat</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strongyle</td>
<td>71.4 ± 603</td>
<td>93.3 ± 582</td>
<td>90.9 ± 713</td>
<td>75</td>
<td>478 ± 212</td>
</tr>
<tr>
<td>Strongylloides</td>
<td>21.4 ± 170</td>
<td>40 ± 143</td>
<td>9.1 ± 83</td>
<td>50</td>
<td>960 ± 996</td>
</tr>
<tr>
<td>Eimeria</td>
<td>21.4 ± 95</td>
<td>20 ± 143</td>
<td>13.6 ± 48</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fasciola</td>
<td>35.7 ± 374</td>
<td>20 ± 470</td>
<td>9.1 ± 83</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Trichuris</td>
<td>14.3 ± 59</td>
<td>0 ± 0</td>
<td>9.1 ± 83</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Moniezia</td>
<td>14.3 ± 83</td>
<td>6.7 ± 166</td>
<td>125 ± 59</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dictyocaulus</td>
<td>14.3 ± 82</td>
<td>0 ± 0</td>
<td>9.1 ± 83</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nematodirus</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>249 ± 117</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Sheep</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strongyle</td>
<td>66.7 ± 570</td>
<td>68.8 ± 677</td>
<td>78.9 ± 389</td>
<td>69.0 ± 1216</td>
<td>52.6 ± 1087</td>
</tr>
<tr>
<td>Strongylloides</td>
<td>3.7 ± 83</td>
<td>6.3 ± 83</td>
<td>5.3 ± 83</td>
<td>17.2 ± 266</td>
<td>5.3 ± 166</td>
</tr>
<tr>
<td>Eimeria</td>
<td>25.9 ± 31</td>
<td>25 ± 220</td>
<td>0 ± 0</td>
<td>6.9 ± 125</td>
<td>31.6 ± 1605</td>
</tr>
<tr>
<td>Fasciola</td>
<td>11.1 ± 166</td>
<td>6.25 ± 83</td>
<td>0 ± 0</td>
<td>6.9 ± 83</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Trichuris</td>
<td>0 ± 0</td>
<td>12.5 ± 83</td>
<td>0 ± 0</td>
<td>6.9 ± 83</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Moniezia</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>6.9 ± 83</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Dictyocaulus</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>6.9 ± 83</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Nematodirus</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>6.9 ± 83</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

\(^1\) Values are significantly different by ANOVA/Tukey’s HSD testing.

Table 3 shows the prevalence and mean EPG counts for GI parasites with respect to sheep breed, agro-ecology and farm management systems. For comparisons of agro-ecology and farm management systems, the data for sheep and goats were combined. No statistically significant differences were found in either prevalence or mean EPG counts for each parasite type between the two agro-ecological zones, with the exception for *Eimeria* and *Strongyle*.

<table>
<thead>
<tr>
<th></th>
<th>Highlands ( N = 124 )</th>
<th>Lowlands ( N = 41 )</th>
<th>NARI ( N = 115 )</th>
<th>Smallholder ( N = 50 )</th>
<th>Priangan ( N = 27 )</th>
<th>H/Halfbred ( N = 83 )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Prevalence (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strongyle</td>
<td>75</td>
<td>68.3</td>
<td>74.8</td>
<td>70</td>
<td>66.7</td>
<td>67.5</td>
</tr>
<tr>
<td>Strongylloides</td>
<td>14.3</td>
<td>9.8</td>
<td>12.2</td>
<td>16</td>
<td>3.7</td>
<td>9.6</td>
</tr>
<tr>
<td>Eimeria</td>
<td>14.5</td>
<td>24.4</td>
<td>13.1</td>
<td>26</td>
<td>25.9</td>
<td>14.5</td>
</tr>
<tr>
<td>Fasciola</td>
<td>6.5*</td>
<td>19.5*</td>
<td>10.4</td>
<td>8</td>
<td>11.1</td>
<td>3.6</td>
</tr>
<tr>
<td>Trichuris</td>
<td>2.4</td>
<td>4.9</td>
<td>1.7</td>
<td>6</td>
<td>0</td>
<td>3.6</td>
</tr>
<tr>
<td>Moniezia</td>
<td>2.4</td>
<td>4.9</td>
<td>3.5</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dictyocaulus</td>
<td>0</td>
<td>4.9</td>
<td>1.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nematodirus</td>
<td>4</td>
<td>0</td>
<td>4.3</td>
<td>0</td>
<td>0</td>
<td>3.6</td>
</tr>
<tr>
<td><strong>EPG</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strongyle</td>
<td>818 ± 1156</td>
<td>551 ± 605</td>
<td>748 ± 942</td>
<td>775 ± 1316</td>
<td>405 ± 570</td>
<td>889 ± 1412</td>
</tr>
<tr>
<td>Strongylloides</td>
<td>278 ± 375</td>
<td>374 ± 582</td>
<td>357 ± 491</td>
<td>189 ± 151</td>
<td>1246</td>
<td>208 ± 160</td>
</tr>
<tr>
<td>Eimeria</td>
<td>696 ± 1492*</td>
<td>133 ± 80*</td>
<td>133 ± 69*</td>
<td>913 ± 1723*</td>
<td>95 ± 32*</td>
<td>948 ± 1796*</td>
</tr>
<tr>
<td>Fasciola</td>
<td>228 ± 283</td>
<td>187 ± 165</td>
<td>152 ± 141</td>
<td>374 ± 362</td>
<td>166</td>
<td>83</td>
</tr>
<tr>
<td>Trichuris</td>
<td>111 ± 48</td>
<td>126 ± 59</td>
<td>125 ± 59</td>
<td>111 ± 48</td>
<td>0</td>
<td>111 ± 48</td>
</tr>
<tr>
<td>Moniezia</td>
<td>138 ± 48</td>
<td>83</td>
<td>104 ± 42</td>
<td>166</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dictyocaulus</td>
<td>0</td>
<td>83</td>
<td>83</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nematodirus</td>
<td>166 ± 102</td>
<td>0</td>
<td>166</td>
<td>0</td>
<td>0</td>
<td>111 ± 48</td>
</tr>
</tbody>
</table>

NARI, National Agricultural Research Institute.
H/Halfbred, Highlands Hybridbred sheep.
*Values are significantly different by either FET or \( t \)-test.
**Fasciola.** The mean EPG for *Eimeria* was higher in the highlands than in the lowlands ($t = 2.1$, $P = 0.045$) and the prevalence of *Fasciola* was higher in the lowlands than highlands (FET, $P = 0.028$). There were no significant differences in prevalence or mean EPG between the institutional and smallholder farm management systems with the exception for *Eimeria*. The mean EPG for *Eimeria* was higher in the smallholder farms than National Agricultural Research Institute (NARI) farms ($t = 3.0$, $P = 0.004$). Similarly, no significant differences were found in both parasite prevalence and mean EPG between sheep breeds, with the exception of *Eimeria* where the mean EPG in the Highlands Halfbred sheep was higher than in Priangan sheep ($t = 2.1$, $P = 0.045$).

**Discussion**

The present study observed 78% of the examined farm animals in PNG being infected with one or more types of GI parasites. Similar infection rates for both sheep and goats have been reported in other developing countries, such as Ethiopia and Kenya (Maichomo et al., 2004; Regassa et al., 2006). This high parasite prevalence may be attributed to poor farm management, e.g. little pasture rest time, poor nutrition and lack of anthelmintic treatment. At all study sites, mixed-species flocks grazed or browsed on natural forage on the same land for most of the time or were moved to other areas with only short pasture rest times. Studies in Fiji, Tonga and Malaysia found that nematodes have short survival times on pasture and suggested rotational grazing as an effective measure for parasite control (Banks et al., 1990; Barger et al., 1994; Cheah & Rajamanickam, 1997).

Natural pasture and shrubs in PNG are often not very nutritious and may contain anti-nutritive factors such as tannin types, which restrict rather than enhance protein availability to the ruminant (Macfarlane, 2000). Signal grass, for example, may cause hepatic dysfunction in farm animals (Macfarlane, 2000). The low-quality feed may result in subsequent malnutrition which may negatively affect the development of acquired immunity against GI parasites (Kyriazakis & Houdijk, 2006). We did not observe significantly lower GI parasite prevalence at study sites with more frequent anthelmintic treatment.

Strongyles were the most abundant parasites detected in this study. Strongyles, especially *Haemonchus* species, are highly fecund, laying up to 5000 eggs/day where environmental factors are favourable (Gupta et al., 1987). The pre-parasitic stages of *Haemonchus contortus* develop and survive better at mean monthly maximum temperatures $\geq 18.3\, ^\circ C$ (Gupta et al., 1987). Our study sites have mean maximum annual temperatures ranging from 18.9 to 31.1$^\circ C$ with only little variation throughout the year, due to their proximity to the equator. Additionally, most study sites were very humid, which is likely to sustain the survival of the free-living stages of *H. contortus*, leading to high pasture contamination.

A previous study reported high prevalence (89%) of *Eimeria* in sheep from three locations in PNG (Varghese & Yayabu, 1985) whereas the present study found a lower prevalence (21–36%) in similar areas. This may be due to the different methodologies used for parasite identification. In the present study a simple flotation procedure was used, while in the previous study a centrifugation/flotation method was used which has been reported to be more sensitive for detection of oocytes in faecal samples (Dryden et al., 2005). Notwithstanding, this is the first study to report *Eimeria* in goats in PNG. We found *Eimeria* in goats in all study sites except Tambul, where only four goats were screened, and Menifo, where no goats were screened.

The trematode *Fasciola* was present in sheep and/or goats from Labu, Ungai-Bena, Baisu and Tambul. A previous study in PNG found *Fasciola* in the area of Aiyura and showed that the sheep there are exposed to continual (low-level) pasture contamination leading to chronic fasciolosis at all times (Owen, 1989). In PNG, in all areas where the intermediate snail host, *Lymnaea* species, exists, acute fasciolosis can occur in the wet season, especially in areas where the land is not well drained and the grazing pressure is high (Owen, 1989).

This is the first study reporting the prevalence of *Dictyocaulus* in sheep and goats in PNG. Some studies have found that goats are more susceptible to *Dictyocaulus* than sheep (Sharma, 1994; Berrag & Urquhart, 1996; Alemu et al., 2006). We found *Dictyocaulus* in 14% of goats examined in Labu (lowland). A similar prevalence has been reported in Ethiopia in areas with an altitude $<1500\, m$ (Alemu et al., 2006).

We found higher overall infection levels in goats compared to sheep. Goats were also infected with a wider spectrum of GI parasites. This contradicts some previous studies that found a lower prevalence in goats (Kanyari et al., 2009; Khan et al., 2010; Abebe et al., 2011), but is in agreement with a number of other studies, which also reported higher parasite prevalence in goats (Regassa et al., 2006; Nwosu et al., 2007; Gadahi et al., 2009; Dagnachew et al., 2011). Hoste et al. (2008) suggested that goats do not develop resistance as efficiently as sheep and this may be an explanation for our findings.

The EPG counts for *Eimeria* in PNG Priangan sheep ($N = 27$) were significantly lower than those in the Highlands Halfbred sheep ($N = 83$). It is difficult to ascertain the mechanisms behind this difference, especially as, coincidentally, most PNG Priangan sheep samples were collected in Labu, where anthelmintic drug treatment was conducted more regularly. Nevertheless, previous studies have shown that some indigenous sheep breeds exhibit higher levels of resistance against GI parasites and this might partially explain the present findings (Baker & Gray, 2004). PNG Priangan sheep are native to tropical climates, as they originated from South-East Asia and have been exposed to GI parasites in PNG for over a century. In contrast, Highlands Halfbred sheep, which are crossbreeds of the PNG Priangan sheep and the temperate Corriedale and Perendale breeds, have only a fraction of this resistance and will therefore be more susceptible to infection. More detailed studies on immunology and feeding behaviour of the different sheep breeds are required to elucidate this problem further.

The information collected in this study is an important update on GI parasite presence in sheep and goats in PNG. Future investigations should include longitudinal studies and larger cohorts to further assess parasite.
epidemiology in the diverse agro-climatic zones in the country. Molecular methods should be used to identify the different species of GI parasites infecting sheep and goats in PNG, thus extending previous studies (Varghese & Yayabu, 1985; Owen, 1988, 1989, 1998).

Acknowledgements

The authors would like to sincerely thank the owners of the herds and logistic support by the National Agricultural Research Institute, Department of Agriculture and Livestock in Eastern Highlands Province, and Divine Word University in Papua New Guinea. This research received no specific grant from any funding agency, commercial or not-for-profit sectors. This study was approved by the Murdoch University Animal Ethics Committee (Permit R2368/10).

References


Appendix C

*Cryptosporidium* species in sheep and goats in Papua New Guinea.
Research Brief

Cryptosporidium species in sheep and goats from Papua New Guinea

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Abstract

Species of Cryptosporidium are extensively recognised as pathogens of domesticated livestock and poultry, companion animals, wildlife, and are a threat to public health. Little is known of the prevalence of Cryptosporidium spp. in humans, domesticated animals or wildlife in Papua New Guinea (PNG). The aim of the present study was to screen sheep and goats for Cryptosporidium using molecular tools. A total of 504 faecal samples were collected from sheep (n = 276) and goats (n = 228) in village, government and institutional farms in PNG. Samples were screened by nested PCR and genotyped at the 18S rRNA and at the 60 kDa glycoprotein (gp60) loci. The overall prevalences were 2.2% for sheep (6/278) and 4.4% (10/228) for goats. The species/genotypes identified were Cryptosporidium hominis (subtype IdA15G1) in goats (n = 6), Cryptosporidium parvum (subtypes IlaA15G2R1 and IlaA15G4R1) in sheep (n = 4) and in goats (n = 2), Cryptosporidium andersoni (n = 1) and Cryptosporidium scrofarum (n = 1) in sheep, Cryptosporidium xiao (n = 1) and Cryptosporidium rat genotype II (n = 1) in goats. This is the first report of Cryptosporidium identified in livestock warrants better care of farm animals to avoid contamination and illness in vulnerable population. The detection of zoonotic Cryptosporidium in livestock suggests these animals may serve as reservoirs for human infection.

1. Introduction

Species of Cryptosporidium are globally distributed, zoonotic intestinal protozoan parasites that cause diarrheal disease in animals and are one of the main causes of serious diarrhoea in children (Kotloff et al., 2013). Clinical effects of Cryptosporidium infection, which include diarrhoea, weight loss and often death in lambs and goat kids, severely impact the economy of sheep and goat farming (de Graaf et al., 1999).

Globally, the prevalence of Cryptosporidium spp. in sheep can vary drastically from <5% to >70% (Robertson, 2009). Although few epidemiological studies have examined Cryptosporidium spp. in goats, it appears that prevalence is similarly variable, with values of <10% to >40% reported (Robertson, 2009). At least eight Cryptosporidium species have been identified in sheep faeces including...
Appendix C

Gastrointestinal pathogens in sheep, goats and fish in PNG

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Cryptosporidium parvum, Cryptosporidium hominis, Cryptosporidium andersoni, Cryptosporidium suis, Cryptosporidium xiaoì, Cryptosporidium fayeri, Cryptosporidium ubiquitum and Cryptosporidium scrofa-rum, with C. xiaoì, C. ubiquitum and C. parvum most prevalent (Ryan et al., 2005; Santin et al., 2007; Fayer and Santin, 2009; Giles et al., 2009; Yang et al., 2009; Robertson, 2009; Diaz et al., 2010a; Wang et al., 2010; Sweeney et al., 2011; Cacciò et al., 2013; Connelly et al., 2013). Three of these species: C. parvum, C. hominis and C. xiaoì have also been identified in goats (Giles et al., 2009; Robertson, 2009; Diaz et al., 2010b).

Sheep and dairy goats were introduced to Papua New Guinea (PNG) in the early 19th century by colonial administrators and missionaries (Quartermain, 2004). There are two predominant breeds of sheep (PNG Priangan sheep and the Highlands Halfbred) and one breed of goat (PNG goat genotype) in PNG (Quartermain, 2004). Currently, sheep and goats are raised in government stations for breeding and distribution to smallholder farms and in research institutional farms. Little is known about Cryptosporidium in sheep and goats in PNG and therefore the aim of the present study was to determine the prevalence and genotypes of Cryptosporidium in these two hosts in PNG.

2. Materials and methods

2.1. Sample collection

Faecal samples from a total of 228 goats and 276 sheep were collected from February 2011 to April 2011 from government, institutional and smallholder farms in a variety of agro-economic zones in PNG.

2.1.1. Farm management

The flocks from the government (Menifo) and institutional (Labu, Baisu and Tambul) farms grazed pasture in fenced areas (20–60 ha) at daytime. At night time, the flocks were kept in houses with wooden, slatted floors in institutional farms and on the ground in the government farm. At the time of sample collection, the combined numbers of sheep and goats in Menifo, Labu, Baisu, and Tambul were 55, 125, 70 and 143, respectively. The subsistence farmers kept few animals, usually less than 20, which grazed free range or were tethered and housed at night on slatted floors or on the ground underneath the farmer’s house. Most animals grazed on native grasses and shrubs. Smallholder farmers also fed their animals with starchy vegetables (mostly sweet potatoes). The animals drank from troughs (sourced from water supply or rainwater tanks), rainwater run-off water or ponds.

2.1.2. Herd health programs

The floors of the resting houses were not swept. The animals were penned on dirty floor, ground or on bare concrete floors. The farmers at the institutions and government farms sheared their sheep, whereas, the smallholder farmers did not and explained that they did not have the resources for it. Most farm managers reported that the most common signs of illness in their animals were diarrhoea and coughing, followed by itching and hair loss. The three large institutional flocks were drenched with benzimidazole (Panacur) nominally at bimonthly intervals. At the time of sample collection, animals had been drenched 2 months previously in Menifo, Labu and Tambul and 6 months previously in Menifo. Most smallholder farmers did not know about causes of diseases in their sheep and goats or the use of anthelmintic drugs for parasite control. For instance, a smallholder farmer reported the death of his entire flock (n = 25) and noticed nematode worms in the gut of a dead sheep.

All animals sampled were adults. Faecal samples were obtained from the rectum of randomly selected animals and examined visually for consistency, mucus and macroscopic parasites. All sample collection methods used were approved by the Murdoch University Animal Ethics Committee (approval number R2368/10). The faecal samples were preserved in 70% ethanol and transported to Murdoch University, Australia, for further analysis.

2.2. DNA isolation and genotyping of Cryptosporidium sp

Total DNA was extracted from 250 mg of faeces using a PowerSoil® DNA Isolation Kit (MO BIO laboratories, Carlsbad, California, USA). All samples were screened for the presence of Cryptosporidi-um sp. at the 18S rRNA locus using a nested PCR as previously described (Morgan et al., 1997). C. parvum and C. hominis-positive isolates were subtyped at the 60 kDa glycoprotein locus (gp60) as described by Sulaiman et al. (2005). All positive isolates were sequenced as previously described (Koinari et al., 2013).

3. Results

Cryptosporidium was detected in 2.2% (6/276; 95% CI 2.8–6.2) of sheep and 4.4% (10/228; 95% CI 2.8–6.2) of goats at the 18S rRNA locus. Three species of Cryptosporidium were detected in sheep, namely C. parvum (n = 4), C. andersoni (n = 1) and C. scrofarum (n = 1). Four species/genotypes were detected in goats; C. hominis (n = 6), C. parvum (n = 2), C. xiaoì (n = 1) and rat genotype II (n = 1) (Table 1). Rat genotype II, C. xiaoì, C. scrofarum and C. andersoni isolates were detected in animals from smallholder farms. The C. hominis isolates were from smallholder (n = 4) and institutional (n = 2) farms, while C. parvum was identified in animals from all three types of farms; government (n = 1), institutional (n = 3) and smallholder (n = 1). Analysis of the gp60 gene identified the presence of two C. parvum subtypes; IIaA15G2R1 (n = 3) and IIaA19G4R1 (n = 2) in sheep and goats and a C. hominis subtype (IdA15G1) (n = 1) in a goat (Table 1). The partial 18S and gp60 nucleotide sequences were deposited in the GenBank database under the accession numbers KJ584567–KJ584584.

4. Discussion

This is first study to identify and molecularly characterise Cryptosporidium in sheep and goats in PNG and analysis revealed a high diversity of Cryptosporidium parasites within these animal populations. The results of the present study complement recent findings of C. parvum in fish from freshwater aquaculture, wild freshwater and wild saltwater, and C. hominis in a wild marine fish in PNG (Koinari et al., 2013). The only other previous study of Cryptosporidi-um in PNG identified Cryptosporidium antibodies in 24% of young children from Goroka (Groves et al., 1994).

Although point prevalences were low for Cryptosporidium in the present study, the true prevalence may be underestimated as only single faecal samples were screened at one time point and intermittent shedding and seasonal variation are common (O’Handley et al., 1999). In addition, only adult animals were screened and prevalences are known to be much higher in younger animals (Santin et al., 2007). Most importantly, the identification of Cryptosporidium in livestock warrants better care of farm animals to avoid contamination and illness in vulnerable populations, as Cryptosporidium spp. are known for causing diarrhoea and mortality in young animals in both natural and artificial infections (de Graaf et al., 1999; Quilez et al., 2008; Giles et al., 2009).

The three species (C. parvum, C. andersoni and C. scrofarum) identified in sheep from the present study have also been reported in sheep in previous studies (Ryan et al., 2005; Santin et al., 2007;
Quilez et al., 2008; Giles et al., 2009). In addition, C. andersoni is frequently reported in cattle and occasionally in humans, while C. scrofarum is commonly identified in pigs (Xiao, 2010). C. ubiquitum is a common species found in sheep in other countries (Ryan et al., 2005; Santin et al., 2007; Wang et al., 2010; Yang et al., 2009); however, it was not identified in the present study.

Three species, C. hominis, C. parvum and C. xiaoi, detected in goats in the present study have also been reported in goats in other studies (Goma et al., 2007; Geurden et al., 2008; Quilez et al., 2008; Giles et al., 2009; Diaz et al., 2010b). For example, molecular analyses confirmed infections with C. hominis and C. parvum in diarrhoeic goat kids in the UK (Giles et al., 2009) and C. parvum in goats in Spain (Quilez et al., 2008). C. xiaoi is commonly reported in sheep (Fayer and Santin, 2009) and occasionally in goats (Diaz et al., 2010b). This is the first report of rat genotype II in goats. Rat genotype II has been reported in house rats in China (Lv et al., 2009), and in the Philippines (Ng-Hublin et al., 2013), brown rats in the Philippines (Ng-Hublin et al., 2013) and in wild black rats in Northern Australia (Paparini et al., 2012). The goat in which rat genotype II was identified was from a smallholder farm in Bena–Bena, PNG. Smallholders usually keep their goats in night houses, which are built very close to their own homes in order to avoid theft. The goat could have acquired this genotype from the house rats; however, further studies are required to confirm this and to determine if the goat was actually infected or just passing oocysts from ingestion of rat faeces. Identification of species such as C. andersoni, C. scrofarum and C. xiaoi in smallholder flocks probably reflects the management system. Typically, these small ruminants are tethered and/or allowed to graze freely on shrubs and grasses along road sides, near homes and gardens, where they share the feeding grounds with other livestock, especially cattle and pigs.

C. hominis and C. parvum are the most common causes of cryptosporidiosis in humans worldwide (Xiao, 2010). In the present study, C. hominis (subtype IdA15G1) was found in goats and C. parvum (subtypes IlaA15G2R1 and IlaA19G4R1) was found in both sheep and goats. Both the C. parvum Ila subtypes and C. hominis Id subtype identified in the present study were previously identified in fish in PNG (Koinari et al., 2013). The C. parvum subtype IlaA15G2R1, has been reported in sheep and goats in previous studies in Belgium, Spain, Brazil, China and Australia (Diaz et al., 2010a; Geurden et al., 2008; Paz et al., 2014; Yang et al., 2014; Ye et al., 2014). C. parvum subtype IlaA15G2R1 is a common subtype in cattle and humans (Feng et al., 2013; Xiao, 2010) in the Americas, Europe, Northern Africa and Asia (Alyousefi et al., 2013; Amer et al., 2010; Brook et al., 2009; Diaz et al., 2010a; Geurden et al., 2009; Helmy et al., 2013; Iqbal et al., 2012; Meireles et al., 2011; Quilez et al., 2008; Rahmouni et al., 2014; Rieux et al., 2013; Santin et al., 2008; Soba and Logar, 2008). It has also been found in yak in China (Mi et al., 2013) and in buffalo in Egypt (Helmy et al., 2013). The C. parvum subtype IlaA19G4R1 was identified in both a goat and a sheep in the present study. Previously, C. parvum subtype IlaA19G4R1 was identified in cattle in Northern Ireland (Thompson et al., 2007) and Australia (Ng et al., 2008) and freshwater fish (tilapia and silver barb) from PNG (Koinari et al., 2013).

These findings suggest that sheep and goats may be important reservoirs of C. hominis and zoonotic C. parvum subtypes in PNG. The detection of C. hominis in goats presumably reflects the very close association between humans and goats. Further research is necessary to characterise the prevalence of various Cryptosporidium species and genotypes in young lambs, goats and cattle and other hosts such as humans to more fully understand the transmission dynamics of Cryptosporidium in PNG.

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### References


Appendix D

Identification of novel and zoonotic *Cryptosporidium* species in fish from Papua New Guinea.
Identification of novel and zoonotic Cryptosporidium species in fish from Papua New Guinea

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A B S T R A C T

There is still limited information on the distribution of Cryptosporidium species and genotypes in fish. The present study investigated the prevalence of Cryptosporidium species in cultured freshwater (n = 132), wild freshwater (n = 206) and wild marine (n = 276) fish in Papua New Guinea (PNG) by PCR screening at the 18S rRNA locus. A total of seven fish (2 cultured freshwater, 1 wild freshwater and 4 wild marine fish) were identified as positive for Cryptosporidium. Specifically, Cryptosporidium was found in four different host species (Nile tilapia, Oreochromis niloticus; silver barb, Puntius gonionotus; mackerel scad, Decapterus macarellus and oblong silver biddy, Gerres oblongus), giving an overall prevalence of 1.14% (95% CI: 0.3–2%, n = 7/614). Of the seven positive isolates, five were identified as C. parvum and two were a novel piscine genotype, which we have named piscine genotype 8. Piscine genotype 8 was identified in two marine oblong silver biddies and exhibited 4.3% genetic distance from piscine genotype 3 at the 18S locus. Further subtyping of C. parvum isolates at the 60 kDa glycoprotein (gp60) locus identified 3 C. parvum subtypes (IIaA14G2R1, IIaA15G2R1 and IIaA19G4R1) all of which are zoonotic and a C. hominis subtype (IdA15G1). The zoonotic Cryptosporidium were identified in fish samples from all three groups; cultured and wild freshwater and wild marine fish. Detection of Cryptosporidium among aquaculture fingerlings warrants further research to gain a better understanding of the epidemiology of Cryptosporidium infection in cultured fish. The identification of zoonotic Cryptosporidium genotypes in fish from PNG has important public health implications and should be investigated further.

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1. Introduction

The apicomplexan protozoan parasite Cryptosporidium infects a wide range of mammals, birds, reptiles and fish, primarily causing diarrhoea in mammals, diarrhoea and/or catarhal respiratory signs in birds and gastritis in reptiles and possibly fish (O’Donoghue, 1995; Ryan, 2010). Cryptosporidium has been described in more than 17 species of both fresh and salt water fish with parasitic stages located deep within and on the surface of the stomach or intestinal epithelium (Alvarez-Pellitero and Sitja-Bobadilla, 2002; Alvarez-Pellitero et al., 2004; Ryan et al., 2004; Murphy et al., 2009; Reid et al., 2010; Zanguee et al., 2010; Morine et al., 2012). In fish, Cryptosporidium can cause high morbidity with clinical signs including variable levels of emaciation, poor growth rates, swollen coelomic cavities, anorexia, listlessness and increased mortality (Murphy et al., 2009).
Currently the only recognised species infecting fish is *Cryptosporidium molnari*, which was identified in gilthead sea bream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) (Alvarez-Pellitero and Sitja-Bobadilla, 2002) and was characterised genetically in 2010 (Palenzuela et al., 2010). *C. molnari* primarily infects the epithelium of the stomach and seldom the intestine (Alvarez-Pellitero and Sitja-Bobadilla, 2002). In 2004, *C. scophthalmi* was described in turbot (*Psetta maxima*, syn. *Scophthalmus maximus*) (Alvarez-Pellitero and Sitja-Bobadilla, 2002; Alvarez-Pellitero et al., 2004). However, no genetic sequences are available for *C. scophthalmi* and it can thus not be considered a valid species due to the high genetic heterogeneity and morphological similarity among *Cryptosporidium* species in fish. In addition to *C. molnari*, a total of 3 species and 10 genotypes have been characterised genetically in fish (Table 1).

Fish are an important part of the diet and a source of income especially for people living on the coast and along the rivers in Papua New Guinea (PNG). Freshwater fish farming began in the 1960s with the introduction of carp and trout species; however, difficulties due to lack of knowledge among farmers impeded farming progress and the spread of its nutritional and financial benefits to rural communities (Smith, 2007). Since 1995, the number of inland aquaculture operations has increased in PNG due to international programmes that involve the expansion of hatcheries, training of farmers and the introduction of new fish species (Smith, 2007). To date, very little is known about the prevalence and genotypes of *Cryptosporidium* in fish or other animals in PNG (Owen, 2005; Koinari et al., 2012, 2013). The present study represents a detailed investigation of the prevalence and genetic characterisation of *Cryptosporidium* in cultured freshwater, wild freshwater and wild marine fish sampled from a number of different locations throughout PNG. It is therefore the first comprehensive study describing the distribution of *Cryptosporidium* species in PNG.

### Table 1

<table>
<thead>
<tr>
<th>Species/genotype</th>
<th>N</th>
<th>Fish host</th>
<th>References</th>
</tr>
</thead>
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<tr>
<td><em>C. molnari</em></td>
<td>&gt;100</td>
<td>Gilthead sea bream and European seabass</td>
<td>Alvarez-Pellitero and Sitja-Bobadilla (2002), Palenzuela et al. (2010)</td>
</tr>
<tr>
<td><em>C. molnari</em>-like</td>
<td>7</td>
<td>Butter bream, madder seaperch, bristle tooth tang, upsidedown catfish, wedge-tailed blue tang and green chromis, golden algae eater</td>
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</tr>
<tr>
<td>Piscine genotype 1</td>
<td>2</td>
<td>Guppy and neon tetra</td>
<td>Ryan et al. (2004), Zanguee et al. (2010)</td>
</tr>
<tr>
<td>Piscine genotype 2</td>
<td>&gt;5</td>
<td>Angelfish, neon tetra and Oscar fish</td>
<td>Murphy et al. (2009), Zanguee et al. (2010)</td>
</tr>
<tr>
<td>Piscine genotype 3</td>
<td>2</td>
<td>Sea mullet</td>
<td>Reid et al. (2010)</td>
</tr>
<tr>
<td>Piscine genotype 4</td>
<td>4</td>
<td>Golden algae eater, kupang damsel, Oscar fish and neon tetra</td>
<td>Zanguee et al. (2010), Morine et al. (2012)</td>
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<tr>
<td>Piscine genotype 5</td>
<td>3</td>
<td>Angelfish, butter bream and golden algae eater</td>
<td>Zanguee et al. (2010)</td>
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<td>Piscine genotype 6</td>
<td>1</td>
<td>Guppy</td>
<td>Zanguee et al. (2010)</td>
</tr>
<tr>
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<td>Gold gourami</td>
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<td>Piscine genotype 7</td>
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<td>Red-eye tetra</td>
<td>Morine et al. (2012)</td>
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<td>Morine et al. (2012)</td>
</tr>
<tr>
<td><em>C. scrofarum</em></td>
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<td>School whiting</td>
<td>Reid et al. (2010)</td>
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<td>Reid et al. (2010)</td>
</tr>
<tr>
<td><em>C. xiaoi</em></td>
<td>1</td>
<td>School whiting</td>
<td>Reid et al. (2010)</td>
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</table>

### 2. Materials and methods

#### 2.1. Sample collection

A total of 614 fish from cultured freshwater, wild freshwater and wild marine environments were collected in PNG between February and August 2011 (Fig. 1). Cultured fish (*n* = 133) included three species, which were collected from four smallholder fish ponds in Kundiawa, Asaro, Mumeng and Bathem (Table 2). Wild freshwater fish (*n* = 205) included six species and were collected from the Ramu and Sepik Rivers, while 276 wild marine fish consisting of 16 species were bought from local fishermen in Bilbil, Madang, Tavana and Pilapila (Table 2). On average, time lag between collection or purchasing and processing of the fish was up to 4 h. All sampling was conducted under Murdoch University Animal Ethics permit R2369/10.

The fish were weighed, measured (length and weight) and dissected. Sections of intestine and stomach were cut using a sterile scalpel blade for each fish, placed in 2 mL Eppendorf tubes and preserved in 70% ethanol for molecular screening. The remaining stomach and intestine were fixed in 10% buffered formalin for histological analysis. All samples were stored at 4 °C in PNG until sample collection was completed. The samples were then transported to Murdoch University, Perth, Australia and stored at 4 °C until analysis.

#### 2.2. DNA isolation

The preserved intestines and stomachs were washed 5 times with water to remove ethanol and the epithelial layers were scraped using a sterile scalpel blade for each fish. DNA was extracted from 25 mg of intestinal and stomach scrapings using a PowerSoil® DNA Isolation Kit (MO BIO laboratories, Carlsbad, California, USA) according to the manufacturer's instructions and incorporating five
Fig. 1. Map of the study sites in Papua New Guinea. Cultured freshwater fish were obtained from Bathem, Kundiawa, Asaro and Mumeng; wild freshwater fish were collected in the Ramu River near Sausi and the Sepik River near Pagwi and marine fish were collected from Bilbil, Madang, Pilapila and Tavana.
freeze–thaw cycles as described previously (Ng et al., 2006) to break open the Cryptosporidium oocysts. DNA was eluted in 50 μL of elution buffer. All extracted samples were stored at −20 °C until required for screening.

### 2.3. Cryptosporidium genotyping and subtyping

All 614 samples from fish were screened for the presence of Cryptosporidium at the 18S rRNA locus as previously described (Morgan et al., 1999). Prevalences were expressed as a percentage of positive samples; with 95% confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rozsa et al., 2000). Isolates that were positive at the 18S locus were subtyped at the 60 kDa glycoprotein (gp60) locus using primers which produce an 850 bp product (Alves et al., 2003); however, amplification was unsuccessful and a shorter (400 bp) product was amplified as described by Sulaiman et al. (2005).

Positive isolates were also amplified at the actin locus. New primers were designed specifically based on actin gene sequences of piscine-derived Cryptosporidium and a semi-nested PCR protocol was used. For the primary PCR, a PCR product of ~392 bp was amplified using the forward primer ActinallF1 (5’-GTAAATATACAGGCAGTT-3’) and reverse primer ActinallR1 (5’-GGTTGGAACAATGCTTC-3’). Each PCR was performed in a reaction volume of 25 μL using 1 μL of DNA, 1× PCR buffer (Kapa Biosystems, Cape Town, South Africa), 2.0 mM MgCl2, 200 μM (each) dNTP (Fisher Biotech, Perth, Australia), 12.5 pmol of forward and reverse primers and 0.5 U of kapa Taq DNA polymerase (Kapa Biosystems, Cape Town, South Africa). Forty-five PCR cycles (95 °C for 30 s, 46 °C for 30 s, 72 °C for 30 s) were performed using a Perkin Elmer Gene Amp PCR 2400 thermocycler with an initial hot start (95 °C for 4 min) and a final extension (72 °C for 7 min). For the secondary PCR, a fragment of ~278 bp was amplified using 1 μL of primary PCR product with forward primer ActinallF2 (5’-CTICATGCTATAATGAG-3’) and reverse primer ActinallR1. The conditions used for the secondary PCR were identical to those for the primary PCR.

Secondary PCR products were separated by gel electrophoresis and purified using a simple tip elution method. Briefly, the PCR product was excised from the gel using a scalpel blade and purified using an in house filter tip method and used for sequencing without any further purification as previously described (Yang et al., 2013). DNA sequencing was performed using the ABI Prism BigDye® terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 3730 DNA

---

**Table 2**

Cultured freshwater, wild freshwater and marine fish species collected in the present study.

<table>
<thead>
<tr>
<th>Cultured freshwater fish</th>
<th>Locations</th>
<th>Asaro</th>
<th>Mumeng</th>
<th>Batem</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nile tilapia (Orechromis niloticus)</td>
<td>Kundia</td>
<td>36</td>
<td>20</td>
<td>27</td>
<td>83</td>
</tr>
<tr>
<td>Common carp (Cyprinus carpio)</td>
<td></td>
<td>11</td>
<td>32</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>Mozambique tilapia (Orechromis massambica)</td>
<td></td>
<td>7</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>36</td>
<td>59</td>
<td>7</td>
<td>133</td>
</tr>
<tr>
<td>Wild freshwater fish</td>
<td>Ramu River</td>
<td>Sepik River</td>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silver barb (Puntius gonioanotus)</td>
<td>13</td>
<td>39</td>
<td>52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highfin catfish (Neorius berneyi)</td>
<td></td>
<td>20</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mozambique tilapia (Orechromis massambica)</td>
<td></td>
<td>15</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pacu (Colossoma bidens)</td>
<td></td>
<td>34</td>
<td>34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indo-Pacific trapon (Megalops cyprinoides)</td>
<td></td>
<td>70</td>
<td>70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Redtail catfish (Phracopterus hemilopeterus)</td>
<td></td>
<td>14</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>13</td>
<td>192</td>
<td>205</td>
<td></td>
</tr>
<tr>
<td>Wild marine fish</td>
<td>Bilil</td>
<td>Madang</td>
<td>Pilapila</td>
<td>Havana</td>
<td>Total</td>
</tr>
<tr>
<td>Bigeye scad (Selar crumenophthalmus)</td>
<td>46</td>
<td>60</td>
<td></td>
<td></td>
<td>106</td>
</tr>
<tr>
<td>Bigeye trevally (Caranx sexfasciatus)</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Blackfin barracuda (Sphyraena genie)</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Coachwhip trevally (Carangoides oblongus)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Indo-Pacific trapon (Megalops cyprinoides)</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Mackerel scad (Decapterus macarellus)</td>
<td></td>
<td>24</td>
<td></td>
<td></td>
<td>29</td>
</tr>
<tr>
<td>Oblong silver biddy (Gorres oblongus)</td>
<td>1</td>
<td></td>
<td>54</td>
<td></td>
<td>55</td>
</tr>
<tr>
<td>Oriental bonito (Sarda orientalis)</td>
<td></td>
<td>4</td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Pinjalo snapper (Pinjalo pinjalo)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Rainbow runner (Elagatis bippinnulus)</td>
<td></td>
<td>5</td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Reef needlefish (Strongylisha incisa)</td>
<td></td>
<td>4</td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Slender pinjalo (Pinjalo lewisti)</td>
<td></td>
<td></td>
<td></td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Spanish mackerel (Scomberomorus maculatus)</td>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Talang queenfish (Scomberoides commersonianus)</td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Wahoo (Acanthocybium solandri)</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Yellow fin tuna (Thunnus albacares)</td>
<td></td>
<td>34</td>
<td></td>
<td></td>
<td>34</td>
</tr>
<tr>
<td>Total</td>
<td>69</td>
<td>51</td>
<td>87</td>
<td>69</td>
<td>276</td>
</tr>
</tbody>
</table>
Analyser instrument. Nucleotide sequences were analysed using FinchTV 1.4.0 (Geospiza, Inc., Seattle, WA, USA; http://www.geospiza.com) and aligned with reference genotypes retrieved from GenBank using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Phylogenetic trees were constructed using additional sequences retrieved from GenBank. Distance estimation was conducted using MEGA5 (Tamura et al., 2011) based on evolutionary distances calculated using the p distance model (Nei and Zhang, 2006) and grouped using neighbour-joining. Parsimony and maximum likelihood analyses were also conducted using the MEGA5 software. Reliabilities for the trees were tested using 1000 bootstrap replications (Felsenstein, 1985) and bootstrap values exceeding 70 were considered well supported (Hillis and Bull, 1993).

### 2.4. Microscopy

Sections of intestinal and stomach tissues fixed in 10% formalin were embedded in paraffin. Histological sections were cut at 5 μm thicknesses, stained with haematoxylin and eosin and examined with an Olympus BX50 light microscope at 400 and 1000 fold magnification.

### 3. Results

#### 3.1. Prevalence of Cryptosporidium in fish hosts

Of the 614 fish sampled, seven (1.14%, 95% CI 0.3–2%) were positive by PCR, of which, two (0.33%, 95% CI 0.09–1.2%) were cultured fingerlings, one (0.16%, 95% CI 0.03–0.9%) was a wild freshwater species and four (0.65%, 95% CI 0.25–1.66%) were wild marine species. Among the fish hosts infected were: two Nile tilapia (Oreochromis niloticus) with a prevalence of 2.4% (95% CI = 0–5.7%; 2/83) from fish ponds in Kundia and Mumeng; one silver barb (Puntius gonionotus) from Sepik River with a prevalence of 1.9% (95% CI = 0–5.7%; 1/52); two oblong silver biddies (Gerres oblongus) from Bilbil and Tavana with a prevalence of 3.6% (95% CI = 0–8.5%; 2/55) and two mackerel scads (Decapterus macarellus) from Pilapila with a prevalence of 6.9% (95% CI = 0–16.1%; 2/29) (Fig. 1).

#### 3.2. Sequence and phylogenetic analysis of the 18S rDNA, gp60 and actin genes

Sequence analysis identified *C. parvum* (5 isolates: Nile tilapia ON36 and ON68, mackerel scad DM17 and DM18, and silver barb PG37) and a novel piscine genotype (2 isolates: silver biddy GO18 and GO55), hereafter referred to as piscine genotype 8 at the 18S rRNA locus (Table 3). The two piscine genotype 8 isolates from silver biddies were genetically identical to each other, but distinct from all isolates previously characterised at the 18S rRNA locus (Table 4). Neighbour-joining, parsimony and maximum likelihood analysis produced similar results and indicated that piscine 8 genotype clustered most closely with the piscine 3 genotype from a sea mullet, while the other five isolates regularly clustered with *C. parvum* (85% bootstrap support) (Fig. 2). Sequences were also obtained for the two piscine genotype 8 isolates at the actin locus. At this locus, sequence information was only available for *C. molnari* and piscine genotype 1 and piscine genotype 8 grouped more closely with *C. molnari* genotypes (7.3–8.5% genetic differences) (Fig. 3 and Table 4).

At the gp60 locus, three subtypes belonging to *C. parvum* (family Id: IlaA14G2R1, IlaA15G2R1, IlaA19G4R1) and a *C. hominis* subtype (family Id: IdA15G1) were identified (Table 3).

#### 3.3. Microscopy

No parasites were observed during microscopic examination of the intestinal or stomach tissues due to substantial autolysis of tissues.

#### 3.4. Nucleotide sequence accession numbers

The unique partial 18S rRNA and actin sequences of piscine genotype 8 from silver biddies were deposited

---

**Table 3**

Species, genotypes and subtypes of *Cryptosporidium* identified in fish in the present study. Sample DM18 was typed as *C. parvum* at the 18S locus but at gp60 locus it was typed as *C. hominis* (IdA15G1) indicating the presence of mixed *C. parvum/C. hominis* in this sample.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Host species</th>
<th>Genotype</th>
<th>Group</th>
<th>18S</th>
<th>gp60</th>
</tr>
</thead>
<tbody>
<tr>
<td>ON36</td>
<td>Nile tilapia</td>
<td>Cultured</td>
<td></td>
<td><em>C. parvum</em></td>
<td>IlaA19G4R1</td>
</tr>
<tr>
<td>ON68</td>
<td>Nile tilapia</td>
<td>Cultured</td>
<td></td>
<td><em>C. parvum</em></td>
<td>IlaA14G2R1</td>
</tr>
<tr>
<td>PG37</td>
<td>Silver barb</td>
<td>Wild freshwater</td>
<td></td>
<td><em>C. parvum</em></td>
<td>IlaA19G4R1</td>
</tr>
<tr>
<td>DM17</td>
<td>Mackerel scad</td>
<td>Wild marine</td>
<td></td>
<td><em>C. parvum</em></td>
<td>IlaA15G2R1</td>
</tr>
<tr>
<td>DM18</td>
<td>Mackerel scad</td>
<td>Wild marine</td>
<td></td>
<td><em>C. parvum</em>, IdA15G1</td>
<td></td>
</tr>
<tr>
<td>GO18</td>
<td>Silver biddy</td>
<td>Wild marine</td>
<td></td>
<td>Piscine genotype 8</td>
<td>–</td>
</tr>
<tr>
<td>GO55</td>
<td>Silver biddy</td>
<td>Wild marine</td>
<td></td>
<td>Piscine genotype 8</td>
<td>–</td>
</tr>
</tbody>
</table>

---

**Table 4**

Percentage of genetic differences between piscine genotype 8 and other *Cryptosporidium* species/genotypes at the 18S RNA and actin loci.

<table>
<thead>
<tr>
<th></th>
<th>18S locus</th>
<th>Actin locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piscine genotype 1</td>
<td>13.0</td>
<td>17.4</td>
</tr>
<tr>
<td>Piscine genotype 2</td>
<td>5.1</td>
<td>Not analysed*</td>
</tr>
<tr>
<td>Piscine genotype 3</td>
<td>4.3</td>
<td>Not analysed*</td>
</tr>
<tr>
<td>Piscine genotype 4</td>
<td>6.3</td>
<td>Not analysed*</td>
</tr>
<tr>
<td>Piscine genotype 5</td>
<td>5.5</td>
<td>Not analysed*</td>
</tr>
<tr>
<td>Piscine genotype 6</td>
<td>13.0</td>
<td>Not analysed*</td>
</tr>
<tr>
<td>Piscine genotype 7</td>
<td>11.8</td>
<td>Not analysed*</td>
</tr>
<tr>
<td><em>C. molnari</em></td>
<td>10.8</td>
<td>7.3–8.5</td>
</tr>
<tr>
<td><em>C. parvum</em></td>
<td>15.4</td>
<td>21.4</td>
</tr>
<tr>
<td><em>C. hominis</em></td>
<td>15.4</td>
<td>21.4</td>
</tr>
<tr>
<td><em>C. bovis</em></td>
<td>17.7</td>
<td>19.0</td>
</tr>
<tr>
<td><em>C. baileyi</em></td>
<td>14.7</td>
<td>19.4</td>
</tr>
<tr>
<td><em>C. muri</em></td>
<td>15.0</td>
<td>19.4</td>
</tr>
<tr>
<td><em>C. andersoni</em></td>
<td>13.8</td>
<td>20.1</td>
</tr>
</tbody>
</table>

* Actin sequences were not available for these genotypes.
Evolutionary relationships of Cryptosporidium piscine-derived isolates inferred by neighbour-joining analysis of p distances calculated from pairwise comparison of 18S rRNA sequences. The percentage of replicate trees in which associated taxa clustered together in the bootstrap test (1000 replicates), are shown at the internal nodes (>50% only) for distance, ML and parsimony (n.s. = not supported). Accession numbers are given in parentheses. Isolates from the present study are marked with asterisk (*).
in the GenBank database under the accession numbers KC807985–KC807988.

4. Discussion

In the present study, the overall prevalence of Cryptosporidium sp. was low (1.14%, 7/614). Previous studies have also reported low prevalences in similar groups of fish (0.8%, 6/709) (Reid et al., 2010) and in ornamental fish (3.5%, 6/171) (Morine et al., 2012) while others have reported higher prevalences (10–100%) mostly among juvenile fish (Alvarez-Pellitero et al., 2004; Sitja-Bobadilla et al., 2005; Murphy et al., 2009; Zanguee et al., 2010).

This study identified three new fish hosts for Cryptosporidium; silver barb (P. gonionotus), mackerel scad (D. maracellus) and oblong silver biddy (G. oblongus). The fourth host Nile tilapia (O. niloticus) could also be a new host since previous studies have detected Cryptosporidium in the same genus but did not identify the species (Landsberg and Paperna, 1986; Paperna and Vilenkin, 1996).

No oocysts or life cycle stages were observed in the infected fish hosts in the present study due to substantial autolysis of tissues, which has been reported as an issue for Cryptosporidium detection in piscine hosts (Zanguee et al., 2010). Fish are known to have a very rapid rate of tissue autolysis compared to homeotherms (Roberts, 2012) and many of the fish were dead for up to 4 h prior to being processed which contributed to the problem. Previous studies have provided histological and electron microscopic evidence of considerable cellular damage associated with several Cryptosporidium species/genotypes that infect fish; C. mohari in the stomach of fingerlings and juveniles of gilt-head sea bream (Alvarez-Pellitero and Sitja-Bobadilla, 2002), piscine genotype 1 in the stomach of a guppy (Ryan et al., 2004) and piscine genotype 3 in the intestine of a mullet (Reid et al., 2010). Piscine genotype 2 was associated with gastric infections in angelfish, with the greatest morbidity and mortality seen in larval and juvenile fish (Murphy et al., 2009). Whether the C. parvum and C. hominis identified in the present study represents
actual or mechanical infections remains to be determined as the oocysts may have been passing through rather than infecting these fish and further research using histological analysis of rapidly preserved tissue specimen is required to confirm this.

The zoonotic Cryptosporidium genotypes identified in this study are of significance to public health. Cryptosporidium parvum subtypes IIA14G2R1, IIA15G2R1 and IIA19G4R1 were found in cultured freshwater (Nile tilapia), wild freshwater (silver barb) and a marine (mackerel scad) fish. The zoonotic C. parvum Ila subtype family has predominately been found in calves and in humans in North America, Europe and Australia (Xiao, 2010). Only one study has previously detected zoonotic C. parvum (subtype Ila18G3R1) in a marine fish (Reid et al., 2010). The presence of Ila subtypes in the fish samples from the present study could be due to waterborne contamination with human and animal waste. Human sewage management systems in PNG villages typically involve dugout toilets near the homes and toilets built over the rivers or seas. No cattle farms were seen in close vicinity to where the samples were collected, whereas dugout toilets, companion animals and/or domesticated poultry and pigs were seen in the environment. Fish ponds, rivers and seas could be contaminated from rainwater runoff and from humans and/or animals bathing in them. A previous study has detected antibodies against Cryptosporidium among children from PNG (Groves et al., 1994), however, no molecular work has been done to confirm the species or genotypes present.

One marine fish (mackerel scad DM18) was identified as C. parvum at the 18S locus while at the gp60 locus it was subtyped as C. hominis IdA15G1R1, indicating that a mixed C. parvum/C. hominis infection was present. This is the first report of C. hominis in fish. The only other marine organism in which C. hominis has been reported was a dugong (Dugong dugon) (Morgan et al., 2000), and its presence probably reflects human sewage contamination of the water.

The novel piscine genotype 8 was identified in two marine silver biddies. At the 18S locus, piscine genotype 8 exhibited 4.3% genetic difference with piscine genotype 3 and 13.8–17.7% genetic difference with other Cryptosporidium spp. (Table 4). At the actin locus, piscine genotype 8 exhibited 7.3% genetic difference with C. molnari 2 and 19.0–21.4% with other Cryptosporidium spp. (Table 4). Based on the differences in the genetic sequences, piscine genotype 8 is unique and may represent a new species; however, further research is required to confirm this.

The present study identified zoonotic C. parvum subtypes in fish species, which are frequently eaten in PNG. Previous studies have not identified conclusive evidence for transmission of Cryptosporidium from fish to humans but one study reported that urban anglers are at a risk for contracting cryptosporidiosis from exposures received while fishing and consuming caught fish (mean probability of infection was nearly one) (Roberts et al., 2007). It is therefore essential that fish for human consumption are handled appropriately to avoid contamination. In addition, a novel Cryptosporidium sp. (piscine genotype 8) was identified based on molecular data but lacks histological details of infection or morphological features of the oocysts, thus future work is required to establish this genotype as a species.

Acknowledgements
We are grateful to the staff of PNG’s National Agriculture Research Institute (NARI), especially Dr. Workneh Ayalew for logistical support and advice, Ms Atmaleo Aguyanto and Mr Densley Tapat for assistance with sampling of cultured fish and staff at the PNG Department of Agriculture and Livestock for their assistance during sampling. We would like to thank Dr. Andrea Paparini for helping with PCR at the actin locus and Dr. Rongchang Yang for helpful discussions.

References


Appendix E

Identification of *Anisakis* species (Nematoda: Anisakidae) in marine fish hosts from Papua New Guinea
Identification of *Anisakis* species (Nematoda: Anisakidae) in marine fish hosts from Papua New Guinea

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ITS
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Zoonotic
Papua New Guinea

**A B S T R A C T**

The third-stage larvae of several genera of anisakid nematodes are important etiological agents for zoonotic human anisakiasis. The present study investigated the prevalence of potentially zoonotic anisakid larvae in fish collected on the coastal shelves of Madang and Rabaul in Papua New Guinea (PNG) where fish represents a major component of the diet. Nematodes were found in seven fish species including *Decapterus macarellus*, *Gerris oblongus*, *Pinjalo lewisi*, *Pinjalo pinjalo*, *Selar crumenophthalmus*, *Scomberomorus maculatus* and *Thunnus albacares*. They were identified by both light and scanning electron microscopy as *Anisakis* Type I larvae. Sequencing and phylogenetic analysis of the ribosomal internal transcribed spacer (ITS) and the mitochondrial cytochrome C oxidase subunit II (cox2) gene identified all nematodes as *Anisakis* typica. This study represents the first in-depth characterisation of *Anisakis* larvae from seven new fish hosts in PNG. The overall prevalence of larvae was low (7.6%) and no recognised zoonotic *Anisakis* species were identified, suggesting a very low threat of anisakiasis in PNG.

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1. Introduction

The family Anisakidae includes parasitic nematodes of marine fauna. They have a worldwide distribution and a complex life-cycle which involves invertebrates, fish, cephalopods and mammals (Chai et al., 2005). Anisakid nematodes can accidentally infect humans who can suffer from several symptoms including sudden epigastric pain, nausea, vomiting, diarrhoea and allergic reaction (Sakanari and Mckerrow, 1989; Audicana and Kennedy, 2008). Most cases of human infection involve anisakid species belonging to the genus *Anisakis* Dujardin, 1845. There are nine described species of *Anisakis*, which are further subdivided into two types. Type I consists of *Anisakis simplex* sensu stricto (s.s.), *A. pegreffii*, *A. simplex*, *A. typica*, *A. ziphidarum* and *A. nascetti* while Type II consists of *A. paggaie*, *A. phy-seteris* and *A. brevispiculata* (Mattucci and Nascetti, 2008; Mattucci et al., 2009). Of these, only *A. simplex* s.s. *A. pegreffii* and *A. phyeseteris* have been shown to cause infection in humans (Mattucci et al., 2011; Arizono et al., 2012).

Anisakid nematodes can be differentiated based on their morphological characteristics and molecular data. According to Berland (1961), larval morphological features including the absence of a ventricular appendage and an intestinal caecum are useful for distinction between several anisakid genera. *Anisakis* Type I or Type II larvae can be identified based on ventriculus length and the presence of a tail spine (or mucron) (Berland, 1961). More recently, polymerase chain reaction (PCR) based tools have been widely used for characterisation of anisakid species at multiple loci, including ribosomal internal transcribed spacer (ITS)
Appendix E

2. Materials and methods

2.1. Parasite collection

A total of 276 whole fresh fish were collected from markets in the coastal towns of Madang and Rabaul from March to August 2011 (Fig. 1). The fish were necropsied and nematodes were collected from the body cavities. The muscles of the fish were thinly sliced and investigated under white light to check for nematode larvae. Nematodes were preserved in 70% ethanol and transported to Murdoch University, Australia, for analysis. The prevalence of anisakids in each fish host was expressed as the percentage of positive samples; with 95% confidence intervals calculated assuming a binomial distribution (Rozsa et al., 2000).

2.2. Morphological analysis

Whole nematodes were cleared in lactophenol for more than 48 h and individually mounted onto microscope slides. The body lengths of the nematodes were directly measured. Images were taken with an Olympus BX50 light microscope equipped with Olympus DP70 Camera at 40/100× magnification. The following features were measured: body width, oesophagus length, ventriculus length and mucron length. Morphological identification was conducted according to keys previously reported (Berland, 1961; Cannon, 1977; Shamsi et al., 2009a,b).

Scanning electron micrographs (SEMs) were taken for representative specimens to study further morphological details. SEMs were obtained on a Phillips XL30 scanning electron microscope at the Centre for Microscopy Characterisation and Analysis at the University of Western Australia. Parasite samples were fixed in 2% glutaraldehyde and 1% paraformaldehyde in PBS for 60 min at 4 °C and washed twice with PBS (pH = 7.4) in 1.5 mL eppendorf tubes. Samples were dehydrated using a PELCO Biowave microwave processor (TedPella Inc., Redding, CA, USA) by passage through increasing ethanol concentrations in water (33%, 50%, 66% and 100%) followed by two washes in dry acetone. Samples were then dried in a critical point dryer (Emitech 850, Quorum Technologies, Ashford, UK), attached to aluminium sample holders and coated with a 5 nm thick platinum coating to enable surface electrical conduction.

2.3. Genetic characterisation and phylogenetic analysis

DNA from individual nematodes was isolated using a DNeasy® Tissue Kit (Cat. No. 69504, Qiagen, Hilden, Germany). The ITS rDNA region was amplified using primers NC5 5′-GTAGTTAACCTGCCGGAAGGATCAT-3′ and NC2 5′-TTAGTTTTTTCTCCCCGT-3′ (Zhu et al., 1998) and the mt-DNA cox2 gene was amplified using primers 210 5′-CACCAACTCTTTAAATTATC-3′ and 211 5′-TTTTCTAGTATATAGTTGRTYTAT-3′ (Nadler and Hudspeth, 2000).

Each PCR was performed in a reaction volume of 25 μL using 1 μL of DNA, 1× PCR buffer (Kapa Biosystems, Cape Town, South Africa), 1.5 mM MgCl2, 200 μM (each) dNTP (Fisher Biotech, Australia), 12.5 pmol of each primer and 0.5 U of kapa Taq DNA polymerase (Kapa Biosystems, Cape Town, South Africa). Negative (no DNA template) and positive (genomic DNA from L3 Anisakis typica larvae) controls were included in all PCRs. Thermal cycling was performed in a Perkin Elmer Gene Amp PCR 2400 thermal cycler at conditions as previously described (Valentini et al., 2006; Kijewksa et al., 2009).

All amplicons were purified using an Ultra Clean® DNA purification kit (MolBio, West Carlsbad, CA, USA).

Fig. 1. Map of the study sites. Samples were collected on the coastal shelves off Madang and Rabaul in Papua New Guinea.
Sequencing was performed using the ABI Prism BigDye® terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 3730 DNA Analyser instrument according to manufacturer's instructions except that the annealing temperature was lowered to 46 °C for the cox2 locus. Sequences were analysed using FinchTV 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com) and compared with published sequences for identification using the National Institute of Health's National Centre for Biotechnology Information Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov). Additional known ITS and cox2 nucleotide sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank) for phylogenetic analysis.

MEGA5 (http://www.megasoftware.net/) was used for all phylogenetic analyses (Tamura et al., 2011). The nucleotide sequences were aligned using MUSCLE (Edgar, 2004), edited manually and tested with MEGA5 model test to find the best DNA model to infer the phylogenetic trees. Phylogenetic analysis with other known anisakid species was conducted using both neighbour-joining (NJ) and maximum-likelihood (ML) analysis for both loci. Evolutionary relationships were calculated using the Kimura two-parameter model for ITS sequences and the Tamura–Nei model for cox2 sequences with Contracaecum osculatum as an outgroup. Reliabilities for both NJ and ML trees were tested using 1000 bootstrap replications (Felsenstein, 1985) and bootstrap values exceeding 70 were considered well supported (Hills and Bull, 1993). The nucleotide sequences were deposited in GenBank under the accession numbers: JX648312–JX648326.

3. Results

3.1. Anisakid prevalence

The overall prevalence of anisakids in fish from PNG was 7.6% (21/276, 95% CI = 0.05–0.11). Anisakid larvae were found in 7 fish species, at prevalences ranging from 2.9% to 100% (Table 1). The larvae were observed mostly within the body cavities of the fish and their intensity ranged from 1 to 6 per infected fish host with the exception of Pinjalo pinjalo, which had an intensity of 120 larvae per fish, with larvae being found in many other body parts including muscles, pyloric region and liver.

3.2. Morphology of Anisakis Type I larvae

Morphological analysis showed that all anisakid nematodes examined were Anisakis Type I larvae. The larvae were white and cylindrical in shape. They measured between 20–36 mm in length and 0.4–0.45 mm in width. SEM revealed that the cuticles were irregularly striated transversely at 5.5 μm intervals. The larva had inconspicuous lips with six papillae, a prominent boring tooth and excretory pore which opened ventrally at the cephalic end (Fig. 2, panels A and E). The mouth opening led to a cylindrical striated oesophagus (length 1.6–2.1 mm), which was followed by a slightly wider ventriculus (length 0.98–1.13 mm). The junction between oesophagus and ventriculus was transverse (Fig. 2, panel B). The ventriculus connected obliquely with the intestine, without a ventricular appendage and intestinal caecum (Fig. 2, panel C). The intestine filled the remaining part of the body. The mucron was distinct and was located at the caudal end (length 17.5–18.0 μm) (Fig. 2, panels D, F and G).

3.3. Sequence and phylogenetic analysis of the ITS region

Amplification of the ITS rDNA generated an approximately 900 bp product. Both neighbour-joining and maximum-likelihood analyses produced trees with similar topology. Neighbour-joining analysis of the ITS nucleotide sequences from the present study with previously reported sequences from GenBank clustered all the Anisakis Type I larvae examined with Anisakis typica (Fig. 3). The ITS nucleotide sequences of all the Anisakis Type I larvae from the present study exhibited 99.1–100% similarities to the published sequence of Anisakis typica (AB432909) found in Indian mackerel (Rastrelliger kanagurta) in Thailand and 96.1–97.6% similarities to the published sequence of Anisakis typica (JQ798962) found in cutlassfish (Trichiurus lepturus) from Brazil. The sequences exhibited 82.7% to 88.7% similarities with other Anisakis species (Table 2).

Amplification of the cox2 gene generated an approximately 629 bp product. As with the ITS locus, neighbour-joining and maximum-likelihood analyses produced trees with similar topology. Neighbour-joining analysis of cox2 nucleotide sequences showed that all isolates clustered broadly with A. typica (DQ116427) but revealed more variation. Two broad groups were produced with subgroup 1 consisting of 5 isolates and A. typica reference

Table 1

<table>
<thead>
<tr>
<th>Fish species</th>
<th>N</th>
<th>Prevalence (CI)</th>
<th>MI ± SD (min–max)</th>
<th>Specimen code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decapterus macarellus (Mackerel Scad)</td>
<td>29</td>
<td>6.9 (0.03 to 0.17)</td>
<td>1</td>
<td>DM23, DM24</td>
</tr>
<tr>
<td>Gerres amblosus (Slender Silver-biddy)</td>
<td>54</td>
<td>3.7 (0.02 to 0.09)</td>
<td>3 ± 0.4 (2–4)</td>
<td>G014, G015</td>
</tr>
<tr>
<td>Pinjalo lewisi (White-spot Pinjalo Snapper)</td>
<td>14</td>
<td>50 (0.2–0.8)</td>
<td>5 ± 0.92 (1–6)</td>
<td>PL1, PL5, PL8, PL9</td>
</tr>
<tr>
<td>Pinjalo pinjalo (Pinjalo)</td>
<td>1</td>
<td>100 (0.2–0.8)</td>
<td>120</td>
<td>PP1</td>
</tr>
<tr>
<td>Scomberomorus maculatus (Spanish Mackerel)</td>
<td>3</td>
<td>33.3 (1–1 to 1.8)</td>
<td>1</td>
<td>SM3</td>
</tr>
<tr>
<td>Thunnus albaces (Yellowfin Tuna)</td>
<td>34</td>
<td>2.9 (0.3 to 0.09)</td>
<td>3</td>
<td>TA3</td>
</tr>
<tr>
<td>Salar crumenophthalmus (Bigeye Scad)</td>
<td>106</td>
<td>6.6 (0.02–0.11)</td>
<td>2.9 ± 0.95 (1–3)</td>
<td>SC76, SC77, SC78, SC88, SC97, SC100, SC102</td>
</tr>
</tbody>
</table>

N is the number of fish sampled, prevalence is the % of infected fish (95% CI in parentheses) and mean intensity (MI) is the mean number of larvae in the infected fish hosts ± SD (range). Where no SD value was given, there was only one observation or all observations were similar so that SD could not be calculated.
sequence (DQ116427), and subgroup II containing 16 isolates (Fig. 4). Based on genetic distance analysis, subgroup I had 98.9–99.3% similarity to A. typica (DQ116427) while subgroup II had 92.4–94.6% similarity to A. typica (DQ116427). The cox2 nucleotide sequences from the present study shared 77.0–87.2% similarity with other known Anisakis species (Table 2).

4. Discussion

Anisakid larvae were found in 7.6% (21/276) of the 7 fish species examined. The intensity of infection was low (1–6)

Table 2
Percentage similarity of the Anisakis species analysed in the present study and their closest relatives.

<table>
<thead>
<tr>
<th>Species compared</th>
<th>% similarity at ITS rRNA locus</th>
<th>% similarity at Cox2 locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. typica</td>
<td>96.1–100</td>
<td>92.4–99.3</td>
</tr>
<tr>
<td>A. ziphidarum</td>
<td>87.5–88.7</td>
<td>82.1–84.3</td>
</tr>
<tr>
<td>A. pegreffii</td>
<td>85.4–86.2</td>
<td>84.7–87.2</td>
</tr>
<tr>
<td>A. simplex s.s</td>
<td>85.6–86.3</td>
<td>84.7–86.7</td>
</tr>
<tr>
<td>A. simplex C</td>
<td>85.8–86.6</td>
<td>84.2–85.8</td>
</tr>
<tr>
<td>Anisakis sp.*</td>
<td>86.5–87.8</td>
<td>Not analysed</td>
</tr>
<tr>
<td>A. nascettii</td>
<td>Not analysed</td>
<td>83.9–86.7</td>
</tr>
<tr>
<td>A. physeteris</td>
<td>82.7–83.9</td>
<td>82.8–84.5</td>
</tr>
<tr>
<td>A. brevispiculata</td>
<td>78.6–80.1</td>
<td>77.0–79.1</td>
</tr>
<tr>
<td>A. poggiae</td>
<td>83.8–84.7</td>
<td>79.3–82.2</td>
</tr>
</tbody>
</table>

At the ITS locus, comparison with A. typica, accession numbers AB432909 and JQ798962 were presented. Anisakis sp.* is conspecific with A. nascettii (Mattiucci et al., 2009).

in all fish hosts except for Pinjalo pinjalo (120) (Table 1). Previous studies have reported wide variation in prevalence and intensity of infection of anisakids in other fish hosts (Costa et al., 2003; Farjallah et al., 2008a,b; Setyobudi et al., 2011). The relatively low infection level found in the present study could be due to the fact that most of the fish hosts sampled were relatively small in size (range 16–49 cm fork length) compared to previous studies. In general, prevalence and parasite burden tends to increase with the size and the age of the fish host (Setyobudi et al., 2011).

All nematodes in the present study were identified morphologically as Anisakis Type I larvae, based on an oblique connection between the ventriculus and the intestine, lack of a ventricular appendage and intestinal caecum, and the presence of a mucron (Berland, 1961; Cannon, 1977). Larvae of A. typica found in cutlassfish (Trichiurus lepturus) from Brazil shared similar morphological characteristics with the A. typica larvae from the present study (Borges et al., 2012).

Phylogenetic analysis of DNA sequences indicated that all examined samples were Anisakis typica. At the ITS locus, all isolates examined formed a single clade with A. typica. The comparison of the ITS nucleotide sequences from this study with sequences previously deposited in Genbank resulted in 96.1–97.6% similarities to A. typica found in cutlassfish (accession no. JQ798962) from Brazil and 99.1–100% similarities to A. typica (accession no. AB432909) from Indian mackerel in Thailand.
At the cox2 locus, whilst the isolates clustered broadly with the reference A. typica genotype, two distinct subgroups (I: 98.9–99.3% similarity and II: 92.4–94.6% similarity) were identified. Previously reported cox2 trees by Valentini et al. (2006) also showed similar genetic divergence within the Anisakis typica clade. Furthermore, the sequence difference of 5.4–7.6% between the subgroup II clade and the reference A. typica sequence is still within the range found between conspecifics in other nematode taxa (Blouin et al., 1998).

According to Mattiucci and Nascetti (2006), Anisakis species form two sister clades and A. typica is grouped within clade I, based on phylogenetic relationships inferred from allozyme and mitochondrial gene markers. In the present study, A. typica clustered within clade I at the cox2 locus, consistent with previously reported phylogenetic trees (Valentini et al., 2006; Mattiucci et al., 2009; Cavallero et al., 2011; Setyobudi et al., 2011). However, at the ITS locus, A. typica did not cluster within clade I and formed a separate group to the two clades. Other studies have shown similar tree topologies at the ITS locus (Kijewska et al., 2009; Cavallero et al., 2011) and according to Cavallero et al. (2011), A. typica could form a distinct lineage (resulting in three clades, rather than two, for the genus Anisakis). It should be noted, however, that the position of A. typica in both the ITS tree and cox2 tree was not well supported (<50% bootstrap support) in our study and therefore more sampling of the species from a wider range...
of hosts and geographical areas is needed to resolve this discrepancy.

The present study identified seven new fish species as hosts for *A. typica*; *Decapterus macarellus*, *Gerres oblongus*, *Pinjalo lewisi*, *Pinjalo pinjalo*, *Selar crumenophthalmus*, *Scomberomorus maculatus* and *Thunnus albacares*. Previous studies have identified *A. typica* in more than 15 different fish hosts, which have an epipelagic distribution in the Atlantic Ocean close to the coast lines of Brazil, Mauritius, Morocco, Portugal and Madeira (Mattiucci et al., 2002; Pontes et al., 2005; Marques et al., 2006; Farjallah et al., 2008a; Iniguez et al., 2009; Kijewska et al., 2009; Borges et al., 2012). *Anisakis typica* has also been found in the Mediterranean Sea close to Tunisia, Libya, Cyprus and Crete, and in the Indian ocean off Somalia (Mattiucci et al., 2002; Farjallah et al., 2008b) and Australia (Yann, 2006). Furthermore *A. typica* has been found in Japan, Taiwan, China, Thailand and Indonesia (Chen et al., 2008; Palm et al., 2008; Umehara et al., 2010). Although it has been hypothesised that *A. typica* has a global distribution that extends from a 30°S to a 35°N latitude (Mattiucci and Nascetti, 2006), a previous distribution model for anisakid species has not included PNG (Kuhn et al., 2011).

In conclusion, all anisakids identified from PNG in the present study were *A. typica*, which has not previously been associated with human infections. Further studies are needed to extend the knowledge of anisakid species distribution in larger fish hosts and other seafood hosts in PNG waters, but the present study results suggest that the danger from zoonotic anisakid species in PNG is very low.

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Appendix E
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Gastrointestinal pathogens in sheep, goats and fish in PNG
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Committee (Permit R2368/10).

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