Investigating parasite patency and phenotypic characterization of *Giardia* spp. infections in canines using proteomic tools
This thesis is presented for the degree of Doctor of Philosophy

In the School of Veterinary and Life Sciences

Murdoch University,

Murdoch

Western Australia

2015
Declaration

I declare that this entire thesis is my own research and contains original work, which has not been published or submitted for examination at any other tertiary education institution.

I, the undersigned author of this thesis understand that Murdoch University will make the contents of this thesis available to other readers through the library and in digital format.

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2015
Abstract

Published research indicates that *Giardia* spp. infections in Canidae, both domestic and wildlife species, are caused by several different subtypes. Four different genetic sequences of *Giardia* spp., termed ‘Assemblages’ infect domestic dogs, two of which also infect humans.

Currently, DNA sequencing is the only method for distinguishing between different types of infections and published studies concerning potential transmission remain inconclusive. Nevertheless, it is well established that infections primarily affect juveniles and immuno-compromised individuals whilst the complexity of definitive diagnosis of symptomatic and asymptomatic *Giardia* spp. infections in dogs is problematic.

The focus of this research programme concerns *Giardia duodenalis*, which infects mammalian species, specifically *Giardia* spp. infections in *Canis lupus familiaris*, or domestic dogs. Faecal sampling was conducted from naturally infected populations over a three-year period, from breeding kennels and from the urban dog population in Perth, Western Australia. Sampling strategies used microscopy and commercially available immuno-antigen tests to record enteric parasites infections and to confirm the earliest time of infection in different breeds of puppies. Results indicated that puppies could be infected with *Giardia* spp. within three weeks after birth and that clinical signs were associated with enteric parasite infections. This additional information detailing the impact of *Giardia* spp. infections on the canine host is an important factor for accurate diagnosis and understanding pathogenesis.

Innovative methods were developed for analysing phenotypic descriptors of *Giardia* spp. extracted from canine faecal material using proteomic tools. Results from this work identified novel proteins and peptides, not previously published for any parasite protein extracted from mammalian faecal samples. This thesis discusses those peptide sequences identified in the context of canine gastro-intestinal ecosystems in
conjunction with host-parasite interactions, and further explores the application and integration of molecular genomic and proteomic data. This work supplements annotation of *Giardia* spp. genomic data and may prove pivotal in accurate differentiation between host genotypes.

Finally, one protein identified during the proteomic analysis, lactoferrin, was further investigated using a commercially available enzyme linked immunosorbent assay (ELISA). Results suggest that elevated levels of faecal lactoferrin occur in dogs with clinical signs of infection.

A critical analysis of the challenges facing veterinarians and medical epidemiologists in diagnosis of enteric parasites in humans and canines is discussed and some important implications for the management and health of domestic dogs are presented.
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Ethics Statement

The research reported in this thesis did not involve the use of animals for scientific purposes and therefore approval was not required from the University’s Animal Ethics Committee. Prior to commencement the committee was informed that this project only involved the opportunistic collection of waste samples from living animals, both healthy and naturally infected, to examine them for the presence of parasites. A statement of research objectives and a sampling strategy was provided. The research sought to improve the knowledge and understanding of the impact of pathogenic parasite infections on the host animal, *Canis familiaris*.

This thesis contains references to historical research experiments conducted on humans and animals, some of which would not meet today’s rigorous ethical standards. Outcomes from the work presented in this thesis may warrant further investigation requiring the use of animals or humans. The author supports the premise that all experimental animal work should strive to reduce, refine, and replace animals wherever possible, and should not be conducted purely for anthropocentric gains but for the overall benefit of our planet and towards sustainable ecosystems.
Acknowledgements

I would like to thank Murdoch University for awarding me with a scholarship that provided a stipend for 3 years. My supervisors invested their confidence in me and I hope that this body of work does justice to their guidance and patience, although I accept responsibility for any omissions or errors.

I would like to acknowledge that I have benefitted and learned from the supervision, advice and research leadership provided by Professor R.C.A. Thompson (Andy) and the Murdoch University Parasitology group. Working with Dr. Richard Lipscombe and the team from the Proteomics International Laboratories I absorbed mass spectrometry tips and techniques, including quality control methods. I appreciate the importance of honesty, trust and ethical behaviour in developing sustainable business and the sacrifices individuals need to make to break into new markets. Intellectual property is assigned to the tangible expression of an idea. Thank you to Dr. Rob Steuart, Curtin University, for shining a light on one potential area for investigation and for securing support for this project.

I am extremely fortunate to have been one member of a team working alongside staff in the laboratories at Murdoch University, Curtin University, The University of Western Australia, the State Agricultural Biotechnology Centre, the Western Australia Institute of Medical Research at the Royal Perth Hospital and in the new laboratories at the Harry Perkins Institute of Medical Research.

I share the anticipation that outcomes from this work may have commercial applications or improve the health and wellbeing of our communities and catchments both in Western Australia and beyond.

My children were rather disgusted that I became known as the ‘Poo Lady’ by staff at a dog shelter in Perth. Regular collection of samples earned me this title and without the cooperation of staff at the shelters and dog breeding facilities in Perth and Bulla, Victoria this work would not have been possible. Thank you John Russell-Cook.

Substantial work and determination were important factors that shaped my involvement in the collaborative efforts that successfully contributed to meeting project expectations. The Australian Society for Parasitology provided incentives to travel and share our research in four states of Australia. Murdoch University and the American Association for Veterinary Parasitology supported an international travel opportunity for me to participate in the Complimentary Alliances: Veterinary, Human and Agrochemical Approaches to Parasiticides Meeting in San Diego, USA.
I would like to acknowledge the traditional owners of the land where this work was conceived and carried out, included are many references to the contributions from communities in the Kimberley region of north Western Australia, more than twenty years ago.

This work would not have been possible without access to key reference materials and online databases provided through the library and IT support services of Murdoch University and other tertiary institutions in Australia and New Zealand. I acknowledge the funding support for researchers who contribute, curate, and maintain electronic resources for the benefit of the research community including PhD research students like myself.

My reasons for embarking on this research were written before I was enrolled. These included ‘Because I believe I can’, ‘Because of my interest in the intersect between animal health, human health and the environment’ and ‘Because educated women should use their education’.

Good companionship inside and outside the university was integral to managing the emotional rollercoaster of PhD candidacy and my friends were there to listen and offer guidance. Thank you to everyone who read parts of this thesis and provided feedback.

I acknowledge the friendship of PhD students at Murdoch Uni, UWA, Curtin Uni. JCU, ANU, Macquarie, Monash and UTS and students from other places who shared their time, jokes and sweet stories. I acknowledge the Lucky break that gave me insight into managing my wellbeing, taking a breath and being grateful for achievements and progress. Solitude was required to find my academic voice.

I acknowledge my family whose unswerving support enabled me to complete this thesis. In October 2012, my mother passed away in Yorkshire, England, and within five months in Surin, Thailand, my mother-in-law passed away too. Both women were teachers and incredibly important in my life and the lives of my family.

My husband, Dr. Prayadt Wangpen, allowed me the freedom to engage with my research and achieve personal growth. I am grateful for his help with improving the layout of the Figures and Tables in this thesis.
Dedication

This thesis is dedicated to Kathleen Jones (nee Byrne) and Kasorn Wangpen (nee Kongsiri) who provided love and leadership to their families and communities. They encouraged all their children and grandchildren including Amarisa and Joseph, to observe, remember and carry forward their aspirations and achievements for future generations.
Live Simply, So that others may Simply Live
Chapter 1

General introduction to *Giardia duodenalis*

*G. duodenalis* is a unicellular parasitic organism, a pathogen of humans and other mammals. The vast amount of literature concerning *Giardia* spp. mainly pertains to *G. duodenalis* that causes human infections, however there are many key features common to all genetic variants. Hereafter, the term ‘*G. duodenalis*’ refers specifically to the infective pathogens of human hosts and its various genotypes, as described in the literature. Consideration of the current and accepted taxonomic nomenclature informed the writing of this thesis, and *Giardia* spp. was selected in preference over *Giardia canis* to describe infections in canines. Where detailed information about canine *Giardia* spp. is absent, facts about *G. duodenalis* reported in the literature, including aspects of the biology, life cycle and structural features are included in this introductory section. In addition, this introduction includes a brief summary of the findings and current theories associated with *G. duodenalis* infections in humans, including how infection occurs, the clinical impacts and host susceptibility. Comments on the modes of transmission, public health implications and cycling of asymptomatic infections in the absence of diagnosis are presented, together with the potential consequences for those individuals and communities at risk.

1.1 Taxonomic Classification

*Giardia* spp. are protozoan parasites - non-fungal eukaryotic organisms capable of surviving in aerobic and anaerobic conditions. Taxonomic classification is problematic as the nomenclature of *Giardia* species has been reclassified and renamed numerous times since the first documented discovery by Leewenhoek in 1681 (Wolfe, 1992). Protozoans are classified within the domain Excavata. Within this domain, parasites closely related to *Giardia* spp. are the Parabasalids (e.g. trichomonads). Classification categories for protozoans include apicomplexans such as coccidians which, are obligate intracellular protozoan parasites of the gastrointestinal tract (Levine, 1973). Within the classic taxonomic characterization scheme *Giardia*, an anaerobic flagellated
protozoan parasite, is classified within the **eukarya kingdom excavata** Domain; **metamonada** Phylum; **diplomonadida** Order; **hexamitidae** Family; **Giardia** Genus; **duodenalis** species (Thompson and Monis, 2011).

The current taxonomic class for **Giardia** is the Hexamitidae, which comprises flagellated organisms. The definitive nomenclature using traditional taxonomic methods has been much revised in recent years and more recent attempts have been made to classify the protist organisms according to clades, based upon ultrastructure, biochemistry and genetics (Cavalier-Smith, 2004, Adl *et al.*, 2005, Hampl *et al.*, 2009). At the present time whilst different host species are susceptible to **Giardia** spp. infections the International Committee for Zoological Nomenclature has confirmed six different **Giardia** species in 2007. Host infections are known for mice, **Giardia muris**; voles and muskrats, **Giardia microti**; budgerigars, **G. psittaci** heron and ibis, **G. ardeae**; frogs, **G. agilis**; and most other mammals, including humans, **G. duodenalis** (syn. **G. intestinalis**, **G. lamblia**). Giardia spp. belongs to a group of unicellular protozoan organisms that have evolved through specialised adaptations to exist within niche environments (Ricklefs, 2010, Niculescu, 2014).

### 1.2 Importance of parasite infections on human health

The World Health Organization (WHO) reports estimate over 200 million reported gastro-intestinal infections each year which, directly cause debilitating disease and death and the subsequent indirect effects of missed educational and employment opportunities – key contributors to poverty, are particularly evident in developing countries (Thompson, 2004, WHO *et al.*, 2006). Infectious parasitic diseases include enteric protozoan infections of the mammalian digestive system that are of particular concern for humans (Thompson and Smith, 2011). The protozoan parasite **Giardia** spp. is ranked eighth in the top ten parasites affecting humans in the world (Buret, 2005). **Giardia** spp. infects a diversity of host organisms, including humans, wildlife and domesticated livestock pets (Buret, 2008).
In less developed countries with large disease burdens, *G. duodenalis* might be considered by some to be relatively minor in comparison to the effects of other parasitic organisms, such as malaria (Nwanguma and Alumanah, 2009). However, the cumulative factors of poverty; conflict and politics; unstable governments and lack of education and health care services result in poor hygiene, untreated water supplies; open sewers shared by wildlife, humans, and livestock thus increase exposure to waterborne infectious diseases, such as *G. duodenalis* (Putignani and Menichella, 2010). This is due to the wide prevalence of the disease throughout the world, but most significantly in countries with large population numbers but low economic status and poverty, poor sanitation, high child mortality and low nutritional status.

During 2004, the number of cases of *G. duodenalis* infections worldwide resulted in the nomination of this illness as one of the world’s most neglected tropical diseases by the WHO (Savioli *et al.*, 2006).

### 1.3 Clinical signs of infection

*G. duodenalis* infections occur when cysts are ingested, trophozoites become attached to the surface of the intestine and feed on the mucus secretions of the intestine (Eckmann, 2003). In humans, symptoms usually appear two weeks after exposure and include: diarrhoea, dehydration, stomach cramps, flatulence, and weight loss (Nash *et al.*, 1987, Robertson *et al.*, 2010). Heavy infection can hinder the body's ability to absorb nutrients. Acute infections are those considered to cause severe or debilitating symptoms in the host, such as diarrhoea, vomiting and abdominal pain (Thompson, 2000). Nonetheless, other symptoms have been reported such as psychotic manifestations that responded to anti-*Giardia* treatment (Heap and Mumford, 1989). Chronic illness associated with *G. duodenalis* infections, have been reported in juveniles as failure to thrive, poor growth rates, lethargy and suppressed appetite in adult hosts (Eligio-Garcia *et al.*, 2005). The clinical appearance of the host small intestine has been associated with loss of brush border membrane integrity and peripheral enzyme function (Thompson, 2004, Scott *et al.*, 2004, Cotton *et al.*, 2011).
Clinical symptoms are the result of pathogenic mechanisms occurring at the host-parasite interface. One of the key features of *Giardia* spp., which contribute to its success as a parasite, is the ability of trophozoites to attach to epithelial cells of the small intestine causing pathogenic changes.

### 1.4 Pathogenicity

Pathogenic effects noted by Chin *et al.* (2003), Troeger *et al.* (2007) and Cotton *et al.* (2011) are associated with brush border of villi in the small intestine and disruption of tight junction of enterocytes in humans. The mechanism of attachment, both in term of the adhesion process, and the signalling pathways to initiate and activate attachment are yet to be confirmed. Trophozoites are able to attach to epithelial cells with ease. Only at the point when signalling occurs, either by reaching sufficient density or numbers; or by reaching receptor points that pathological changes occur, resulting in disruption of tight junctions (Scott *et al.*, 2002) and cell leakage (Buret *et al.*, 2002, Cotton *et al.*, 2011) ultimately resulting in clinical signs in the host.

More recently, researchers have demonstrated an impact upon cell apoptosis caused by different *G. duodenalis* isolates on enterocytes of the small intestine through disruption of tight junction at *zona occluda* (Chin *et al.*, 2003, Koh *et al.*, 2012). This is important as it may explain some of the differences between asymptomatic and symptomatic infections.

### 1.5 Prevalence of *Giardia duodenalis* in a global context

Prevalence rates of *G. duodenalis* infections in humans varies greatly from less than 1% to more than 50% depending on the location of the population and prevailing mode of *G. duodenalis* transmission. Reports indicate that 50% of all diarrhoeal deaths are due to acute watery diarrhoea, and 35% are due to persistent diarrhoea (WHO, 1992). Although the mortality rate from diarrheal disease is decreasing 500 million young children under 5 years old living in developing countries (excluding China) are still expected to suffer from 1,400 million episodes of diarrhoea per year. *G. duodenalis* poses significant health implications in densely populated countries of the world such
as in India with prevalence rates of 3-20% (Traub et al., 2002) or reported cases in China exceeding 28 million annual infections in humans (Feng and Xiao, 2011). Variable prevalence rates of 4.8 – 9.7 % have been reported between Chinese urban and rural communities (Dib et al., 2008). At least 16,747 infections occur per year in technologically advanced countries, such as the United States of America (USA) (Office of Surveillance, 2013) and incidents of infection appear to be seasonal (Esch and Petersen, 2013).

G. duodenalis is comprised of a genetically diverse group of isolates which infect humans and other mammals. These divergent Giardia spp. genotypes were first recognized in humans by molecular techniques and termed Assemblages A and B (Mayrhofer et al., 1995) and later confirmed by Cacciò et al. (2005). Phylogenetic groupings based upon regions of nucleotide homology using comparative DNA sequence data have characterized the differences between Giardia spp. isolates as genetic Assemblages (Monis et al., 1999, Lasek-Nesselquist et al., 2009, Thompson and Monis, 2012). The term ‘Assemblage’ is now widely accepted by parasitologists to describe the different genotypes according to phylogenetic analysis from multiple gene loci.

Eight different genotypic groupings of G. duodenalis (described in 1.3) have been characterized according to similarity of genetic characteristics, are known to infect different host species and designated as genetic Assemblages A – H (Monis et al., 2003, Caccio and Ryan, 2008) as shown in Table 1.1.

<table>
<thead>
<tr>
<th>G. duodenalis genotype</th>
<th>Host species</th>
<th>Proposed species nomenclature</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (human)</td>
<td>Giardia duodenalis</td>
<td></td>
</tr>
<tr>
<td>B (human)</td>
<td>Giardia enterica</td>
<td></td>
</tr>
<tr>
<td>C and D (canine)</td>
<td>Giardia canis</td>
<td></td>
</tr>
<tr>
<td>E (ungulates)</td>
<td>Giardia bovis</td>
<td></td>
</tr>
<tr>
<td>F (feline)</td>
<td>Giardia cati</td>
<td></td>
</tr>
<tr>
<td>G (rats)</td>
<td>Giardia simondi</td>
<td></td>
</tr>
<tr>
<td>H (marine seals)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Adapted from (Thompson and Monis, 2011)
Collection of evidence for a potential Australian marsupial assemblage is ongoing (Thompson et al., 2010). Reclassification of *G. duodenalis* as 6 different species, has been proposed to replace those currently designated as Assemblages A-G (Monis et al., 2008).

Published data has reported the presence of mixed *G. duodenalis* assemblages after laboratory testing identified more than one genetic group (Caccio and Ryan, 2008, Lebbad et al., 2011). The increasing evidence that more than one *Giardia* spp. genotype is able to infect mammalian species, either as one genotype, or as multiple or mixed infections confuses genetic characterization and there is the possibility of transfer of genetic material (Birky, 2005, Lasek-Nesselquist et al., 2009).

A review of molecular epidemiology of *Giardia* species examined global data from humans, wild and captive animals, including marine species, livestock, and domestic pets, providing further verification that giardiasis is a zoonotic disease (Feng and Xiao, 2011). The reviewers assert that some critical omissions remain in the research, including perspectives on the impacts of disease originating from zoonotic parasites. Moreover, that evidence for transmission dynamics requires rigorous longitudinal studies, and clear associations between the distribution of the particular *Giardia* species responsible for infection of humans and endemicity.

Prevalence data alone cannot extend the body of knowledge to adequately inform members of the public, health professionals and government institutions with the information necessary to appreciate the risks of such infections and prevent large-scale outbreaks and the spread of asymptomatic infections.

### 1.6 Infection routes

Only viable cysts are capable of survival in aquatic environments and enable infections to occur through direct person-to-person contact or contaminated water and food by the faecal-oral route. All these circumstances arise from host organisms excreting cysts into the environment. A specialized cell wall construction provides a stable arrangement to preserve cyst structure as inactive under cool, moist conditions until
ingestion and exposure to favourable conditions. Within another host organism ingested cysts develop into active trophozoites they are able to cause patent infections. The features of *Giardia*, which contribute to the successful invasion of a wide range of host organisms appears to depend on efficient transformation from motile trophozoite into a resistant impenetrable cyst.

Mycoplasma-like structures were first shown attached to the outer surface of *Giardia* cysts (Feely *et al.*, 1988) and trophozoites isolated from rats were discovered to be capable of ingesting bacteria (Sogayar and Gregorio, 1989). Intact *Giardia* cysts are ingested by other microorganisms as described for *Entamoeba* sp. (Logan *et al.*, 1980) and may also be parasitized themselves, confirmed for *Giardia* lamblia virus, a 36nm double stranded ribonucleic acid virus (Wang and Wang, 1986, De Jonckheere and Gordts, 1987).

1.7 Eukaryotic protozoan model organism

Whilst *Giardia* spp. infections are rarely life threatening this eukaryotic protozoan is an important organism to study in more detail because 1) It has unique and complex structural and metabolic features. Considered as a protist, *Giardia* spp. lack mitochondria, have a simplified endoplasmic reticulum and golgi apparatus and are binucleated. 2) It is unicellular with a simple lifecycle, easily cultured in-vitro and can be used as a model for similar parasitic organisms. 3) Unanswered questions remain concerning the indirect effects of subclinical, or asymptomatic, *Giardia* spp. infections and failure to thrive in the host species 4) The complexity of the host interactions and differences between *Giardia* spp. infections in various host species is undocumented. However, these unicellular parasitic organisms may have actually always been present and co-evolved in parallel with other organisms in the ecosystem and adapted as the environmental conditions changed thus leaving migrants to the region uniquely vulnerable to infection (Ricklefs and Bermingham, 2002).
1.8 Lifecycle of *G. duodenalis* in mammalian species

A recent review by (Luján and Svärd, 2011) brings together global expertise in *G. duodenalis* epidemiology and biology that details and discusses the value of *G. duodenalis* as a model organism. Detailed explanations of *G. duodenalis* life cycle and cellular metabolism have recently been reviewed (Dawson *et al.*, 2011, Jarroll *et al.*, 2011). A summary of the *G. duodenalis* lifecycle, as it is known to occur in human hosts, is presented in Figure 1.2.

1.8.1 Cellular structures

*G. duodenalis* has a simple, lifecycle, with two distinctive morphological forms the non-motile cyst and the motile, reproductive trophozoite that transition within the gastrointestinal tract of the host organism (Adam, 2001). Key features of unicellular *Giardia* spp. are binuclear cells and absence of mitochondria in this protozoan parasite. *Giardia* cysts are thin-walled impenetrable cysts capable of surviving in the environment for long periods, whilst remaining infective for up to 6 months (Deregnier *et al.*, 1989).

1.8.2 *Giardia* spp. cyst

Cysts are excreted in the faeces, sometimes hundreds of thousands (Wallis and M., 1986). These microscopic structures are specially adapted to be able to withstand a wide range of environmental conditions, subject to the limiting factor of water, as they are highly susceptible to desiccation (Van Herk *et al.*, 2004). Some of the distinguishing biological features are explored in more detail with respect to diagnostics in Chapter 3 and Chapter 5.

![Giardia spp. cyst](Figure 1.1 Giardia spp. cyst)
*Giardia* spp. cysts, clearly visible under light microscopy at X400 magnification have evident microtubule structure running the entire length of the cyst with paired nuclei to either side, as depicted in Figure 1.1. Median bodies, unique microtubule structures, are visible in the centre of the cyst. More detailed electron micrograph studies provide comprehensive descriptions of trophozoite and cyst ultrastructures (Erlandsen *et al.*, 1989, Benchimol and De Souza, 2011).

The non-motile *G. duodenalis* cysts are 8-14µm long and 6-10µm wide, with two or four nuclei, axostyle and crescent shape fragment of the ventral disc structure (Smith and Mank, 2011). Host ingestion of *Giardia* cysts occurs by the faecal-oral route. Binucleate or tetranucleate cysts excreted in faecal samples have the potential to excyst and produce four daughter trophozoites in a new host. Cysts are induced into excystation following exposure to low pH by gastric acid to form an intermediate excyzoite (Bernander *et al.*, 2001) and ultimately mature trophozoites in the proximal region of the small intestine Douglas *et al.* (1988). Encystation occurs due to conditions prevailing in the proximal small intestine, alkaline pH, in the presence of bile secretions or cholesterol depletion and (Reiner, 1995, Lauwaet *et al.*, 2007) or in the presence of carbonic acid (Feely *et al.*, 1991). The process of encystation is a key survival mechanism of the parasite and the specializations enable the dissemination of excreted cysts across a range of environmental conditions.

### 1.8.3 Trophozoite (vegetative stage)

Excystation occurs in the duodenal and jejunal portions of the small intestine. In general, trophozoites are seldom observed exterior to the body cavity, and if present in fresh faecal samples disintegrate rapidly. The free form, vegetative, anaerobic, trophozoite is binucleate and pyriform typically measuring 12-15µm in body length, 5-9µm wide and 2-4µm thick (Benchimol and De Souza, 2011). The trophozoite has a distinctive ventral disc and eight flagella (Harris *et al.*, 2000). A complex internal organization comprises four cytoskeletal components; ventral disc; median body; kinetosomes; flagella; and fibrils.
1.8.4 *In vitro* culture

The value of *in vitro* experiments has been and continues to be important for understanding species differences in trophozoite localization, and gastrointestinal conditions conducive to excystation or encystation. Gillin and Reiner (1982) have demonstrated the conditions supporting optimal *in vitro* growth of *G. duodenalis* (Portland strain) to include thiol reducing agents, neutral pH, 35.5 - 37°C temperature in the presence of serum globulins, and 200-300 mOsmol/kg ionic composition. In addition, some physiochemical features of the gastrointestinal tract such as polyanionic mucins may influence survival and pathogenicity of *G. duodenalis* (Hansen and Fletcher, 2008).

Despite advances using *in vitro* culture experimentation (Davids *et al.*, 2011) the majority of the information has been limited to only a few human infective strains of *Giardia* spp. There is a need to conduct comparative research using genetic variants isolated from other host species. Whilst the literature also poses questions about the long-term viability of *in vitro* *Giardia* spp. cultures and clonal lines and the encystation and excystation methods used (Lauwaet *et al.*, 2007, Ratner *et al.*, 2008, Solarczyk, 2009). The suitability of these methods may not adequately reflect the *in vivo* conditions and interpretation of results must be carefully considered.

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Chapter 1 General Introduction

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1.8.5 Life cycle

Different morphological forms exist as trophozoites (enteric) and cysts (environmental). This aspect of the lifecycle becomes important with respect to distinct structural features, which are discussed further in the following section and in Chapter 5.

Additional intermediate morphological forms reported to occur during excystation and encystation lifecycle stages include excyzoite (Bernander *et al.*, 2001) and encyzoite (Reiner, 2008).

1.8.6 *Giardia* spp. life cycle transitions

Table 1.2 Summary of key features in *Giardia* life cycle transitions

<table>
<thead>
<tr>
<th>Morphological form</th>
<th>Physiological actions / functions</th>
<th>Unknown mechanisms</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYST</td>
<td>Entry into digestive tract</td>
<td>One genotype or more than one present?</td>
<td>(Lauwaet <em>et al</em>., 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Signalling to induce excystation.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Excystation</td>
<td>What proportion excyst?</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control point mediation</td>
<td></td>
</tr>
<tr>
<td>TROPHOZOITE</td>
<td>Replication</td>
<td>Signalling to induce replication</td>
<td>(Sagolla <em>et al</em>., 2006)</td>
</tr>
<tr>
<td></td>
<td>Mitosis and Cytokinesis</td>
<td></td>
<td>(Bernander <em>et al</em>., 2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Reiner, 2008)</td>
</tr>
</tbody>
</table>

Figure 1.2 Life cycle of *G. duodenalis*
Table 1.2 Continued from Page 35

<table>
<thead>
<tr>
<th>Morphological form</th>
<th>Physiological actions / functions</th>
<th>Unknown mechanisms</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adhesive disk</td>
<td>Attachment to epithelium of duodenum and jejunum</td>
<td>What is the specific location of attachment? Position in the digestive tract? At apical surface (villar epithelium)? Or at mucosal base of crypts? Does localization vary between host species or <em>G. duodenalis</em> genotype?</td>
<td>(Erlandsen and Feely, 1984) (Gillin and Reiner, 1982) (Sousa et al., 2011)</td>
</tr>
<tr>
<td>Unknown?</td>
<td>Do trophozoites encyst because they did not attach? Or, do attached trophozoites detach and then proceed to encystation? What signals trophozoites to detach? Host or parasite control point?</td>
<td>(Dawson, 2011a) (Carranza and Lujan, 2010) (Hansen and Fletcher, 2008)</td>
<td></td>
</tr>
<tr>
<td>Host mucosal secretions</td>
<td>Encystation</td>
<td>What signals encystation? What location in the digestive tract? Is this a host specific variable?</td>
<td>(Arguello-Garcia et al., 2009)</td>
</tr>
<tr>
<td>Cyst wall construction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYST</td>
<td>Impervious</td>
<td>Organelles</td>
<td></td>
</tr>
<tr>
<td>External environment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In vitro culture Assemblages A and B only</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.8.7 Signaling pathways

*Giardia* spp. signalling pathways as depicted in Table 1.2 involve a range of molecular types, including phosphatase and kinase enzymes (Lauwaet *et al.*, 2007, Davids *et al.*, 2008). There are specialized ESV and intraflagellar (IFT) protein trafficking mechanisms (Hehl and Marti, 2004, Dawson and House 2010) and trophozoite responses to host lipid components of steroid compounds, such as cholesterol (Yichoy
et al., 2011). In addition, research has demonstrated similar functional proteins in *G. duodenalis* to those seen in plants and algae for *Giardia* cell wall protein regulation Ankarklev et al. (2010) and DNA binding, (Sun et al., 2006) together with calcium as a key regulator in synaptic vesicle recycling and fusion pore formation (Südhof, 2004). Within the context of the microflora of the gastro-intestinal tract, inter species signaling or quorum sensing may exist between *Giardia* spp. and other parasites, or with selected bacterial species. These types of factors, which may prove critical to establishment of active infection, are important to consider in relation to the interactions within the mucosal epithelium, likely biofilm composition, and *Giardia* secretory products.

1.8.8 Cell cycle regulation and synchronisation

The regulation of *Giardia* spp. cell cycle is only partially understood, and various factors make this a challenging and unique organism to understand. *Giardia* spp. is considered a protist possessing features of both prokaryotic and eukaryotic organisms. The results from *in vitro* culture studies shown in Table 1.2 using a DNA replication inhibitor, aphidicolin, has demonstrated that *Giardia* cell cycle regulation of genes, such as histone, occurs at the transcriptional level (Reiner et al., 2008). Reiner’s work has confirmed that DNA replication is not dependent on cell growth and that a control point exists at G2/M phase of DNA replication, the absence of double stranded DNA effectively impedes cell division.

Flow cytometry studies of cell synchronisation suggest that *G. duodenalis* undergoes three life cycle stages, regulated by transcriptional processes, over a six hour time period (Troell and Svärd, 2011). Post-translational modification has been demonstrated to occur in cyst wall and trophozoites by mono- and poly-ubiquitinylation modifications at lysine residues (Nino and Wasserman, 2010).
1.8.9 *G. duodenalis* organelles and cytoskeletal structure

The following section describes in more detail the cellular and subcellular organelles unique to *G. duodenalis* that have been investigated using *in vitro* culture techniques and worthy of closer scrutiny both from diagnostic and therapeutic perspectives (Aguilar-Diaz *et al.*, 2011). A comprehensive review published by Luján and Svärd (2011) contains more details.

1.8.10 Organelles and molecular mechanisms

Four key elements occur in the encystation process in the lower portion of the digestive tract; detection and triggering expression of specific genes; synthesis and transport of the cell wall proteins (CWPs) into ESVs (encystation-specific vesicles); (Reiner *et al.*, 1990) assembly of the extracellular cyst wall; and nuclear division and DNA replication (Hehl and Marti, 2004). The *G. duodenalis* cyst cell wall encloses cell contents comprising nuclei and other organelles. On the cyst surface variant specific proteins (VSP) possess unique antigenic properties for evasion of the host immune system (Nash *et al.*, 1983). The cyst functions as the principle infective form of *G. duodenalis* and a later section comments on literature describing *in vivo* studies of artificial *Giardia* infections induced in experimental animals using *Giardia* spp. cysts.


Electron microscopy work demonstrated a fibrillar extracellular cyst matrix contained extensive protein–protein interactions (Erlandsen *et al.*, 1989) and since confirmed to be proline and glycine amino acids within leucine rich repeats, the critical elements for
retaining cyst wall structure (Lujan et al., 1995). Molecular carbohydrate interactions of the cyst wall coupled with protein structures contribute to providing a highly insoluble cyst wall structure (Gerwig et al., 2002).

1.8.11 Cyst wall proteins

Four cyst wall proteins have been identified. One has been identified as a lectin (Chatterjee et al., 2010) and is similar to other cell wall proteins (Gillin et al., 1987, Luján et al., 1997, Sun et al., 2003) with a structural conformation that contains leucine rich repeats a coding motif common in protozoans, (Lujan et al., 1995, Kedzierski et al., 2004b). The fourth, is a cysteine rich non-variant specific protein HCNCp (Davids et al., 2006). The cell wall matrix of *G. duodenalis* consists of an outer protofilamentous layer and an inner two-layered membrane but the final fibrillar extracellular matrix observed in electron microscopy points to a highly organized structure involving extensive protein–protein interactions (Hehl et al., 2000). Golgi type functions in ESV have been demonstrated by the use of epitopes and monoclonal antibodies where proteolytic processing of CWP 2 develops into two parallel protein sorting and secretion routes entailing alternate reaction kinetics (Konrad et al., 2010). Subsequent protein aggregation is regulated by biophysical conditions of acidic pH and calcium ions, and the outcome from an elaborate study depicts the molecular integration steps for construction of cyst cell wall, shown in Figure 1.3 (Chatterjee et al., 2010). The steps involved in cyst cell wall manufacture are critical for conferring cell wall integrity and it’s impermeable membrane. Disruption of the process would result in thicker membrane but lacking its impervious properties (Chatterjee et al., 2010).
1.8.12 Encystation specific vesicles (ESV)

Encystation specific vesicles (ESV) identified by (Reiner et al., 1989) are believed to have a specific role in synthesis and transportation of cyst wall proteins ready for assembly and construction of the cyst wall (Hehl et al., 2000). Visible protrusions have been documented on the outer surface of trophozoites at commencement of the encystation stage during formation of ESV (Bittencourt-Silvestre et al., 2010). These encystation specific vesicles package and transport proteins from the endoplasmic reticulum to sites for cell well construction (Marti and Hehl, 2003). Intracellular control of vesico-tubular transport during encystation occurs by cytosolic recognition, with Rab protein and cytoskeletal actin guiding newly manufactured ESV to location for synthesis of cyst cell walls (Castillo-Romero et al., 2010).

The process of encystation is a key survival mechanism of the parasite and the organism’s specialisation enable the dissemination of cysts across a range of environmental conditions. Low levels of cysts can induce infections, and molecular genetic technologies are able to determine Giardia genotype from a single cyst (Ankarklev et al., 2012). G. duodenalis appears to have a minimal endomembrane system, with true early/late endosomes lacking, and a fused endosomal compartment (peripheral vacuole). Principle features of the endocytic system - are (i) multiple routes from the surface, (ii) several functionally differentiated endosomal structures including
the early endosome, the recycling endosome and late endosomes, (iii) integration with a degradation pathway, variously termed the lysosome or vacuole, and (iv) close integration with the Golgi complex and the trans-Golgi network and consequently exocytic traffic (Field et al., 2007, Rivero et al., 2012). The control of the endocytic system is partially mediated by the Rab proteins, and integrated with the protein and lipid kinase system (Rivero et al., 2013). There is evidence that endosomal trafficking indicates a specific role for phosphatidylinositol (PI) lipid kinases (Field et al., 2007).

### 1.8.13 Excystation

A *Giardia* specific cysteine protease, cathepsin B initiates excystation (Ward et al., 1997). *In vitro* studies have successfully demonstrated excystation of *G. duodenalis* cysts, although varying results have been reported by different research groups, and may also be dependent upon the host species of origin, and the maturity of the cysts (Lauwaet et al., 2007). Excystation is dependent upon dephosphorylation of cyst wall proteins, Giardial acid phosphatase is one of the proteins responsible for this and localizes to the soluble transmembrane fraction (Slavin et al., 2002). Whilst reference to host proteases is proposed by (Samuelson and Robbins, 2011) the role of these proteins is not yet fully understood. Aguilar-Díaz et al. (2011) compared the cyst structure of *G. duodenalis* to that of the free-living amoeba *Acanthamoeba castellani* suggesting similarities between the architecture and packaging of cyst wall structures and the existence of a double envelope of ectocyst and endocyst structure with an intervening space. The authors suggest that between the envelopes, at the point of contact, an ostiol exists from which an operculum can be observed for exit of binucleated trophozoite supporting similar observations made for encysting *G. duodenalis* trophozoites (Hirukawa et al., 1998, Midlej and Benchimol, 2009, Fouque et al., 2012).

### 1.8.14 Trophozoite

The free form vegetative anaerobic trophozoite has a binucleate, pyriform shape, broad anterior and narrow posterior typically measuring 12-18μm in body length, 6-8μm wide
and 2-4μm in thickness with eight flagella arranged in four lophotrichous pairs that are attached to basal bodies at anterior and posterior lateral positions at ventral and caudal positions, with all flagella arranged in the caudal direction (Nohynkova et al., 2006). Two nuclei are situated bilaterally to the midline in the broad anterior. The cytoplasm is studded with glycogen granules and peripheral vacuoles, free ribosomes and vesicles are present (Gillin et al., 1996). Mitochondria are absent, although mitosomes, putative golgi complexes and lysosomal vacuoles, all believed to form part of a transport mechanism, are present (Abodeely et al., 2009). Structural bodies are integral to differentiation process and flagella synthesis occurs at the same time as degradation (Dawson and House, 2010). The motile trophozoites migrate along the proximal duodenum as free trophozoites or attach and colonize the duodenal epithelial layer. Complex interactions control the Giardia spp. cell cycle and life cycle and a ubiquitin transfer process has been proposed to control glucosamine phosphate production during cyst cell wall formation (Lopez et al., 2002). Protein phosphatase 2A (PP2A) locates to the cell wall and is associated with loss of trophozoite motility and adhesion during encystation process (Lauwaet et al., 2007). In other eukaryotic organisms phosphatase enzymes reverse regulatory control exerted by protein kinases. (Porter and Sale, 2000).

Mitosomes have been shown to play an important role in the Fe-S metallo protein complexes (Tovar et al., 2003) and a proteomic analysis of the mitosomal fraction of Giardia trophozoites supports a biosynthetic role in assembly of the iron-sulphur cluster assembly. The study identified 139 proteins, 44 of which could be localized by combining iTraQ mass spectrometry data with bioinformatic analysis. Thus, confirmation of redox proteins together with chaperone proteins and protein transport functions by ToM40 indicates the possibility of ion and metabolite exchange across the outer membrane. However, TIM proteins (inner membrane transport) were absent (Dolezal et al., 2005, Jedelsky et al., 2011). Peripheral vacuoles have lysosomal and endosomal functions Lanfredi-Rangel (2003) and Touz et al. (2002) have demonstrated
that *Giardia* possess a uniquely divergent transmembrane cysteine protease displaying homology to other eukaryotic cathepsins, which cleaves cyst wall protein 2 (CWP 2) disrupting cyst wall synthesis.

A lectin-affinity chromatography approach combined with PAGE mass spectrometry to investigate the glycoproteins of *Giardia* trophozoites and cysts demonstrated that proteins with glycine rich repeats were more common in cysts and contained more unique membrane proteins in contrast to trophozoites. More endoplasmic reticulum (ER) folding associated proteins, lysosomal and secretory proteins with leucine rich repeats were present in trophozoites (Ratner *et al.*, 2008). These differences in protein trafficking processes are important for understanding how-host-parasite interactions occur and molecular mechanisms for evasion of the host immune system.

### 1.8.15 Flagella

*G. duodenalis* trophozoites are tetrakonts, with four pairs of flagella, these are positioned as ventral; anterior; caudal and posteriolateral pairs. Flagella are composed of axonemes and cross linking coiled coil protein structures and similarity to myosin type structures were described in early studies (Holberton *et al.*, 1988). The role of flagella are primarily for motility, fluidity of movement, both in terms of speed and specialization to interact with host digestive system and external environment, and survival within aqueous conditions. Paraflagellar rods are closely associated with flagellar structures and have been described for ventral flagella structure (Benchimol and De Souza, 2011) and similar dense rod structures linked to anterior and posterior flagella. The anterior axoneme flagellar structure has distinctive striated fibres or ‘marginal plate’ whereas caudal flagella have distinctive microtubule structures described as ‘funis’ (Elmendorf *et al.*, 2003). Since the original estimates of at least 20 cytoskeletal proteins Holberton and Ward (1981), Crossley and Holberton (1983) the number of confirmed identities has increased to include 32 ventral disk associated proteins (Hagen *et al.*, 2011) and 278 protein kinases (Manning *et al.*, 2011). More recently, results from electron microscopy spatial 3D whole trophozoite models have
endorsed the work performed 40 years earlier by Crossley and Holberton (1983). This preliminary 3D imaging research is expected to be of importance for establishing localized functions for specific proteins and proteinaceous complexes (Schwartz et al., 2012).

1.8.16 Cell signaling – flagellar mechanisms

Flagella are also likely to have a key biological role for signal mediation either between trophozoite and host, or between individual trophozoites, in addition to transition to the cyst form. Signaling may occur through protein trafficking by intraflagellar transport proteins known as ‘trains’ (Engel et al., 2009). Sloboda (2009a) used video-enhanced differential interference contrast (DIC) light microscopy to describe how intraflagellar transport complexes fall into two distinct categories, according to length and morphology and protein assembly.

The role of flagella may be key in establishing Giardia infections and signaling responses, be they immune or pathophysiological. Importantly, flagellar development occurs over 3 cell cycles with eight flagella of trophozoites having chronologically different development stages, until reaching maturation at completion of the fourth cell cycle (Dawson et al., 2011). The caudal flagella signalling of G. duodenalis has been associated with PP2A and nek kinase, although the precise pathway remains to be determined, although likely to be chemotactic processes (Lauwaet et al., 2007). The unique organelle components of Giardia spp. basal bodies may provide an option for two different mechanisms of flagellar protein trafficking, for morphogenesis and maturation (Briggs et al., 2004).

1.8.17 Molecular components of intraflagellar transport

The role of deflagellation and regeneration has been investigated in green algae cells of Chlamydomonas reinhardtii, a bikont (flagellated organisms with 2 flagella). Cells were exposed to an acidic pH shock, which induces flagellar abscission (Dentler, 2005, Pazour et al., 2005, Engel et al., 2009). The key structural components for IFT include tubulin dimers, membrane proteins, and pre-assembled dynein arms and radial spokes.
Engel et al. (2009) used Green Fluorescent Protein (GFP) to tag IFT proteins and measured IFT rates as a function of flagellar length. Cytoplasmic factors limit IFT formation and a significant role has been proposed implicated for flagella in the interaction with extracellular matrix proteins. Engel (2011) reports that the size of IFT proteins is inversely proportional to flagellar length. This technique has been used in recent experiments on G. duodenalis to confirm that trophozoite attachment to epithelium of the small intestine is not dependent upon flagellar motility (House et al., 2011).

Considering that the flagellar proteome of green algae contains nearly one hundred proteins that have conserved homologues in humans but whose homologues have not yet been characterized in any organism, this highlights the need for further research on these type of proteins (Pazour et al., 2005). Ninety per cent of the genes examined were induced by deflagellation, suggesting primary functions in cilia or flagella although only ten percent of them have been identified (Ostrowski et al., 2002). G. duodenalis is a non-ciliated organism, with a unique array of eight flagella, although differences in associated cell structures such as the marginal groove or striated fibres have been documented for different Giardia species (Friend, 1966, Sogayar and Gregorio, 1989). Flagellar length has been noted by Binz (1996) as one significant morphological feature differentiating between Giardia spp. isolated from various host and geographical locations. Further details of recent discoveries concerning the structural components and specialized functions of Giardia spp. flagellar proteins have been reviewed (Benchimol and De Souza, 2011). O-linked glycosylation of proteins is a unique feature of Giardia spp. (Banerjee et al., 2009). A definitive explanation of the interactions between cytoplasmic or organelle proteins and structural elements of the microtubules, such as those described for acid phosphatase and adaptin proteins remain under investigation (Rivero et al., 2012). The cell differentiation and encystation processes are dependent upon rearrangement of microtubule structures, but it is unclear how the genetic information directs protein expression and trafficking to support new cell growth.
or cyst formation.

These mechanisms are of key importance in establishing *Giardia* infections through successful attachment to the epithelial cell wall of the small intestine. Hence, it is important to understand the molecular features conferring the structural and infective functions of cysts and trophozoites transitioning between the two life cycle stages.

### 1.9 Detection of *Giardia* spp. infections

Detection and characterization of *G. duodenalis* has been reported from all continents of the world (Lu *et al.*, 1998, Leonhard *et al.*, 2007, Caccio and Ryan, 2008). Early research reports the detection of *Giardia* using light microscopy and whilst prevalence rates and host species were identified, development of DNA sequencing and genetic characterization did not become readily available until the 1990’s (Ford, 2005). Attempts to understand the differences between *Giardia* spp. infections in humans and other host species commenced using isoenzyme electrophoresis (Homan *et al.*, 1992, Mayrhofer *et al.*, 1995). Genetic characterization of *Giardia* spp. isolates using DNA sequencing methods (Hopkins *et al.*, 1997, Monis *et al.*, 2009) have been developed as research tools and routine diagnostic methods remain principally faecal flotation and light microscopy. Diagnosis is not straightforward and an entire chapter (Chapter 2) of this thesis is devoted to investigating this further.

Currently diagnostic tests for *Giardia* spp. are not able to alert medical practitioners or veterinarians to potentially zoonotic strains. Nor do the tests define or guide industry acceptance of what constitutes normal or acceptable levels of infection and what is considered a high prevalence rate within a population or severe infection levels in individuals, even though they may be asymptomatic. Hosts frequently experience recurrent bouts of infection and intermittent shedding of cysts poses challenges for diagnosis.

Gastrointestinal tract infections are caused by a variety of pathogens; the clinical practitioner relies on patient history and clinical diagnostics to eliminate infections of bacterial or viral origin. In addition, the use of correct sampling and diagnostic methods
for investigation of intestinal protozoa, such as *Giardia*, *Blastocystis* sp.; *Entamoeba* sp.; *Diaentomoeba fragilis* in the differential diagnosis of IBS are important (Savioli et al., 2006, Stark et al., 2007). The routine use of light microscopy for diagnosis, based on the presence or absence of intact cysts or trophozoites in faecal samples is gradually being replaced with molecular detection methods for copro-antigenic protein. Unlike genetic characterization using faecal DNA fragments of *Giardia*, detection of *Giardia* proteins reveal phenotypic characteristics.

### 1.10 Public health

In a global context, the neglected diseases initiative provides an important focus for governments in the 21st century to better understand zoonoses (WHO et al., 2006). There is much interest in biological differences between mammalian species to better understand parasitic ecosystems and aspects of human health. (Pedersen and Fenton, 2006).

### 1.11 *Giardia canis* – a literature review

The earliest reports of *Giardia* spp. infections in dogs can be traced back 90 years to Pennsylvania in the United States of America (Hegner, 1922). A comparative study conducted by Hegner (1922) demonstrated differences between three different *Giardia* spp. isolates from rabbit, human and canine hosts, confirming an earlier study by Bensen in 1908, that three isolates of *Giardia* spp. were discernible by morphological features (Grant and Woo, 1977). At this time, trophozoites from rabbit hosts were named *Giardia duodenalis*, those from humans *Giardia lamblia* and those from puppies were *Giardia canis*. Morphological differences in size and shape were considered as constant heritable characteristics after examining more than fifty specimens from each host. *G. canis* trophozoites and cysts were both slightly larger than those for *G. lamblia* specimens from human hosts.

Differences in the morphologically distinct features of trophozoites found to infect dogs, have been mentioned by Barlough (1979) and Binz (1996). Given the recent
publication of the *Giardia* genome, (Hamnes *et al.*, 2007, Morrison *et al.*, 2007, Aurrecoechea *et al.*, 2009, Jerlstrom-Hultqvist *et al.*, 2010) it is timely to advance the body of knowledge to include phenotypic descriptors exclusive to one host species, other than human.

### 1.11.1 Prevalence and epidemiology

Tracing the origins of *Giardia* spp. infection is important in the understanding the spread of infection and potential management strategies; details of studies conducted in human hosts are outlined in Section 1.5.

### 1.11.2 Molecular epidemiology of canine *Giardia* spp. infections

The first molecular identification of *Giardia* spp. assemblages C and D was from dogs living in remote indigenous Australian communities (Monis *et al.*, 1998) and now consistently referred to as host specific genotypes (Thompson *et al.*, 2000, Eligio-Garcia *et al.*, 2005, Wielinga *et al.*, 2011). Since 1998, most molecular epidemiological studies indicate that dogs are infected with host specific *Giardia* spp., although the ability to detect potentially zoonotic genotypes is becoming more common, both in dogs and in other wildlife species (Carmena *et al.*, 2012). Subsequent studies have demonstrated that the majority of *Giardia* spp. infected dogs harbor *Giardia* spp. Assemblages C and D, confirmed by genotyping at 18srDNA and beta giardin gene loci (Palmer *et al.*, 2008, Upjohn *et al.*, 2010, Uehlinger *et al.*, 2013).

This concept of host specific *Giardia* spp. corroborates the earlier identification and naming of *G. canis*. The predominance of *Giardia* spp. genotypic assemblages C and D infections in canines is associated with both symptomatic and asymptomatic cases (Barutzki and Schaper, 2003, Barutzki *et al.*, 2007, Claerebout *et al.*, 2009, Upjohn *et al.*, 2010, Uehlinger *et al.*, 2013). The other *Giardia* spp. genetic isolates commonly isolated from dogs are assemblages A and B, both of which are known to infect humans (Van Der Giessen *et al.*, 2006). Research in different laboratories around the world, has demonstrated the existence of four different genetic assemblages of *Giardia*
duodenalis infecting dogs (Homan et al., 1992, Mayrhofer et al., 1995, Monis et al., 2009). However, to complicate matters further there are increasing reports of mixed infections in other species and the possibility of genetic changes to the organism, over time (O'Handley et al., 2000, Fontanarrosa et al., 2006, Ankarklev et al., 2012).

The use of genetic tools has both strengths and weaknesses and increasingly researchers are reporting the need for improved genetic characterization methods, such as routine use of Multi Locus Genotyping (MLG) in preference to single gene loci (Caccio et al., 2008, Wielinga et al., 2011, Jimenez-Cardoso et al., 2012). On occasions, amplification of DNA from Giardia positive faecal samples misses nearly 30% of samples containing Giardia cysts. This is problematic because the molecular techniques of PCR and sequencing are unable to verify the presence of mixed assemblages even when positive results have been confirmed by microscopy (Inpankaew et al., 2007, Traub et al., 2009, Covacin et al., 2011).

1.11.3 Prevalence and geographical distribution of canine Giardia spp. infections

This section summarizes the prevalence of Giardia spp. infections in canines whilst discrete factors of importance are addressed in more detail in section 1.2.10 for clinical signs of infection, in section 1.2.21 for management and husbandry and the implication of concurrent parasite infections is introduced briefly in section 1.2.23 and continued in chapter 3. The literature reviewed in this section includes reports from all continents based upon data collected or sourced from a variety of grouped, roaming and individually housed dogs and puppies from both small local studies to large cross-border surveys. The publications reviewed used many different diagnostic methods: a range of dog population types, variation in sampling, transportation and storage procedures, and different genetic tools and genetic analysis alignment methods. Some of these inconsistencies are also noted by (Uehlinger et al., 2013) as potential error factors for comparison of canine intestinal parasite prevalence rates. The geographic
and demographic extent of the literature reviewed covered natural pristine wild environments/landscapes and bushland to urban slums.

Reaching conclusions concerning the prevalence rates of _Giardia_ spp. infections in canines presents challenges for researchers and clinicians as the figures reported are dependent upon the sensitivity and specificity of different diagnostic tests used (Upjohn _et al._, 2010), and their accuracy in determining the apparent or true prevalence rates (Geurden _et al._, 2008). Different criteria and approaches used in the research studies hinder an accurate assessment of the prevalence and epidemiology of _Giardia_ spp. infections. Nevertheless, the material reviewed here provides an opportunity to examine infections rates in terms of chronology and trends in detection of _Giardia_ spp. infection rates as well as a critical analysis of the diversity of research approaches required to resolve questions concerning the impact of _Giardia_ spp. infections in dogs.

Whilst many studies include samples from humans and other animals many are reported as single species (dog only) studies making this of importance to veterinarians, companion animal industry, breeding facilities and dog welfare agencies and working dog and pet owners. An indication of longitudinal variation in parasite prevalence rates, in published reports from Europe Australia and Asia, suggest that overall parasite burdens have reduced over time for _Giardia_ spp. although an increasing rate for detection of other protozoan infections (Swan and Thompson, 1986, Bugg _et al._, 1999, Itoh _et al._, 2009 Solarczyk, 2009).

Seven main factors are indicative of the intricacies of determining prevalence rates of _Giardia_ spp. in canines and other species. (1) Consistently higher prevalence rates were reported in studies using copro-antigen tests for diagnosis, rather than microscopic detection of _Giardia_ spp. cysts. (Carlin _et al._, 2006, Epe _et al._, 2010, Olson _et al._, 2010, Upjohn _et al._, 2010, Mircean _et al._, 2012). (2) The highest prevalence rates were reported for puppies and juvenile dogs up to 12 months of age, at 16%, 26%, and 46%, 78% (Batchelor _et al._, 2008, Epe _et al._, 2010, Itoh _et al._, 2011, Uehlinger _et al._, 2013) respectively. (3) Intensive housing situations repeatedly reported higher
prevalence rates with frequent detection of mixed genotype infections (Swan and Thompson, 1986, Traub et al., 2004, Huber et al., 2005, Claerebout et al., 2009, Uehlinger et al., 2013). (4) Zoonotic Infections were reported more frequently in densely populated settlements such as Assemblage A in Bangkok, (Traub et al., 2009) and Assemblages A and B, in USA (Covacin et al., 2011) although consistency in the methods of detection may resolve the actual published prevalence rates. (5) Difficulties with reliable molecular detection of Assemblage B isolates from faecal samples from dogs, and humans was common (Traub et al., 2003, Souza, 2007). These difficulties were frequently reported, despite using Multi Locus Genotyping (MLG) (Lalle et al., 2005, Wielinga and Thompson, 2007, Volotao et al., 2007, Caccio et al., 2008, Huey et al., 2013). These problems have been attributed to two key factors, i) multiple infections and ii) allelic sequence heterogeneity (ASH) (Caccio and Ryan, 2008). (6) In situations where both humans and dogs were sampled, the Giardia spp. prevalence in dogs was consistently higher than in humans (Capelli et al., 2003, Traub et al., 2004, Itagaki et al., 2005, Solarczyk, 2009, Traub et al., 2009). (7) Overall, widespread parasitism in dogs occurs in rural areas, with an extensive range of infecting species (Traub et al., 2004, Palmer et al., 2008, Himsworth et al., 2010, Mircean et al., 2012).

1.12 Zoonoses

The literature reports that Giardia spp. occurs in most Canidae both domestic and wildlife species and that several different subtypes are present (Trout et al., 2006, Thompson et al., 2009). The Giardia spp. genotypes confirmed in infected Canidae are assemblages A, B, C and D, as outlined in Section 1.5.

Microscopically it is difficult to differentiate between the various infective forms of G. duodenalis. However, at a molecular level, genotyping work has demonstrated that different subtypes of Giardia spp. exist. The different sub-types correspond with the host species and have been described by Monis et al. (2009) and Thompson and Monis (2011).
Giardia spp. infects domesticated livestock, household pets and, both captive wildlife and those in natural environments. The rapid and high rates of transmission observed between individuals in a community and the fact that individual hosts respond differently to parasitic invasion, with asymptomatic hosts, has contributed to a proposal for zoonotic transfer of Giardia spp. from dogs (Thompson, 2000, Traub et al., 2004).

Multiple reports exist proposing the zoonotic potential G. duodenalis, although this remains to be proven (Faubert, 1988, Bemrick and Erlandsen, 1988, Monis and Thompson, 2003, Ryan and Caccio, 2013).

Determining the mode of transmission between dogs (intra species) of the different Giardia spp. subtypes has not been established. In addition, if the Giardia spp. subtypes found in dogs are capable of infecting humans (inter species transmission) this knowledge will be of medical and veterinary importance, because of the increased risk of exposure and infections in humans. The potential epidemic spread of gastrointestinal disease would have major implications for public health policy, animal management and, prophylactic measures and treatment for both humans and dogs.

The absence of consistency in research tools or approaches impedes the direct analysis and comparison research results reporting prevalence rates and genetic characterization of Giardia spp. in dogs.

1.13 Transmission

Transmission cycles of G. duodenalis are still not fully understood, the fact remains that whilst host specific genotypes exist many non-human species are infected with potentially zoonotic genotypes causing public health concerns (Buret et al., 1990, Abe et al., 2005, Ryan and Caccio, 2013). The potentially zoonotic Giardia assemblages A and B that commonly infect humans have been implicated in cross species transmission (Thompson, 2000, Thompson et al., 2008, Palmer et al., 2008, Ballweber et al., 2010). This suggestion for zoonotic transmission is further supported by data from multi-locus genotyping Giardia spp. infected canine faecal samples with 79% Assemblage A Traub et al. (2009) 69% Assemblages A and B Covacin et al. (2011)
and 100% Assemblage A (Marangi et al., 2009, Jiménez-Cardoso et al., 2012). This data when considered in the context of western communities which permit dogs to lick human faces and share sleeping space does pose serious implications for animal management and the spread of infectious diseases (Westgarth et al., 2007).

One biological interpretation proposed by Faubert (1988), is that Giardia spp. is zooanthroponotic. This view supported by (Snel et al., 2009) proposes that a similar scenario exists for Giardia spp. as previously described for Cryptosporidium sp. transmission where there is involvement of two transmission cycles, zoonotic by one species, and anthroponotic transmission by a different species, (Morgan et al., 1998). Other studies in wildlife species have also suggested that anthroponotic transmission of Giardia spp. can occur (Teichroeb et al., 2009, Johnston et al., 2010). There is a good indication that the sylvatic cycle is an important facet of Giardia spp. survival in a diverse range of environmental conditions, with the capacity to shed infective cysts in excrement entering water courses destined for human and domestic animal consumption (Thompson et al., 2009, Ash et al., 2010, Johnston et al., 2010, Thompson et al., 2010). The non-human host species include canines both wild and domestic animals (Beck et al., 2011, Levecke et al., 2011).

Transmission mechanisms have been investigated using cross species infection and association with clinical signs of infection. Gerbils have been shown to be susceptible to the Giardia spp. isolates that infect humans, in addition to those infecting beavers, guinea pigs, dogs, pronghorn, rats, and sheep. Results from references studies examining the action of Giardia spp. in gerbils carried out by Moss et al. (1990), Moss et al. (1991), Mohammed and Faubert (1995), and Rivero et al. (2010) have been applied to other species including humans. The infective dose used for the successful infection of gerbils from in vitro cultures of G. duodenalis did not affect numbers of cysts excreted (Amorim et al., 2010) discounting the suggestion of increased cyst dissemination. Belosevic and Faubert (1987) demonstrated that sera from mice could immobilize and lyse trophozoites, although it remains unknown exactly what
component of sera produces this type of lethal defence mechanism. Davies and Hibler (1979) recommended the need for cross species transmission studies following the ‘beaver fever’ incident that contaminated waterways in North America. A gap remains in the knowledge of *Giardia* spp. biology as this study indicated that not all mammals were capable of being artificially infected with *Giardia* spp. of beaver origin, but dogs and humans were, and conversely a number of animals were susceptible to artificial infection by *Giardia* spp. of human origin. The critical issue concerning the extensive studies conducted in mice and gerbils is that some findings are not directly transferable to human physiology.

There is the possibility that the wildlife infective pool is not confined solely to *Giardia* spp. in beavers, that it could also include herbivore or rodent species in the cycle (Mills and Childs, 1998, Fernandez-Alvarez *et al.*, 2013). In addition, the landmark study by Davies and Hibler (1979) also includes comments on the location of a dog kennels close to the watercourse and defecation by humans directly into the waterways. This suggests that *Giardia* spp. infections in beavers may have originated from non-wildlife sources.

The general view is that *Giardia* spp. has a simple direct lifecycle, although mechanical transmission of cysts to dogs has been discussed by (Barlough, 1979) proposing that flies or cockroaches serve as potential mechanical vectors. This opinion is supported by additional evidence for transmission of *Giardia* spp. cysts by flies (Graczyk *et al.*, 2003, Szostakowska *et al.*, 2004) even though the potential for establishing active infection would still requires ingestion of cysts in sufficient numbers equating to an infective dose (Rendtorff, 1954). Physical transfer on material objects, such as housing construction and bedding may also play a role in facilitating transmission of cysts due to the adhesive properties (Dumetre *et al.*, 2012). The fur of dogs has been shown to contribute to transfer of viable *Toxocara canis* eggs (Overgaauw *et al.*, 2009) and indirect transport of *Giardia* spp. cysts by rotifers or other metazoan plankton can occur within aquatic environments (Jurgens, 1997, Trout, 2002). Vertical transmission occurs
in parasitic protozoans, such as transplacental route for *Toxoplasma* sp. in developing foetus or as described in sexually transmitted diseases (STD), *Trichomonas* sp. or *Candida* sp. during parturition from contact with mucus membranes (Carlier *et al.*, 2012) and the lactogenic transmission of *Toxocara canis* via the mammary glands (Martinez-Carrasco C. *et al.*, 2007). Since *Giardia* spp. trophozoites are not intracellular parasites, no evidence has been presented to indicate that *Giardia* spp. may be transmitted by these mechanisms, although the potential for maternal transmission to their puppies is high. Transmission can occur during the neonatal suckling period when the adhesive outer layer of infective cysts may become attached to the maternal fur and gain entry to the digestive tract through the nuzzling action of blind puppies (Overgaauw *et al.*, 2009, Dumetre *et al.*, 2012). Additionally there is the possibility that composition of the nasal secretion or maternal saliva might contain substances to promote cyst activation (Klein, 2004, Piña-Vázquez *et al.*, 2012).

### 1.14 Domestic dogs (Canis familiaris) as host species

Few detailed studies have focused on the impact of *Giardia* spp. infection in domestic dogs. Most studies report on comparative prevalence rates for dogs and humans or other species (Berrilli *et al.*, 2004, Lebbad *et al.*, 2008, Minvielle *et al.*, 2008) or as retrospective studies of clinical cases presenting at veterinary clinics (Batchelor *et al.*, 2008, Gates and Nolan, 2009). However, some early studies in indigenous communities were able to clearly link infections between dogs and humans (Hopkins *et al.*, 1997) by grouping DNA fragment sequences detected by PCR into four groups. One group of faecal DNA samples included homologous DNA sequences from humans and dogs. Some startling results from a recent survey of canine faecal samples from an indigenous community revealed high levels of exposure to *Giardia* spp. infection. Environmental samples that were collected from households in several neighbourhoods that owned dogs, and from landfill sites, showed more than half were positive for *Giardia* with faecal cyst counts in excess of 50,000 per gram of faeces (Himsworth *et al.*, 2010). This type of data has also been shown to be true for Indian
and Thai communities with intensive dog populations, particularly in rural areas of low economic living in close proximity to humans (Traub *et al.*, 2009).

Analysis of *Giardia* spp. infections in domestic dog populations, such as shelters and kennels, by sequencing of the 18s rRNA and beta giardin genes identified the majority of infections to be host specific canine assemblages C and D 76.4%, 93.5%, 98.9%, 100%, (Upjohn *et al.*, 2010), (Claerebout *et al.*, 2009), (Palmer *et al.*, 2008), (Souza *et al.*, 2007) respectively. These high rates of *Giardia* spp. infections reported in dogs directly influences the likelihood of human exposure to infection, either from direct animal contact or from infected excrement.

1.15 **Public health implications of canine host specific infections**

Canines are known to be susceptible to *Giardia* infection, as reported for wolves, foxes, and African painted dog (Hamnes *et al.*, 2007, Bajer, 2008, Ash *et al.*, 2010, Sousa *et al.*, 2011). There is the possibility that co-evolution of host and parasite occurred suggesting that different canid species are likely to be infected with similar genetic strains of *Giardia* spp. Despite the large amount of genetic data available it remains unclear if different species within the same family of Canidae are infected by *Giardia* spp. of the same genetic origin.

Canines as domestic dogs (*C. familiaris*) are important animals in the lives of ordinary Australians. Pet ownership figures indicate that 36% of Australia households own a dog, 3.41 million dogs, one of the highest rates of ownership of companion animals in the world. (Australian Companion Animal Council, 2010). Some of the current public health concerns include dog excreta in public open space and the implications for transmission of pathogenic parasite infections including *Giardia* spp. (Rinaldi *et al.*, 2008, Balassiano *et al.*, 2009, Wang A. *et al.*, 2012). Authors of a public health review of the significance of *Giardia* spp. infections in pets, including dogs suggest that study design is an important factor for realizing the significance of *Giardia* spp. infections in dogs (Ballweber *et al.*, 2010). Recommendations included the need for trifecta approach including biological, molecular and epidemiological datasets; together with
longitudinal sampling and multilocus genotyping or case control investigations considering spatial and temporal variations (Ballweber et al., 2010).

1.16 Genetic predisposition of host

The canine genome (domestic dog) is now fully published (Lindblad-Toh et al., 2005), the domestication and selective breeding of *Canis lupus familiaris*, has given rise to the uniquely wide phenotypic diversity of dogs known in the 21st century. Each breed is recognized by distinctive phenotypic traits, the results of genetic inheritance. It is now established that particular dog breeds selected for distinct phenotypic traits are known to carry genetic pre-disposition to diseases such as hip dysplasia and irritable bowel disorder (Famula, 2012, Simpson, 1998) or compromised innate immune receptor mechanisms, as reported for Boxer dogs (Simpson, 2010).

The link between exposure to *Giardia* spp. infection, and occurrence of other diseases may be caused by a compromised immune system, in a similar manner to psoriasis and *G. duodenalis* infections in humans (*Demodex* sp. infections in dogs) (Singh and Dimri, 2014). Researchers suggest that hereditary predisposition to immune system dysfunction, and the ability of T-cells to recognize or react to parasite dog leucocyte antigen system is more commonly observed in some particular dog breeds, such as Labrador retrievers (It et al., 2010). Host immune system responses are discussed further in Section 1.2.13. The genetic basis of canine disease explains some inherited factors for disease attributed to the intensive and extensive selection and inbreeding (Akey et al., 2010, Mellersh, 2012). There is increasing suggestion that some breeds of dog exposed to *Giardia* spp. infections may be more likely to display clinical signs of infection (Itoh et al., 2005, Fontanarossa et al., 2006, Hamnes et al., 2007). If this fact can be verified the cause of changes in pathogenicity also need to be confirmed. The cause of clinical signs may be attributed to host genetic deficiency in clearing *Giardia* spp. infection rather than variation in parasite genetics or the interaction between host and parasite (Lymbery and Thompson, 2012).
Chapter 1 Giardia canis

Research conducted in the context of the microbial ecosystem of the host gastrointestinal tract requires consideration of the co-evolution and adaptation mechanisms of both the parasite (G. duodenalis) and host (C. familaris) and the potential for genetic predisposition and co-speciation. The phylogeny and virulence of mucosal bacterial gene profiles infections of gastro-intestinal tract in dogs may also infect humans and that lineage-specific pathoadaptation may occur (Simpson et al., 2006). Confirmation of individual or breed specific genetic susceptibility would assist veterinarians to make quicker and more accurate diagnosis, and that special husbandry and management practices may be advised for particular breeds, possible anti/protozoal drugs or vaccination or oral immune boosts.

The phylogeny and virulence of Giardia spp, infections may also be subject to pathoadaptation in a similar manner to bacteria of the mucosal gastro-intestinal tract. Studies have confirmed phenotypic differences for in vitro growth rate kinetics for Giardia spp. (Plutzer et al., 2010) and the variation of surface proteins is an existing adaptation strategy for trophozoites to evade host defences (Nash, 1985, Nash, 2013).

1.17 Host- parasite interactions

This review directs attention to the parasitisation of one species, Canis lupus familiaris, the domestic dog whilst also considering the prevalence of Giardia spp. in many wild canids (captive and non-captive). Dogs as the host species, whilst domesticated, have a close genetic relationship with critically endangered wild canids, such as African Painted Dogs. As such, their continued survival in the wild depends upon the minimization of health threats, such as introduced human pathogens (Ash et al., 2010). Based upon genetic analysis and taxonomy, when does one infer the point at which a wild animal becomes domesticated? Is this after ‘n’ reproductive generations or when there are observable genotypic and phenotypic alterations? Additional information is required concerning the phenotypic expression in terms of proteins unique to specific Giardia spp. genotypes. Identification of phenotypic characteristics of Giardia spp. isolated from dogs may produce unique identifiers assigned to Giardia spp. infections.
The potential for using unique phenotypic biomarkers to distinguish between zoonotic and host specific *Giardia* spp. strains and further understand the nature of symptomatic or asymptomatic infections dogs infected with *Giardia* spp. is of value for assessing the impact on the host.

Veterinarians also need to be aware that many dogs harbour asymptomatic *Giardia* infections (Zajac *et al.*, 2000, Lymbery, 2004) and understand the limitations of commercial copro-antigen tests. Undetected asymptomatic infections contribute to the spread of infection and none of the currently available tests can detect if *Giardia* infections are likely to cause clinical signs. These types of asymptomatic infections can only be captured if routine sampling of healthy animals is carried out, using more than one diagnostic tool, and further research is needed into the consequences of asymptomatic *Giardia* infections and potential reservoirs of circulating genotypes.

### 1.18 High risk groups

*Giardia* spp. has been identified in all continents and *Giardia* ssp. infections are common in young and immuno-compromised dogs (Feng and Xiao, 2011). Swan and Thompson (1986) first reported *Giardia* ssp. infections in dog shelters and breeding establishments in Australia. A pool of infectious *Giardia* ssp. cysts may persist in intensive housing situations as a reservoir of disease, whether or not clinical signs develop. Many reports identified the incidence of *Giardia* spp. infection to be much higher in puppies under the age of 12 months (Rimhanen-Finne *et al.*, 2007, Epe *et al.*, 2010, Itoh *et al.*, 2011, Mircean *et al.*, 2012). Unlike many bacterial or viral gastrointestinal diseases, *Giardia* ssp. does not induce life-threatening conditions, but is easily spread and has a debilitating effect on young host organisms, especially at critical development stages or immuno-compromised individuals (Thompson, 2000, Sackey *et al.*, 2003). Puppies and immuno-suppressed dogs become especially susceptible to infection, due to the immune system's ability to respond to infectious challenges. Disease occurs when cysts are ingested, usually by drinking contaminated
water. Parasites then attach to the surface of the intestine and feed on the mucus secretions of the intestine.

Discussion concerning the severity of acute \textit{Giardia} spp. infections in humans and animals has identified that clinical signs of infection are associated with diarrhoea and vomiting. Conversely, chronic \textit{Giardia} infections are more likely to be reported as a failure to thrive, slow growth and stunting and, anorexia (Thompson and Lymbery, 1996, O'Handley \textit{et al}., 1999, Berkman \textit{et al}., 2002, Savioli \textit{et al}., 2006, Geurden \textit{et al}., 2010, Cotton \textit{et al}., 2011).

Four main groups of dogs have been identified as being at high risk of \textit{Giardia} spp. infection i) those under the age of one year (puppies and juveniles), (Bemrick, 1964, Swan and Thompson, 1986, Itoh \textit{et al}., 2011, Mircean \textit{et al}., 2012). Even at low prevalence (2.5%) higher prevalence rates were reported for puppies (6%) (Becker \textit{et al}., 2012). ii) those dogs kept in intensive rearing or production groups, (Bugg \textit{et al}., 1999, Itagaki \textit{et al}., 2005, Itoh \textit{et al}., 2005, Miro \textit{et al}., 2007, Katagiri and Oliveira-Sequeira, 2008, Claerebout \textit{et al}., 2009, Itoh \textit{et al}., 2011) iii) stray or free-roaming dogs are considered to be important contributors to the spread of infectious disease, including \textit{Giardia} spp. (Slater, 2001, Mirzaei, 2010, Mukaratirwa and Singh, 2010, Becker \textit{et al}., 2012). iv) in situations with poor husbandry in lower economic areas with reduced accessibility to education (Traub \textit{et al}., 2009, Gates and Nolan, 2009). A small number of reports are provided for incidence of \textit{Giardia} infections in working dogs, such as assistance animals, farm or detector dogs (Papazahariadou \textit{et al}., 2007, Bajer \textit{et al}., 2011).

Factors contributing to physiological stress include pregnancy, lactation, weaning, movement and rehousing, neutering, and drug interventions, such as deworming are likely to affect susceptibility to parasite infection, if exposed. One social survey of parasitism in rural households showed that 99% of all dogs tested harboured parasites with up to 16 different species being identified, with more than 28% of dogs having greater than 3 concurrent infections (Traub \textit{et al}., 2002). Although the prevalence of
Giardia was low, there were high rates of coccidian infections. Similar results were reported in a survey of shelter dogs, where Giardia spp. infections were present in 46% of all concurrent infections (Miro et al., 2007).

In urban areas with prophylactic anthelminthic treatments, the number of parasitic species reduced with less helminthes and nematodes, but increased protozoan infections (Bugg et al., 1999). Additionally, in urban areas, more potentially zoonotic genotypes are recorded from urban populations than rural ones, where host specific genotypes C and D predominate (Itagaki et al., 2005, Dubna et al., 2007). This polyparasitic type of infection is discussed further in Section 1.2.18.

Risk factors of infection identified for dogs grouped in intensive shelter facilities were considered to be at risk of developing Giardia spp. infections due to communal housing; construction design and materials of housing facility and the quarantine policy and implementation (Ho et al., 2006, Mircean et al., 2012). The public health implications are important, from local quarantine to international policy, lack of adherence to policy facilitates the spread of disease, as described for importation of dogs in Taiwan where 16.8% were carrying parasitic infections (Ho et al., 2006). Therefore analysis of the intersect between the rights and responsibilities of companion animal and working dog owners, public health officials, wildlife conservationists and animal welfare staff is key to minimizing transmission of parasites, such as G. duodenalis. Inclusion of dog shelters and breeding facilities in parasitology research is important because of possible transmission to humans, particularly children, as well as for increasing awareness that immuno-compromised individuals may be exposed and succumb to seemingly benign parasite infection. Many research reports have been published concerning Giardia spp. infections in canines over the last decade however, only one study specifically addressed public education concerning dog health (Palmer et al., 2008). A separate article provided an assessment of professionals ability to accurately diagnose Giardia infections (Gates and Nolan, 2009a).
1.19 Clinical signs of infection in dogs
Primary signs of acute *Giardia* spp. infections in dogs include; frequent bouts of diarrhea with blood or mucus in pale coloured or bile-stained faeces and malodorous stools (Barlough, 1979) with vomiting in 17% of infected cases (Olson *et al.*, 2010). Secondary signs of chronic *Giardia* spp. infections in dogs include; deficiency of fat-soluble vitamins and associated dry skin and poor hair coat condition (Barlough, 1979); weight loss and failure to gain weight (Carlin *et al.*, 2006); anemia and change in leukocyte count (Rosa *et al.*, 2007). In addition, asymptomatic infections of *Giardia* spp. may be present although not associated with any clinical or subclinical signs of infection (Irwin, 2002).

The clinical signs of infection evident in dogs have been attributed to host specific *G. duodenalis*. infections with assemblages C and D (Berrilli *et al.*, 2004, Barutzki *et al.*, 2007). In contrast, asymptomatic *Giardia* spp. infections in a survey of household dogs in Europe have been associated with mainly assemblage A infections (Leonhard *et al.*, 2007). Whilst in Brazil and Japan, the cause of diarrhoea in dogs was attributed to host specific *G. duodenalis* infections (Itagaki *et al.*, 2005, Souza *et al.*, 2007).

1.20 Pathophysiology and Intestinal inflammation
Simpson *et al.* (2006) established that mucosal microflora are contributing factors for granulomatous colitis in Boxer dogs, and it is likely that pathogenic enteric protozoa are capable of provoking immune responses in genetically susceptible individuals. Intestinal inflammation has been defined as a causative factor of clinical signs in *Giardia* spp. infections. The detailed understanding of canine digestive tract accepts that the canine digestive function is similar to that of humans, with the exceptions for essential amino acids requirements. Historically, wild canines, such as *Canis lupus* spp. the genetic ancestors of *Canis familiaris* were mainly carnivorous and the contradictory evidence for gastric pH in canines remains unexplained (Akimoto *et al.*, 2000, Sagawa *et al.*, 2009). The importance of bile secretion and digestive function is an important aspect of *Giardia* spp. metabolism (Farthing *et al.*, 1983, Farthing *et al.*, 1985) and its
role in canines might explain the disparity in genetic identification of *Giardia* spp. infections between host species.

The microbial ecosystem of the gastrointestinal tract may play a role in enabling *Giardia* spp. populations to establish an infection although this would be influenced by diet and secretion of digestive juices and immune factors (Hegner, 1924, Tsuchiya, 1931).

A key feature of *Giardia* spp. is the biochemical and structural transformation from inactive cysts to motile, flagellated trophozoites and the subsequent ability to achieve attachment to host cells of the duodenal lining. The mucosal lining of the gut may have microclimate (or niche conditions) that is conducive to infections.

Both anecdotal reports and earlier studies have alluded to the severity of clinical signs from *Giardia* spp. infections with other protozoan parasites, such as the coccidian’s *Cystoisospora* sp. and *Sarcocystis* sp. (Little et al., 2009). However there is limited commentary that seriously addresses the causes, interactions, and impacts of polyparasitism in dogs. It is apparent that the interface of host-parasite interactions is critical for successful infective strategy by this protozoan parasite.

Martinez and Baquero (2002) present an evolutionary and ecological review of pathogenicity of bacteria by examining virulence and drug resistance mechanisms. Their assertion is that horizontal gene transfer can explain some of the acquired pathogenic characteristics developed from co-existence with the host species within an evolutionary context. Furthermore a range of factors are predicted to contribute to the evolution of virulence and the potential impacts of novel therapeutics, although emphasizing that hygiene approaches to reducing host-host transmission are important anti-virulence strategies. This type of scenario might also apply to the evolution of *Giardia* spp. infections where differences in host specificity and pathogenicity could be explained as the consequence of co-evolutionary processes. Linking clinical signs with microbial gut composition and processes is likely to be a key to understanding the pathogenicity of different *Giardia* spp. genotypes. Further research may extend our
current understanding of inflammatory processes in healthy and infected dogs. Some of the largest studies on *Giardia* spp. infections have been conducted in Europe, including thousands of dogs over a number of years examining both companion animals and breeding populations (Barutzki and Schaper, 2003, Epe *et al.*, 2010). Many of the parasite prevalence rates are reported as an overall figure and as subsets of juvenile dogs less than 12 months of age (Batchelor *et al.*, 2008, Epe *et al.*, 2010). Alternatively, Claerebout *et al.* (2009) reports differences in prevalence rates for household pets, veterinary clinical cases and dogs in kennels although no differences in *Giardia* spp. prevalence rates were due to breed or sex.

All the studies in Table 1.3 report on clinical signs associated with *Giardia* spp. infections in dogs and genotyping of *Giardia* spp. suggest that mixed infections with assemblage A are present in asymptomatic infections (Leonhard *et al.*, 2007, Barutzki *et al.*, 2007). Reports indicate that host specific *Giardia* spp. (assemblages C and D) are associated with the majority of symptomatic infections in dogs (Claerebout *et al.*, 2009). Assemblage B has been detected in canine faeces, but less frequently than other Assemblages except those reported from Africa and USA (Ash, 2010, Covacin, 2010).

Table 1.3 Clinical signs associated with *Giardia* spp. infections in dogs

<table>
<thead>
<tr>
<th>Country</th>
<th>Summary</th>
<th>Clinical signs and source of samples</th>
<th>Findings</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK</td>
<td>4526 dogs over 2 years</td>
<td>Symptomatic cases</td>
<td><em>Giardia</em> spp. common parasite, particularly in some high risk groups.</td>
<td>(Batchelor <em>et al.</em>, 2008)</td>
</tr>
<tr>
<td>Canada</td>
<td>107 dogs</td>
<td>Asymptomatic cases</td>
<td>Vaccination did not reduce infections</td>
<td>(Anderson <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td>Belgium</td>
<td>1159 dogs over 3 years</td>
<td>Breeding, household, symptomatic cases mainly C and D assemblages</td>
<td>Mainly zoonotic assemblage A in household dogs. Presence of other parasites contributing to symptoms</td>
<td>(Claerebout <em>et al.</em>, 2009)</td>
</tr>
<tr>
<td>Europe</td>
<td>8685 dogs over 2 years</td>
<td>Symptomatic dogs</td>
<td>Differences between countries 25% dogs infected, most noticeable in young, Gender, age and difference between breeding and shelter facilities.</td>
<td>(Epe <em>et al.</em>, 2010)</td>
</tr>
</tbody>
</table>

Number of symptomatic cases twice as many as asymptomatic cases.
1.21 Asymptomatic infections and failure to thrive

A study of symptomatic dogs using a single commercial copro-antigen diagnostic test suggest that 43% of dogs under 12 months old are infected with *Giardia* spp. (Epe et al., 2010) although no additional information is available to assess causes of clinical signs. Most cases of *Giardia* spp. infection in dogs are confirmed in those displaying clinical signs, although some studies have investigated asymptomatic dogs as shown in Table 1.3. The consequences of asymptomatic infections in dogs may have a direct impact on the host with implications for transmission to other host species (Thompson, 2004). The absence of datasets on asymptomatic infections and possible cycling of infection between hosts make this an important consideration for research into *Giardia* infections in canines.

1.22 Immune function

Reviews detailing the immune responses to *G. duodenalis* infections in non-canine host species published by Faubert (2000) and Singer (2011) supplement the knowledge of antigenic variation found in *G. duodenalis* trophozoites (Nash, 2011). The following section integrates information known about mammalian immune responses to...
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*G. duodenalis* infections together with information available in the literature that focuses on the canine immune system and responses to *Giardia* spp. infections.

1.23 **Features of the mucosal tract**

The intricate and specialized system of cells and organs of the gastrointestinal tract are responsible for sifting and sorting through dietary contents to harness nutrients and energy sources and discard waste products. The ability to recognize and respond to xenobiotic agents, in particular parasites is an important function. The inner surface of the duodenum and colon is coated with secreted mucus, and localized immunoglobulin secretion is predominantly IgA (Heddle and Rowley, 1975, Reinholdt and Husby, 2004). Mucosal immune responses in dogs have been shown to be influenced by age, genetic factors, diet, gut flora and weaning (Hall, 2013). Composition of the gut microflora directly affects host physiology (Kinross *et al.*, 2011, Lee and Hase 2014) including host immune responses (Round and Mazmanian, 2009, Tang, 2011, Maynard *et al.*, 2012). Studies have demonstrated that molecular patterns of microbial and pathogenic organisms (MAMP /PAMP) invoke immune responses in colonic epithelial cells of canine hosts (Swerdlow *et al.*, 2006).

1.24 **Canine immune system**

Dogs acquire immune status in two distinct phases, firstly in healthy dogs immunoglobulin acquisition occurs *in-utero*, immunoglobulin G (IgG) is transferred via the placenta and, IgA is present in colostrum (Snoeck *et al.*, 2006). Schafer-Somi *et al.* (2005) reported that IgM is the first immunoglobulin to be produced as a response to immune challenge. Clinical data from German shepherd dogs (German *et al.*, 2000) presenting with gastro-intestinal tract infection demonstrated that immunoglobulins (A, G and M) circulate at detectable levels in saliva and serum, regardless of age, sex or disease status. Nevertheless, studies indicate an apparent deficiency of IgA secreted from duodenal mucosal lining in some breeds of dog (German *et al.*, 1998). Whilst relatively few markers have been investigated for cats and dogs differences are known to exist between species for intraepithelial lymphocytes and expression of surface IgM.
1.24.1 Canine immune response mechanisms

Whilst there is ever increasing information and understanding of the human and murine immune responses to injury and infectious disease as defence mechanisms there remains limited knowledge on other common species, such as companion animals, including dogs, (*C. familiaris*) (Linde *et al.*, 2008). A study of pyoderma in dogs using polyacrylamide gel electrophoresis (PAGE) identified patterns of IgG in response to bacterial infection (Neuber *et al.*, 2008). Infections rarely resolved spontaneously, with recrudescence episodes and evidence that dogs cannot effect an appropriate immune response to eliminate the organism, despite forming part of normal microflora, of nares, oropharynx, and anal ring. This type of response has also been observed in dogs infected with *Giardia* spp. (Gates and Nolan, 2009).

The alpha defensins, anti-microbial proteins of the human and murine immune systems are absent in cattle and dogs (Shanahan *et al.*, 2011). To date, the immune systems of dogs are known to possess three different beta defensins, one cathelicidin (K9CATH) and a hormonal peptide canine hepcidin (Fry *et al.*, 2004, Sang *et al.*, 2005, Sang *et al.*, 2007) but no published studies have yet confirmed intestinal tract AMP (antimicrobial peptides). In the context of *Giardia* spp. and its ability to infect a wide range of species plus frequent cross species infections a detailed understanding of host response mechanisms is essential. Singer, (2011) suggests further studies into anti-*Giardia* spp. effector mechanisms in humans whilst Patil *et al.* (2004) outlines the phylogenetic relationships of cross-species beta-defensins demonstrating distinct evolutionary lineage groupings for dog and rodent specific; primate and dog specific; rodent specific and dog specific defensins. This suggests that host specific infectious weaponry may provide a link to understanding the reasons for differences between host specific and cross species infections.

Proposed mechanisms for mammalian innate immune responses to MAMP /PAMP are suggested to occur through membrane bound Toll receptors and cytosolic nucleotide
binding sites distinctive leucine rich repeats (LRR) as described by Basset et al., (2003), Inohara and Nunez (2003), Akira et al. (2006). These LRR are a unique feature of protozoan parasites, associated with immune responses (Boceta et al., 2000, Mcguinness et al., 2003, Kedzierski et al., 2004b, Loftus et al., 2005, Daher et al., 2006) and form an integral component of Giardia cell wall proteins (Lujan et al., 1995, Luján et al., 1997). Evidence for the expression of IL-7 induced in canine cell lines is associated with lipopolysaccharide (LPS) of bacterial organisms (Swerdlow et al., 2006) and host microbiota. These types of cellular and molecular processes, in conjunction with markers of infection and further exploration of possible health links may uncover implications for other gastro-intestinal diseases, such as IBD or Crohn's Disease (Elphick and Mahida, 2005).

1.25 Mucosal immune responses to Giardia spp. infections

There are differing opinions regarding the ability of Giardia to cause mucosal inflammation, and it's role in host defence mechanisms against the enteric parasite as mentioned by (Fisher et al., 2013). Responses to G. duodenalis infections have been attributed to a reduction in size of the microvillus, reduced enzyme expression and activity and the absence of mucosal inflammation (Eckmann, 2003), although infectious challenge stimulated production of circulating immunoglobulins, IgA, IgG, and IgM. In addition the B cell (humoral) and T cell (cell-mediated) responses are induced by nitric oxide levels; antimicrobial (parasitic) peptides and, paneth cell secretions such as cryptidin and defensin (Elphick and Mahida, 2005, Ankarklev, 2012).

Are IL-6, dendritic cells or mast cells activated during Giardia infection? How do genes that encode VSP vary in their antigenic mechanisms? Answering these questions, posed by Roxstrom-Lindquist et al. (2006) concerning G. duodenalis infections in humans may enhance our understanding of the spread of infections and treatment interventions in canines. Dendritic cells are the key intermediaries within the gastro-intestinal tract directing immune responses resulting in either oral tolerance or protective immunity (Mowatt, 2005). Sites for dendritic cell activity in the intestine, the
lamnna propria and Peyer’s patches, can initiate t-cell responses and associated cytokine mediation of inflammation (Cerutti et al., 2011). Functions for the host cell defences include host cell receptors. Pathogenic organisms can target this receptor molecule to gain advantage and interact with TLR signaling in the gastro-intestinal tract (GIT), inducing dendritic cell production and pro-inflammatory cytokines. Inhibition of pro-inflammatory cytokine production is one outcome from G. duodenalis infections (Singer, 2011).

The consequences for hosts infected with Giardia spp. are contingent upon active inflammation of the gastro-intestinal wall occurring simultaneously with a Giardia spp. infection (Singer, 2011). The primary cause of gastrointestinal tract inflammation may not necessarily be induced by Giardia trophozoites. Accordingly, if Giardia spp. infections occur alone then tolerance is likely to be induced. However, in the presence of co-infections a potential exists for initiation of mechanisms for conferring protective immunity. Experimental immunization of mice with Giardia spp. antigens induced IL-4 and IL-6 responses to Giardia (Singer, 2011). Intra-species genetic differences of mice were considered to affect host resistance to of G. duodenalis and authors assumed that the bacterial microflora were the most likely causative factors (Singer and Nash, 2000).

1.26 Tolerance or conferred immunity?

The complexity of oral tolerance mechanisms include systemic interactions between the Peyer’s patches and epithelial cells of the gastric lymphoid tissue (GALT) within the intestinal wall. Responses to antigen presenting cells require interaction between mucosal immunoglobulin (secretory IgA) and systemic responses (Strobel and Mowat, 1998). Experimental methods for boosting immune system responses to parasites demonstrated that oral tolerance could be induced in dogs (Deplazes et al., 1995). Ingestion of combined antigens extended the immune response (elevated levels of IgG and IgE) three fold, in comparison to ingestion of single antigen. This work reinforces the premise that ingested parasite proteins play a role in enhancing immune responses
through the production of antibodies. Olson et al. (2002) suggest that oral tolerance may develop in hosts experiencing signs of chronic *Giardia* spp. infections, due to *Giardia* spp. antigens in the gut. This concept has been used to investigate an oral vaccine to prevent *Giardia* spp. infections in dogs and as detailed immune characteristics of GALT are further understood they continue to be proposed as an effective target for oral vaccines (Kunisawa et al., 2012).

TLR 4 mRNA, present in the canine stomach and small intestine, is responsive to lipopolysaccharides and peptidoglycans (Swerdlow et al., 2006, Burgener et al., 2008). Polymorphism of the TLR 5 gene in canines has shown hyper-responsiveness to flagellin and whilst inflammatory bowel disease manifestations are similar in dogs and humans, some differences in histopathology have been reported (Cerquetella et al., 2010, Kathrani et al., 2012). In humans when hyper-sensitization of the gut occurs, such as from gluten intolerance, it can lead to coeliac disease or IBS (Powrie, 2012).

The complexities of immune system responses remain poorly understood, and the increasing incidence of adverse food reactions (allergies) and relationship with gut microflora are under medical and veterinary investigation (German, 2001, Hooper et al., 2002, Cobo and Chadee, 2013, Beirão et al., 2014, Lee and Hase, 2014).

### 1.27 Other immune responses

Additional host responses to *G. duodenalis* infections in humans include the activity of lysozyme and phospholipase A, and lactoferrin as a component of the host innate immune system (Eckmann, 2003, Ordaz-Pichardo et al., 2013). Lacto-transferrin an iron-binding protein, secreted by leukocytes, is present in plasma and secretions (milk, mucus, bile) (Levay and Viljoen, 1995) although previously undetected in milk from dogs (Masson and Heremans, 1971). It is an important component of granulocytes, with multiple functions, including bacteriostatic action, depriving bacteria of iron essential for growth (Bellamy et al., 1992, Farnaud and Evans, 2003) and improves cellular immune responses and faecal bacterial populations (Levay and Viljoen, 1995, Adlerova et al., 2008, Hellweg et al., 2008.).
Mammalian lactoferrin receptors are understood to play a crucial role in mediating multiple functions of the protein and evidence of expression has been shown in the infant small intestine and in other tissues (Liao et al., 2007, Pang et al., 2014). A team of researchers recently reviewed the relationship of iron as an intermediary between lactoferrin and pathogenic protozoa concluding that some parasites require holo-lactoferrin as an iron source for in vitro growth and alluded to potential immunogenic properties being valuable as drug targets (Ortiz-Estrada et al., 2012). Notably, significant differences in the immune responses between mice and humans, have been recently published (Snoeck et al., 2006, Merrifield et al., 2011) and investigation into other species such as dogs, may reveal more details of variation across mammalian species. Limited information is available concerning the innate immune system responses to *Giardia* spp. infection in dogs, although it is certainly worthy of further examination. Some researchers suggest that the initial host immune response is provoked by bacterial infection, and *Giardia* trophozoites persist, undetected, enabling host infection (Batt et al., 1984, Muller and Von Allmen, 2005). Increasing pH in the gastro-intestinal tract has been associated with bacterial infections and might affect the ability for *Giardia* spp. trophozoites to become established. Conflicting evidence presented in the literature concerns the capacity for *Giardia* spp. infection to provoke inflammatory responses (Kohli et al., 2008, Hanevik et al., 2011, Buret and Cotton, 2011). The action of signalling peptides and diet have been linked to allergic dermatitis in dogs. (Halliwell, 2009). Understanding the concept of immunosuppression, particularly in grouped housing situations, such as shelters is critical to appreciation of the impact of *Giardia* and other clinical signs of the disease syndrome, such as failure to thrive or association with opportune infections (Thompson and Smith, 2011).

### 1.28 Vaccination

There have been several approaches to studying immune responses to *Giardia* spp. in pursuit of effective vaccine development, reviewed by (Lee et al., 2011). Although infective mechanisms affect the host immune system in different ways, it was initially
considered simple to produce a vaccine against *Giardia* spp.. Olson (1998) found evidence of antibody-specific reactions to *Giardia* cysts and trophozoite antigens. These findings later resulted in a commercial *Giardia* vaccine for dogs and cats (Olson *et al.*, 2000). Vaccination may only offer protective immunity in the short term, and unlikely to be specific for genotypes infecting dogs. Manufacture of the vaccine was prepared from axenic culture of *G. duodenalis* originally isolated from sheep. The antigenic properties for *Giardia* spp. infections in sheep are different to those in dogs.

In addition to vaccines trialed in dogs, researchers have also investigated vaccines against human *Giardia* infections. Most recently, Rivero *et al.* (2010) developed a human vaccine based upon blocking the production of membrane surface proteins of *G. duodenalis* trophozoites. The integral membrane proteins are cysteine-rich and possess varying extracellular amino-acid terminal regions. These regions of variation (Variant Specific Surface Protein, VSP) serve as a unique adaptive mechanism enabling *Giardia* spp. trophozoites to withstand challenges from host immune responses and persist to attach to the epithelium, causing infection. Infection of gerbils using a human isolate of *Giardia* established that protection could be conferred by vaccination with a modified live VSP vaccine including all of its 190 surface proteins, (Rivero *et al.*, 2010). The host immune response thus renders the protozoan surface antigens ineffective in attaching to the epithelium, thereby reducing the severity of reaction and the likelihood of establishing infection. Whilst adaption and variation may be multiple and frequent, the principle mechanism for this vaccine activity is antigenic variation of trophozoite surface proteins and is a key defence mechanism of all *Giardia* species, including infections in canines. The VSP constitutes an extensive range of immune proteins that are located on the external surfaces of *Giardia* spp. trophozoites and Nash (2011) described the specific features of these proteins in more detail.

**1.29 *Giardia* spp. localization in the canine digestive tract**

Early studies into dietary effects on *Giardia* spp. infections in canine hosts, concluded that carnivorous hosts did not harbour *Giardia* spp. or protozoan infections probably
due to high numbers of putrefactive bacteria in the intestinal tract (Hegner, 1924). In contrast, rats fed on vegetarian diets were infected with *Giardia* spp. and other protozoa accompanied by high numbers of acidophilous bacterial species. Artificial infection of cats with *Giardia* spp. was unsuccessful.

Localization of *G. canis* trophozoites and cysts were compared together with bacterial composition of gut contents at various intestinal sections from 4 puppies receiving protein or vegetarian diets (Tsuchiya, 1931). The results confirmed those of Hegner (1922) that the lower portion of small intestine and jejunum are the optimal locations for trophozoite development where they are both larger and fully formed than in other locations in the absence of bacterial species. Protein diets appeared to inhibit trophozoite establishment in the small intestine, with many more cysts observed in the ileum and bowel region. Tsuchiya (1931) suggests that it may not be diet *per se* but the chemical characteristics of food source, which may influence trophozoites to thrive, the intestinal conditions, such as pH, specific gravity or viscosity may be less important than food chemistry or bacterial species.

### 1.30 Management of *Giardia* infections in dogs

To the current day, *Giardia* spp. remains capable of infecting a wide variety of hosts on a global scale with limited means of control or remediation. Prevention of *Giardiasis* depends on good sanitation. Standard concentrations of chlorine for water treatment do not kill the infective form of *Giardia* (Schantz, 1991).

#### 1.30.1 Treatment

Whilst veterinary pharmaceutical treatments are used to eliminate parasitic organisms from the gastrointestinal tract, this often occurs without full knowledge of the exact cause of clinical signs, either in terms of the physiology of the pathogenic organism and life cycle events, or specific processes occurring in the gastro-intestinal tract. The rationale for treating suspected or confirmed *Giardia* spp. infections in dogs also includes public health concerns, namely, prevention of potential zoonotic transmission (Thompson, 2000, Thompson *et al.*, 2008).
Giardia spp. treatments include the use of nitroimidazoles, such as metronidazole, which have long been reported as effective against G. duodenalis (Barr et al., 1993, Barr et al., 1994, Barr et al., 1998). An alternative drug, a benzimidazole has also been used a treatment in dogs. Adverse effects of the benzimidazole, albendazole, led to the use of metronidazole, which was consistent with in vitro findings of Giardia spp. susceptibility (Cedillo-Rivera and Munoz, 1992, Farbey et al., 1995) to nitroimidazoles. However, metronidazole resistance is of increasing concern in humans, and possibly in dogs (Busatti et al., 2009, Tejman-Yarden et al., 2011). Some alternative therapeutic approaches examined bacitracin zinc, which has been found to be effective against Trichomonads and Giardia spp. (Andrews et al., 1994), and ethnobotanical surveys identified two medicinal herbs with active compounds against Giardia spp. One of herbs was reported to have a 98% success rate against G.muris. (Gardner and Hill, 2001). Consistent results from an early promising use of probiotics to reduce Giardia cyst excretion have not been forthcoming (Simpson et al., 2009, Benyacoub et al., 2005).

The drugs metronidazole and tinidazole have been reported for use of as antiparasitics for treatment of Giardia infections in dogs by (Zajac et al., 1998, Villeneuve et al., 2000, Decock et al., 2003, Hamnes et al., 2007) and using fenbendazole (Payne et al., 2002). Fenbendazole (Febantel) is currently recommended as a preferred treatment method for Giardia infections in dogs (ESCCAP, 2011). The use of combined treatments using praziquantel and fenbendazole for control of Giardia in shelter dogs compared by Ortuno and Castella (2011) showed that no significant difference in prevalence rates was detected following treatments. In contrast, a study reporting the use of a similar, commercially available, product in naturally infected dogs Montoya et al. (2008) showed that faecal cyst excretion by infected dogs ceased after three days of treatment. On further investigation, Bowman (2009) demonstrated that this treatment only remained effective for a short time, possibly due to re-infection, and also that untreated dogs spontaneously ceased to shed cysts in faeces.
As a follow on from successful \textit{in vivo} and \textit{in vitro} studies published for anti-protozoal activity of ronidazole, against \textit{Giardia} spp. in mice, the effects of this drug was studied in kenneled Beagle dogs (13-19 months old) naturally infected with \textit{Giardia} spp. This research explored the effectiveness of ronidazole, a nitroimidazole drug, against \textit{Histomonas} sp. and \textit{Trichomonas} sp., as described in previous studies, in a new trial with dogs. The study used drug therapy combined with environmental disinfection and elimination of infective cysts from fur coats using shampooing (Fiechter \textit{et al.}, 2012). Unfortunately this study was inconclusive, whilst the incidence of infection was reduced it could not be attributed to action of therapeutic drugs when combined with chemical and manual methods for parasite elimination.

Current anti-protozoal treatments reviewed by (Astelbauer and Walochnik, 2011) are approaching half a century of use and managing \textit{Giardia} spp. infections may require improved efficacy for existing benzimidazole drug treatments (Miro \textit{et al.}, 2007).

\textbf{1.31 Animal husbandry}

An important factor for reducing infection rates, and clinical signs relies upon mechanical or physical methods for removal of infectious agents. Husbandry and management practices, such as type and frequency of manual cleaning and sterilisation practices using chemical or UV light disinfection or desiccation (Li \textit{et al.}, 2007) are important for eradication of infective cysts from the environment. Other factors that need to be considered are diet, housing construction, animal movement and transportation (Mircean \textit{et al.}, 2012).

\textbf{1.31.1 Behaviour and interaction between humans and animals}

A survey conducted in the UK of dog owner behavior illustrates how management and husbandry practices may affect the potential for transmission of infectious parasites between dogs and their owners (Overgaauw \textit{et al.}, 2009). Results from the survey identified three commonly accepted pet-owner interactions including 1) dogs licking the face of household members (60%), 2) dogs sleeping in the bedroom (33%) and 3) dogs sleeping on the human’s bed (14%). These incidences of close physical contact
and shared sleeping space are considered conducive to the transmission of infectious zoonotic diseases (Westgarth et al., 2007).

The cultural values and beliefs of dog owners are important in the way that dogs interact with each other, with humans and with the wider environment. Different nationalities religious and ethnic groups, in addition to socio-economics and demographics, affects the attitudes of people towards animals and in turn, behavioural responses of the animal, both wild and domesticated. Examples in the literature acknowledging this approach to understanding Giardia spp. infections include (Gates and Nolan, 2009, Volotao et al., 2007). In addition, Giardia infections in dogs are common and there are certain behavioural aspects of dogs such as coprophagy that may directly influence exposure to the parasite and likelihood of infection (Askew, 2003, Borg and Graat, 2006).

1.31.2 Therapeutic intervention

There is an increasing suggestion that drug treatment interventions are ineffective in preventing rapid re-infection, spontaneous clearing or non-responsive to treatment in asymptomatic infections (Saffar et al., 2005). The concept of alternative transmission and infection routes must be considered, as Overgaauw et al. (2009) suggest. Mechanical means of transmission via fur or bedding may allow dog – dog transmission or dog to other host organism (Overgaauw et al., 2009).

1.31.3 Physiological stress factors

In addition, stress factors may also increase the likelihood of Giardia spp. infection, either physiological stress during growth and development stages, such as weaning, in juveniles, and mature females during pregnancy and lactation or from increased exposure to infectious challenges. Appropriate changes to management practices need to be implemented accordingly. Approaches to solving some of these practical interventions and recognizing the different parasite infections include both education of veterinary clinic clients and pet owners, as discussed in Sections 1.12 and 1.19.
1.32 Co-infections with other enteric parasites

It is well established that concurrent parasite infections occur in domestic dogs, and those previously associated with *Giardia* are other protozoans, such as *cryptosporidium*, coccidian’s, and nematodes. A longitudinal study of pure bred domestic dogs by Hamnes *et al.* (2007) monitored the prevalence and intensity of infection of two closely associated water borne parasites, *Cryptosporidium* sp. and *Giardia* spp. the only other parasite reported in the study was the detection of *Toxocara canis*. Patency of infection was associated with the age of the animal, although lack of genotyping data greatly limited any observations on the transmission dynamics.

Prevalence data is showing increased rates of protozoal infections in dogs, including *Giardia* spp. It is important to study and know more about the impact of protozoan parasite infections in dogs and how they are transmitted and ecological impacts.

The role of polyparasitic infections, predominantly protozoan infections, and the significance of these concurrent infections are yet to be explained. The prevalence of *Giardia* spp. co infections is discussed in chapter 3. A summary of published research is outlined in Chapter 3, to comment on instances where polyparasitism may actually mask the true cause of clinical signs or contribute to inaccurate diagnosis.

The ability to resolve questions concerning the impact of *Giardia* infections in dogs, and importantly recognizing that knowledge of the parasite genotype alone, is insufficient may require a new approach. What may actually be required is a broader picture encompassing host-parasite interactions, phenotypic data, and ecosystem biology that address physiology concurrent infections.

1.33 Importance of understanding *Giardia canis* infections

Research using dogs as a model host is essential to verify the role of this domestic animal in the transmission of *G. duodenalis*. Records for cellular and immune response to *Giardia* spp. infections that occurs in different host species are incomplete. Developing research approaches to examine the interaction or co-location of humans and dogs are a key to understanding aspects of management practices that affect the
incidence of *Giardia* spp. infections. Artificial infection experiments and studies of naturally infected populations remain necessary for understanding and resolving *Giardia* spp. transmission routes. Findings from this type of study will help explain the epidemiology and potential impacts of disease outbreaks on canine and human hosts. Many epidemiological studies include genotyping but lack association with clinical signs of infection. Absent from the literature are clinical case studies on individual dogs infected with *Giardia* spp. treatment schedules, parasite clearance and frequency of re-infection or recrudescence and associated signs (and severity) of infection.

Studies designed to clarify the conditions that enable *Giardia* spp. infections to flourish and any synergistic effects between parasites are important. Molecular characterization of *Giardia* spp. infections which, contribute to pathogenicity and clinical signs requiring veterinary or medical intervention, as reports of canine protozoal infections has increased in affluent human settlements. Seeking answers to questions relating to the numbers of *Giardia* spp. cysts excreted and confirm any association with potential zoonotic transmission mechanisms.

Studies that consider the overall microbial environment of the gut and cellular interactions within digestive processes are required to address episodes of chronic diarrhoea in dogs and possible association with impaired endocrine or digestive function and parasite burden. Intestinal parasites are suspected of contributing to inflammatory bowel disease in humans and this could be the same situation for dogs.

Critical analysis of *Giardia* spp. diagnostic tests is timely and the consequences of asymptomatic infections remaining undiagnosed needs to be evaluated. The majority of published studies refer to snapshot sampling methods, but there is a need for more detailed longitudinal studies. Reliable molecular detection of Assemblage B, in faecal samples from dogs, remains problematic, despite the increased use of multi-locus genotyping both in terms of understanding characteristics of host specific infections and to understand the characteristics of *Giardia* spp. infections in other species.
Research using dogs as a model host cannot be underestimated, both in terms of understanding characteristics of host specific infections and the importance of this domestic animal in the potential transmission routes of \textit{G. duodenalis} genotypes.

1.34 Research hypothesis

That \textit{G. duodenalis} infections in humans are the same as \textit{Giardia} spp. infections in dogs.

Knowledge concerning the clinical impacts and frequency of zoonotic transmission is undetermined, for further understanding of the impacts of \textit{Giardia} infections in canines broad based studies are needed. Examination of the literature \textit{Giardia} spp. infections reported in domesticated dogs and in \textit{Canid} species in wild and captive wildlife populations raised the following specific research questions to be addressed in this thesis.

1. When is the earliest time that patent \textit{Giardia} spp. infections can be detected? How long is the pre-patent period?

2. What tests are available for accurate diagnosis of \textit{Giardia} spp. infections in dogs?

3. Can \textit{Giardia} spp. proteins be detected in canine faeces using proteomic techniques?

4. Can novel proteins or peptides be detected in canine faeces be used to differentiate between different genotypes of \textit{Giardia} spp.?

5. What host-parasite interactions can be detected using proteomic techniques for the \textit{Giardia} spp. infected canine host?

6. Do lactoferrin levels in canine faecal samples change when clinical signs of parasite infection are observed?
Chapter 2

Comparative diagnostics for *Giardia* spp. infections in canine faecal samples

2.1 Introduction

Enteric protozoan infections in dogs go largely unrecognized by the majority of pet owners, especially in Australia where there is a high level of pet ownership. Recent studies have found that parasite infection occurs in over twenty percent of dogs, the majority by the protozoan parasite, *G. duodenalis* (Palmer et al., 2008).

Molecular studies have advanced understanding of the epidemiology of *G. duodenalis* infections, in addition to identifying some knowledge gaps in terms of transmission and potentially zoonotic genotypes (Thompson et al., 2008). Overall, substantial differences in study design and methodology have obscured taxonomic differentiation between host specific and cross species *G. duodenalis* infections in dogs. These differences include host population studies Eligio-Garcia L. et al., (2005) and diagnostic approaches, with the latter based solely on results using microscopy (Hamnes et al., 2007, Claerebout et al., 2009, Batchelor et al., 2008) and others on copro-antigen test results (Epe et al., 2010, Olson et al., 2010, Rishniw et al., 2010). Epidemiological studies reporting *G. duodenalis* infections using genotyping methods are available, although these are not consistent in the choice of gene loci for PCR analysis (Itagaki et al., 2005, Lalle et al., 2005, Leonhard et al., 2007, Upjohn et al., 2010, Covacin et al., 2011). Few studies use a combination of all three techniques for diagnosis (Barutzki et al., 2007, Traub et al., 2009). Veterinary clinicians have been unable to apply findings from molecular research work at a diagnostic level and remain reliant upon faecal flotation and light microscopy for routine laboratory practices, although there is increasing use of copro-antigen test kits (Carlin et al., 2006, Epe et al., 2010). Currently diagnostic *G. duodenalis* tests lack the precision or resolution to alert medical practitioners or veterinarians to potentially zoonotic strains. Nor do they define or guide
industry acceptance of what constitutes normal or acceptable levels of infection, particularly because many infections are asymptomatic.

Research has demonstrated the existence of four genetic assemblages of *G. duodenalis* in dogs: C and D (*G. canis*), B (*G. enterica*) and A (*G. duodenalis*) (Monis *et al.*, 2009, Thompson and Smith, 2011, Thompson and Monis, 2011). Concurrent parasite infections occur in canines (polyparasitism), and those previously reported to be associated with *G. duodenalis* are other protozoans, such as *Cryptosporidium* sp. coccidians, and nematodes (Kirkpatrick, 1988, Hopkins *et al.*, 1993, Dubey, 2009, Payne and Artzer, 2009). The host response to different parasites and the order of exposure to infection may affect host overall well-being and clinical manifestations (Keusch and Migasena, 1982) and virulence (Taylor *et al.*, 1998, Wammes *et al.*, 2010).

There is increasing evidence that multiple sub-genotypes of *Giardia* spp. are able to infect mammalian species. Hopkins *et al.* (1997) and Traub *et al.* (2004), indicated that genetically similar *Giardia* spp. isolates occur in dogs and humans in the same household, whilst data from a recent study in Europe, Epe *et al.* (2010) does not support the idea that the more dogs, the higher likelihood of human infection. Differences between genetically distinct *Giardia* spp. infections of asymptotically infected dogs in Germany (Leonhard *et al.*, 2007) suggest that domestic pets are more likely to carry zoonotic infections when compared to dogs in shelter situations. Knowledge concerning the clinical impacts and frequency of zoonotic transmission is inconclusive, although some dogs appear to be infected with host specific types of *Giardia* spp. other have been found to be infected with genotypes considered to be of zoonotic potential (Barutzki and Schaper, 2003, Barutzki *et al.*, 2007, Claerebout *et al.*, 2009).

Diagnostic tests for *Giardia* spp. in dogs are generally performed using fresh faecal matter and the quality of the sample is important for consistent and reliable results. Some limitations of these diagnostic methods are dependent upon microscopic detection of excreted cysts, however, recrudescence, sporadic cyst shedding and
differing levels of infection can complicate when and how much sample will yield a positive diagnosis (Hewlett et al., 1982). In cases of asymptomatic *Giardia* spp. infection and intermittent shedding of cysts, a lack of urgency exists for diagnosis and even less likelihood of follow up treatment. Although undetected infections may also pose serious consequences (T.D.R. Diagnostics Evaluation Expert Panel, 2010).

Reported comparative analyses of diagnostic methods with molecular techniques are largely confined to research studies based upon light microscopy and copro-antigen tests (Batchelor et al., 2008, Olson et al., 2010, Rishniw et al., 2010). Results from zinc sulphate floatation and light microscopy have been compared to IFAT and PCR (along with prevalence rate) for dogs and humans in Thailand. Different prevalence rates were reported, depending on the type of test used, as low as 8% using light microscopy for detection of *Giardia* spp. cysts and up to 56% in dogs detecting *Giardia* spp. antigen using ELISA (Traub et al., 2009).

A European wide diagnostic study Epe et al. (2010) reported a prevalence rate of 25% from a copro-antigen test survey of more than 8000 symptomatic dogs. Results from this study confirmed earlier findings from a retrospective study of clinical cases of gastro-intestinal disease in dogs in the UK of a true prevalence rate of 21% which takes into account the reported sensitivity and specificity of the diagnostic test (Batchelor et al., 2008).

A study of healthy Australian adult dogs using zinc sulphate centrifugal flotation and light microscopy for the diagnostic method indicated that 10 - 20% of dogs are infected with *Giardia* spp. with up to 42% infection rates in puppies, and higher prevalence rates in intensive housing situations such as kennels or shelters (Thompson et al., 2008).

### 2.1.1 Host specific infections and public health implications

The host specific assemblages C and D have been reported to be associated with clinical signs of infection in dogs (Berrilli et al., 2004, Claerebout et al., 2009). The other *Giardia* spp. positive samples isolated from dogs are assemblages A and B, both of which infect humans (Van Der Giessen et al., 2006). These potential zoonotic
assemblages are common in humans and have been implicated in cross species transmission (Thompson, 2000, Thompson et al., 2008). This suggestion for zoonotic transmission is further supported by data from multi-locus genotyping Giardia spp. infected canine faecal samples with 79% assemblage A (Traub et al., 2009), 69% (assemblages A and B) (Covacin et al., 2011) 100% assemblage A (Marangi et al., 2009, Jimenez-Cardoso et al., 2012).

Challenges for accurate diagnosis and the need for genotyping studies to contribute to understanding transmission dynamics in a global context were identified by Ballweber et al. (2010) in a public health review of the significance of Giardia spp. infections in pets, including dogs. The authors suggest that study design is an important factor for realizing the significance of Giardia spp. infections in dogs. Recommendations included the need for a trifactor approach including biological, molecular and epidemiological datasets; together with longitudinal sampling and multi-locus genotyping or case control investigations considering spatial and temporal variations.

In recognition of the increasing concern of potential zoonotic reservoirs and potential transfer of Giardia spp. infection from dogs to humans, this study examined two different high-risk groups, both from high-density populations in breeding establishments and shelter facilities. Risk factors for infection include: neonates and juveniles; poor nutritional status or eating patterns and resultant gut microflora populations and pH; poor sanitation and hygiene conditions; poorly managed animals with lack of prophylactic anti-parasite programs, intensive housing situations and dogs during periods of physiological stress (Klein, 2004, Thompson et al., 2009, Thompson and Smith, 2011)

2.1.2 Methods for diagnosis of Giardia spp. infection

A variety of methods used by different laboratories to confirm patent Giardia spp. infections from faecal samples are well published. In veterinary clinics the gold standard for detection of Giardia spp. cysts in faecal samples is considered to be density gradient flotation and light microscopy following either salt flotation (Rishniw et
zinc sulphate flotation (Zajac et al., 2002) or sucrose flotation (Dryden et al., 2006). However, others prefer to use direct smear using Lugol’s iodine or immunofluorescent antibody test (Cellabs, Australia). Detection of Giardia spp. antigen is also performed using commercially available immunochromatographic test kits and enzyme linked immunosorbent assay (ELISA) (Hopkins et al., 1993). For the purposes of this comparative study microscopy and copro-antigen tests were used to detect clinical cases of Giardia spp. infections. Molecular methods using PCR were used to confirm the presence of Giardia spp. infections and to explain the epidemiology of these infections occurring in dogs.

2.1.3 Hypothesis
That different diagnostic methods can detect Giardia spp. infections in dogs with equal accuracy.

2.1.3.1 Experimental design
The accuracy of diagnosing Giardia spp. infections was investigated in groups of dogs by comparing different diagnostic methods. Canine faecal sampling was conducted from naturally infected groups of dogs as part of a longitudinal study. Faecal samples were collected and examined for the presence of Giardia spp. cysts and reactivity to commercial copro-antigen tests. High risk groups were identified as dogs under the age of one year (puppies and juveniles), (Swan and Thompson, 1986) and dogs kept in intensive rearing or production groups (Itagaki et al., 2005, Claerebout et al., 2009, Itoh et al., 2009). Diagnostic tests were assessed by calculation of the negative and positive predictive values relative to results obtained by the light microscopy gold standard.

2.1.3.2 Sampling strategy and sample collection
Private breeding facilities and dog shelters were approached for their cooperation. One dog-breeding establishment was in Victoria, Australia, and all other samples originated from metropolitan locations in Perth, Western Australia. Samples were obtained as freshly voided faeces. Third party collections (labeling and dispatch) were transported
variable distances and times before reaching laboratory diagnostic and processing facilities. For the litters of puppies samples were collected on a weekly basis from the age of 4 weeks. Samples were pooled from litters of puppies, rather than from individual animals.

2.1.3.3 Data analysis methods

Faecal samples were collected from four different groups of dogs during this study and the prevalence data was determined for each study group. The group of purebred Labrador retrievers from Victoria Australia, were from a government owned dog breeding facility known to have prior and ongoing clinical signs of diarrhea and vomiting and confirmed diagnosis with *Giardia* spp. This group was considered to represent symptomatic infections with collection of canine faecal samples from adult dogs and puppies.

The second two groups of dogs were both from privately owned dog-breeding facilities in Western Australia, one was Labrador retriever, and the other was miniature schnauzer. The majority of canine faecal samples were from puppies under 3 months old.

A fourth group included canine faecal samples from dog shelter facilities owned by an animal welfare organization. The dogs were mainly adult dogs without clinical signs of gastro-intestinal infection and faecal samples were collected from new dogs entering the facility.

2.2 Methods

Faecal flotation and light microscopy is the standard diagnostic method for diagnosis of *Giardia* spp. infections in canines (Blagburn, 2006) and research publications report the use of light microscopy as the most frequent faecal diagnostic method performed in studies of *Giardia* spp. infections in dogs (Dryden *et al*., 2006). Light microscopy methods were considered to form the gold standard diagnostic method throughout this study.
2.2.1 Sample collection

Samples were kept refrigerated at 4°C and generally arrived at the lab for processing within 48 hours of collection. Samples were processed on arrival and an aliquot of 0.2g dispensed and stored in ethanol for future DNA extraction, if required.

2.2.2 Microscopic examination

An adapted centrifugal zinc sulphate flotation method was used to visualize parasitic eggs and cysts (Bugg et al., 1999). Firstly, zinc sulphate solution was prepared by dissolving 330g of zinc sulphate in deionized water to make 1 litre of solution and mixed thoroughly before checking for a specific gravity of 1.18. Each sample was processed by transferring 2g of fresh canine faeces into a 10ml centrifuge tube. Deionized water was added to the 10ml mark and thoroughly mixed using an applicator stick. The faecal mixture was centrifuged at 2000 rpm for 2 minutes, and filtered through gauze if the fecal material contained debris and other particulates. The supernatant was discarded and zinc sulphate solution (1.18 SG) was added to the sedimented faecal material and thoroughly mixed using an applicator stick.

The mixture was centrifuged at 2000 rpm for 2 minutes and allowed to come to rest without applying the brake. A sterile pipette was used to transfer 50 microlitres of liquid from the surface to a clean microscope slide. A coverslip was applied and immediately viewed under light microscope using 100x and 400x magnification for the presence of *Giardia* spp. cysts or trophozoites.

2.2.3 Immuno-antigen tests

Three different immuno-antigen tests were used in this study, methods are described for each test as follows,

2.2.3.1 *Giardia* Enzyme Linked Immunosorbent Assay (ELISA)

Standard operating procedures for a routine commercial diagnostic screening test made available for use in this study by Antech Diagnostics were followed to detect *Giardia* spp. antigen using spectrophotometry. Attention was paid to consistent
incubation time intervals and to ensuring reagents and materials reached room
temperature prior to use.

The principles of this test, a sandwich ELISA, used assay plates coated with primary
immobilized antibodies against *Giardia* spp. cyst antigens. Dilution buffer was added to
each well in 50µl aliquots together with 50µl of solubilized faecal samples. Each plate
was incubated at room temperature for 60 minutes to allow *Giardia* spp. antigen –
antibody binding. Then the well contents were washed five times with wash buffer
blotted dry. A conjugated secondary antibody (anti-*Giardia* spp. cyst enzyme
conjugate) was added to each well and incubated for 30 minutes. The amount of
conjugate that was able to successfully bind to form an antibody-antigen complex was
representative of the quantity of *Giardia* spp. antigen in each sample. The conjugate
was removed by washing five times and 2 drops of chromogen was added for
development of a colorimetric reaction that was measured using a spectrophotometer
plate reader at 450 and 620nm wavelength.

Positive and negative control standards were included in duplicate wells on the same
plate for each batch of test samples. All samples were tested in duplicate. Diagnostic
criteria for confirmation of *Giardia* spp. were determined based upon performance
characteristics of the control samples. For positive confirmation control samples must
have yellow coloration and an absorbance value greater than 0.5. The negative control
appeared almost colorless with an absorbance value of less than 0.08.

**2.2.3.2 Copro-antigen test 1**

The copro-antigen test 1 used in this study has been developed by IDEXX laboratories
(IDEXX Laboratories, USA) from an immunosorbent assay and requires the action of
an enzyme conjugate. Results can be obtained within eight minutes, although to
preserve the efficacy of the test, components must be kept refrigerated prior to use.

The manufacturers state that the test identifies *Giardia* species, which infect dogs, that
is *G. duodenalis*. Detection of *Giardia* spp. antigen in fecal samples indicates the
animal has ingested *Giardia* spp. cysts, may be actively infected, and may be shedding (excreting) cysts.

Manufacturers instructions for the SNAP *Giardia* antigen test (IDEXX Laboratories, USA) were followed, as described here. A test kit swab was immersed in a fresh faecal sample. The faecal-coated swab was then flooded with a conjugate-bound antibody solution and mixed. Five drops of the solution were transferred onto a sealed test device, containing a secondary antibody. The solution flowed across the device until observed in the activation circle. Immediately the lower part of the device was pressed until an audible “snap” was heard. Coloured reaction spots in the results window determine correct operation of the test and were interpreted to confirm positive and negative results, in comparison to two control spots. In positive samples, where *Giardia* spp. antigen was present in the faecal sample, the reaction between the substrate solution and conjugate-bound antibody generated a blue colour.

### 2.2.3.3 Copro-antigen test 2

Copro-antigen test 2 used in this study is a one step immunochromatographic test performed at room temperature. This test has been developed by VetAll Laboratories, (VetAll Laboratories, Korea) from an immunochromatographic assay and is based upon direct sandwich binding of antigen-antibody conjugate on a nitrocellulose pad. Results can be obtained after 5-10 minutes, and kits remain stable at room temperature (2 – 30°C for 2 years). The manufacturers state that the kit contains antibodies with two different epitopes for specific antigen binding. A test kit swab was immersed in a fresh faecal sample, to collect a maximum of 30mg faeces. The faecal-coated swab was then immersed in a buffer solution and mixed. Four drops (100µl) of the solution supernatant were transferred onto a sealed test device, containing a nitrocellulose conjugate pad, using the dropper provided. A gold colloid complex migrated along the pad membrane to meet a second monoclonal antibody. A red/purple band appeared to confirm correct operation of the test, and a second band appeared if the test was positive. The results window became invalid after ten minutes.
2.2.4 Molecular methods
The methods used for DNA extraction and genetic detection of *Giardia* spp. are outlined as follows,

2.2.4.1 DNA extraction

DNA was extracted from fresh faecal samples using the Maxwell® 16 Tissue DNA Purification Kit (Promega, Madison, USA) with the Maxwell® 16 SEV Instrument (Promega). Water, ultrapuregrade (FisherBiotechPerth, Australia), was added to samples to further dilute the extracts and reduce inhibiting factors.

2.2.4.2 Genotyping methods - nested PCR gene loci

2.2.4.2.1 Nested PCR for 18S rDNA

This PCR technique is established as a sensitive method for genetic confirmation of *Giardia* spp. infections and is valid for epidemiological and genetic characterization studies (Hopkins *et al.*, 1997, Morgan, 2000, Thompson and Monis, 2004). The external forward and external reverse primers,

(RH11) 5’-CATCCGGTCGATCTGCC-3’ and

(RH4) 5’-AGTCGAACCCTGATTCTCCGCCAGG-3’, used in the primary reaction were developed and described by (Hopkins *et al.*, 1997) to amplify a 292 bp region of the small subunit of ribosomal RNA. The secondary reaction internal forward and reverse primers were (GiarF) 5’-GACGCTCTCCCAAGGAC-3’ and (GiarR) 5’-GTCGAACCCTGATTCTCCG-3’ developed by (Read C. *et al.*, 2002) to amplify a 130 bp product. The PCR reaction was performed in 96 well plates along with a positive control and pre- and post-reaction negative controls. Each reaction consisted of 1 μl of extracted DNA, 2.0 mM MgCl₂, 2.5 μl PCR reaction buffer (67 mM Tris-HCL, 16.6mM (NH₄)₂SO₄ 4.5% TritonX-100, 0.2mg/ml gelatin), 500 μl, 4 of each dNTP, 10 pmol of each primer, 0.55 units of TAQ DNA polymerase (Fisher Biotec, Perth Australia), 2.4% cresol and 5% DMSO was added to each reaction, plus ultrapure water to a total volume 25 μl. The primary reaction, modified from (Hopkins *et al.*, 1997) used the
following conditions, denaturing step of 95 °C for 5 min, then 40 cycles of 95 °C for 30 s, 53 °C for 30 s and 72 °C for 45 s, followed by a final extension of 72 °C for 7 min. The conditions for nested reaction were the same as for the primary reaction, except that the annealing temperature was increased to 56 °C.

### 2.2.4.2.2 Nested PCR for β Giardin gene

A 753 bp fragment was amplified using the forward primer G7 and the reverse primer G759, as previously described (Caccio, S. M., 2002). Secondary PCR reaction primers, developed to amplify a 511 bp fragment by (Lalle et al., 2005), used a forward primer of 5’-GAACGAACGAGATCGAGGTCCG-3’ and a reverse primer of 5’-CTCGACGAGCTTCGTGTT-3’. The PCR reaction was performed in 96 well plates along with a positive control and pre- and post-reaction negative controls. Each reaction consisted of 1 μl of extracted DNA, 2.5 mM MgCl₂, 2.5 μl 1X reaction buffer (67 mM Tris-HCL, 16.6mM (NH₄)₂ SO₄, 4.5% TritonX-100, 0.2mg/ml gelatin), 500 μM, of 4 dNTP, 10 pmol of each primer, 0.55 units of TAQ DNA polymerase (Fisher Biotec, Perth Australia), 2.4% and 5% DMSO was added to each reaction, plus ultrapure water to a total volume 25 μl.

Amplification conditions were modified from. Caccio (2002) and Lalle et al. (2005) as follows the primary reaction was initiated with a denaturing step of 95 °C for 5 minutes, then 40 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 45 seconds, followed by a final extension of 72 °C for 7 minutes. Conditions for the nested reaction were the same as those in the primary reaction except that the annealing temperature was increased to 56 °C.

### 2.2.4.2.3 Nested PCR for Glutamate Dehydrogenase (GDH) gene

This is the third locus that was used for Giardia spp. genotyping, the amplified fragment is 450 bp which is larger than the more sensitive multiple copy 18S gene but is important for genotyping as it can distinguish between the two subgroups of assemblage A and B (Read et al., 2004). The Forward primers use were
5’TCAACGTYAAYCCYGGYTTCCGT3’ and 5’CAGTACAACTCYGCTCTCGG3’ and the reverse primer used was 5’GTTRTCCTTGACATCTCC3’.

The PCR reaction was performed in 96 well plates along with a positive control and pre- and post-reaction negative controls. Each reaction consisted of 1 μl of extracted DNA, 3.0 mM MgCl₂, 2.5 μl 1X reaction buffer (67 mM Tris-HCL, 16.6mM (NH₄)₂SO₄ 4.5% TritonX-100, 0.2mg/ml gelatin), 500 μM, of 4 dNTP, 10 pmol of each primer, 0.55 units of TAQ DNA polymerase (Fisher Biotec, Perth Australia), 6μl of 0.1% cresol and 5%DMSO was added to each reaction, plus ultrapure water to a total volume 25 μl.

Amplification conditions were modified from Read et al. (2004) and are as follows: the primary reaction was initiated with a denaturing step of 95 °C for 5 minutes, then 40 cycles of 95 °C for 30 seconds, 50 °C for 30 seconds and 72 °C for 45 seconds, followed by a final extension of 72 °C for 7 minutes. The nested reaction was altered by an increase in annealing temperature to 53 °C.

2.2.5 Visualization of gene products

Aliquots of 10 μl of PCR product were electrophoresed in a 2% agarose gel, containing 0.2% Sybrsafe TAE buffer (242g/l Tris, 57.1g/l ethanoic acid, 18.6g/l EDTA) for 30 minutes or 1 hour depending upon size of the PCR product and visualized using a UV transilluminator.

2.2.6 Sequencing of *Giardia* spp. positive canine faecal samples

2.2.6.1 DNA sequencing method from PCR product

PCR products from faecal samples were analysed by direct sequencing using the Sanger method, capillary sequencing protocol Sanger (1951) and carried out using Applied Biosystems (ABI) 3730 XL analyzer.

2.2.6.2 DNA purification

The secondary products from nested PCR reactions were purified using a commercial product, based upon magnetic beads AMPure. A known volume (10-15μl) of PCR
product was transferred to a new plate, ready for sequencing. The AMPure bead solution was mixed thoroughly and then 1.8 volumes were added to the PCR product. The solution was mixed well using a pipette and incubated for 5 minutes at room temperature. The entire microtitre plate was then placed on the magnetic base for 5 minutes. The plate remained on the magnetic base whilst the supernatant was removed and discarded using a pipette. The DNA fragments bound to the AMPure beads were retained by magnetic force to sides of the wells. The next step required addition of 200μl of 70% ethanol to each well for 30 seconds. The ethanol was discarded, and 200μl of 100% ethanol was added for 30 seconds. Finally, all traces of ethanol were removed from the plate at room temperature for 5 minutes.

The microtitre plate was removed from the magnetic base and wells were washed with 40μl of ultrapure water by repeated pipette action.

2.2.6.3 Sequencing reaction

Sequencing was performed using both Forward and Reverse primer for each sample. 1μl of Dye Terminator was mixed with 1.5 μl of 5X buffer and this solution was used in 2.5μl volumes in reaction wells with 6.5μl of sample DNA template and 1μl of each primer. The reaction plate was sealed with film and placed in a thermocycler with the following conditions: 1 cycle 96°C for 2 minutes; 25 cycles (denaturation) 96°C for 10 seconds; (annealing) 56°C for 10 seconds, (extension) 60°C for 4 minutes; 1 cycle at 14°C and held at this temperature to complete the reaction.

Post-reaction ethanol precipitation

After removing the 96 well PCR plate from the thermocycler, 1μl 125mM EDTA was added to each well. The following solutions were added sequentially to each well 1μl of 3M sodium acetate (pH4.6) and 25μl 100% ethanol. The plate was sealed and covered with aluminum foil to minimize exposure of dye terminator to UV light. The plate was placed on a vortex mixer for 15 seconds and then left to stand at room temperature for 15 minutes, then centrifuged for 30 minutes at 3000g. The plate was inverted, blotted.
dry and returned to the centrifuge for pulse spin. After removing from the centrifuge, 35 μl of 70% ethanol was added to each well and mixed by vortex for 15 seconds. The plate was covered with foil, and kept on the bench for 15 minutes before centrifuging at 1650g for 15 minutes. The plate was inverted to discard all residual ethanol and then stored at 4°C prior to sequencing.

2.2.6.4 DNA sequence analysis and genotyping

DNA sequences were viewed as chromatographs using Finch TV or Sequencher. DNA sequence identity of purified product was confirmed by rapid search using NCBI BLAST (www.ncbi.nlm.nih.gov/BLAST/).

The term ‘assemblage’ (Monis et al., 1999) in common usage for phylogenetically grouped *Giardia* spp. isolates has been further characterized by Wielinga and Thompson (2007) for *Giardia* spp. isolates infecting dogs, and these were imported from GenBank and used as reference sequences. Sequence alignment was carried out by Clustal W. Genotyping was confirmed by alignment of nucleotide sequences, using Sequencher (Gene Codes Corporation, Michigan, USA), to reference sequences. The differences between nucleotides at positions 60 and 120, identified by Hopkins et al. (1993) with SNP occurring at positions 60 and 111 Robertson et al. (2006) were compared to phylogenetic alignment data retrieved from GenBank and confirmed by (Monis et al., 1998, Wielinga and Thompson, 2007).

Genotyping into assemblages A-E as described by Monis et al. (2003) was carried out according to the published method Hopkins et al. (1997) using base pair alignment tools to distinguish between isolates based on base pair changes of the 18S rDNA gene. Sequencing at the 18S gene locus was performed with the reverse primer, both forward and reverse primers were used for sequencing at β Giardin and GDH gene loci.

2.3 Results

Results for diagnosis of *Giardia* spp. infections in canine faecal samples are presented here. *Giardia* spp. detection by a centrifugal zinc sulphate flotation and light
microscopy method was considered the gold standard diagnostic, to which all other methods were compared. Results include prevalence rates and an analysis of the sensitivity and specificity of three different copro-antigen diagnostic tests. Molecular identification of *Giardia* spp. by PCR is reported for three different gene loci and *Giardia* spp. genotyping results are presented for the different groups of dogs tested, including Labrador retriever puppies from breeding facilities.

### 2.3.1 Light microscopy *Giardia* diagnostic test results for Victoria and WA

Results shown in Table 2.3.1 include all canine faecal samples tested for *Giardia* spp. infection using the centrifugal zinc sulphate flotation and light microscopy gold standard. In total there forty-four samples were diagnosed as positive for *Giardia* spp. infection from a Victoria Labrador retriever breeding facility. Fifty-two positive samples were from a Labrador retriever breeding facility, 4 positive samples from miniature schnauzer breeding facility and 6 positive samples were from dog shelter facilities all located in Western Australia.

<table>
<thead>
<tr>
<th>Group</th>
<th>Location</th>
<th>N</th>
<th>POSITIVE</th>
<th>NEGATIVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breeder (Labrador retriever)</td>
<td>Bulla, Victoria</td>
<td>155</td>
<td>44</td>
<td>111</td>
</tr>
<tr>
<td>Breeder (Labrador retriever)</td>
<td>Perth, Western Australia</td>
<td>86</td>
<td>52</td>
<td>34</td>
</tr>
<tr>
<td>Breeder (miniature schnauzer)</td>
<td>Perth WA</td>
<td>22</td>
<td>4</td>
<td>18</td>
</tr>
<tr>
<td>Shelters</td>
<td>Perth WA</td>
<td>135</td>
<td>6</td>
<td>128</td>
</tr>
</tbody>
</table>

It was noted that *Giardia* spp. infected faecal samples were easily identifiable by a sweet smelling odour that was not present in uninfected samples. In total three hundred and ninety-eight canine faecal samples were collected during from puppies and dogs aged 15 - 1277 days of age. Some samples contained both trophozoites and cysts, although only cysts were detected in the majority of positive samples. Microscopy results confirmed that *Giardia* spp. Infections were frequently concurrent with, or preceeded by, other protozoan parasites, the significance of these concurrent infections is discussed further in Chapter 3.
2.3.2 Prevalence and infection rates

The numbers of samples tested (N) are given for each test, and all prevalence values (%), were determined using the gold standard test.

Table 2.3.2. Prevalence data

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Prevalence</th>
<th>Confidence Interval (CI) (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labrador retrievers Victoria</td>
<td>155</td>
<td>28.4%</td>
<td>21.4 – 36.2</td>
</tr>
<tr>
<td>Labrador retrievers WA</td>
<td>86</td>
<td>60%</td>
<td>48.9 – 70.5</td>
</tr>
<tr>
<td>Miniature schnauzer WA</td>
<td>22</td>
<td>18.2%</td>
<td>6.5 - 36.9</td>
</tr>
<tr>
<td>Dog shelters WA</td>
<td>135</td>
<td>4.4%</td>
<td>2.0 – 8.6</td>
</tr>
</tbody>
</table>

Prevalence rates shown in Table 2.3.2 were calculated from the number of *Giardia* spp. infected faecal samples using Quantitative Parasitology 3.0, which assumes a binomial parasite distribution. In total 20.1% (99% CI 15.07 – 25.92) of Australian dogs tested, from a total sample size of 398, were confirmed with *Giardia* spp. infections after light microscopy detection of cysts in faecal material. A higher prevalence of 28.4% (99% CI 21.4 – 36.2) was recorded for a large breeding facility of Labrador retrievers in Victoria. High prevalence rates were recorded for puppies less than 6 months of age from Labrador retriever breeding facilities in Western Australia 60% (99% CI 48.9 – 70.5). A lower prevalence rate of 18.2% (99% CI 6.5 -36.9) was recorded for miniature schnauzer puppies from breeding facilities in Western Australia. In contrast, faecal samples collected from asymptomatic, mainly adult, dogs housed in shelter situations, in Perth metropolitan area of Western Australia, showed the lowest *Giardia* spp. infection rate of 4.4%, (99% CI 2.0 – 8.6).

2.3.2.1 Longitudinal diagnostic testing for *Giardia* spp. infections in Labrador retriever puppies in Western Australia

This study collected faecal samples from litters of puppies, over a three-year period.

During 2011, thirty-nine pooled faecal samples were collected on a daily basis from 2 litters of puppies aged 35-49 days old and examined using the light microscopy gold standard. *Giardia* spp. cysts were observed in twenty-nine samples, as shown in Table 2.3.3. Results from observation of other enteric parasites were recorded and these are
presented in Chapter 3. Copro-antigen tests were not performed during the first year of data collection, due to time constraints.

Table 2.3.3 Longitudinal diagnostic test results using light microscopy gold standard

<table>
<thead>
<tr>
<th>Year</th>
<th>N</th>
<th>Positive</th>
<th>Negative</th>
<th>Age</th>
<th>Prevalence (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>39</td>
<td>29</td>
<td>10</td>
<td>35-49</td>
<td>73.7% (0.569-0.866)</td>
</tr>
<tr>
<td>2012</td>
<td>27</td>
<td>8</td>
<td>19</td>
<td>29-50</td>
<td>29.6% (0.138-0.502)</td>
</tr>
<tr>
<td>2013</td>
<td>20</td>
<td>15</td>
<td>5</td>
<td>14-85</td>
<td>75.0% (0.509-0.914)</td>
</tr>
<tr>
<td>2011-13</td>
<td>86</td>
<td>52</td>
<td>34</td>
<td>14-85</td>
<td>60.0% (0.489-0.705)</td>
</tr>
</tbody>
</table>

2.3.3 Comparison of diagnostic methods for different groups of dogs

A comparative analysis of *Giardia* spp. diagnostic tests is presented in this section. Sensitivity, specificity, positive predictive value and negative predictive values were calculated for each diagnostic test, as described by Morgan et al., (1998).

2.3.4 Comparison of three different diagnostic copro-antigen tests for *Giardia* spp. infections in two Labrador retriever breeding facilities

The sensitivity of copro-antigen test 1 was determined as 0.65 and specificity of 0.82 with a negative predictive value of 82%, for samples tested from Victoria, shown in Table 2.3.4. Samples from WA tested with copro-antigen test 1 gave a test sensitivity of 0.60 and specificity of 1.0. The positive predictive value calculated for copro-antigen test 1 for detection of *Giardia* infections indicates that 100% of all *Giardia* spp. infections in the WA breeding facility were likely to be detected. In contrast, in the Victorian population only 50% of infections were likely to be detected.

<table>
<thead>
<tr>
<th>Location</th>
<th>Test</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIC</td>
<td>Copro-antigen test 1</td>
<td>0.65</td>
<td>0.82</td>
<td>50%</td>
<td>82%</td>
</tr>
<tr>
<td>WA</td>
<td>Copro-antigen test 1</td>
<td>0.60</td>
<td>1.0</td>
<td>100%</td>
<td>76%</td>
</tr>
<tr>
<td>VIC</td>
<td>Copro-antigen test 2</td>
<td>0.50</td>
<td>0.50</td>
<td>60%</td>
<td>40%</td>
</tr>
<tr>
<td>WA</td>
<td>Copro-antigen test 2</td>
<td>0.75</td>
<td>0.60</td>
<td>82%</td>
<td>50%</td>
</tr>
<tr>
<td>VIC</td>
<td>ELISA antigen test</td>
<td>0.33</td>
<td>0.43</td>
<td>56%</td>
<td>23%</td>
</tr>
<tr>
<td>WA</td>
<td>ELISA antigen test</td>
<td>0.41</td>
<td>1.0</td>
<td>100%</td>
<td>24%</td>
</tr>
</tbody>
</table>

Ten canine faecal samples from Victoria were tested with both copro-antigen test 1 and 2 for the presence of *Giardia* spp. infections, results are shown in Table 2.3.5
Table 2.3.5 Comparison of individual copro-antigen test results to microscopy results

<table>
<thead>
<tr>
<th>Sample</th>
<th>Parasites detected</th>
<th>Microscopy</th>
<th>Copro-antigen Test 1</th>
<th>Copro-antigen Test 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Giardia</em> spp.(1+)</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td><em>Giardia</em> spp.(&lt;1+)</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>No parasitic organisms detected</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td><em>Giardia</em> spp.(1+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td><em>Giardia</em> spp.(3+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Sarcocystis sp.(&lt;1+)</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td><em>Giardia</em> spp.(&lt;1+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td><em>Giardia</em> spp.(2+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td><em>Giardia</em> spp.(1+)</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td><em>Giardia</em> spp.(1+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

8+ /2-  8+ /2-  6+4-

2.3.4.1 *Giardia* spp. ELISA test

Seventy-four samples in total from a shelter population and two Labrador retriever breeding facilities (WA and Victoria) were tested using ELISA copro-antigen plate assay test. Calculated values for sensitivity and specificity, and the negative and positive predictive values are shown in Table 2.3.6.

2.3.4.2 ELISA test for breeding facilities in WA and Victoria

Twenty-six canine faecal samples from Labrador retrievers in breeding facilities were tested for *Giardia* spp. infections using a commercial ELISA test, over half of the samples tested gave negative results. The sensitivity and specificity values of the ELISA tests relative to the gold standard test shown in Table 2.3.6 indicate that the ELISA test has low sensitivity (0.33, 0.41) and low efficacy (23%, 24%) in detecting *Giardia* spp. negative samples for both groups of dogs tested. The specificity (1.0) and PPV (100%) for detection of *Giardia* spp. infections in samples from WA using ELISA were much higher than the values obtained from Victoria (0.43, 56%).

Table 2.3.6 Sensitivity and specificity values for ELISA test relative to microscopy

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labrador retriever WA</td>
<td>0.41</td>
<td>1.0</td>
<td>100%</td>
<td>24%</td>
</tr>
<tr>
<td>Labrador retriever VIC</td>
<td>0.33</td>
<td>0.43</td>
<td>56%</td>
<td>23%</td>
</tr>
<tr>
<td>Shelter WA</td>
<td>0.17</td>
<td>0.65</td>
<td>12.5%</td>
<td>72%</td>
</tr>
<tr>
<td>All Groups Tested</td>
<td>0.35</td>
<td>0.63</td>
<td>58%</td>
<td>42%</td>
</tr>
</tbody>
</table>
The sensitivity values for *Giardia* spp. ELISA across all the groups tested were very low ranging from 0.17 -0.41, shown in Table 2.3.6. Also, some of the ELISA positive samples were also tested positive with other *Giardia* spp. diagnostic tests. Fifteen samples produced a positive result by light microscopy, nine tested positive with copro-antigen test 1, and 4 tested positive with both light microscopy and copro-antigen test 1.

### 2.3.4.3 Longitudinal testing for *Giardia* spp. infections by two copro-antigen tests

In 2012 twenty-seven canine faecal samples were tested for *Giardia* spp. infection, from puppies aged 29 - 50 days old using copro-antigen test 1, and 4 positive test results were recorded. Three samples that tested positive by microscopy for parasites were negative by copro-antigen test 1. Two of these samples were confirmed *Giardia* spp. co-infections with *Cystoisospora* spp. by microscopy. During 2013, nineteen canine faecal samples from one litter of puppies were collected from individual puppies over consecutive weeks between the ages of 68-85 days. Eight positive test results recorded using light microscopy gold standard were also tested with copro-antigen test 1. Two positive test results and six negative test results were obtained. Sensitivity, specificity, negative and positive predictive values are shown in Table 2.3.7.

<table>
<thead>
<tr>
<th>Year</th>
<th>Puppy Age (days)</th>
<th>Number tested</th>
<th>Prevalence</th>
<th>Test 1</th>
<th>Gold Standard</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>2012</td>
<td>29-50</td>
<td>27</td>
<td>14.8%</td>
<td>29.6%</td>
<td>0.67</td>
<td>1.0</td>
<td>100%</td>
<td>91%</td>
<td></td>
</tr>
<tr>
<td>2013</td>
<td>14-85</td>
<td>20</td>
<td>42.1%</td>
<td>75.0%</td>
<td>0.53</td>
<td>1.0</td>
<td>100%</td>
<td>42%</td>
<td></td>
</tr>
<tr>
<td>2012-13</td>
<td>14-85</td>
<td>86</td>
<td>26.0%</td>
<td>60.0%</td>
<td>0.60</td>
<td>1.0</td>
<td>100%</td>
<td>76%</td>
<td></td>
</tr>
</tbody>
</table>

The same faecal samples were also tested with copro-antigen test 2 however, due to time constraints only 3 samples were tested in 2012. Fourteen samples were tested in 2013 and the results are shown in Table 2.3.8.
Table 2.3.8 Sensitivity and specificity values for copro-antigen Test 2

<table>
<thead>
<tr>
<th>Year</th>
<th>Puppy Age (days)</th>
<th>Number tested</th>
<th>Prevalence</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Test 2</td>
<td>Gold Standard</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2012</td>
<td>29-50</td>
<td>3</td>
<td>66.7%</td>
<td>29.6%</td>
<td>nc*</td>
<td>nc</td>
<td>nc</td>
</tr>
<tr>
<td>2013</td>
<td>14-85</td>
<td>14</td>
<td>64.3%</td>
<td>75.0%</td>
<td>0.80</td>
<td>0.75</td>
<td>89%</td>
</tr>
<tr>
<td>2012-13</td>
<td>14-85</td>
<td>17</td>
<td>58.8%</td>
<td>60.0%</td>
<td>0.75</td>
<td>0.60</td>
<td>82%</td>
</tr>
</tbody>
</table>

*nc not calculated

One microscopy positive sample was tested with copro-antigen test 2 and a positive result was obtained. Further testing of this sample produced positive results across all three copro-antigen tests and positive by PCR at the 18s gene locus.

2.3.5 Molecular genetics of *Giardia* spp. infected faecal samples from all dogs tested in this study

Three hundred and ninety eight canine fecal samples were analysed using polymerase chain reaction (PCR) during this study. Sixty-four (16%) of the samples were confirmed positive for *Giardia* spp. at 18s gene locus. Positive samples were further analysed by PCR at beta giardin and GDH gene loci. The PCR products were sequenced and the results are reported in Table 2.3.9 where sequencing was successful. For some samples the initial attempt to obtain DNA sequences were unsuccessful, and DNA extraction was repeated from faecal samples preserved in ethanol and sequences were successfully produced. Table 2.3.10 displays a summary of the results from genotyping *Giardia* spp. infected faeces based upon gene loci and Table 2.3.11 shows results for each individual gene locus.

Table 2.3.9 Percentage of samples successfully sequenced for each gene loci

<table>
<thead>
<tr>
<th>Gene locus</th>
<th>Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>18s gene locus</td>
<td>42</td>
<td>55</td>
</tr>
<tr>
<td>Beta giardin gene locus</td>
<td>18</td>
<td>23</td>
</tr>
<tr>
<td>GDH gene locus</td>
<td>17</td>
<td>22</td>
</tr>
</tbody>
</table>

In total 44% of samples were positive at only one gene locus, 39% at 2 gene loci and 17% positive at all three gene loci tested. The majority of those samples positive using all three gene loci were those with host specific infections, assemblage C or D.
Table 2.3.10 Percentage of samples successfully sequenced at one or more gene loci.

<table>
<thead>
<tr>
<th></th>
<th>1 gene locus</th>
<th>2 gene loci</th>
<th>3 gene loci</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>28</td>
<td>25</td>
<td>11</td>
</tr>
<tr>
<td>%</td>
<td>44</td>
<td>39</td>
<td>17</td>
</tr>
</tbody>
</table>

Table 2.3.11 Results of genotyping *Giardia* spp. at three gene loci

<table>
<thead>
<tr>
<th>Number of gene loci sequenced</th>
<th>18sDNA</th>
<th>BETA</th>
<th>GIARDIN</th>
<th>GDH</th>
<th>Number of gene loci sequenced</th>
<th>18sDNA</th>
<th>BETA</th>
<th>GIARDIN</th>
<th>GDH</th>
<th>Number of gene loci sequenced</th>
<th>18sDNA</th>
<th>BETA</th>
<th>GIARDIN</th>
<th>GDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+*</td>
<td>-</td>
<td>-</td>
<td></td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
<td>3</td>
<td>A</td>
<td>D</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
<td>3</td>
<td>A/C/D</td>
<td>C</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>A</td>
<td>-</td>
<td>-</td>
<td></td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
<td>3</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>A</td>
<td>-</td>
<td>-</td>
<td></td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
<td>3</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>A</td>
<td>-</td>
<td>-</td>
<td></td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>A</td>
<td></td>
<td>3</td>
<td>D</td>
<td>D</td>
<td>D</td>
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</tr>
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<td>1</td>
<td>A</td>
<td>-</td>
<td>-</td>
<td></td>
<td>2</td>
<td>A</td>
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<td>-</td>
<td></td>
<td>3</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>A/D</td>
<td>-</td>
<td>-</td>
<td></td>
<td>2</td>
<td>A</td>
<td>+</td>
<td>-</td>
<td></td>
<td>3</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td></td>
<td>2</td>
<td>A</td>
<td>+</td>
<td>-</td>
<td></td>
<td>3</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td></td>
<td>2</td>
<td>A/D</td>
<td>+</td>
<td>-</td>
<td></td>
<td>3</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>D</td>
<td>-</td>
<td>-</td>
<td></td>
<td>2</td>
<td>C</td>
<td>-</td>
<td>C</td>
<td></td>
<td>3</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>D</td>
<td>-</td>
<td>-</td>
<td></td>
<td>2</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td></td>
<td>3</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>D</td>
<td>-</td>
<td>-</td>
<td></td>
<td>2</td>
<td>D</td>
<td>+</td>
<td>-</td>
<td></td>
<td>3</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>D</td>
<td>-</td>
<td>-</td>
<td></td>
<td>2</td>
<td>D</td>
<td>+</td>
<td>-</td>
<td></td>
<td>3</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>D</td>
<td>-</td>
<td>-</td>
<td></td>
<td>2</td>
<td>D</td>
<td>D</td>
<td>-</td>
<td></td>
<td>3</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>C</td>
<td>-</td>
<td></td>
<td>2</td>
<td>-</td>
<td>D</td>
<td>D</td>
<td></td>
<td>3</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>C</td>
<td>-</td>
<td></td>
<td>2</td>
<td>-</td>
<td>D</td>
<td>D</td>
<td></td>
<td>3</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>C</td>
<td>-</td>
<td></td>
<td>2</td>
<td>-</td>
<td>D</td>
<td>D</td>
<td></td>
<td>3</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td></td>
</tr>
</tbody>
</table>

* denotes PCR positive result, failure to obtain sequence

Host specific *Giardia* assemblages were successfully genotyped at 18s, beta giardin and GDH gene loci. Sequencing of *Giardia* spp. positive PCR product using the beta giardin locus was unsuccessful in genotyping *Giardia* assemblage A, but successful genotyping was confirmed at 18s and GDH loci.
2.3.5.1 Molecular genetics of *Giardia* spp. in dogs from shelter facilities in Perth

Seventeen samples were confirmed positive by PCR using the 18s gene locus and 15 of these were successfully sequenced. Genotyping showed that 3 samples were *G. duodenalis* assemblage A, 8 samples were *G. duodenalis* assemblage D, 3 samples were *G. duodenalis* assemblage C and one contained mixed *G. duodenalis* assemblages A/D.

2.3.5.2 Molecular genetics of *Giardia* spp. infections in breeding populations of Labrador retrievers

Genotyping results for faecal samples tested from Victoria and WA breeding facilities by PCR at 18s, beta *Giardia* spp. and GDH gene loci are shown in Table 2.3.12. Forty-four samples from Victoria were confirmed positive by microscopy. Twenty-nine samples that returned a positive result by PCR at any gene locus were successfully sequenced and genotyped. Five of these previously tested negative by light microscopy, as no *Giardia* cysts were observed. Only 7 samples were considered successfully genotyped using multi-locus genotyping (at 3 gene loci).

<table>
<thead>
<tr>
<th>Genetic characterization</th>
<th>WA</th>
<th>VIC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2011, n=39</td>
<td>2012, n=27</td>
</tr>
<tr>
<td>PCR positive</td>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td>Successfully genotyped</td>
<td>8/15</td>
<td>3/4</td>
</tr>
<tr>
<td>Assemblage A</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Mixed Assemblage</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Assemblage C</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Assemblage D</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

2.3.5.3 *Giardia* spp. genotypes in adult male dogs in a breeding population of Labrador retrievers, Victoria

Faecal samples tested for *Giardia* spp. infection from working dogs showing signs of ill thrift and failure to gain weight. Genotyping results are shown in Table 2.3.13, six dogs in the group were infected with host specific *Giardia* assemblage C/D (150 -250 days old) and the results were confirmed at all 3 gene loci. Two samples tested showed mixed infections with *Giardia* assemblage A whilst the other 4 dogs were infected with only host specific *Giardia* assemblage C and/or D.
Table 2.3.13 Genotyping results for working dogs

<table>
<thead>
<tr>
<th>Gene loci</th>
<th>Working dogs N=6</th>
</tr>
</thead>
<tbody>
<tr>
<td>18s</td>
<td>Beta Giardin</td>
</tr>
<tr>
<td>A</td>
<td>D</td>
</tr>
<tr>
<td>A/D</td>
<td>D</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>C/D</td>
<td>D</td>
</tr>
<tr>
<td>D</td>
<td>D</td>
</tr>
</tbody>
</table>

2.3.5.4 Molecular genetics – comparison of polymerase chain reaction (PCR) with the light microscopy gold standard for Victorian dogs

The positive predictive value for PCR results relative to the gold standard indicates that up to 100% of all *Giardia* spp. infections, are likely to be detected, as shown in Table 2.3.14 In the litter with the lowest prevalence rate (29.6%) the PPV was lowest at 75%.

Table 2.3.14 Sensitivity and specificity of PCR against the standard for dogs tested in WA

<table>
<thead>
<tr>
<th>Year</th>
<th>Puppy Age</th>
<th>Number Tested</th>
<th>Prevalence</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>35-49</td>
<td>39</td>
<td>38.5%</td>
<td>0.52</td>
<td>1.0</td>
<td>100%</td>
<td>42%</td>
</tr>
<tr>
<td>2012</td>
<td>29-49</td>
<td>27</td>
<td>14.81%</td>
<td>0.50</td>
<td>0.95</td>
<td>75%</td>
<td>87%</td>
</tr>
<tr>
<td>2013</td>
<td>68-85</td>
<td>20</td>
<td>50%</td>
<td>0.60</td>
<td>0.80</td>
<td>90%</td>
<td>40%</td>
</tr>
<tr>
<td>2011-13</td>
<td>29-85</td>
<td>86</td>
<td>31%</td>
<td>0.54</td>
<td>0.94</td>
<td>93%</td>
<td>60%</td>
</tr>
</tbody>
</table>

Results are shown in Appendix 1.

2.3.6 *Giardia* spp. genotypes in Labrador retriever breeding dams and litters of puppies

Faecal samples from more than 15 breeding dams were examined during this study. *Giardia* spp. genotyping results for six dams are reported in Table 2.3.15 below where data was collected for both puppies and maternal dam.

Samples positive for *Giardia* spp. infection by microscopy were confirmed positive by PCR at the 18s gene locus and beta giardin gene locus and both genotyped to Assemblage A. The other samples were PCR positive samples (18s gene locus) not previously confirmed using light microscopy gold standard.
Table 2.3.15 Genotyping results for *Giardia* spp. infections in breeding dams and litters of puppies

<table>
<thead>
<tr>
<th>Gene loci</th>
<th>Positive results by group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Breeding Dams n=11</td>
</tr>
<tr>
<td>18s</td>
<td>BG</td>
</tr>
<tr>
<td>A</td>
<td>9</td>
</tr>
<tr>
<td>A/C</td>
<td>1</td>
</tr>
<tr>
<td>A/D</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>D</td>
<td>D</td>
</tr>
</tbody>
</table>

The samples from the litters of young puppies and breeding dams were much easier to genotype at all 3 loci than the other groups of samples. The results were consistent even when DNA re-extractions and PCR reactions were repeated on faecal samples that had been frozen for 12 months or more.

Genotyping results for faecal samples from juvenile dogs (aged over 12 weeks) were mainly host specific assemblages C and or D, shown in Table 2.3.16.

Table 2.3.16 Genotyping results for juveniles (over 3 months old)

<table>
<thead>
<tr>
<th>Number of dogs</th>
<th>18s</th>
<th>BG</th>
<th>GDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>C</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>A/D</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

2.3.7 Summary of the performance of four *Giardia* spp. faecal diagnostic tests relative to light microscopy in two breeding populations of dogs

A comparative summary of the different diagnostic tests used to determine *Giardia* spp. infections in two different populations of dogs is shown in Table 2.3.17.
Table 2.3.17 Summary of sensitivity and specificity tests for *Giardia* spp. diagnostic tests

<table>
<thead>
<tr>
<th>Diagnostic Tests</th>
<th>Test performance</th>
<th>Sample Source (State)</th>
<th>WA</th>
<th>VICTORIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference Standard</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light Microscopy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Prevalence</strong></td>
<td></td>
<td></td>
<td>60%</td>
<td>28.6%</td>
</tr>
<tr>
<td><strong>n</strong></td>
<td></td>
<td></td>
<td>n=86</td>
<td>n=155</td>
</tr>
<tr>
<td>Test 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td></td>
<td></td>
<td>0.6</td>
<td>0.65</td>
</tr>
<tr>
<td>Specificity</td>
<td></td>
<td></td>
<td>1.0</td>
<td>0.82</td>
</tr>
<tr>
<td>PPV</td>
<td></td>
<td></td>
<td>100%</td>
<td>50%</td>
</tr>
<tr>
<td>NPV</td>
<td></td>
<td></td>
<td>76%</td>
<td>82%</td>
</tr>
<tr>
<td><strong>n</strong></td>
<td></td>
<td></td>
<td>n=46</td>
<td>n=155</td>
</tr>
<tr>
<td>Test 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td></td>
<td></td>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td>Specificity</td>
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<td>0.60</td>
<td>0.60</td>
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<tr>
<td>PPV</td>
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<td>82%</td>
<td>60%</td>
</tr>
<tr>
<td>NPV</td>
<td></td>
<td></td>
<td>50%</td>
<td>60%</td>
</tr>
<tr>
<td><strong>n</strong></td>
<td></td>
<td></td>
<td>n=17</td>
<td>n=10</td>
</tr>
<tr>
<td>ELISA</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td></td>
<td></td>
<td>0.45</td>
<td>0.33</td>
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<tr>
<td>Specificity</td>
<td></td>
<td></td>
<td>1.0</td>
<td>0.43</td>
</tr>
<tr>
<td>PPV</td>
<td></td>
<td></td>
<td>100%</td>
<td>56%</td>
</tr>
<tr>
<td>NPV</td>
<td></td>
<td></td>
<td>24%</td>
<td>23%</td>
</tr>
<tr>
<td><strong>n</strong></td>
<td></td>
<td></td>
<td>n=26</td>
<td>n=22</td>
</tr>
<tr>
<td>DNA PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td></td>
<td></td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
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<td>0.94</td>
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<td>93%</td>
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<td>NPV</td>
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<td>60%</td>
<td></td>
</tr>
<tr>
<td><strong>n</strong></td>
<td></td>
<td></td>
<td>n=86</td>
<td></td>
</tr>
</tbody>
</table>

n= number of samples tested

2.4 Discussion

2.4.1 Comparison of *Giardia* spp. diagnostic tests

The diagnostic standard used throughout this study, centrifugal zinc sulphate flotation and light microscopy had low values for sensitivity (0.31) and specificity (0.74). The Positive Predictive Value (48%) suggests that using this method of diagnosis for this dog population (breeding facility with *Giardia* spp. prevalence rate of 28.6%) would detect less than half of *Giardia* spp. infected dogs, similar to (Geurden et al., 2008, Gates and Nolan, 2009a). However the accuracy of this method for confirming dogs without *Giardia* spp. infections was relatively poor, the Negative Predictive Value was only 58%, much lower than the value (81-95%)
Chapter 2 Comparative Diagnostics

reported by (Geurden et al., 2008).

In summary, the performance of the four different Giardia spp. diagnostic tests shown in Table 2.3.16, shows that the positive predictive value PPV, was consistently higher for the WA population, for all tests used, and an overall higher Giardia spp. prevalence rate suggesting a high level of confidence in diagnostic accuracy. There were apparent differences in the types of Giardia spp. infections diagnosed in dogs from WA and Victoria. Results for copro-antigen test 2 and ELISA indicate that these tests were not able to accurately confirm uninfected samples.

Data in the Table 2.3.5 shows test results for a small subgroup of ten samples (from a larger group) tested for Giardia spp. with copro-antigen tests 1 and 2. Results for these ten samples using copro-antigen test 2 indicated fewer positive test results than copro-antigen test 1, relative to light microscopy. The sensitivity and specificity values calculated for test 2, as shown in Table 2.3.4 were calculated to be 0.5 and 0.5 respectively, relative to the gold standard. Calculation of the positive predictive value indicates that 60% of Giardia spp. infections are likely to be detected using test 2 in canine faecal samples containing Giardia spp. cysts and 40% of test results can accurately confirm the absence of Giardia spp. infection.

2.4.1.1 Individual and pooled faecal samples

The levels of test sensitivity, indicating the effectiveness of the test to detect infections, was highest for samples collected from individual puppies (0.8), and lowest for the litter of puppies aged 29-49 aged days. The highest PPV for light microscopy gold standard diagnostic (76%) was for puppies aged 35-49 days where pooled faeces were collected from mixed litters of puppies, and lowest (27%) for the litter of puppies which were older and sampled individually.

Nevertheless, despite the fact that samples came from the same breeding facility, the puppies were from different litters. The level of exposure reflected by the disease status of the dam together with genetic pre-disposition may exert higher levels of influence on infection rates in newborn puppies.
At lower prevalence rates, reduced specificity and positive predictive value for the copro-antigen tests suggests that genetic confirmation by PCR methods is important.

2.4.1.2 Comparison of test standard (Light Microscopy) with immuno-antigen tests

Previously published results have compared copro-antigen tests with molecular genotyping or microscopy methods (Rimhanen-Finne et al., 2007), but few studies have compared different immuno-antigen tests for Giardia spp. diagnostics in dogs (Geurden, et al., 2008).

Three copro-antigen tests used in this study showed variability in sensitivity and specificity values, summarized in Table 2.3.17. Increased detection rate using microscopy and copro-antigen tests included both dogs that are excreting cysts and those dogs that were infected and not excreting intact cysts, but where the presence of a cyst wall antigen was detected. This is likely to indicate the excystation transition phase to trophozoites has occurred, or is occurring.

For the longitudinal diagnostic testing of a breeding population of Labrador retriever puppies (WA) the prevalence rate over three years was 60%, a high rate of infection. Combined with light microscopy, a combined diagnostic test series produced consistently higher scores for sensitivity specificity and PPV and NPV than if immuno-antigen test was used alone. Similar results were also found for the Copro-antigen test 2 when used in series with light microscopy. It is worth noting that all the positive test results for Copro-antigen test 2 were from faecal samples with no signs of clinical infection (asymptomatic). More tests would need to be performed on dogs experiencing clinical signs of infection.

The results in Table 2.3.5 indicate that the ELISA plate assay was most effective at detecting Giardia spp. positive samples when the prevalence rate was high (100% PPV) and less effective at low prevalence levels, in shelter dog group (12.5% PPV). The frequent occurrence of false negative results was reflected in low NPV values (23 – 72%).
When results from all groups of dogs were considered as a combined group, the ELISA test failed to perform as well as any of the other immune-antigen tests giving a PPV of 41-58%. Results from the ELISA test used to diagnose *Giardia* spp. infections in faecal samples from Western Australian puppies with high prevalence rate showed high sensitivity and in the absence of false negatives gave 100% NPV. However, the NPV for the Victorian group was only 25%, most likely due to the presence of low levels of antigen caused by asymptomatic or recurrent infection. This low efficacy in excluding non-infected individuals could be attributed to the presence of *Giardia* spp. antigen in most samples, regardless of clinical signs of infection, or to the diagnostic test preferential detection of one genotype over another. So, for the case of mixed infections, one genotype may be detected by immuno-antigen reaction but another genotype may remain undetected by other tests.

### 2.4.2 Molecular diagnostics

The confirmed detection of *Giardia* spp. DNA in 16% (64/398) of all samples and 28% (18) of these genetic positives were negative by light microscopy has confirmed the findings of other researchers that diagnosis by light microscopy alone is an underestimate (Palmer *et al.*, 2008). Furthermore, only five samples that tested negative by light microscopy and returned positive results by DNA sequencing were confirmed positive using the copro-antigen test, which suggests that as many as 13% of all *Giardia* spp. infections remain undiagnosed using current diagnostic methods.

#### 2.4.2.1 Comparison of the test standard with polymerase chain reaction (PCR)

In the litter with the lowest prevalence rate (29.6%) the PPV was lowest at 75%. The NPV was 40-87%. The failure to genotype all samples at multi-locus genes from canine faecal samples is common (Covacin *et al.*, 2011, Gomes *et al.*, 2011). No assemblage B isolates were confirmed by sequence analysis by any of the three gene loci used in this study. PCR is an important tool for genetic characterization and understanding epidemiology and transmission routes. However, is it actually possible to separate
epidemiological and clinical studies as suggested by Geurden et al. (2008), when the presence of clinical signs are unclear and the diagnosis unsubstantiated.

2.4.2.2. Discordant diagnostic results

The source of samples was blinded during this study to minimize biased interpretation of results. The data presented here supports an explanation from a comparative study to detect Giardia spp. infections in humans for discrepant results which occur when microscopy results are positive and immunoassay results are negative (Johnston et al., 2003). The authors suggest this may occur due to low levels of Giardia spp. cyst intensity whilst also noting that positive controls in commercial kits were far more intensely coloured than results for positive test samples, even those with highest cyst intensity, as was the case here. One study, Gates and Nolan (2009a), focused attention on technical troubleshooting. Maybe such small quantities of faecal material, 1g (less than 0.5 % of total stool from large dogs) are not sufficiently representative, as it is unlikely that parasitic cysts are evenly distributed throughout a stool sample. Whilst sporadic cyst shedding is a reality facing diagnostic technicians, it is unconfirmed if multiple sampling strategies actually generate a more accurate picture of Giardia spp. infections (Rimhanen-Finne et al., 2007).

The results from this study confirm that detection of antigenic protein occurs less frequently than observation of parasitic Giardia spp. cysts by light microscopy. There were differences in diagnostic test results using the different copro-antigen tests. As the details of the antigens included in the commercial diagnostic tests are unknown, it is possible that one test is preferentially detecting a particular genetic isolate.

If the antigen is only expressed during trophozoite replication or attachment it is possible that any cysts which have been ingested and failed to become active would be excreted. This explanation could account for the false negative results reported, which were more frequent than false positive results, similar to (Dryden et al., 2006).
The number of cysts observed did not appear to influence copro-antigen test results, but increased numbers of cysts in faecal samples may be associated with the ability to elicit PCR results.

### 2.4.3 Gold standard diagnostic validation

Standards for reporting diagnostic tests in a medical context have been reviewed for methods lacking a gold standard Rutjes et al. (2007). The authors recommend validation of tests by statistical methods, clinical data or reference standard development. The comparative diagnostic analysis reported for Giardia spp. infections in the current study was performed in the absence of a gold standard. Attempts to determine the accuracy of the light microscopy gold standard using clinical signs of infection were inconclusive as other parasites were also detected by microscopy. A gold standard diagnostic requires a study which generates a two by two table to express test results as Disease Positive with clinical infection and Disease Negative in the absence of clinical infection (Greenhalgh, 1997). Accurate assessment of clinical (and subclinical) signs is an important factor for appropriate development of *Giardia* spp. diagnostic tools. Of critical importance is the impact of infection in the population under study. Many studies refer to asymptomatic infections, assuming no detrimental consequences caused by the infection.

In addition to the diagnostic methods described in this study another interesting factor associated with canine faecal samples positive for *Giardia* spp. was a distinctive odour. Whilst frequently referred to as malodorous, (Carlin *et al.*, 2006, Caccio and Ryan, 2008) the *Giardia* spp. positive faecal samples could be identified when the pots were opened, due to a distinctive “sweet odour” not present in other samples. Whilst this observation is neither a proven or preferred diagnostic method, it is worthy of further investigation. Olfactory detection methods have reported for other infectious and intestinal diseases using Enose (Bijland *et al.*, 2013).
2.4.4 Longitudinal data

The collection of faecal samples from litters of puppies at the same breeding facility over a three-year period clearly demonstrated ongoing *Giardia* spp. infections at higher rates than published for the wider dog population.

Longitudinal diagnostic testing of puppies showed a prevalence rate at one WA Labrador retriever breeding facility of 60%. This is a high rate of infection, the only other published prevalence rate that exceeds those reported here were for puppies in remote Canadian communities (74%) (Himsworth *et al.*, 2010). These high infection rates are indicative of high levels of exposure to cysts and reinfections in dogs, either because they are housed together, or due to poor levels of hygiene. Another possible explanation is the genetic pre-disposition of the host breed, such as reported by Upjohn *et al.* (2010) for purebred Rottweilers.

Reinfections are likely to be occurring within the facility and the lower rates of infection in the second year could be due to chance sampling from uninfected puppies, when using pooled sampling methods or an uninfected maternal dam. The timing of collection and age of the dogs sampled is also an important factor and is discussed more in the context of patent infection in Chapter 3.

This study has shown the importance of longitudinal studies (Hamnes *et al.*, 2007) and has shown to be valuable for monitoring infections in puppies, as recommended by Ballweber *et al.* (2010). Whilst this study was able to conduct repeated sampling of individual puppies, unfortunately it was unable to monitor presence of parasite infections in maternal dams over time pre- and post-consecutive whelping. Point prevalence studies are limited in their ability to capture dynamics of parasite infections and host-parasite interactions.

2.4.4.1 Prevalence and infection rates

The prevalence of *Giardia* spp. Infections in Labrador retriever puppies (60%) aged 14-56 days was nearly three times higher than for miniature schnauzers (18.2%)
aged 14-72 days. Prevalence rates for different populations of dogs tested in this study varied according to characteristics of the populations being investigated. The differences were attributed to different geographical locations, breeds and ages of dogs, management practices and potential exposure to *Giardia* spp. and other enteric parasite infections. Prevalence rates for *Giardia* spp. Infections differed between dogs from two different purebred breeding facilities.

### 2.4.4.2 Subpopulations

Prevalence rates for different subpopulations of dogs showed that *Giardia* spp. Infections were highest in young Labrador retrievers 75% (14-85 days) and this supports the findings of others (Itoh *et al.*, 2005, Batchelor *et al.*, 2008, Epe *et al.*, 2010, Uehlinger *et al.*, 2013). The overall higher rates of infection in litters of puppies was considerably higher with almost 100% infection rate in some litters at certain points in time.

Many studies reporting overall prevalence rates may create a misleading picture of *Giardia* spp. epidemiology if the differences which exist within different discrete groups of dogs, one considers as suggested by (Carlin *et al.*, 2006).

### 2.4.5 Host pre-disposition

There are already indications that in human infections two different European isolates of *Giardia* spp. exist, of Polish and Belgian origin, (Homan *et al.*, 1992, Mayrhofer *et al.*, 1995, Van Keulen *et al.*, 2002). Different distributions of the different genetic assemblages that occur in humans of dogs have been reported from studies in the USA (Nash and Keister, 1985) and a predominance of sub-genotype A in remote Australian communities (Hopkins *et al.*, 1997).

#### 2.4.5.1 Breed differences

Diagnosis of *Giardia* spp. Infections by the light microscopy gold standard was much lower in faecal samples from the miniature schnauzer breeding facility (18.2%) although this is located in the same catchment area (within 20 kilometres) as the
Labrador retriever breeding facility (60% infected) in Western Australia. Similar studies found conflicting results, Hamnes et al. (2007) examined Giardia spp. Infections in purebred dogs including Labrador retrievers, but obtained no results for differences between breeds whereas Fontanarrosa et al. (2006) suggested higher infection rates in purebred dogs. The differences may be due to different host genetics, or may be due to husbandry and management system for large groups of dogs. It was confirmed during this study that some dogs imported for breeding purposes might be a contributing factor for introducing Giardia spp. Infections. Facility management may be an influencing factor, however the propensity for host pre-disposition and possible parasite co-evolution should be considered. Further detailed studies would be required, including statistical validation, to determine if breed differences do exist, which affects host susceptibility to Giardia spp. Infections.

2.4.5.2 Dogs from shelter facilities

The Giardia spp. Infection rate in shelter dogs was lower than expected and could be due to the sampling method, as most were dogs older than 12 months and many were new to the shelter, similar to results found by Upjohn et al. (2010) and Swan and Thompson (1986) (14.4%). This suggests that they could be lost dogs from well cared for homes. Nevertheless it is more likely that those dogs resident at the shelter for longer periods, genuine ownerless strays or young or sick dogs at the shelter, would be more likely to have higher infection rates. Most infections successfully sequenced were shown to be host specific assemblages.

2.4.6 Geographical and ecological variation

Survival of Giardia spp. in the environment may pose a risk, recent reports have already identified the changes in distribution of some parasites as an impact of climate change (World Health Organization, 2003). Despite environmental factors and exposure to infection this study supports the view that some hosts may be more susceptible than others to particular Giardia spp. Genotypes.
Some researchers have recognized that geographic and climatic variations on a global scale have a consequence for localized weather patterns, habitat, and ecology, including for parasites such as *Giardia* spp. (Leonhard et al., 2007, Ballweber et al., 2010, Covacin et al., 2011). *Giardia* spp. prevalence rates might be affected by seasons, or climate throughout the world as found for *Cryptosporidium* spp. (Kirkpatrick, 1988, Hamnes et al., 2007, Britton et al., 2010, Lal et al., 2012). It is apparent that the occurrence is not as marked as for *Cryptosporidium* spp. or *Plasmodium* spp., infections although with appropriate tools for of spatial, temporal and weather related information is being compiled for *Giardia* spp. Infections in dogs (Fontanarrosa et al., 2006). Monitoring the patterns of infectious outbreak may be performed using a systematic parametric framework similar to the one described by Naumova et al., (2007). Research of this nature is important because of the variation in environmental and climatic conditions, human population density, and pet ownership in different countries. Inclusion of more than a single parasite species may improve the validity and application of data models (Viney and Graham, 2013). Parasitic organisms are major health concerns for communities where conditions are conducive to spread of infectious disease, including climate, large populations, recent industrialization and large-scale deforestation (Wilcox and Colwell, 2005, Bates et al., 2008, Wilkes et al., 2011, Nuwagaba and Namateefu, 2013). *Giardia* spp. Infections can directly cause debilitating disease and death in children and contribute to the subsequent indirect effects of missed educational and employment opportunities – key contributors to poverty, are particularly evident in developing countries (Thompson, 2004, World Health Organization et al., 2006).

### 2.4.7 Genotyping of *Giardia* spp. Infections in adult dogs

In total, 65% of samples confirmed positive for *Giardia* spp. By microscopy also tested positive using PCR methods. This correlates well with PCR positive test results for canine faecal samples from USA (62%) (Covacin et al., 2011). The limited success for multi-locus sequence analysis, only 7 samples, indicates that genotyping at one locus
may produce unreliable results, although the reasons for this remain unexplained. Only one dog showed infection with potentially zoonotic specific genotype, assemblage A. Results from this study for asymptomatic dogs found that sequence alignments to assemblage A at 18SrDNA were unconfirmed using multi-locus genotyping. This indicates that mixed infections were frequent and that none of the adult male dogs were infected with only zoonotic *Giardia* spp. isolates.

### 2.4.8 Clinical signs of infections in adult male dogs

This study has demonstrated that testing for *Giardia* spp. infections in individual adult male dogs displaying sub-clinical signs, such as loss of appetite, lacklustre body condition and weight loss, or failure to gain weight, generated positive results, similar to clinical cases reported for *G. duodenalis* infections in children (Ajjampur *et al.*, 2009). Recognition of sub-clinical signs of infection is important and is discussed further in Chapter 3. These include signs such as anorexia, failure to thrive or general ill-thrift (Table 2.3.12) which present sporadically, not necessarily on consecutive days.

The ‘asymptomatic' infections detected here were successfully genotyped and shown to be exclusively host specific *Giardia* spp. assemblages C and D, although the presence of mixed assemblage sequences was also confirmed in two samples. Detection of *Giardia* spp. infections in the absence of classical signs of infection, such as diarrhea, vomiting and flatulence, suggests an ongoing problem of asymptomatic *Giardia* spp. infections within this group of dogs. This is similar to the reports of abdominal pain rather than diarrhoea in humans infected with *G. duodenalis* (Hussein *et al.*, 2009). Numerous studies report the incidence of asymptomatic infection, but few have pinpointed the significance and implications of circulating asymptomatic *Giardia* spp. infections within populations of dogs. Determining the cause of symptomatic cases of infection becomes more important in understanding the impact of asymptomatic infections.

The faecal collection methods used and potential limitations to the design of this study
include (1) the maternal dam – her willingness for puppies to be handled and her persistence on removal of excreta whilst the puppies were young, and therefore unavailable for sample collection purposes. (2) Pooled samples from litters may randomly miss *Giardia* spp. infected puppies. (3) The aim of regular and multiple faecal sample collections was to overcome inaccuracies posed by sporadic cyst excretion (but difficult to check that this was actually improved). (4) The use of stool consistency as an indicator of clinical signs of infection. The results show that samples collected from individual puppies, rather than pooled samples gave higher Negative Predictive Values.
Chapter 3

Enteric parasite infections in dogs – determination of the pre-patent period and clinical signs of infection

3.1 Introduction

Protozoan parasite infections affect young dogs (*Canis familiaris*), with higher prevalence rates reported in intensive housing situations, such as kennels or shelters, where rapid and high rates of transmission can occur among individuals (Bajer et al., 2011). There is little published data documenting the earliest pre-patent for enteric protozoan infections in dogs, such as *Giardia* spp. especially in canines; published data focuses on infections in humans. Questions that remain to be answered concern the correlation of confirmed parasite infections, host exposure to *Giardia* cysts, and the presence of clinical signs of infection. What role do these sub-clinical infections have in the circulation of infectious organisms and potential zoonotic transmission cycles?

3.1.1 Patency of *Giardia* infection

Transmission studies using *G. duodenalis* trophozoites and cysts to artificially infect domestic and wildlife species (including dogs) have shown the pre-patent period to be of 5-22 days and that infections may last as long as six months and that apparent host re-infection and cyclic cyst excretion occurs (Davies and Hibler, 1979, Hewlett et al., 1982). Artificial infection studies in humans established that variation in pathogenicity occurs between *G. duodenalis* species. Clinical signs may occur after patency is first observed, where the average pre-patent period was recorded as 7.5 days (Nash et al., 1987).

3.1.2 Clinical signs of infection in dogs

Some studies have suggested that clinical signs of *Giardia* spp. infection in dogs are caused by infection of host specific *Giardia* spp., assemblages C and D (Barutzki et al., 2007). It is important to know which genetic *Giardia* spp. strains can cause infection in
humans, which produce clinical signs and are the cause of subsequent re-infection or
the transmission potential to other individuals or species. Cross-species studies are
needed which can assess both development of patent infections and determine the
genotype. It is important to understand the pre-patent period, patency duration,
evidence of cyclic cyst excretion to establish the opportunity and frequency of *Giardia*
spp. re-infection, which may vary according to *Giardia* spp. genotype or between host
species.

### 3.1.3 Prevalence of canine enteric parasites

It is common for dogs to be infected by more than one parasite at one point in time, a
number of recent studies report on multiple parasite infections, concurrent with *Giardia*
spp. infection in dogs; Batchelor *et al.* (2008) and Hamnes *et al.* (2007) in Europe and
and *Cystoisospora* sp., infections in Japan, with half of all dogs having multiple parasite
infections, raises questions concerning the source of infections as 88.3% of dogs were
housed indoors. Reported results were based upon fresh faecal examination and
copro-antigen tests, although genetic characterization was absent. The prevalence of
*Giardia* spp. and *Cystoisospora* infections was significantly higher in symptomatic dogs
(7.9%) than asymptomatic cases (1.6%).

A study conducted in India indicated that dogs were infected with multiple parasite
infections, with 28.7% infected with 3 concurrent parasite species and were suggested
to serve as indirect reservoirs of parasite infection (Traub *et al.*, 2002). This study in
India confirmed patent infections in 99% of dogs tested with identification of 16 different
parasite species. *Giardia* spp. infection rates were low, and 9% of dogs were infected
with coccidian species. Significantly higher infection rates were observed in young
dogs which is in agreement with results from the longitudinal study by Hamnes *et al.*
(2007) investigating multiple parasite infections of *Giardia* spp., *Cryptosporidium* sp.
and helminths in dogs, during the first year of life. Results from this study indicated that
13.6% of dogs were suspected of being re-infected with *Giardia* spp. and
Cryptosporidium sp. between different sampling dates, although there was no indication if other protozoan species were present. Similar evidence for re-infection rates were published by Gates and Nolan (2009b).

A national survey of 1400 asymptomatic Australian dogs by Palmer et al. (2008) reported that protozoan infections were common (16% of all dogs surveyed). Detection methods used in the study used light microscopy to confirm more than 10 parasite species in faecal samples. Multiple parasite infections were confirmed in 4.1% of all dogs tested, although the authors support the view that prevalence rates are likely to be underestimated (Gates and Nolan, 2009a). This study confirmed earlier work using per rectum random sampling of dogs in Buddhist communities of Thailand (Traub et al., 2009) where sophisticated molecular diagnostic tools determined the prevalence of Giardia spp. infections to be high (56.8% in dogs and 20.3% in humans). In contrast, Inpankaew et al. (2007) reported 7.9%. Giardia spp prevalence rates in dogs using zinc sulphate flotation and light microscopy methods.

3.1.4 Significance of concurrent infections

The majority of parasitology research has focused on detection and control of parasite species in isolation. There have been minimal attempts to address parasite infections at a community or catchment level. Stoll (1947) alluded to the parasitization of communities, although mainly in the context of helminth infections, with the notable absence of protozoan parasites.

This study uses the term mixed or concurrent infections in acknowledgement of synonymous expressions common in other epidemiological settings describing ‘polyparasitism’ or multi-parasitism. Steinmann et al. (2008) argues from a systematic biology standpoint that multi-parasitism has not been fully accounted for as morphological differentiation between many known parasite species is not possible, suggesting that well recognized parasites might actually form a complex of several species, distinguishable only by the use of molecular tools.
Houk et al. (2013) are of a similar opinion when they refer to *Cystoisopora* "ohioenis-like" parasitic organisms due to the inability to distinguish between different species by morphology, definitive confirmation of *Cystoisospora* species is not possible by microscopy alone. A wide range of factors influence the survival of parasite communities and the interrelationships between different parasites affect pathogenicity (Petney and Andrews, 1998).

Keusch and Migasena (1982) presented polyparasitism as a ‘biological norm’ in the human context and reflect upon the ideas of Buck *et al.* (1978) who proposed that parasite infections are self-limiting by host, population size and ecological factors. This idea is also supported by research indicating that males of mammalian species are more frequently parasitised by multiple species than females, suggesting some type of protection mechanism for unborn and young offspring (Budischak *et al.*, 2012). Murray *et al.* (1978) suggested that parasites compete for access to the host, and inevitably one species wins at the expense of the other species.

### 3.1.5 Pathogenicity and concurrent infections

The effect of multiple parasite infections may exert synergistic impact on the host. This may occur due to cooperative interactions between parasites to overcome the host immune defence system. There is strength in this concept, as it is known that *Giardia* spp. is not invasive, but has a specialised attachment mechanism and subsequent disruption of the membrane barrier inducing cell leakage (Cotton *et al.*, 2011, Buret and Cotton, 2011). This attachment mechanism is still far from being fully understood, with cell biologists supporting either a physical mechanism, ligand binding to surface proteins; or immunologic secreted protein interactions (Ortega-Pierres *et al.*, 2011).

However, phenotypic alteration first became apparent in polyparasitized hosts when Blom *et al.* (1979) showed elevated levels of alpha-1-acid glycoprotein, alpha-1-antitrypsin and ceruloplasmin in serum from human patients with concurrent helminth infections. These acute-phase protein reactants present in serum suggested an associated inflammatory reaction although the functional significance and metabolic
consequences were unknown. Lefèvre et al. (2009) discuss the cascade effects of phenotypic alterations in parasitized hosts suggesting that parasite survival mechanisms extend beyond the physiology of individual hosts. Parasites are attributed with the potential to modify host population ecology, competition processes, food web structures and metabolic flows of energy and nutrients.

3.1.6 Transmission routes and artificial infection studies

Questions concerning the reliability of results from artificial transmission studies which, were unable to provide assurance for the absence of prior, or accidental, infections of *Giardia* spp. This directed an interest in using specific pathogen free (SPF) hosts. Consequently, Mongolian gerbil (*Meriones unguiculatus*) studies confirmed that patent infections could develop from human, dog, cat and, sheep isolates of *Giardia* spp. (Gasser et al., 1987, Visvesvara et al., 1988).

Experimental infection studies in Mongolian Gerbils (*M. unguiculatus*) with *Giardia* spp. isolates of human origin successfully demonstrated 100% infection rates for one isolate, however not all gerbils in the experiment became infected leading researchers to conclude that only certain *Giardia* spp. isolates had infective capability (Visvesvara et al., 1988). Prepatent period variation occurs between hosts infected with *Giardia* spp., both interspecies and intraspecies infections, in a similar manner to that reported for *Cryptosporidium* sp. (Xiao et al., 2004).

These artificial infection studies clearly demonstrate the infective capability of cysts and trophozoites and that inter species infections can occur. What is not clear from this data – is the specific *Giardia* genotype responsible for infection. The early work using artificial infection studies precede the start of genetic characterization methods, and the phylogenetic alignment work for establishing *Giardia* genotype assemblages (Andrews et al., 1989, Mayrhofer et al., 1995, Ey et al., 1996, Hopkins et al., 1997).

Whilst artificial infection of dogs with *Giardia* cysts and trophozoites is achievable infection is not always successful, even in young dogs (Hewlett et al., 1982). The lack of success in artificial infection could be due to host immunity, but could equally be
attributed to the *Giardia* genotype of the infective cyst or trophozoite. That is, genetic variation of cysts or trophozoites isolated from different hosts. *Giardia* spp. cysts isolated from humans are most likely to be genotypic assemblages A or B, unlike the host specific canine assemblages C and D.

### 3.1.7 Risk factors for canine parasite infection

The first reports of *Giardia* spp. infections in dogs in Western Australia usefully demonstrated that higher prevalence rates occur in younger dogs, or those dogs considered to be stressed or on a diet of poor nutritional value, such as puppies from breeding facilities or from dog shelters (Swan and Thompson, 1986). Furthermore, Swan and Thompson suggest that *Giardia* spp. may not be the cause of clinical signs as the incidence of clinical signs did not correlate with *Giardia* spp. infections, although the study failed to detect or report on the presence of any other parasites.

Thompson (2000) raised the issue of *Giardia* spp. re-emerging as an infectious disease with potential zoonotic origins. Whilst risk factors include host and parasite elements, environmental factors such as population density, environmental pollution and quality of pet care and animal welfare are also critical factors contributing to the incidence of infectious parasitic disease (Gates and Nolan, 2009). The patterns of cyclic excretion of *Giardia* spp. cysts may also be critical factors in transmission. In recognition of the increasing concern of potential zoonotic reservoirs and potential transfer of *Giardia* spp. infection either to or from dogs to humans this study examined two different high-risk groups. Risk factors for infection include; neonates and juveniles; poor nutritional status or eating patterns and resultant gut microflora populations and pH; poor sanitation and hygiene conditions; poorly managed animals with lack of prophylactic anti-parasite programs, intensive housing situations and dogs during periods of physiological stress (mating, pregnancy, whelping). The groups studied were puppies from breeding facilities and dogs kept at dog shelter facilities.
3.1.8 Environmental and economic factors

Findings from different countries appear to support data from a retrospective study by Gates and Nolan (2009) identifying socio-cultural factors as an important factor influencing the likelihood of parasite infections in dogs. Dog ownership in high density population areas, synonymous with lower income households, were more frequently infected than those from low density housing areas. Contrasting parasite profiles occur in animals from different socio-economic groups. Experimental evidence has shown that there is predominantly less diversity in parasite species in wealthier communities (Traub et al., 2002). The literature reports that Giardia spp. infections are common in intensive, well cared for or semi-domestic dogs (Gates and Nolan, 2009) and this observation is supported by other research studies (Itoh et al., 2009, Itoh et al., 2011).

An epidemiological study of parasite infections in stray and pet dogs in Spain, by coprology and necroscopy examination, (Martinez-Carrasco C. et al., 2007), noted 25% of dogs carried patent infections and that variation occurred with seasonality and climate; Cystoisospora sp. infections were reported at ten times higher levels than Giardia spp.

3.1.9 Host pre-disposition

Host characteristics, such as breed and age; are also associated with higher prevalence rates of multiple parasite infections, where juvenile dogs under 6 months of age have been shown to be infected with 4 parasite species, where Giardia spp. and Cystoisospora co-infections were common (Hamnes et al., 2007, Batchelor et al., 2008).

There is a similarity in infection rates between the two protozoan pathogens Giardia spp. and Tritrichomonas species with similar distinctive morphological features, including three or more anterior flagella, and musco-flagellate features for invasion and disruption of the mucosal epithelial membranes of gastro-intestinal or reproductive tract. These two pathogens infect intensively housed or stressed domestic pets, in both cats and dogs (Payne and Artzer, 2009). Accordingly, as many questions concerning
excretion of *Giardia* spp. cysts and transmission mechanisms currently remain unanswered. Veterinarians and pet owners need to be aware of the potential risks from zoonotic disease, or implications of anthroponotic transmission (Zajac et al., 2000, Thompson et al., 2008). If, indeed the incidence of protozoan infections of more affluent communities in humans and domestic pets is increasing either in prevalence, or in priority for medical or veterinary intervention. It is important to better understand the conditions enabling parasite populations to flourish as well as any synergistic effects between microorganisms contributing to pathogenicity and clinical signs.

Knowledge about the patency and duration of infection serves a useful purpose in identifying appropriate intervention and treatment points. Also, other influencing factors, such as concurrent infections, nutritional status or genetic pre-disposition of the canine host may be critical in the development of clinical signs.

### 3.1.10 Hypothesis

That pre-patent infection period can be determined in dogs and that longitudinal sampling and testing can provide evidence of *Giardia* cyst excretion patterns.

That *Giardia* spp. infections cause clinical signs of infection in dogs.

### 3.2 Methods

Investigation of the earliest patent *Giardia* spp. infections, pre-patent period, duration of patency to determine cyclical cyst excretion and recrudescence infections was performed by collection and examination of canine faecal samples for the presence of parasites, as part of a longitudinal study. Microscopy was used as the reference standard in this study to confirm patent parasite infections. The results are presented as temporal dynamics of parasite patency and sequence of infection. Phenotypic analysis is reported in Chapters 4 and 5.
3.2.1 Sampling strategy

High-risk groups were identified as dogs less than one year of age (puppies and juveniles), dogs kept in intensive rearing or production groups, or in situations with poor husbandry standards. Private breeding facilities and dog shelters were approached for their cooperation. One dog-breeding establishment was in Victoria, Australia and all other samples were collected from metropolitan locations in Perth, Western Australia, as described in Chapter 2. Samples were collected from brood bitches and litters of pups to assess potential transmission, both pre and post whelp.

3.2.2 Sample collection

Samples were kept refrigerated at 4°C and generally arrived at the lab for processing within 48 hours of collection. Samples were processed as soon as possible and an aliquot dispensed and stored in ethanol for future use.

For the litters of puppies samples were collected on a weekly basis from the age of 2 weeks. Samples were pooled from litters of puppies, rather than from individual animals. Following positive confirmation of *Giardia*, samples were collected on a daily basis to monitor patency and duration.

3.2.3 Assessment of clinical signs

Stool consistency was the method used to assess the presence or absence of clinical signs of infection as determined by record of faecal stool form, ‘normal’ or ‘mucus or diarrhoeic’.

3.2.4 Diagnosis of *Giardia* infection

The experimental methods used in this study rely on the standard diagnosis of *Giardia* spp. infections by detection of cysts in faecal samples using centrifugal flotation and light microscopy methods, as described in Chapter 2, section 2.2.2.
3.2.5 Grading of infection

*Giardia* spp. infected faecal samples were graded using a relative scale to represent the quantity of cysts observed. Samples with low cyst intensity graded (1+), those graded moderate (2+) and (3+) and those with high cyst intensity were graded (4+).

3.3 Results

Results presented here describe the pre-patent infection periods for enteric parasites detected in faecal samples obtained from dogs (including puppies and juveniles). This section includes a comparative analysis of enteric protozoan parasites in two naturally infected populations from dog breeding facilities in different geographical areas. The following characteristics of parasite infections in canine hosts were determined:

1. *Giardia* spp. earliest patent infections and pre-patent period
2. Association between parasite patency and presence or absence of clinical signs of infection
3. Temporal dynamics and sequence of enteric parasite infections
4. Zoonotic and host specific genotypes of *Giardia* infections
5. *Giardia* spp. cyst intensity

3.3.1 *Giardia* spp. infections and pre-patent period in canine hosts

The pre-patent period for *Giardia* spp. infections and the presence of concurrent parasite infections were determined by examination of fresh faecal samples. Data from shelters and breeding kennels, in Perth, Western Australia and Bulla, Victoria revealed that 20.1% of adult and juvenile dogs were infected with *Giardia* spp., as outlined in Chapter 2, section 2.3.2.

3.3.2 Determination of the pre-patent period of *Giardia* spp. infections in puppies from breeding facilities

The earliest detection of *Giardia* spp. infections was confirmed in Labrador retriever puppies at 17 days (2 weeks) of age from a Victorian breeding facility. Patent *Giardia* spp. infections were also detected in different litters of Labrador retriever puppies from
a breeding facility in Perth, Western Australia at 35 days (5 weeks) 42 days (6 weeks) and 62 days (9 weeks) of age. Miniature schnauzer puppies in Perth, Western Australia were confirmed with *Giardia* spp. infections at 58 days of age.

### 3.3.2.1 Labrador retriever breed, Bulla, Victoria

*Giardia* spp. cysts were recovered from pooled faecal samples collected from Labrador retriever puppies in Victoria on four occasions when puppies were aged 17, 28, 45 and 67 days old, as shown in Table 3.3.1.

### 3.3.2.2 Longitudinal sampling of Labrador retrievers, Perth, W.A.

Over a three-year period, canine faecal samples were collected from the same Labrador retriever breeding facility in Western Australia. In 2011, *Giardia* spp. cysts were recovered from pooled faecal samples collected from puppies on two occasions when puppies were aged 35 days and 42 days. In 2012, *Giardia* spp. positive pooled faecal samples were confirmed by microscopy on one occasion when puppies were 30 days of age, shown in Figure 3.3.6.

In 2013, *Giardia* spp. cysts were recovered from faecal samples collected from individual puppies 72 days of age, shown in Figure 3.3.3 and Figure 3.3.8.

### 3.3.2.3 Miniature schnauzer breed

*Giardia* spp. cysts were recovered from pooled faecal samples collected from miniature schnauzer puppies in Western Australia on one occasion when puppies were aged 58 days of age, shown in Figure 3.3.4.

### 3.3.3 Concurrent parasite infections in Labrador retriever puppies

Infections detected in faecal samples by gold standard microscopy occurred as single parasite species infections or as co-infections with *Giardia* spp. Notably, all concurrent infections were associated with *Giardia* spp. that is, no co-infections were recorded for *Sarcocystis* sp. and *Cystoisospora* sp. in the absence of *Giardia* spp. Faecal samples were collected from separate litters of Labrador retriever puppies and examined for enteric parasites infections by microscopy.
3.3.3.1 Concurrent parasite infections, Litter 1

Results from the examination of twenty-seven pooled faecal samples from one litter of Labrador retriever puppies (29-50 days of age) are shown in Figure 3.2. More than half (65.2%) of all positive samples were infected with Cystoisospora sp. cysts, and 26.1% of samples were infected with Giardia spp. cysts, a small number (8.7%) were infected with Toxocara canis. No other types of parasitic organisms were detected for this litter of puppies. No parasite infections were detected in seven faecal samples and 23 parasite infections were recorded in total.

Table 3.2 Enteric parasites detected in litters of Labrador retriever puppies in Perth

<table>
<thead>
<tr>
<th>All Parasites detected</th>
<th>Perth litter 1 27 pooled faecal samples</th>
<th>Perth litter 2 20 individual faecal samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>Sarcocystis sp.</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Cystoisospora sp.</td>
<td>15</td>
<td>65.2</td>
</tr>
<tr>
<td>Toxocara canis</td>
<td>2</td>
<td>8.7</td>
</tr>
<tr>
<td>Giardia spp.</td>
<td>6</td>
<td>26.1</td>
</tr>
<tr>
<td>Total parasite infections</td>
<td>23</td>
<td>100</td>
</tr>
</tbody>
</table>

The 23 infections shown in Table 3.2 included single and concurrent species of protozoan parasites. The proportion of four types of infections that are shown in Table 3.2.1 include Giardia spp. (16.7%) and Cystoisospora sp. (66.7%) and co-infections (16.7%) of both Giardia spp. and Cystoisospora sp., a total of 18 protozoan infections.

Table 3.2.1 Concurrent and single parasite infections detected in two litters of puppies

<table>
<thead>
<tr>
<th>Single species of enteric protozoan parasites and Giardia spp. co-infections</th>
<th>Perth litter 1 27 individual faecal samples</th>
<th>Perth litter 2 20 individual faecal samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Sarcocystis sp.</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Cystoisospora sp.</td>
<td>12</td>
<td>66.7</td>
</tr>
<tr>
<td>Giardia spp.</td>
<td>3</td>
<td>16.7</td>
</tr>
<tr>
<td>Giardia spp. co-infections</td>
<td>3</td>
<td>16.7</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>100</td>
</tr>
</tbody>
</table>

3.3.3.2 Concurrent infections Litter 2

Results from the examination of 20 individual faecal samples collected from one litter of Labrador retriever puppies (68-85 days of age) are shown in Table 3.2. More than half
(60%) of all the positive samples were infected with *Giardia* spp. cysts, 28% of samples were infected with *Sarcocystis* sp, and 8% infected with *Cystoisospora* sp. A small number (4%) of samples were infected with *Toxocara canis* eggs, no other types of parasitic organisms were detected for this litter of puppies and three samples were uninfected.

The 26 parasite infections included both single species and concurrent enteric protozoan infections as shown in Figure 3.2.1 *Giardia* spp. (41.2%) and *Sarcocystis* sp. (11.8%) and *Giardia* spp co-infections (47.1%). The co-infections present with *Giardia* spp. were *T. canis* (28.5%) and *Cystoisospora* sp. (71.5%).

### 3.3.3.3 Multiple litters of Labrador retriever puppies, Victoria

Results from microscopic examination of 155 faecal samples from maternal dams and multiple litters of Labrador retriever puppies are shown in Table 3.3 with 74 negative test results. The samples positive for parasite infections indicated that 37% were infected with *Giardia* spp., 40.7% infected with *Cystoisospora* sp. and 22.2% were infected with *Sarcocystis* sp. No other types of parasitic organisms were detected and 48% of samples were uninfected. In total, 81 of 155 samples (52%) from breeding dams and puppies tested positive for enteric protozoan parasites.

<table>
<thead>
<tr>
<th>All Parasites detected</th>
<th>Bulla, Victoria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td><em>Sarcocystis</em> sp.</td>
<td>18</td>
</tr>
<tr>
<td><em>Cystoisospora</em> sp.</td>
<td>33</td>
</tr>
<tr>
<td><em>Toxocara canis</em></td>
<td>0</td>
</tr>
<tr>
<td><em>Giardia</em> spp.</td>
<td>30</td>
</tr>
<tr>
<td>Total</td>
<td>81</td>
</tr>
</tbody>
</table>

These samples infected with parasites were both single species enteric protozoan parasites as shown in Table 3.3.1 *Giardia* spp. (23.9 %) and *Sarcocystis* sp. (16.4%) and co-infections (20.9%) with *Giardia* spp. The co-infections present with *Giardia* spp. were *Sarcocystis* sp. (28.5%) and *Cystoisospora* sp. (71.5%).
Table 3.3.1 Concurrent and single parasite infections in multiple litters of puppies

<table>
<thead>
<tr>
<th>Single species of Protozoan Parasites and <em>Giardia</em> spp. co-infections detected</th>
<th>Bulla, Victoria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>Sarcocystis sp.</td>
<td>11</td>
</tr>
<tr>
<td>Cystoisospora sp.</td>
<td>26</td>
</tr>
<tr>
<td><em>Giardia</em> spp.</td>
<td>16</td>
</tr>
<tr>
<td><em>Giardia</em> spp. co-infections</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>67</td>
</tr>
</tbody>
</table>

3.3.4 Temporal dynamics of *Giardia* spp. infections and concurrent enteric protozoan infections in dogs

The dynamics of parasite detection in faecal samples was consistent across groups of puppies, regardless of breed and location. The first onset of parasitic infection occurred in puppies as young as 17 days after birth and the order of infection commenced with *Cystoisospora* sp., followed by *Giardia* spp. and *Sarcocystis* sp. Generally, when *Sarcocystis* sp. was detected, *Giardia* spp. was absent and *Cystoisospora* sp. was frequently found as a co-infection with *Giardia* spp. At the weaning stage (4-6 weeks) the number of *Giardia* spp. cysts (cyst intensity) in faecal samples increased considerably. The results are presented below as timelines of patent infection.

3.3.4.1 Parasite infections in puppies in breeding facilities

In all puppies diagnosed with patent *Giardia* infections, the initial diagnosis recorded faecal samples with low cyst intensity, rising to higher cyst intensities at later dates, described in more detail in Section 3.3.5.

3.3.4.2 Labrador retriever (Victoria)

Pooled faecal samples were collected from litters of puppies aged over 14 days, the first patent *Giardia* spp. infection was recorded at 17 days, *Cystoisospora* sp. was also detected in puppies of the same age, as shown in Figure 3.3.1. Both of these parasites continued to be detected until puppies were aged 40 days. In puppies of this age, and at 44 days of age *Sarcocystis* sp. was detected as a single species infection. Both *Giardia* spp. and *Cystoisospora* sp. were detected between 50 – 60 days of age. Single
species parasite infections of *Sarcocystis* sp. were detected in puppies at 60 days of age, and *Sarcocystis* sp. infections were not detected in puppies older than 62 days of age, unlike *Giardia* spp. and *Cystoisospora* sp. that continued to be detected until puppies were aged over 70 days of age.

![Graph showing frequency of parasite infections](image)

**Figure 3.3.1 Sequence of patent parasite infections in Labrador retrievers Bulla, Victoria**

**3.3.4.3 Labrador retriever (Perth)**

Pooled faecal samples were collected from one litter of eight puppies from the age of 2 weeks during January - February 2013. The first parasite infection was recorded when the puppies were 29 days of age, shown in Figure 3.3.2. This was a concurrent infection of *Cystoisospora* sp. and *Toxocara canis*. *Cystoisospora* sp. was the parasite most frequently detected, as a single species infection at 35 and 36 days of age. *Giardia* spp. and *Cystoisospora* sp. were both present in faecal samples collected from puppies aged 37 days of age. Both *Giardia* spp. and *Cystoisospora* sp. were observed as concurrent infections at 42 and 44 days after birth up until 50 days of age.
Chapter 3 Clinical signs and temporal dynamics

Individual faecal samples were collected to determine levels of *Giardia* spp. infection and sequence of patency in one litter of four Labrador retriever puppies, in Perth Western Australia, from the age of 14 days. No parasite infections were detected by microscopy during the first 30 days after birth. At 30 days of age *Toxocara canis* eggs were observed, as shown in Figure 3.3.3. After 70 days of age *Giardia* spp. infections dominated, with high levels of cyst excretion and *Cystoisospora* sp. co-infections reduced. Only one type of concurrent infection, *Giardia* spp. with *Sarcocystis* sp occurred in puppies over 75 days of age.

Figure 3.3.2 Sequence of patent parasite infections in Litter 1 of Labrador retriever puppies

Figure 3.3.3 Sequence of patent parasite infections in Litter 2 of Labrador retriever puppies
3.3.4.4 Observation and sequence of patent parasite infections in miniature schnauzer puppies Perth, Western Australia

Pooled faecal samples were examined from one litter of ten miniature schnauzer puppies aged 14 days until 72 days of age. No parasitic organisms were detected when puppies were aged 14-40 days of age. At 40 days of age Sarcocystis sp cysts were observed in faecal samples. The sequence of patent parasite infection and temporal dynamics of parasite patency are shown in Figure 3.3.4 No other parasites were detected until puppies were 57 days (aged 8 weeks) when Cystoisospora sp. was detected. The earliest observation of Giardia spp. patency was in puppies aged 58 days. Giardia spp. cysts were not detected again until puppies were aged 69 days (10 weeks). Sarcocystis sp. was not detected at the same time as Giardia spp. and the numbers of Giardia spp. cysts observed increased greatly over a two-week period from 58 – 72 days.

![Figure 3.3.4 Sequence of patent parasite infections and temporal dynamics in one litter of miniature schnauzer puppies in Perth, June 2011](image-url)
3.3.5 Enteric parasite infections and association with clinical signs of infection in Labrador retriever dogs from breeding facilities

Comparisons were made between the presence or absence of clinical signs of infection in dogs, based upon stool consistency, to determine the likelihood of parasite infections being associated with clinical signs in Labrador retriever dogs, using chi squared analysis, as shown in Table 3.4.

Table 3.4 Clinical signs and enteric parasite infections in Labrador retriever puppies from Victoria

<table>
<thead>
<tr>
<th>Parasites detected</th>
<th>Victoria Breeding Facility (Total tested) N</th>
<th>Clinical signs of infection</th>
<th>( \chi^2 ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Symptomatic 68</td>
<td>Asymptomatic 87</td>
<td></td>
</tr>
<tr>
<td>Sarcocystis spp.</td>
<td>11</td>
<td>6</td>
<td>5)</td>
</tr>
<tr>
<td>Cystoisospora spp.</td>
<td>26</td>
<td>19</td>
<td>7)</td>
</tr>
<tr>
<td>Giardia spp.</td>
<td>30</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td>Giardia co-infections</td>
<td>14</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>No parasitic organisms</td>
<td>74</td>
<td>22</td>
<td>52</td>
</tr>
</tbody>
</table>

3.3.5.1 Likelihood of *Giardia* spp. and enteric parasite infections being associated with clinical signs of infection

The results shown in Table 3.4 suggest that in the Victorian breeding facility dogs and puppies were more likely to display clinical signs when they were infected with enteric protozoan parasites. Dogs infected with *Giardia* spp. (single and concurrent infections) were statistically significant \( \chi^2 = 3.86 \) p<0.05 and all co-infections were statistically more likely to show clinical signs of infection, \( \chi^2 = 3.94 \). A higher likelihood was calculated for coccidian infections (both single and concurrent infections) \( \chi^2 = 14.47 \) p<0.05, p<0.01 and for all parasite infections, as single species (*Giardia* spp. and coccidians, excluding concurrent infections) \( \chi^2 = 10.48 \) p<0.05, p<0.01.

3.3.5.2 Likelihood of *Giardia* spp. and enteric parasitic infections being associated with clinical signs of infection in Labrador retriever dogs from Perth, WA

To compare parasite infections with clinical signs of infection three different litters of Labrador retriever puppies were tested over a three-year period in Perth, Western
Australia. Due to the low numbers of samples, it was not appropriate to use a chi-squared test for statistical validation on a litter-by-litter basis therefore results for all litters were combined. Details of parasite infections detected for each litter are shown in the Appendices, puppies were aged between 14 and 85 days old.

Table 3.5 Clinical signs and enteric parasite infections in 3 litters of Labrador retriever puppies from in Perth, Western Australia

<table>
<thead>
<tr>
<th>Parasites detected</th>
<th>Number (N)</th>
<th>Symptomatic</th>
<th>Asymptomatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td>85</td>
<td>54</td>
<td>31</td>
</tr>
<tr>
<td>Sarcocystis spp.</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Cystoisospora spp.</td>
<td>12</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Giardia spp.</td>
<td>37</td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td>Giardia co-infections</td>
<td>14</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>No parasitic organisms</td>
<td>20</td>
<td>14</td>
<td>6</td>
</tr>
</tbody>
</table>

The likelihood of concurrent (co-infections) parasitic infections being associated with clinical signs of infection in dogs was tested using chi-squared analysis and no significant results were obtained.

3.3.5.3 Likelihood of Giardia infections being associated with clinical signs of infection in dogs from two Labrador retriever breeding facilities

Results from all canine faecal samples tested from two breeding facilities (in WA and Victoria) were combined to analysis of the likelihood of parasite infections being associated with clinical signs in dogs from a two Labrador retriever breeding facilities was performed using chi-squared analysis, shown in Table 3.5. Details of all samples tested for parasite infection and clinical signs of infection are included in Appendix 2.

Table 3.6 Association between faecal parasite detection and clinical signs of infection in Labrador retriever breeding facilities

<table>
<thead>
<tr>
<th>Parasite detection</th>
<th>VICTORIA</th>
<th>WA</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=155)</td>
<td>(n=85)</td>
<td>(n=240)</td>
</tr>
<tr>
<td>Detection of parasites</td>
<td>10.48 (0.01)</td>
<td>NS</td>
<td>9.72 (0.01)</td>
</tr>
<tr>
<td>Any coccidian parasite</td>
<td>14.47 (0.001)</td>
<td>NS</td>
<td>8.67 (0.01)</td>
</tr>
<tr>
<td>All single species infection</td>
<td>14.25 (0.001)</td>
<td>NS</td>
<td>11.63 (0.001)</td>
</tr>
<tr>
<td>Single coccidian species</td>
<td>14.25 (0.001)</td>
<td>NS</td>
<td>12.17 (0.001)</td>
</tr>
<tr>
<td>Giardia spp. infection</td>
<td>3.86 (0.05)*</td>
<td>NS</td>
<td>4.5 (0.05)*</td>
</tr>
<tr>
<td>Concurrent parasite infections</td>
<td>3.94 (0.05)</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

* single and concurrent infections
Determination of any association between detection of *Giardia* spp. infection - single or concurrent infections with clinical signs of infection was performed using chi-squared analysis. There was no association with clinical signs of infection for the combined group.

These results indicate that clinical signs of gastro-intestinal upset in dogs, such as loose stool form or mucus in the stool, are significantly associated with enteric protozoan parasite infection \( \chi^2 = 9.72, p<0.05, p<0.01 \); and also for infections of single parasite species (excluding co-infections) \( \chi^2 = 11.63, p<0.05, p<0.01 \). Dogs were significantly more likely to display clinical signs if infected with a single species coccidian parasite than those dogs with no parasitic infections \( \chi^2 = 12.17, p<0.05, p<0.01 \)

### 3.3.6 Genotyping results for *Giardia* spp.

From the *Giardia* spp. positive faecal samples, 46 out of 64 (72%) were successfully genotyped as host specific *Giardia* spp. assemblages C and or D. Eighteen of these (28%) were successfully genotyped as *Giardia* spp. assemblage A and eight (3.5%) were considered to be of mixed assemblage both A and (host specific) C and D.

<table>
<thead>
<tr>
<th>Symptomatic</th>
<th>Mixed genotype A / C / D</th>
<th>Host specific genotype C and/or D</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>15</td>
</tr>
</tbody>
</table>

Genotyping results by performing PCR at 3 different loci, were successfully amplified at all 3 loci and sequences obtained from 2 of 18 (11%), 4 samples (22%) successful at 2 loci and 12 samples (61%) successfully sequenced from a single, 18s, gene loci.
3.3.6.1 Litters of puppies – maternal infections and puppies infected with enteric protozoa and the presence of clinical signs of infection

Five of the six mothers were confirmed with patent parasite infections by light microscopy from 4 – 44 days post-whelp and results for each litter are presented here.

Litter #1 The maternal dam did not show clinical signs of infection and faecal samples collected at 2 and 15 days post-whelp were free from parasite infections. Puppies appeared to have clinical signs, such as loose stools, at 16 days of age but no parasites were detected by microscopy.

Litter #2 The maternal dam displayed clinical signs of infection and she was confirmed positive for parasite infections on 4 out of 5 sampling occasions. Infection with Sarcocystis sp. was confirmed at 8 days post-whelp, no infections detected during subsequent 2 -3 weeks. Sarcocystis sp. was detected again at 38 days post whelp and Cystoisospora sp. infection after 42 days. Puppies were confirmed with Cystoisospora sp. infections at 108 days old accompanied by clinical signs of diarrhoea.

Litter #3 The maternal dam was confirmed with concurrent infections of Giardia spp. and Sarcocystis sp. and showed clinical signs at 37 and 44 days post whelp. At 45 days old puppies were confirmed with parasite infections; either Cystoisospora sp. alone or as concurrent infections with Giardia spp.

Litter #4 The maternal dam was negative for parasitic organisms pre-whelp but positive for Cystoisospora sp. at 23 and 27 days post whelp. Puppies were positive for Giardia spp. at 17 days of age and for Cystoisospora sp. at 36 days old.

Litter #5 The maternal dam had a history of clinical signs and she was confirmed positive for Giardia spp. infection 4 and 15 days post whelp. Puppies were confirmed with Cystoisospora spp. infection at 15 days old.

Litter #6 The maternal dam was positive for Cystoisospora sp. infection 28 days post whelp although clinical signs were absent. Puppies were also positive for Cystoisospora sp. infection at 28 days old although none showed clinical signs.
3.3.6.2 Puppies infected with enteric protozoa and clinical signs of infection

All the puppies with loose stool consistency (symptomatic cases) were infected with host specific *Giardia* spp. confirmed by multi-locus genotyping. The majority of those puppies considered as asymptomatic cases were infected with host specific *Giardia* spp., although one faecal sample contained assemblage A genotype, as shown in Table 3.8.

Table 3.8 Combined genotyping data for seven litters of Labrador retriever puppies infected with *Giardia* spp.

<table>
<thead>
<tr>
<th>Litter</th>
<th>Gene loci</th>
<th>Clinical Signs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18s</td>
<td>BG</td>
</tr>
<tr>
<td>1</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>2</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>3</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>4</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>5</td>
<td>A</td>
<td>D</td>
</tr>
<tr>
<td>6</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>7</td>
<td>C/D</td>
<td>D</td>
</tr>
</tbody>
</table>

3.3.7 Grading of infection

Results are presented in charts showing the frequency of samples that were confirmed with *Giardia* spp. based on similar numbers of cysts (cyst intensity). The data displayed as a timeline, according to the age of the puppy when faecal samples were collected.

3.3.7.1 Intensity of *Giardia* spp. infections in Labrador retriever puppies

The majority of samples were considered to have low cyst intensity (75%), 14% were graded moderate (2+, 5%) and (3+, 9%) and samples with high cyst intensity occurred in puppies over four weeks of age (11%) as shown in Figure 3.3.5.
Figure 3.3.5. *Giardia* spp. cyst intensity and frequency of detection from multiple litters of puppies in one Victorian breeding facility at different time intervals. The intensity of cysts excreted was lower in younger puppies (20 – 40 days) infected with *Giardia* spp. and peak intensity occurred once puppies reached 45 days of age, as shown in Figures 3.3.5, 3.3.6, 3.3.7 and 3.3.8.

Figure 3.3.6 *Giardia* spp. cyst intensity and frequency of detection for two litters of Labrador retriever puppies at 35 and 42 days old at a Perth breeding facility.

Figure 3.3.7 *Giardia* spp. cyst intensity and frequency of detection for Litter 1, Perth.
3.4 Discussion

3.4.1 Pre-patent period for *Giardia* spp. infection

Results from this study have established that patent *Giardia* spp. infections occurred at 17 days (2 weeks) of age in Labrador retrievers from the Victorian breeding facility and at 35 days (5 weeks) 42 days (6 weeks) and 62 days (9 weeks) in Labrador retrievers in Perth, and at 57 days (8 weeks) in miniature schnauzer puppies in Perth. These results confirm that the pre-patent period is 17 days or less, as the precise day of ingestion of *Giardia* spp. cysts could not be determined. At this young age the transmission to neonatal host is expected to be of maternal origin or from an environmental source. Despite the fact that this study was relatively small, the data suggests that differences in the pre-patent period may exist between breeds of dogs. Differences in infection rates between the dog breeds may be due to husbandry or management factors, but more likely to arise from unique phenotypic diversity of the host species, an outcome of the selective breeding of *Canis lupus familiaris*, into the dog breeds of the 21st century.

Similar studies have also shown that endoparasite infections in dogs can occur at an early age and that more than one parasite species causes infection, (Barlough, 1979, Bugg *et al.*, 1999, Traub *et al.*, 2002, Inpankaew *et al.*, 2007, Martinez-Carrasco C. *et al.*, 2007, Batchelor *et al.*, 2008, Rinaldi *et al.*, 2008, Thompson and Smith, 2011, Itoh *et al.*, 2011). A recent retrospective study of more than 2000 dogs in Germany...
confirmed high levels of enteric protozoan infection in puppies under 12 months of age, where infections commenced in puppies aged 3 weeks (Barutzki and Schaper, 2013). In addition, other studies have demonstrated different physiology between different breeds of dogs (German et al., 2000) and their responses to infection.

### 3.4.2 Concurrent parasite infections

In this study detection of *Cystoisospora* sp. was common, and was the most frequently detected enteric protozoan, often present as concurrent infections with *Giardia* spp. This is in contrast to *Sarcocystis* sp. which was seldom concurrent with *Giardia* spp. infections, although a small number of samples were concurrently infected with all three parasite species.

Maternal transmission of parasites, via the placental route, for *T. canis*, appears within first three weeks (Glickman and Schantz, 1981). Other parasites do not appear as quickly, and the results reported here demonstrate a pattern in which *Cystoisospora* sp. infection precedes *Giardia* spp. infections, and that concurrent infections commonly occur. This sequence maybe linked to development of the innate immune system. When the host responds to infectious challenge often through antigen recognition by M cells (Gebert, 1997, Jung et al., 2010) this initiates production of cellular immune responses (Maynard et al., 2012). Development of M cells in the follicle associated epithelium of Peyer’s patches commences in utero with a rapid increase in numbers after birth, and there is a species variation in the frequency of M cells (Brandtzaeg and Pabst, 2004).

Characteristics of *Giardia* spp. infected maternal immune system may directly influence embryonic development of foetal immune system affecting vertical and horizontal transmission as described in other parasite life cycles (Dunn and Smith, 2001). Distinctly different physiologies have been suggested to be associated with variable cyst excretion levels and the suggestion of action by a specific anti-*Giardia* immunoglobulin (Danciger and Lopez, 1975). In addition to potential in utero events there is also the role of colostrum and other nutritional factors in maternal milk, which
provide protection during the early stages of life (Hennart et al., 2002, Hellweg et al., 2008, Liao et al., 2012, Rodriguez et al., 2015,).

The salient points concerning sequence of parasite infection concerns physiological and immune responses of the neonatal dog when exposed to different enteric parasites and other microbial organisms and the consequent development of the innate immune system. Does early exposure confer a level of immunity, and capacity for host clearance of subsequent infection?

These results suggest that different types of enteric protozoan infections, including co-infections, are associated with clinical signs. Patent infections with Cystoisospora sp. either as single species infection or concurrently with Giardia sp. are more likely to be associated with clinical signs, than patent infections of single species Giardia spp. infections. This in turn, emphasizes the importance of understanding more clearly the synergies and conflicts of multiple infections (Lymbery and Thompson, 2012).

### 3.4.3 Clinical signs of infection

Collection of faecal stool consistency data as an indicator of clinical signs of infection did reveal some associations between parasite infections and differences were noted between groups of dogs in different geographical locations. Statistical analysis indicated that the likelihood of dogs in the Victorian breeding facility displaying clinical signs of infection was associated with coccidian parasite infection ($\chi^2$ value = 14.7). These results suggest that coccidian infections are the most likely cause of clinical signs of infection in the Victorian group, both singly and as concurrent infections. The results also indicate that infections with a single species of parasite are likely to be a factor contributing to clinical signs in infected dogs ($\chi^2$ value = 10.4). The results also suggest that concurrent parasite infections could also be associated with clinical signs of infection ($\chi^2$ value = 3.94). The association of clinical signs with Giardia spp. infections was not evident and the potentially zoonotic genotypes of Giardia spp. did not appear to have a clinical impact on the canine host.
In the Western Australia group single parasite infections, including coccidian species were not associated with clinical signs of infection, and no association with concurrent infections was apparent. Nevertheless, an association between the presence of *Giardia* spp. infections and clinical signs of infection was determined ($\chi^2$ value = 4.25). The most likely conclusion for this group is that *Giardia* spp. infections are contributing to clinical signs of infection and these infections are predominantly assemblage D genotype. Consequently, if parasite infection is the cause of clinical signs, as unformed stools, the cause is due to host specific *Giardia* spp. infections.

By combining the data as a larger sample size for a group of 240 Labrador retrievers the statistical analysis indicated that concurrent parasite infections were not associated with clinical signs of infection. The results show that parasite infections that include detection of *Giardia* spp. are more likely to be associated with clinical signs of gastrointestinal upset in dogs, such as loose stool form or mucus in the stool ($\chi^2$ value = 4.5).

### 3.4.4 Zoonotic and host specific genotypes - Are clinical signs of infection associated with particular *Giardia* spp. genotypes?

Results from this study are inconclusive. Whilst data shown in Table 3.7 suggests that asymptomatic *Giardia* spp. infections in dogs are associated host specific genotypes, these samples were not confirmed to contain *Giardia* spp. cysts by microscopy. In addition, the results for more robust genotyping across multiple gene loci, shown in Table 3.8 suggests that host specific infections occur in dogs with clinical signs of infection as assessed by stool consistency. The presence of mixed infections demonstrated that dogs are host to multiple genetic assemblages of *Giardia* spp. Increasing reports of zoonotic assemblages in domestic animals, such as cats and cattle, and in wildlife species suggests that further work is required to fully understand transmission routes and consequences of pathogenic infections.
3.4.4.1 Age and development of the neonatal canine host

Since this study has shown that zoonotic isolates can infect puppies from a young age there is a strong indication that mechanical transmission may occur from maternal dam to neonatal puppies, causing infection within the first few days of life.

During weaning transitions from maternal milk to solid food in young mammals it is reported that the intestine undergoes a profound transition induced by blood circulating insulin levels or suckling reflexes. These mediate signaling of fucose biosynthesis and subsequent fucosylation of glycoconjugates of intestinal mucins and down regulation of sialylation processes (Becker and Lowe, 2003). Furthermore, these authors refer to impaired fucose glycosylation as the cause of phenotypic variation in mice and its association with inflammatory bowel disease and episodes of diarrhoea.

The importance of these findings in puppies has relevance for Giardia spp. infections in children. There are reports of cognitive impairment in very young children (Berkman et al., 2002, Simsek et al., 2004) and whilst the incidence of acute diarrhea is higher in countries with lower levels of development it has proven difficult to attribute the cause as Giardia spp. infections (Hollm-Delgado et al., 2008).

3.4.4.2 Host specific Giardia spp. infections

These may be explained by the carbohydrate moiety of the cell wall glycoproteins, where the host is able to recognize the particular polysaccharide polymers. Determining the relative composition of the cyst cell wall may be beneficial for establishing any variation between zoonotic and host specific Giardia spp. isolates. It is already established that the galactose content of CWP is higher in G. duodenalis than G. muris isolates (Jarroll and Paget, 1995). Evidence of how host genotype and phenotype expression may be of relevance to the establishment of infectious disease of the mucosal tract, such as Giardia spp. infections, and associated clinical signs can be reflected in results from a study of mannose binding lectins in neonates in Poland (St. Swierzko et al., 2009). This study demonstrated the existence of a rare gene haplotype pre-disposing infants to respiratory infections, the rare gene expression is
considered to be of Slavonic origin occurring in individuals with Polish, Czech and Brazilian ethnic heritage. This information concerning potential pre-disposition of hosts to infection may contribute to the explanation of geographical or breed variations reported in *Giardia* spp. infections described in Chapter 1 and 2.

### 3.4.5 Transmission of *Giardia* spp. infections in breeding dams and litters of puppies

*Giardia* spp. genotyping results for the group of Labrador retriever breeding dams indicated that they were positive with assemblage A infections. Individual dams sampled on different dates returned the same genotyping results. Genotyping results from the dams and litters indicated that if the dam was host to assemblage A *Giardia* spp. infections the pups would also be confirmed with assemblage A infections. Results from one litter of puppies detected *Giardia* spp. and *Cystoisospora* sp. Infections during the period 38 – 56 days after birth. Faecal samples collected from the maternal dam during this time were positive for *Toxocara canis* at 27 days post-whelp. *Cystoisospora* sp. was not detected, but 3 copro-antigen tests were positive for *Giardia* spp. infection 54 days post-whelp, although *Giardia* spp. cysts were not observed by microscopy.

These results demonstrate the potential for young puppies, that when exposed, are susceptible to infection from an early age with host specific *Giardia* spp. The immune responses may contribute to clearance of infections, even when re-exposed to host specific infections. However, in litters of puppies where the maternal dam was confirmed with *Giardia* spp. assemblage A infections pre- or post-whelping the puppies were also confirmed with assemblage A infections, although this study was unable to determine if immune responses contributed to clearance of these infections or if immunity was conferred to individuals for preventing re-infections.

The faecal samples from this group of puppies were much easier to genotype at all 3 loci than the other groups of samples. The results were consistent even when DNA re-extractions and PCR experiments were repeated on faecal samples that had been
frozen for 12 months or more. This could be explained by faecal DNA sequences closely matching the primer sequences and the possibility of nucleotide substitutions in other infections causing difficulties with sequencing.

Parasite infections carried by the maternal dam are likely to affect the incidence of infections in puppies and the genetic type of *Giardia* spp. Infections. Factors at weaning, such as age, type of food and prophylactic treatments could also account for intra-breed variations. These factors are also related both to geography, physical location and husbandry practices.

The juvenile dogs in co-housing situation with litter mates or other kennel mates were infected mainly with host specific assemblages C and or D, as shown in Table 2.3.16, some of which failed to amplify at 18S gene locus but were successfully amplified and sequenced at beta giardin and GDH loci. The reasons for poor results using the 18S gene locus are unclear.

### 3.4.5.1 Asymptomatic infections

Data collected in this study showed that asymptomatic infections were common in older dogs, raising questions over the significance of these infections: 1) the impact on the general health and well being of the dog 2) possible transmission routes and reservoirs of infection.

### 3.4.5.2 Disruption of transmission routes

Detection of *Giardia* spp. infections in puppies less than three weeks of age in this study has confirmed earlier reports of multiple concurrent parasitic infections. This raises questions concerning the influence of patent parasite infections upon development of neonatal host immune system. What factors determine when infections become acute or when the host develops chronic infections in the absence of immune protection? How are the interactions and association with other parasitic species important during concurrent infections? Knowledge of where and when susceptible hosts become vulnerable to disease through exposure to infectious parasitic forms is critical for disruption of transmission routes. Applying networking principles for
examining factors influencing the transmission of parasites require detailed and ongoing recording of the activity of susceptible hosts and frequency and methods of exposure to infection sources.

Whilst researchers advocate the use of networks in ecological studies for understanding transmission of wildlife parasites, the same principles and methods could be applied to studying ecological consequences of protozoan infections in naturally infected domestic animals, such as domestic dogs (Godfrey, 2013). In fact *Giardia* spp. is highly suited to this type of approach, which is most appropriate in parasites with a simple life cycle. Inclusion of elements that consider spatial proximity may be applicable. Research questions and approaches may include 1) social interactions and behavioural studies. Do *Giardia* spp. infected dogs affect host behaviour? Both the infected individual and others closely associated, such as littermates. 2) Use of shared space. What are the implications of housing and management situations, where animals are separated in time but not in access (or contact) to a physical space. 3) The value of point prevalence studies. Parasite transmission as a dynamic (rather than static) process signals the usefulness of longitudinal evidence, particularly in the context of multiple genetic strains of *Giardia* spp. so called host specific and zoonotic, and the consequences of co-infection by other protozoan parasites (Beldomenico and Begon, 2010). Networks imply relationships and construction of both direct and indirect networks may prove useful to determine if directional influences exist.

Three levels of investigation using networking models might provide greater understanding of transmission processes useful for managing the spread of parasitic disease as described by (Godfrey, 2013). Firstly, longitudinal studies in individual dogs from birth, and asymptomatic mature dogs with confirmed *Giardia* spp. infection. Secondly, examination of dyadic relationships could be performed which could be either bitch to pup or interactions between littermates. Thirdly, closer analysis of the population as a whole, could include both behavioural and environmental factors to
determine possibility of vertical or horizontal transmission routes and where reservoirs of infection may be critical in symptomatic outbreaks, such as introduction of individuals from external communities or ecosystems.

Could classical *Giardia* spp. transmission routes require further examination? Whilst infection routes have been considered to be solely by faecal-oral transmission, earlier studies have suggested the possibility of vector based routes (insects) and also the possibility of physical transfer (Barlough, 1979). Puppies have direct contact with mammary gland, fur and bedding, any one of which has the potential to serve as a conduit for transfer of *Giardia* spp. cysts.

### 3.4.6 Grades of infection

The levels of cyst intensity in faecal samples recorded in this study demonstrate the critical time point of *Giardia* spp. cyst shedding that occurred in puppies aged 45-85 days of age. This reflects the period in which there is maximum opportunity of infectious exposure, which is generally at the weaning and immediately post-weaning stage. In all puppies diagnosed with patent *Giardia* spp. infections, the initial diagnosis was confirmed in faecal samples graded as 1+, rising to higher numbers of cysts at later dates. *Giardia* infections in the groups of dogs studies indicates that the majority were graded as low (1+) cyst excretors (75%), with one or more cysts present 14% graded as moderate excretors and 11% were considered to have high faecal content of *Giardia* spp. cysts.

Parasitologists are still unclear if the numbers of *Giardia* cysts excreted in faeces is indicative of symptomatic or asymptomatic infection, or if higher cyst excretion is related to increased infection rates. For diagnostic purposes, it has been calculated that 50 cysts/g faeces for bovine samples is the minimum level of sensitivity (Uehlinger *et al.*, 2006). Routine diagnostics at Murdoch University using zinc sulphate flotation and light microscopy confirm positive samples to be any sample with one or more *Giardia* spp. cysts are observed. Active infections can be produced by ingestion of low
numbers of cysts in humans and gerbils (Rendtorff, 1954, Amorim et al., 2010) hence the reason for accepting low cyst observations.

Different research studies have reported results of Giardia infection in different units, some as cysts/microliter (Kohli et al., 2008) or number of Giardia cysts/g faeces (Hamnes et al., 2007, Rosa et al., 2007, Duffy et al., 2013). Accordingly, quantification of faecal cyst excretion does provide some indication of the extent that the new hosts may become exposed to infection. Future studies examining the quantification of faecal cyst excretion should also take into account the infectious potential of cysts, or their viability. Are those cysts excreted in small numbers at the onset of patent infection equally infectious to those excreted in higher numbers at the weaning period? In addition, one study in gerbils has shown that detection of the earliest patent infection (low numbers of cysts) is associated with elevated faecal immunoglobulin levels. No change in serum immunoglobulin levels was observed at this early point of infection however during the period of high cyst excretion both faecal immunoglobulins and serum immunoglobulins levels were raised (Amorim et al., 2010).

Importantly, results from these experiments demonstrate that on numerous occasions Giardia spp. was not shown to be the cause of clinical signs of infection, which is a cause for concern. If this is case, then much of the earlier published literature reporting clinical impacts of Giardia spp. infections in dogs are of limited value if the presence of other parasites was not determined using microscopy methods. This is the only method that can exclude other parasites as a cause of clinical signs. These findings also reduce the value of the IFAT as a gold standard Giardia spp. diagnostic test as proposed by Mekaru et al. (2007), Rimhanen-Finne et al. (2007), Rishniw et al. (2010) and Uehling et al. (2013).
Chapter 4

Canine faecal protein extraction – detection of *Giardia* spp. proteins using proteomic tools

This chapter describes and analyses approaches and outcomes from the different methods used for the proteomic analysis of *Giardia* spp. infected canine faecal material. The different methods developed in the current study are described according to the sequential experimental steps that were required prior to analysis of mass spectral data. Each stage formed an element in an iterative process for establishing appropriate methods of sample preparation.

Two work flow diagrams are included, one to introduce the various stages in each experiment and another to provide an overview of the analytical framework. Summaries of the experimental data are provided in figures and tables. Results and interpretation of the biological significance of the data are detailed in Chapter 5 with supplementary data contained in Appendix 3.

### 4.1 Introduction

Enteric protozoan infections are common in young dogs. Diagnostic tools remain ineffective for differentiating between host specific and potentially zoonotic *Giardia* spp. infections. Information is lacking for transmission cycles and for the implications of symptomatic and asymptomatic infections. A glycoprotein, 65kDa in size, was first identified and characterized from human faeces using counter-immunoelectrophoresis (CIE) and latex agglutination tests, and named *Giardia* Specific Antigen (GSA) by (Rosoff and Stibbs, 1986). Subsequent production of the commercially available ProSpecT *Giardia* microplate assay for diagnosis of *G. duodenalis* infections was based upon detection of GSA 65 (Anderson *et al.*, 2004). Detection of GSA 65 antigen in faecal samples has been considered a reliable and sensitive diagnostic technique for confirming active *G. duodenalis* infections in humans (Vidal and Catapani, 2005). Since this glycoprotein was easily detected and stable under variable conditions it is likely that the same, or similar, protein might be present in faeces from dogs infected with
*Giardia* spp. The importance of confirming protein expression will inform metabolic processes because in the case of *Giardia* species a detailed understanding of cellular metabolism is not yet fully described. A deeper understanding of host-parasite interactions is a critical area of work for the prevention, management of infections and therapeutic intervention of infected hosts. Using a proteomics approach may enhance current genomic characterization studies and drive capacity building for the differentiation between *G. duodenalis* genotypic assemblages (Humphrey-Smith *et al.*, 1997, Steuart, 2010).

Proteomics enables integration of biochemical and biological data to complement that of genomic and transcriptomic data. It produces insights into developmental regulation aspects of *Giardia* spp. metabolism and the interaction processes within the host gastro-intestinal tract. Specialized genetic techniques such as expressed sequence tags have greatly improved the quality of genetic data, whilst proteomic techniques have become available for describing protein expression. Proteomic techniques include standardization of sample preparation methods (D'imperio *et al.*, 2010) and high resolution methods for detection of low abundance proteins when comparing two organisms with highly similar gene sequence (Jungblut *et al.*, 1999).

Detailed protocols are available for separation of proteins based upon physical and chemical characteristics as well as cellular or organelle location (Pedersen *et al.*, 2003, Mastro and Hall, 1999). Here the work sought to provide a preliminary investigation into the ability to detect *Giardia* spp. proteins and peptides using methods described for similar experiments, such as 2D LC MS/MS (Blonder *et al.*, 2006, Hustoft *et al.*, 2014). The investigation was carried out on *Giardia* spp. infected canine faecal samples, using a proteomic approach, whilst also seeking biomarkers to differentiate between *G. duodenalis* genotypes.

Canine faecal samples were collected from dogs naturally infected with *Giardia* spp. Only those samples containing high numbers of *Giardia* spp. cysts were used in the
proteomic experiments. Faecal samples were first homogenized and the total faecal protein was extracted, details are provided in Section 4.2.

Resolubilized protein fractions were digested into peptide fragments and separated using a strong cation exchange column. Proteome enrichment methods using commercially available kits were unsuccessful in improving the proteome coverage, and methods were revised to overcome initial low protein yields. Fractions were collected and spotted in preparation for MALDI-TOF/TOF mass spectrometry analysis.

The spectral data generated was analysed using database-matching algorithms to identify known G. duodenalis proteins, in addition to identification of unique or hypothetical proteins. Further critical examination of acquired spectral data identified proteins of host origin providing evidence of responses to G. duodenalis infection in dogs.

4.1.1 Sample preparation for proteomic analysis - Introduction

Proteome research in non-medical fields has reported a wide range of protein extraction protocols, which rely on the chemical characteristics of the target proteins, such as those in soil (Benndorf et al, 2006) that require alkaline conditions. Wilmes and Bond (2006) describe the metaproteome and Wilmes et al. (2008) outline proteomic methods for analysis of wastewater sludge.

Medical research reporting the use of proteomic tools has mainly used blood and blood products, such as serum or plasma, as the biological samples of choice. Whilst other biological samples, tissues and excreted body fluids such as urine, and faecal material are not commonly used due to the complexity and variability between and within individuals (Jiang et al., 2008).

Metaproteomics is a specific type of proteomic approach that examines whole microbiota communities and measures and identifies changes to protein profiles as they occur, (Wilmes and Bond, 2006). A study by Klaassens et al. (2007) demonstrated the use of 2D PAGE and mass spectrometry methods in identifying faecal proteins.
from the gastrointestinal tract of infants using a metaproteomics approach. A similar investigation of the metaproteomics of microbiota present in the human distal gut used a non-targeted shotgun mass spectrometry method to successfully illustrate potential interactions between the human microbiome and host organism (Verberkmoes et al., 2009). The potential of a novel proteomic approach to identify host proteins from faecal samples has been demonstrated in a murine model by Oleksiewicz (2004).

Proteomic analysis of proteins from biological samples commonly uses changes in protein conformational structure. These structural changes can be revealed by reduction and alkylation reactions (Gilar et al., 2004), and enzymic proteolysis for peptide cleavage assisting with protein characterization (Phillips and Fletterick, 1992). These principles formed the basis of the methodology used in this study.

The workflow diagram below in Figure 4.1 provides an overview of the main steps included for sample preparation to extract and identify Giardia spp. proteins in Giardia spp. infected canine faecal samples. Details for each step are described in Methods Section 4.2.
Figure 4.1 Workflow Diagram
4.2 Methods

4.2.1 Samples
Fresh canine faecal samples, previously confirmed as positive for *Giardia* spp. by light microscopy, were processed within 1-7 days of arrival at the laboratory and stored at 4°C or at -20°C if longer than 7 days. Details of the diagnosis and grading of *Giardia* spp. cyst numbers are described in Chapters 2 and 3.

Some clinical data was recorded concerning the source of samples - dog breed, sex, age and stool form. No other parasitic organisms were observed by microscopy and all samples tested contained high numbers of *Giardia* spp. cysts.

4.2.2 Faecal protein extraction
In total, four different canine faecal samples were tested, and processing of each sample used different surfactants and buffers with the aim of maximizing the amount of *Giardia* spp. proteins recovered. This is summarized in Table 4.1 and detailed below.

Table 4.1 Summary of sample preparation methods

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Physical processing</th>
<th>Extraction Buffer</th>
<th>Precipitation Solvent</th>
<th>Resolubilization Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fresh homogenate</td>
<td>At 4°C PBS solution, Protease inhibitor EDTA pH 7.4</td>
<td>Acetone -20°C</td>
<td>Room temp 0.5M TEAB solution</td>
</tr>
<tr>
<td>2</td>
<td>Frozen sample Freeze thaw (x3)</td>
<td>At 4°C PBS solution Protease inhibitor EDTA pH 7.4</td>
<td>Acetone -20°C</td>
<td>Room temp 50mM Tris HCl, 0.5% Triton X solution pH5.6</td>
</tr>
<tr>
<td>3</td>
<td>Frozen sample Freeze thaw (x8)</td>
<td>At 4°C 50mM Tris/ 0.5% SDS solution</td>
<td>Acetone -20°C</td>
<td>Room temp 0.5M TEAB 2.0% SDS solution</td>
</tr>
<tr>
<td>4</td>
<td>Fresh homogenate</td>
<td>At 4°C PBS solution, Protease inhibitors, pH 7.4</td>
<td>Acetone -20°C</td>
<td>Room temp 50mM Tris HCl/ 0.5% Triton X solution pH5.6</td>
</tr>
</tbody>
</table>

Performed at room temperature

Experiment 3 was performed on two different canine faecal samples although the same methods were followed, as described in 4.2.4.
4.2.2.1 Aqueous phase extraction 1

In Experiment 1 the whole fresh faecal sample (1g) was emulsified with 4ml distilled water (and filtered through gauze or filter paper if large particulate matter was present) and then centrifuged at 2000g for 2 minutes. The supernatant was discarded and the faecal material was resuspended in phosphate buffered saline (PBS) (136mm NaCl, 13.4mM KCl, 10.1mM Na$_2$HPO$_4$, 1.76mM KH$_2$PO$_4$ in deionized water containing 10mM EDTA) as described by Oleksiewicz (2004). After thorough stirring the mixture was left to settle on the bench at 23°C for 5 minutes, and then centrifuged at 5000g for 20 minutes (4°C).

4.2.2.2 Freeze thaw extraction

• In Experiment 2, 0.2g of whole frozen faecal sample was extracted in 1ml of extraction buffer containing phosphate buffered saline in deionized water with 0.5% (v/v) TritonX-100 and protease inhibitor (Roche Complete Mini). This faecal suspension was then subjected to three, 5 minute freeze-thaw cycles by alternate immersion in liquid nitrogen and boiling water.

• In Experiment 3, whole frozen canine faecal extract was extracted in 5ml of 50mM Tris HCl containing 0.5% (w/v) SDS solution and mixed by vortex, then subjected to eight cycles of freeze thawing prior to acetone precipitation.

4.2.2.3 Aqueous phase extraction 2

In Experiment 4 proteins were extracted from 1g of whole fresh canine faecal sample using 5ml of extraction buffer containing PBS (without EDTA) solution and protease inhibitor (Roche Complete Mini).

4.2.3 Protein precipitation

Protein was precipitated from all samples using the same method by decanting each sample supernatant into a 50ml centrifuge tube. Five to six volumes of ice-cold acetone were added to the contents of the tube to precipitate the proteins, and then inverted several times before cooling the contents to -20°C for 4-12 hrs. Then, working on ice,
the faecal precipitate was centrifuged at 2000g for 5 minutes (4°C). The acetone fraction was decanted and discarded. Complete removal of residual acetone was achieved by a second centrifugation step at 1000g for 20 seconds (4°C) and the residual pellet was transferred to 1.7ml micro centrifuge tube.

4.2.4 Protein resolubilization

Different methods were tested to resolubilize each of the precipitates to maximize protein yield.

- Experiment 1
  The protein fraction was resuspended in 200µl of resolubilization buffer, 0.5M triethylammonium bicarbonate (TEAB) solution. To maximize resolubilization the protein was mixed using a bath sonicator for 15 mins and also mixed by vortex. An additional 200µl TEAB solution was added until all solid had resolubilized and further mixed by vortex. The protein fraction was separated by gel electrophoresis. Excised gel bands were reduced, alkylated and digested as described in 4.2.6.2 prior to further analysis.

- Experiment 2
  Following acetone precipitation the residual protein pellet was resolubilized in 200µl 0.5M TEAB solution containing 2% (w/v) SDS solution. The protein sample was reduced, alkylated and digested as described in 4.2.7.1 prior to further analysis.

- Experiment 3
  The protein fraction was resolubilized at room temperature in 0.5M TEAB solution containing 2.0% (w/v) SDS solution. The protein sample was reduced, alkylated and digested as described in 4.2.7.1 prior to further analysis.

- Experiment 4
  The protein fraction was resolubilized in 300µl of resolubilization buffer containing 50mM Tris HCl and 0.5% Triton X-100, pH5.6, by gradual increases in the volume of buffer, in combination with prolonged vortex and pulse spins, to ensure complete
protein solubilization. The protein sample was reduced, alkylated and digested as described in 4.2.7.1 prior to further analysis.

4.2.5 Protein quantification

This was determined using the Bradford protein assay by colorimetric detection at 595nm.

4.2.6 Protein separation by gel electrophoresis - Introduction

Sodium Dodecyl Sulphate (SDS) Polyacrylamide Gel Electrophoresis (PAGE)

Two dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (2D SDS-PAGE) separates proteins based upon size and charge. The first stage separation isoelectric focusing (IEF) occurs across a pH gradient and protein separation is based upon the isoelectric point (pI), whereas the second dimension further separates proteins based upon their molecular mass.

Application of these principles for protein separation using SDS PAGE techniques has been successful for identifying the protein profile of *Giardia* spp. trophozoites (Smith *et al.*, 1982, Capon *et al.*, 1989). Protein differences observed in *Giardia* spp. isolated from different species using alloenzyme electrophoresis were observed in earlier studies (Meloni and Thompson, 1987). Further work using 2D SDS PAGE coupled with western blot successfully identified immunoreactive proteomics of *G. duodenalis* from serum samples of *Giardia* spp. infected human patients (Palm *et al.*, 2003).

An investigation into proteomic expression of the *Giardia* spp. genome carried out using cultured trophozoites identified an isolate specific protein, alpha 2 giardin as an assemblage A protein (Steuart *et al.*, 2008). A comparative 2D SDS PAGE study that examined *in vitro* induction of *Giardia* spp. cyst formation from cultured trophozoites reported dramatic changes in protein expression during encystation (Kim *et al.*, 2009).

In addition to *Giardia* spp. proteins identified from trophozoites and cysts, PAGE is a frequently performed technique for identification and characterization of wide range of biological materials and has been used in parasitology to document the proteome of
Leishmania viannia and Cryptosporidium spp. (Truong and Ferrari, 2006, Gongora et al., 2003). Two dimensional PAGE methodology enabled researchers to effectively analyse and identify 10 discrete parasitic proteins of Entamoeba histolytica (Leitsch et al., 2005). Use of PAGE for the identification and characterization of excretory-secretory products of Giardia spp. has provided a clearer understanding of the parasite biology in addition to host immunologic responses (Nash and Keister, 1985). The early work on phenotypic characterisation using SDS PAGE techniques has served to improve understanding of Giardia spp. metabolism and immunoreactive proteins (Palm et al., 2003).

The early electrophoretic studies on Giardia spp. infections in humans furthered the understanding of biological and biochemical functions of Giardia spp. proteins although limited information is available specifically concerning Giardia spp. infections in dogs. Depending upon the gel size and pH gradient used, approaches using two dimensional SDS PAGE can resolve more than 5000 proteins simultaneously and detect and quantify proteins at picogram level (Gorg, 2004). Detection of low abundance proteins is dependent upon pre-fractionation methods and high sensitivity mass spectrometry.

In this study polyacrylamide gel electrophoresis was used as an initial step to visualize proteins from canine faecal extract that contained Giardia spp. cysts, following the methods described by Steuart et al. (2008) for electrophoretic separation of Giardia spp. trophozoite sonicated material. Tris tricine gels were used to visualize smaller proteins and peptides. In experiment 1 proteins extracted from whole faecal extract were separated by one dimensional gel electrophoresis using Tris Tricine 10%-20% Peptide Criterion gel (Bio-Rad) and stained with Coomassie Brilliant Blue G-250 (Sigma) as described by (Candiano et al., 2004).

### 4.2.6.1 Gel band excision and destain

Each faecal protein extract separated within the gel was excised. Gel bands were cut into small pieces and destained by adding 25 µl of 25 mM ammonium bicarbonate in 50% (v/v) acetonitrile to each gel band and incubating at 37°C for 45 minutes. The
liquid was removed and discarded and the process repeated until the gel bands became transparent.

4.2.6.2 Reduction and Alkylation of gel band samples

Each separate gel band, containing faecal protein extract, was reduced with 50 µl of 10mM dithiothreitol (DTT) in 50 mM ammonium bicarbonate and incubated for 15 min at 56°C.

Alkylation was performed by addition of 50 µl of 50 mM iodoacetamide (IAM) in 50mM ammonium bicarbonate and incubated for 20 min in the dark at room temperature before washing with water and drying by centrifugation under vacuum.

4.2.6.3 Enzymic Digestion of gel bands

Enzymic digestion was performed by adding 10 µl of 0.1µg/µl bovine trypsin (Roche Diagnostics) to each gel band and incubated overnight at 37°C. Peptides were extracted using 50 µl of 99% (v/v) acetonitrile and 1% (v/v) trifluoracetic acid solution into clean tubes. The extracted peptide samples were then dried under vacuum before mass spectrometry analysis.

4.2.7 Protein separation by High Performance Liquid Chromatography (HPLC)

4.2.7.1 Reduction and Alkylation of protein samples in solution

Protein supernatant from Experiments 2, 3, 4, 5 were reduced and alkylated as follows: The volume of sample solution used was dependent upon the protein concentration, and adjusted accordingly to perform reduction and alkylation based upon 100µg of protein. An aliquot of supernatant (100 µg of protein) was reduced by adding 10µl of 50mM Tris (2carboxethyl)phosphine (TCEP). The contents were mixed thoroughly by vortex and centrifuged briefly for 5 seconds before incubation at 60°C for 1 hour. Alkylation was performed immediately by addition of 5µl of 200mM methylmethanethiosulphate (MMTS) to the sample solution and thoroughly mixed by
Chapter 4 Proteomic Methods Development

vortex. The sample was centrifuged briefly for 5 seconds and incubated at room temperature for 10 minutes.

4.2.7.2 Enzymic digestion of protein samples in solution

Enzymic digestion of the sample was performed by adding 5µl of 0.2mg/ml solution of trypsin sequencing grade (Roche) (1:100 ratio) followed by addition 10µl of acetonitrile to each sample and incubated overnight at 37°C. The final samples containing 100µg of digested protein was dried by centrifugation under vacuum and stored at -80°C prior to cation exchange using High Performance Liquid Chromatography (HPLC). Samples and controls were resuspended in 100 µl of Buffer A 10 mM potassium dihydrogen orthophosphate (KH$_2$PO$_4$), 10% (v/v) acetonitrile (pH 3.0).

4.2.7.3 Two Dimensional Liquid Chromatography (2D LC)

4.2.7.3.1 High Performance Liquid Chromatography system.

Peptides were separated by strong cation exchange chromatography on an Agilent 1100 HPLC spectrum analyzer system (Agilent Technologies, Germany). Each sample was processed separately. Sample volumes of 99 µl, were injected onto a HPLC column 5 micron 30 nm 4.6x100 mm (PolyLC Inc.) at 25ºC. The samples were run under linear gradient, using eluents: Buffer A: 10 mM potassium dihydrogen orthophosphate (KH$_2$PO$_4$), 10% (v/v) acetonitrile (pH 3.0) and Buffer B: 10 mM potassium dihydrogen orthophosphate (KH$_2$PO$_4$), 1 M potassium chloride (KCl), 10% (v/v) acetonitrile (pH 3.0). A column wash was run pre- and post- sample application and blank runs were run prior to running each sample on each occasion and subsequently subtracted from the sample run. Spectral traces were recorded on an Agilent 1100 UV detector at 214 nm wavelength controlled by Chromelon software, over 50 min run time. For each run, 40 eluted fractions were collected into glass vials using an autosampler at 4ºC with a flow rate of 0.5 mL/min. After viewing the chromatograph trace peak heights were recorded, shown in Table 4.3, and the area under the curve (AUC) was calculated. The AUC value represents the total peptide content this was then divided into 8 areas of equal peptide content for the
next stage of separation. Each of the eight, newly combined, fractions was desalted on a solid phase extraction column, Strata-X 33 µM polymeric reversed phase, (Phenomenex) following the manufacturers’ instructions to remove salts and impurities. The eluted samples were dried under vacuum and refrigerated prior to chromatographic separation.

Second Dimension Reverse Phase nanoLC: Peptide solutions were loaded onto a C18 pre-column and then separated on a C18 PepMap100, 3 µm column (Dionex, USA) using the nano HPLC system Ultimate 3000 (Dionex, USA). A gradient of 10-40% acetonitrile in 0.1% formic acid at a flow rate of 300 nl/min was used. The eluent was mixed with matrix solution 5mg/ml CHCA (α-cyano-4-hydroxycinnamic acid) for spotting. Each fraction was spotted over 180 wells on a 384 well Opti-TOF plate (AB SCIEX, USA) using a Probot Micro Fraction Collector (LC Packings, USA). Quality control protein standards (BSA solution) were spotted on wells for calibration.

4.2.7.3.2 Total protein content

The protein content of the canine faecal samples was quantified and is shown in Table 4.3 in µg/µl, together with the amount of protein (µg) loaded onto the HPLC column.

Total protein content was calculated as follows:

Digested protein extract was resolubilised in 100µl of Buffer A x protein content (µg/µl) determined by Bradford assay.

For example, in sample 1 the amount used in the first dimension separation was 100 x 7.7µg/µl. That is 770µg of protein loaded onto column.

The second dimension using reverse phase chromatography required a target load of 100µg protein on the column. The appropriate volume to load, based upon 9µl resuspension in 2% (v/v) acetonitrile and 0.05%(v/v) trifluoracetic acid, was calculated by referring to the AUC from the first dimension chromatograph trace. The protein yields for all samples are shown in Table 4.3.
4.2.7.3.3 Chromatographic peptide separation

The chromatographic separations for the five experiments conducted with peptide mixtures from different protein extracts of canine faecal samples are shown in the results.

4.2.8 Mass spectrometry - Introduction

Different types of mass spectrometer have different capabilities. Simple instruments will measure a set of molecular masses for the intact peptides in a mixture. This study used an instrument with MS/MS capability that provides additional amino acid sequence information by recording the fragment ion spectrum of a peptide. For complex mixtures, such as canine faecal protein extracts, enzymic digestion and separation by chromatography are necessary steps prior to MS/MS analysis. Trypsin cleaves polypeptides at the basic amino acid residues of arginine and lysine, resulting in positively charged peptides which are retained on the strong cation exchange HPLC column, performed under acidic conditions (pH 3.0). Further separation using reverse phase chromatography enables greater separation of peptides increasing sensitivity for mass spectrometry and reducing background contaminants that hamper detection of lower abundant peptides (Gilar et al., 2004).

MS/MS spectra from individual peptides were measured and the experimental mass values were then compared with theoretical peptide mass or fragment ion mass values, obtained by applying peptide cleavage rules to the entries in a comprehensive primary sequence database. When the closest match or matches were identified from database sequences as a "hypothetical" protein the aim was to determine the precise amino acid sequence. This was achieved by extracting those database sequences entries that exhibited the closest homology, possibly occurring as equivalent proteins from similar species.

The separated protein fragments are further analysed using mass spectrometry methods which in these experiments used laser ionization (Matrix Assisted Laser
Desorption Ionization MALDI) with Time of Flight (TOF) mass analysis based upon mass-charge ratio (m/z) and TOF /TOF fragment analysis mass spectrometry.

Researchers have successfully used MALDI-TOF/TOF mass spectrometry in a wide range of medical applications and this technique has been used in the development of a rapid simple method to differentiate between morphologically similar species of plants (Perera et al., 2005).

Current diagnostics for Giardia spp. based upon microscopy are unable to distinguish between different genotypes of Giardia spp. and the proteomic approach used here aimed to identify proteins associated with Giardia spp. infections in dogs. Faecal proteins were extracted and precipitated, and further separated using two dimensional liquid chromatography (2D LC) and applied mass spectrometry analysis using MALDI-TOF/TOF techniques. The resultant mass spectra were analysed and compared to existing protein database entries. An overview of the workflow for mass spectrometry data analysis is provided in Figure 4.2.
4.2.8.1 Mass spectrometry separation and analysis of peptides – plate spotting

Samples were resuspended in 10 µl of 2.0% ACN and 0.5% TFA solution, and spotted in duplicate onto a 384 well Opti-TOF plate (AB SCIEX, USA), 0.6 µl sample and 0.6 µl matrix solution (10 mg/ml α-cyano-4-hydroxycinnamic acid CHCA).
4.2.8.2 Mass spectrometry analysis

Peptides were analysed on a MALDI-TOF/TOF mass spectrometer (Model 4800 or 5800 AB SCIEX, USA) operated in reflector positive mode. MS data were acquired over a mass range of 800-4000 m/z and for each spectrum a total of 400 laser shots were accumulated.

Precursors for MS/MS analysis was automatically generated by the instrument software (4000 Series Explorer Software, AB SCIEX) according to the following selection criteria: minimum signal-to-noise ratio, 10; precursor mass tolerance between spots, maximum outlier error ±50 ppm; maximum 20 peaks per spot; maximum precursors 40 per spot. MS/MS spectra were generated by 1 kV collisions with ambient air and accumulation of maximally 10,000 laser shots. Stop conditions for MS/MS were defined as a minimal number of five fragment peaks with S/N ratio of 100 with at least 20 subspectra accumulated.

4.2.8.3 Spectral evidence

The manufacturer’s data analysis software (ProteinPilot) was used for matching mass spectra against the following databases using the Paragon algorithm (AB SCIEX). Spectral data were matched to theoretical fragment ion patterns to determine peptide presence. Spectral data were also matched to a specialized database with parasite protein information for *G. duodenalis* assemblages A and B (GiardiaDB) and to a universal protein database, details are given in section 4.2.10.1. Confidence scores for peptides determined by pattern matching were only accepted if there was 95% or higher certainty of accurate identification. One or more peptides were used to confirm protein identification. Protein matches were accepted at 95% or above confidence levels.

4.2.9 Bioinformatics

Over many years information has accumulated concerning *Giardia* spp. biology, and the significance of this protozoan as a model organism has been recognized by parasitologists in a recent publication containing a summary of the current knowledge.
Genomic data has been published for *G. duodenalis* genomes WB and GS, and P15 (Morrison *et al.*, 2007). This data forms one of the fundamental sources of reference data for researchers, and is also integral to this study.

Bioinformatics for systems biology is approached from two perspectives; 1) describing the biological system and applying a model to relate the different processes; 2) collection of data, recording observations and then explaining the processes which are occurring to construct a model.

The term model implies representation or display of structure or process, and use of model organisms (e.g. *Aradopsis* sp., *C. elegans*, *Drosophila* sp.) as simpler organisms may serve to depict events occurring in more complex organisms. As a model organism, *G. duodenalis* represents eukaryotes, unicellular protists and parasitic pathogens. Biological experiments that rely on existing knowledge are exposed to erroneous interpretation of data, depending upon the stringency of baseline data accuracy and validation.

### 4.2.9.1 Genomic and proteomic reference databases

Prior to commencing this project searches were conducted using the keywords ‘*G. duodenalis*’ ‘Proteomics’ and ‘Canine faecal protein’. The most frequently accessed web-based material was from www.ncbi.nlm.nih.gov and www.Giardiadb.org (EuPathDB).

The Universal Protein Resource (UniProt) www.UNIPROT.org is a comprehensive resource for protein sequence and annotation data. A universal database selected for this study available online provides an alternative source of reference protein sequences, and has been developed jointly by the Ludwig Institute for Cancer Research, www.ludwigcancerresearch.org/. The LudwigNR database contains high quality annotated and non-redundant protein sequences, derived from experimental results, computed features and scientific conclusions. This database is updated
regularly and the most recent version used in this study, contained 12,595,433 entries (5.31GB), 2013.

Pattern matching software developed and commonly used in publications includes Mascot (Matrix Science, London UK) and Sequest, (Thermo Finningan, USA). Sequence alignment or pattern matching software depends upon algorithms that combine data from peptide detection to construct protein identifications.

4.2.9.2 Spectral data from two dimensional LC MALDI experiments

This pattern matching software used in this study for analysis of the raw spectral data, matched ion fragmentation patterns to amino acid sequence data from specific databases. Spectral data from two dimensional LC MALDI-TOF/TOF experiments were submitted to Protein pilot protein matching software, using the parameter set Sample Type:ID; Cys alkylation: MMTS; Digestion: Trypsin; Instrument 4800 or 5800; Special factors: None; Species: None; ID Focus: Biological Modifications; Database G. duodenalisAandB_130801; Search Effort: Thorough; Results Quality: Detected Protein Threshold (0.05); Run False Discovery Analysis.

False discovery detection settings were applied to all analyses performed and any matches to reverse sequences discarded (Shilov et al 2007, Lu et al 2008). Sample spectra were matched to 3 different databases Database 1) and 2) were genomic databases for G. duodenalis assemblages A and B (G. duodenalisAandBcombined.fasta) G. duodenalis DBv 3.1 (7/05/13); Database 3) served as a decoy database – a universal genomic databases LudwigNR_Q113_genericforward.fasta (10/01/13).

4.2.9.3 Data analysis

The proteins were non-redundant (nr) which refers to database entries that have been manually curated to provide only one entry per protein product; variants are annotated in entry and cross-referenced to other databases. The majority of the protein sequences in the database are derived from the translation of genetic coding sequences from public nucleic acid databases.
Whilst *Giardia* spp. isolates originated from canine (domestic dog) faecal samples, there is currently no genomic data available for host specific *Giardia* spp. strains infecting canines. *G. duodenalis* genome information was published by Morrison et al. (2007) and genetic sequence and annotations are now available for assemblages A, B and E. It has been established that 91% homology exists between these three *G. duodenalis* genomes (Jerlstrom-Hultqvist et al., 2010). In the absence of genomic data for host specific *Giardia* spp. assemblages C and D existing genomic reference databases were used for proteomic analysis. The *G. duodenalis* genomic databases used for protein matching against the mass spectral data were obtained from http://giardiadb.org/ (Aurrecoechea et al., 2009). Assemblages A and B were used as the reference sequences DB v 2.0 (16/11/2009) v 2.1 (15/7/2010) and v3 (11/3/2013) and contain 9663 entries for protein-coding genes.

### 4.3 Results

#### 4.3.1 Sample preparation

Sample preparation methods to map the canine faecal proteome are summarized in Table 4.2, showing the numbers of parasite and host proteins detected by LCMS analysis.

Four faecal samples from different dogs (i) – (iv), were used in the proteomic experiments. Experiment 3 was performed by the same method, on two different canine faecal samples (ii) and (iii).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Physical processing</th>
<th><em>Giardia</em> spp. Parasite proteins</th>
<th>C. familiaris Host proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fresh sample (i) homogenate</td>
<td>3</td>
<td>81</td>
</tr>
<tr>
<td>2</td>
<td>Fresh sample(ii) Freeze thaw (x3)</td>
<td>2</td>
<td>120</td>
</tr>
<tr>
<td>3.1</td>
<td>Frozen sample (ii) Freeze thaw (x8)</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>3.2</td>
<td>Frozen sample (iii) Freeze thaw (x8)</td>
<td>4</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>Fresh sample (iv) homogenate</td>
<td>6</td>
<td>3</td>
</tr>
</tbody>
</table>
4.3.2 Gel separation

Results from the separation of faecal protein extract showed distinct bands, in Figure 4.3, that were visible between 25-100kDa for *Giardia* spp. infected canine faecal samples containing high and moderate cyst numbers.
<table>
<thead>
<tr>
<th>Lane</th>
<th>Amount (µg)</th>
<th>Sample Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10-250 kDa</td>
<td>Protein Marker</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>BSA Protein Std</td>
</tr>
<tr>
<td>3</td>
<td>20µg</td>
<td>canine faecal sample – no Giardia spp. cysts</td>
</tr>
<tr>
<td>4</td>
<td>40µg</td>
<td>canine faecal sample – no Giardia spp. cysts</td>
</tr>
<tr>
<td>5</td>
<td>100µg</td>
<td>canine faecal sample – no Giardia spp. cysts</td>
</tr>
<tr>
<td>7</td>
<td>100µg</td>
<td>canine faecal sample – moderate numbers of Giardia spp. cysts</td>
</tr>
<tr>
<td>8</td>
<td>50µg</td>
<td>canine faecal sample – moderate numbers of Giardia spp. cysts</td>
</tr>
<tr>
<td>9</td>
<td>20µg</td>
<td>canine faecal sample – moderate numbers of Giardia spp. cysts</td>
</tr>
<tr>
<td>11</td>
<td>100µg</td>
<td>canine faecal sample – high numbers of Giardia spp. cysts</td>
</tr>
<tr>
<td>12</td>
<td>50µg</td>
<td>canine faecal sample – high numbers of Giardia spp. cysts</td>
</tr>
<tr>
<td>13</td>
<td>20µg</td>
<td>canine faecal sample – high numbers of Giardia spp. cysts</td>
</tr>
<tr>
<td>14</td>
<td>10-250 kDa</td>
<td>Protein marker</td>
</tr>
</tbody>
</table>
4.3.3 HPLC separation

Table 4.3 Protein yields, quantity used for peptide separation and Area under Curve

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Amount of protein loaded on column (µg)</th>
<th>Area under Curve (AUC) milli absorbance units/min</th>
<th>Maximum peak height (mAU)</th>
<th>Protein content (µg/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 sample(i)</td>
<td>70</td>
<td>2520</td>
<td>350</td>
<td>7.7</td>
</tr>
<tr>
<td>2 sample (ii)</td>
<td>129</td>
<td>1369</td>
<td>615</td>
<td>0.43</td>
</tr>
<tr>
<td>3 sample (ii)</td>
<td>95</td>
<td>2020</td>
<td>270</td>
<td>1.28</td>
</tr>
<tr>
<td>3.1 sample(ii)</td>
<td>61</td>
<td>3567</td>
<td>505</td>
<td>0.97</td>
</tr>
<tr>
<td>3.2 sample (iii)</td>
<td>100</td>
<td>1528</td>
<td>150</td>
<td>3.69</td>
</tr>
</tbody>
</table>

Calculation of protein content is shown in section 4.2.7.3.2.

Figure 4.4 displays an example of an analytical HPLC trace obtained from 1st dimension separation and an example of the second dimension chromatography traces are shown in Figure 4.5. The results of peptide separation using UV detection at 214nm are presented as area under the curve (mAU/min) and maximum peak height (mAU).

The area under the curve and maximum peak heights are shown in Table 4.3 for all samples. Some traces showed separation into smaller peaks at later elution times, however in general all samples were visible as one large peak, with limited resolution into discrete peptide peaks.
Figure 4.4 An example of the analytical HPLC trace of 1st dimension separation of peptides in canine faecal protein extract (ii) in Experiment 2.
Figure 4.5 An example of second dimension reverse phase chromatography trace of peptide mixture from 8 fractions eluted by cation exchange chromatography of canine faecal extract (i) digested proteins and peptides. The maximum absorbance values ranged from 10-38mAU.
4.3.4 Mass spectral data

For each dataset the number of peptides identified are reported in tables below, and protein(s) identified containing the peptide sequences where confidence levels are determined by specific algorithms of the software.

4.3.4.1 Spectra matched to peptide sequences within G. duodenalis database for assemblages A and B

When the same mass spectral data was submitted to a universal protein database, in this instance Ludwig database, a different set of potential peptide and protein matches are generated. In column four the percentage of total spectra that were assigned to peptide sequences is given. The percentage of total spectra is the same for both databases because the spectral evidence does not change, only the confidence matching based upon mass and ion score.

4.3.4.2 Spectra matching to universal protein database (LudwigNR) using ProteinPilot.

The mass spectral data analysed using a universal protein database detected peptides at a high level of confidence. Many of these were of host origin. A summary of the parasite and host proteins and peptides detected is presented in Table 4.4. Details for individual samples are shown in subsequent tables.

Table 4.4 Total spectral matches

<table>
<thead>
<tr>
<th>Confidence Threshold</th>
<th>Proteins Detected</th>
<th>Distinct Peptides</th>
<th>% Total Spectra</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GiardiaDB</td>
<td>Universal DB</td>
<td>GiardiaDB</td>
</tr>
<tr>
<td>&gt;99%</td>
<td>1</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>&gt;95%</td>
<td>6</td>
<td>3</td>
<td>32</td>
</tr>
<tr>
<td>&gt;66%</td>
<td>10</td>
<td>8</td>
<td>45</td>
</tr>
</tbody>
</table>

The tables below represent data summaries from analysis of mass spectra data as determined using Protein pilot pattern matching software.

Results generated by the protein matching software were assigned a rank value for each protein detected, where N=1 is the most confident protein match relative to other
proteins identified. The results were examined for true *G. duodenalis* protein matches and any proteins matching to other organisms were excluded. For example, in Table 4.5 matched proteins ranked N=1 to N=3 were of host origin. All proteins matched were based upon detection of peptides at 95% confidence or higher.

Tables 4.5 - 4.9 display the mass spectra matched to *G. duodenalis* proteins contained within GiardiaDB database. The tables include the total number of proteins and peptides identified, with footnotes accompanied by ranked scores for each confident *G. duodenalis* protein match. Higher ranked proteins that are not displayed were excluded during the validation stage described above.

### 4.3.4.3 Whole faecal sample (i)

Table 4.5 spectral matches for fresh faecal homogenate sample

<table>
<thead>
<tr>
<th></th>
<th><em>G. duodenalis</em> database</th>
<th>Ludwig database</th>
<th>Total Spectra</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spectra identified</td>
<td>259</td>
<td>1178</td>
<td>9958</td>
</tr>
<tr>
<td>Proteins detected</td>
<td>14</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>Distinct peptides</td>
<td>231</td>
<td>930</td>
<td></td>
</tr>
</tbody>
</table>

Best matching *G. duodenalis* protein

N=4 Confidence score 66% match to hypothetical protein

### 4.3.4.4 Whole faecal, freeze thaw (x3) sample (ii)

Table 4.6 Spectral matches for freeze thaw sample

<table>
<thead>
<tr>
<th></th>
<th><em>G. duodenalis</em> database</th>
<th>Ludwig database</th>
<th>Total Spectra</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spectra identified</td>
<td>215</td>
<td>887</td>
<td>2152</td>
</tr>
<tr>
<td>Proteins detected</td>
<td>45</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>Distinct peptides</td>
<td>202</td>
<td>727</td>
<td></td>
</tr>
</tbody>
</table>

Best matching *G. duodenalis* protein

N=2 Confidence score >99% match to histone methyl transferase
4.3.4.5 Freeze-thaw faecal extract (ii)

Table 4.7 Spectral matches for freeze thaw sample

<table>
<thead>
<tr>
<th></th>
<th>G. duodenalis database</th>
<th>Ludwig database</th>
<th>Total Spectra</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spectra identified</td>
<td>188</td>
<td>642</td>
<td>5196</td>
</tr>
<tr>
<td>Proteins detected</td>
<td>20</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>Distinct peptides</td>
<td>145</td>
<td>387</td>
<td></td>
</tr>
</tbody>
</table>

Best matching *G. duodenalis* proteins
N=1 confidence score 99% hypothetical protein
N=2 confidence score 66% Nek kinase (matched two *G. duodenalis* isolates, A and B)

Protein identifications were accepted with 66% confidence score because of a peptide assignment at 99% confidence level and because no other homologous peptide was found in the universal protein database.

4.3.4.6 Freeze-thaw disrupted cysts faecal extract (iii)

Table 4.8 Spectral matches for freeze-thaw disrupted cysts

<table>
<thead>
<tr>
<th></th>
<th>G. duodenalis database</th>
<th>Ludwig database</th>
<th>Total Spectra</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spectra identified</td>
<td>113</td>
<td>436</td>
<td>5219</td>
</tr>
<tr>
<td>Proteins detected</td>
<td>16</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>Distinct peptides</td>
<td>92</td>
<td>289</td>
<td></td>
</tr>
</tbody>
</table>

Best matching *G. duodenalis* proteins
N=2 confidence score 99% signal recognition particle receptor protein
N=4 confidence score 95% hypothetical protein
N=5 confidence score 66% hypothetical protein

4.3.4.7 Faecal homogenate protein extract (iv)

Table 4.9 Spectral matches for faecal homogenate protein

<table>
<thead>
<tr>
<th></th>
<th>G. duodenalis database</th>
<th>Ludwig database</th>
<th>Total Spectra</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spectra identified</td>
<td>171</td>
<td>278</td>
<td>3576</td>
</tr>
<tr>
<td>Proteins detected</td>
<td>55</td>
<td>139</td>
<td></td>
</tr>
<tr>
<td>Distinct peptides</td>
<td>134</td>
<td>212</td>
<td></td>
</tr>
</tbody>
</table>

Best matching *G. duodenalis* proteins
N=1 confidence score 99% hypothetical protein
N=2 confidence score 99% mismatch repair protein (matched two G. duodenalis isolates, A and B)

N=3 confidence score 99% axoneme central apparatus protein (matched two G. duodenalis isolates, A and B)

N=4 confidence score 99% hypothetical protein

N=5 confidence score 99% pyruvate flavodoxin oxidoreductase (matched two G. duodenalis isolates, A and B)

N=6 confidence score 99% coiled coil protein

Table 4.10 Comparative summary of the spectral data for each of the 2D LC experiments.

<table>
<thead>
<tr>
<th>Spectral data</th>
<th>Giardia database matching</th>
<th>Universal database matching</th>
<th>Ratio of parasite: host proteins detected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of spectra identified</td>
<td>% Spectra assigned to G. duodenalis protein</td>
<td>Number of spectra identified</td>
</tr>
<tr>
<td>Experiment</td>
<td>Total number of spectra</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9958</td>
<td>259</td>
<td>2.6</td>
</tr>
<tr>
<td>2</td>
<td>2152</td>
<td>215</td>
<td>9.9</td>
</tr>
<tr>
<td>3</td>
<td>5196</td>
<td>188</td>
<td>3.6</td>
</tr>
<tr>
<td>4</td>
<td>5219</td>
<td>113</td>
<td>2.1</td>
</tr>
<tr>
<td>5</td>
<td>3576</td>
<td>171</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Confidence scores with threshold set at 95% confidence.

4.3.5 Examination of raw spectral traces

The mass spectra were analysed to determine an amino acid sequence and therefore protein identity and fraction. This was achieved by; 1) pattern matching to G. duodenalis proteins, 2) pattern matching to all known proteins as described above and 3) de novo sequencing. Proteins identified by G. duodenalis database matching were investigated further by examination of individual spectral peaks. Each spectral peak was analysed by comparison with proteins matched using the Ludwig database to determine if there was an equal or better match than the G. duodenalis protein identified. An example of fragmentation patterns and peptide assignment are shown in
Figures 4.6 and 4.7 for GiardiaDB and universal DB respectively. The peptides generated and matching to peptide sequences in Ludwig database were then examined by BLAST search against Uniprot and NCBI databases to identify if any protein matches occurred with a higher degree of confidence.

The same spectra were submitted to two different databases for peptide matching, different peptide sequences were assigned to the spectra according to the database used. Figure 4.6 shows that the peptide sequence determined by mass spectra matching to GiardiaDB entries is KNSILNTLK.

Figure 4.7 shows the peptide sequence assigned by pattern matching to the universal protein database. The peptide sequence determined by mass spectra matching to universal protein database entries is QLDFTLSPK.

Only one confident b ion was detected for this peptide and is not matched to a protein in the universal protein database. The total ion score of 4 is less than the total ion score of 6 for spectra matching to Giardia database. A higher ion score increases the confidence of accurate protein identification. The conclusion from spectral analysis accepts that there is no evidence for any other (non-Giardia) protein.
Fragmentation evidence for (Giardia DB) assignment of spectra

In the mass spectrometer laser ionization causes ion collision and breakage of peptide bond resulting in 2 types of daughter ions - b ion fragments and y ion fragments. Each of the fragments consists of an amino acid sequence.

At the lower end of the spectrum (in the table) b ion with a mass of 129 indicates lysine as the terminal amino acid (mass of glutamine is 128, with positive charge).

At the lower end of the spectrum (in the table) Y ion with mass of 147 indicates lysine as the terminal amino acid (128 plus the mass of water and hydrogen ion).

Diamino mass of lysine and asparagine is 242.

Knowing the mass of a b ion allows calculation of the y ion and vice versa. The different fragment sizes are shown as spectral traces below and in exact mass value in the table. This spectra shows the mass for amino acids as they fragmented from 1 – 9 different mass scores indicating peptide of 9 amino acid residues.

Difference in mass between b4 and b3 is 113 (mass of leucine is 113).

Difference in mass between y7 and y6 is 87 (mass of serine is 87).

Figure 4.6 Annotated ms spectra for mismatch repair protein.
Figure 4.7 Fragmentation evidence for universalDB assignment of spectra
Results for the parasite and host faecal proteomes are described in Chapter 5. Summary tables for the parasite faecal proteome present protein identifications in Table 5.2 and peptide identifications in Table 5.3. Summary tables for the host faecal proteome protein identifications are shown in Tables 5.4 – 5.7.

4.3.6 Conclusion

Faecal protein analysis using a proteomic approach has for the first time been successful in the identification of parasite proteins associated with *Giardia* spp. infections in canines. The approach of focusing on aqueous phase proteins was appropriate although variation in the types and numbers of *Giardia* spp. proteins was noted. Further work is required to determine if sample preparation methods influence the types of proteins identified for example different buffers and surfactants may be affecting the integrity of proteins.

The iterative process used here to inform subsequent steps in protein extraction, separation and identification worked well over time. This is a preliminary study and these methods form a firm base for continued work on protein extraction methods and optimisation for parasite protein profile.
Chapter 5

Host-parasite faecal proteome mapping for the canine host infected with *Giardia* spp. parasite

5.1 Introduction

This chapter examines the results from mass spectral data reported in Chapter 4 in the context of the faecal proteome, protein function and host-parasite biology.

5.1.1 Host species investigated

Early studies investigating transmission of *Giardia* spp. were conducted in humans and animal models such as mice or Mongolian gerbils, not specifically for *Giardia* species infecting dogs. These studies provided important fundamental data necessary for further understanding of the biological and biochemical functions of *Giardia* proteins (Holberton and Ward, 1981, Jarroll et al., 1989) and disease processes in humans (Nash and Keister, 1985, Nash et al., 1987).

The research described in this thesis aimed to improve the wider understanding of host-parasite interactions, particularly how *Giardia* spp. infections affect dogs.

5.1.2 Proteomic approach to phenotypic characterization of *G. duodenalis*

Protein analysis using proteomic tools may hold the key for differentiating between the various *G. duodenalis* sub-types and provide the opportunity to extend descriptors beyond the genome sequencing and genotyping of *G. duodenalis* to the phenotypic expression of protein at the cellular level.

Whilst molecular investigations into *Giardia* spp. have focused on genetic analysis, work has already advanced at the proteomic level to elucidate *Giardia* spp. protein expression. To date there is increasing data concerning basal bodies (Lauwaet et al., 2011), protein kinases Manning *et al.* (2011), mitosomes Jedelsky *et al.* (2011) and mitosomal transport proteins of *Giardia* spp. Dagley *et al.* (2009). Kim *et al.* (2009) reported the dramatic change in protein profiles of *Giardia* cysts and trophozoites
sourced from in vitro culture during encystation or excystation periods in *Giardia* spp. using a proteomic approach.

5.1.3 **Biomarkers of infection**

Medical research criteria, together with convenience and patient preference have influenced the type of biological samples required for scientific testing, with blood products and urine being the most frequently used (Xu and Veenstra, 2008, Steiner, 2014). However, advances in scientific understanding combined with changes in human disease patterns have prompted researchers to investigate wider use and application and use of biological samples. Examples of this type of research are the use of faeces for isolation of biomarkers, such as haemoglobin in human colorectal cancer, calprotectin and lactoferrin in inflammatory bowel disease (IBD), or elastase in canine exocrine pancreatic insufficiency (Battersby *et al*., 2005, Ang *et al*., 2011, Lamb and Mansfield, 2011). Although faecal samples are proving useful in biomarker investigations and proteomic profiling, there remain some limitations due to the complexity and diverse composition arising from dietary and host proteins, in addition to dynamic and variable microbial content (Zoetendal *et al*., 2011).

5.1.4 **Ecosystem of the digestive tract**

Conditions of the mammalian gastro-intestinal tract are affected by host species, genotype and physiology, host diet and immunological status (disease, vaccination etc.) (Round and Mazmanian, 2009). In fact, unique and specialized ecosystems of the gastro-intestinal tract exist, which contains anaerobic or microaerophilic microbial populations of bacteria, fungi, and parasites, within fluids of the intestinal lumen and have been estimated to contribute up to ten times the number of cells in a human body (Hattori and Taylor, 2009, Qin *et al*., 2010, Shen *et al*., 2010). The functional efficacy of a particular organism in the complex microbial environment of the GIT is dependent on its numerical abundance, survival, competitiveness, and metabolic activity (Fitzsimons *et al*., 2003). Completion of the digestive process, which provides nutritional benefits both to host and microbial members of the ecosystem, generates waste products, urine
and faeces. Faecal material contains waste products from digestive tract processes and faecal protein composition includes dietary proteins, host epithelial cells and secretory proteins, and other microbial proteins (bacterial, fungal, parasite, or viral) (Eckburg et al., 2005). The dark coloration of faecal matter is due to the end products from breakdown of bilirubin (Liu et al. 2008) and this and other components can be responsible for inhibition of PCR reactions in genetic studies.

5.1.5 Faecal proteomics
Proteomic analysis of faecal material is a recent method and one approach has been used to examine microbial populations of the gastro-intestinal tract. A metaproteomics study by Klaassens et al. (2007) has demonstrated the use of 2D PAGE and mass spectrometry methods for identifying faecal proteins from the gastrointestinal tract of infants. A similar metaproteomic investigation of the microbiota present in the human distal gut used a non-targeted shotgun mass spectrometry method to successfully study functional gene expression in illustrating potential interactions of the gut microbial ecosystem with the human host (Verberkmoes et al., 2009).

5.1.6 Hypothesis
That novel proteins or peptides detected in canine faeces be used to differentiate between different genotypes of Giardia spp. That faecal proteome data can detect host-parasite interactions

5.2 Results for faecal proteome mapping
Presented here are the proteins identified by matching peptide assignments introduced in Chapter 4.

5.2.1 Mapping the faecal proteome
Experimental work investigating parasite proteins in infected faecal material has not been performed before, and it presented significant challenges because Giardia cysts form only a small portion of total faecal proteins. Validation of all proteins identified was essential and only proteins identified from peptides at the highest confidence level
(including screening for false positives) were included in the final proteome map. Some proteins identified did not meet validation criteria and were excluded from the final proteome map.

As each sample was unique in origin, from different locations, different individual dogs (diet, host genetics, physiology and symptomology), together with variation in the processing methods, analysis of the results could not be meaningfully combined. The objective of this study was for mapping and single sample analysis was considered appropriate. Detailed results are presented as follows: 1) a summary of the individual samples analysed in this exploratory study; 2) the parasite faecal proteome map; 3) species composition of proteins detected in the faecal sample; 4) the host faecal proteome map.

5.2.1.1 Summary of canine faecal samples investigated

A summary of all faecal samples used for proteomic analysis are show in Table 5.1. Sample processing methods are detailed in Chapter 4. All protein samples were obtained from *G. duodenalis* infected fresh canine faeces, and processed immediately following diagnostic techniques. Canine faecal samples, numbered 1-5 originated from *G. duodenalis* infected dogs, including 2 symptomatic dogs and 2 asymptomatic dogs. Chapter 2 describes in more detail the methods used for collection of diagnostic and clinical information.

<table>
<thead>
<tr>
<th>Season collected</th>
<th>Age (days)</th>
<th>Location</th>
<th>Management</th>
<th>Clinical info (stool form)</th>
<th>Giardia spp. cyst intensity</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Spring)</td>
<td>283</td>
<td>Melbourne Victoria</td>
<td>breeder</td>
<td>Symptomatic</td>
<td>4</td>
<td>D</td>
</tr>
<tr>
<td>2 (Spring)</td>
<td>730</td>
<td>Perth, WA</td>
<td>shelter</td>
<td>Asymptomatic</td>
<td>4</td>
<td>D</td>
</tr>
<tr>
<td>3 (Autumn)</td>
<td>42</td>
<td>Perth, WA</td>
<td>shelter</td>
<td>Symptomatic</td>
<td>3+</td>
<td>D</td>
</tr>
<tr>
<td>4 (Autumn)</td>
<td>42</td>
<td>Perth, WA</td>
<td>breeder</td>
<td>Symptomatic</td>
<td>3+</td>
<td>D</td>
</tr>
<tr>
<td>5 (Summer)</td>
<td>2190</td>
<td>Perth, WA</td>
<td>shelter</td>
<td>Asymptomatic</td>
<td>3+</td>
<td>C/D and A</td>
</tr>
</tbody>
</table>

* no other parasites were observed by light microscopy gold standard
5.2.2 *Giardia* spp. (parasite) faecal proteome

Results presented in Table 5.2 include only those proteins identified with at least one matching peptide at 99% confidence. Most protein identifications also included matches to other peptides at lower confidence levels, which supported acceptance of the database matched protein, and in the absence of contrary evidence from *de novo* sequencing methods.

Homologous proteins, with different accession numbers were confirmed when peptides were matched to those of *G. duodenalis* assemblage A and assemblage B, such as axoneme central apparatus protein, as shown in Table 5.2.

Several proteins identified were based upon detection of unique peptides, where peptides were assigned to only one protein and not to any other protein, host, parasite or other organism contained in the database described in Chapter 4.

Two unique peptides were identified in one protein (hypothetical protein) and one unique peptide in one each of four proteins (mismatch repair protein, axoneme central apparatus protein, hypothetical protein and coiled-coil protein).
Table 5.2 *Giardia duodenalis* proteins identified (Parasite Faecal Proteome Map)

<table>
<thead>
<tr>
<th>Number</th>
<th>Protein name</th>
<th>Protein Score</th>
<th>Protein Accession Number</th>
<th>Gene accession number</th>
<th>Giardia Assemblage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hypothetical protein</td>
<td>2.0</td>
<td>A8BBE2</td>
<td>GL50803_117192</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>Histone methyltransferase MYST1</td>
<td>2.0</td>
<td>C6LVL9</td>
<td>GL50581_2825</td>
<td>B</td>
</tr>
<tr>
<td>3</td>
<td>Nek Kinase</td>
<td>0.62</td>
<td>C6LMX7</td>
<td>GL50581_75</td>
<td>B</td>
</tr>
<tr>
<td>(3)</td>
<td>Nek Kinase</td>
<td>0.62</td>
<td>A8BVL0</td>
<td>GL50803_92498</td>
<td>A</td>
</tr>
<tr>
<td>4</td>
<td>Signal recognition particle receptor</td>
<td>2.0</td>
<td>C6LN45</td>
<td>GL50581_145</td>
<td>B</td>
</tr>
<tr>
<td>5</td>
<td>Hypothetical protein</td>
<td>1.64</td>
<td>C6LN73</td>
<td>GL50581_174</td>
<td>B</td>
</tr>
<tr>
<td>6</td>
<td>Hypothetical protein</td>
<td>3.18</td>
<td>C6LS33</td>
<td>GL50581_1569</td>
<td>B</td>
</tr>
<tr>
<td>7</td>
<td>Hypothetical protein</td>
<td>2.02</td>
<td>C6LSL4</td>
<td>GL50581_1753</td>
<td>B</td>
</tr>
<tr>
<td>8</td>
<td>Mismatch repair protein</td>
<td>2.0</td>
<td>C6LTM7</td>
<td>GL50581_2123</td>
<td>B</td>
</tr>
<tr>
<td>(8)</td>
<td>Mismatch repair protein</td>
<td>2.0</td>
<td>A8B6C1</td>
<td>GL50803_34058</td>
<td>A</td>
</tr>
<tr>
<td>9</td>
<td>Axoneme central apparatus protein</td>
<td>2.0</td>
<td>C6LYT9</td>
<td>GL50581_3967</td>
<td>B</td>
</tr>
<tr>
<td>(9)</td>
<td>Axoneme central apparatus protein</td>
<td>2.0</td>
<td>A8BQ10</td>
<td>GL50803_16202</td>
<td>A</td>
</tr>
<tr>
<td>10</td>
<td>Hypothetical protein</td>
<td>2.0</td>
<td>A8B8B6</td>
<td>GL50803_6625</td>
<td>A</td>
</tr>
<tr>
<td>11</td>
<td>Pyruvate-flavodoxin oxidoreductase</td>
<td>1.68</td>
<td>E2RTZ1</td>
<td>GL50803_114609</td>
<td>A</td>
</tr>
<tr>
<td>(11)</td>
<td>Pyruvate-flavodoxin oxidoreductase</td>
<td>1.6</td>
<td>C6LZB6</td>
<td>GL50581_4147</td>
<td>B</td>
</tr>
<tr>
<td>12</td>
<td>Coiled-coil protein</td>
<td>1.54</td>
<td>C6LY87</td>
<td>GL50581_3758</td>
<td>B</td>
</tr>
<tr>
<td>13</td>
<td>Hypothetical protein</td>
<td>2.64</td>
<td>C6LUY7</td>
<td>GL 50581_2589</td>
<td>B</td>
</tr>
<tr>
<td>14</td>
<td>tRNA/rRNA cytosine C5 methylase</td>
<td>1.30</td>
<td>A8BA69</td>
<td>GL 50803_3985</td>
<td>A</td>
</tr>
<tr>
<td>15</td>
<td>Coiled coil protein</td>
<td>4.5</td>
<td>C6LZ39</td>
<td>GL 50803_4068</td>
<td>B</td>
</tr>
<tr>
<td>16</td>
<td>Hypothetical protein</td>
<td>2.0</td>
<td>A8B278</td>
<td>GL 50803_37864</td>
<td>A</td>
</tr>
</tbody>
</table>

The results presented in Table 5.2 include proteins detected over a threshold score, where the cut off value is 1.3; all scores equal and above represent highly confident protein matches (95% and above).
### 5.2.2.1 Peptides contributing to *Giardia* spp. protein identification

Sequences and confidence scores for each peptide identified are shown in Table 5.3.

<table>
<thead>
<tr>
<th>N</th>
<th>Peptide Sequence</th>
<th>Peptides matched</th>
<th>Ion Score</th>
<th>Protein Accession Number</th>
<th>Gene Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>KCGIYSSRR</td>
<td>1 @99%</td>
<td>8</td>
<td>Hypothetical protein A8BBE2</td>
<td>GL50803_117192</td>
</tr>
<tr>
<td>2</td>
<td>SQLFNIR</td>
<td>1 @99%</td>
<td>10</td>
<td>Histone methyltransferase MYST1 C6VL9</td>
<td>GL50581_2825</td>
</tr>
<tr>
<td>3</td>
<td>LSYDEIVR</td>
<td>1 @99% 4@&lt;1%</td>
<td>8</td>
<td>Nek Kinase C6LMX7</td>
<td>GL50581_75</td>
</tr>
<tr>
<td>(3)</td>
<td>LSYDEIVR</td>
<td>1 @99% 8@&lt;1%</td>
<td>8</td>
<td>Nek Kinase A8BVLO</td>
<td>GL50803_92498</td>
</tr>
<tr>
<td>4</td>
<td>GVSANLEGK</td>
<td>1 @99% 7@&lt;1%</td>
<td>8</td>
<td>Signal recognition particle receptor C6LN45</td>
<td>GL50581_145</td>
</tr>
<tr>
<td>5</td>
<td>HNDDLELLEK</td>
<td>1 @99% 5@&lt;1%</td>
<td>7</td>
<td>Hypothetical protein C6LN73</td>
<td>GL50581_174</td>
</tr>
<tr>
<td>6</td>
<td>SLQGGGPR VSSDSLQ KTEK (2) NLNL1FIR</td>
<td>3 @99% 1@91.6% 9@&lt;1%</td>
<td>7</td>
<td>Hypothetical protein C6LS33</td>
<td>GL50581_1569</td>
</tr>
<tr>
<td>7</td>
<td>RDRLIOQ FHYVR</td>
<td>1 @99% 1@21.6% 13@&lt;1%</td>
<td>6</td>
<td>Hypothetical protein C6LSL4</td>
<td>GL50581_1753</td>
</tr>
<tr>
<td>8</td>
<td>KNSILNLTK</td>
<td>1 @99% 7@&lt;1%</td>
<td>6</td>
<td>Mismatch repair protein C6LTM7</td>
<td>GL50581_2123</td>
</tr>
<tr>
<td>(8)</td>
<td>KNSILNLTK</td>
<td>1 @99% 7@&lt;1%</td>
<td>6</td>
<td>Mismatch repair protein A8BB6C1</td>
<td>GL50803_34058</td>
</tr>
<tr>
<td>9</td>
<td>EAAAWALGYAR</td>
<td>1 @99%</td>
<td>5</td>
<td>Axoneme central apparatus protein C6LYT9</td>
<td>GL50581_3967</td>
</tr>
<tr>
<td>(9)</td>
<td>EAAAWALGYAR</td>
<td>1 @99%</td>
<td>5</td>
<td>Axoneme central apparatus protein A8BQ10</td>
<td>GL50803_16202</td>
</tr>
<tr>
<td>10</td>
<td>EMQRIVAQR</td>
<td>1 @99%</td>
<td>5</td>
<td>Hypothetical protein A8B8B6</td>
<td>GL50803_6625</td>
</tr>
<tr>
<td>11</td>
<td>LGAGKNSVR</td>
<td>1 @99%</td>
<td>5</td>
<td>Pyruvate-flavodoxin oxidoreductase E2RTZ1</td>
<td>GL50803_114609</td>
</tr>
<tr>
<td>(11)</td>
<td>NSVRLVAGGR LGAGKNSVR</td>
<td>1 @97.9% 1 @14.5% 4 @&lt;1%</td>
<td>7</td>
<td>Pyruvate-flavodoxin oxidoreductase C6LZB6</td>
<td>GL50581_4147</td>
</tr>
<tr>
<td>12</td>
<td>ESMLKKTPLR</td>
<td>1 @99% 6@ &lt;1%</td>
<td>7</td>
<td>Coiled-coil protein C6LY87</td>
<td>GL50581_3758</td>
</tr>
<tr>
<td>13</td>
<td>PKSSQKR/ RAIDALNEIR EWPDPDSQENVPR ATLEQLFFKLNHEQL</td>
<td>2@99% 1@94.2% 1@13.3% many@&lt;1%</td>
<td>7</td>
<td>Hypothetical protein C6LUY7</td>
<td>GL50581_2589</td>
</tr>
<tr>
<td>14</td>
<td>EYEPEINDEADR</td>
<td>1@98.8% 2many @&lt;1%</td>
<td>7</td>
<td>tRNA/rRNA cytosine C5 methylase A8BA69</td>
<td>GL50803_3985</td>
</tr>
<tr>
<td>15</td>
<td>HDMHLSLEQSSK LSHSAQSVALLQEQVQSR TEPSCORKEGEQTILLDR IDKLNSVTAFTKATR</td>
<td>4@99% 22@&lt;1%</td>
<td>13</td>
<td>Coiled coil protein C6LZ39</td>
<td>GL50581_4068</td>
</tr>
<tr>
<td>16</td>
<td>EMQRIVAQR</td>
<td>1 @99%</td>
<td>5</td>
<td>Hypothetical protein A8B278</td>
<td>GL50803_37864</td>
</tr>
</tbody>
</table>
Twenty-two unique amino acid sequences provided positive *Giardia* spp. peptide identifications. Those protein matches in Table 5.2 that were underpinned by multiple peptide matches (sequences shown in Table 5.3) at high quality provide greater confidence of the protein identification. Some peptide sequences appear in more than one entry, which indicates either a conserved sequence, or redundant entries in the database. The most accurate peptide assignments determined by Protein Pilot software report the likelihood of an individual spectra matching to that protein sequence. The ion score is determined at the peptide level only as described in Figures 4.6 and 4.7. The top ion score reflects the most accurate spectral match to a peptide sequence.

### 5.2.2.2 Peptide alignment by comparison of amino acid sequences

This section contains comparison of the amino acid sequence alignment for proteins matching to different *G. duodenalis* genotypic assemblages obtained from database searching.

An * (asterisk) indicates positions which have a single, fully conserved residue.

Point accepted mutations are illustrated as follows:

- A : (colon) indicates conservation between groups of strongly similar properties
- A . (period) indicates conservation between groups of weakly similar properties
- A   (space) indicates residue differences due to discrepant sequence

The red circles mark consecutive amino acid insertions or deletions. The yellow circles mark amino acid point mutations. Peptide sequences detected by mass spectrometry are highlighted.
A comparison using uniprot protein database BLAST search with clustal omega of Nek kinase proteins detected in sample 3 showed 92% identity between sequences from *G. duodenalis* Assemblage A and B shown in Figure 5.1. The peptide detected consists of an amino sequence (LSYDEIVR) indicating an area of homology between the two *G. duodenalis* strains.

**C6LMX7 Nek Kinase**, *G. intestinalis* (strain ATCC 50581 / GS clone H7) (G. lamblia) GL50581_75

**A8BVL0 Nek Kinase**, *G. intestinalis* (strain ATCC 50803 / WB clone C6) (G. *duodenalis* lamblia) GL50803_92498

![Figure 5.1 Amino acid sequence of Nek kinase for two *G. duodenalis* isolates](image)
A comparison using clustal omega from uniprot protein database of axoneme central apparatus proteins detected in sample 5, shown in Figure 5.2, gave 99.8% identity between the two *G. duodenalis* sequences for Assemblage A and B with 501 identical positions and 1 similar position, with a point mutation occurring at position 9. The peptide identified using proteomic analysis in this study is highlighted in yellow for amino acid positions 145 – 156. This is an area of homology between the two *G. duodenalis* strains.

C6LYT9 **Axoneme central apparatus protein** *G. intestinalis* (ATCC 50581 / GS clone H7) (*G. lamblia*) GL50581_3967

A8BQI0 **Axoneme central apparatus protein** *G. intestinalis* (ATCC 50803 / WB clone C6) (*G. lamblia*) GL50803_16202

![Figure 5.2 Amino acid sequence of Axoneme Central Apparatus protein for two *G. duodenalis* isolates](image)
C6LTM7 **Mismatch repair protein** G. intestinalis (strain ATCC 50581 / GS clone H7)
(G. lamblia) GL50581_2123

A8B6C1 **Mismatch repair protein** G. intestinalis (strain ATCC 50803 / WB clone C6)
(G. lamblia) GL50803_34058

Figure 5.3 Amino acid sequence of Mismatch repair protein for two *G. duodenalis*
isolates
A comparison of pyruvate flavodoxin oxidoreductase protein amino acid sequence detected in experiment 5, showed 96.3% identity between the sequences from *G. duodenalis* Assemblage A and B with 1207 identical positions and 40 similar positions, shown in Figure 5.4.

Figure 5.4 Amino acid sequence of pyruvate flavodoxin oxidoreductase protein for two *G. duodenalis* isolates
Figure 5.4 Amino acid sequence of pyruvate flavodoxin oxidoreductase protein for two *G. duodenalis* isolates

E2RTZ1 **Pyruvate-flavodoxin oxidoreductase** *G. intestinalis* (strain ATCC 50803 / WB clone C6) (*G. lamblia*) GL50803_114609
C6LZB6 **Pyruvate-flavodoxin oxidoreductase** *G. intestinalis* (strain ATCC 50581 / GS clone H7) (*G. lamblia*) GL50581_4147

Two different peptides identified by proteomic analysis in this study are highlighted, one in yellow (NSVRLVAGGR) and one in pink (LGAGKNSVR). Two point mutations occur in these peptides identified at positions 365 and 367 for the two overlapping peptides (LGAGKNSVR) and (NSVRLVAGGR). The two *G. duodenalis* strains WB and GS have homologous sequence for NSVRLVAGGR peptide. There are different amino acid sequences with evidence for two point mutations, suggesting that these may constitute phenotypic differences between the *G. duodenalis* strains WB and GS or specific sequences for canine *Giardia* spp. isolates detected in this study. *Giardia* spp. protein identifications were detected in this exploratory study and a wider discussion on the function of proteins that were detected is presented here. These include metabolic, cytoskeletal and genetic replication proteins and provide a deeper insight into *Giardia* interactions in the canine gastro-intestinal tract.

A comparison using clustal omega from uniprot protein database of mismatch repair proteins detected in sample 5, showed 78.3% identity between sequences from *G. duodenalis* Assemblage A and B. Areas of homology occurred between the amino sequences for *G. duodenalis* Assemblages A and B in the peptide identified (KNSILNTLK).

### 5.2.3 *Canis familiaris* (Host) Faecal Proteome

#### 5.2.3.1 Universal protein database matching

Spectra were analysed and matched to peptide entries in the Ludwig protein database. *G. duodenalis* proteins were not detected, but a number of peptides matched to proteins (at high level of confidence) of other organisms. Results were sorted
according to the most confident matching protein, host species and confidence score. The results presented here include proteins detected over a threshold score, where the cut off value is 1.3; all scores equal and above represent highly confident protein matches (95% and above).

Host proteins detected from 5 different faecal protein extracts are described in Tables 5.3 – 5.7, originating from *Giardia* spp. infected dogs. In total 256 host proteins at 95% confidence level were detected and identified by matching spectral traces to sequences in a universal protein database (Ludwig nr version) and those specific to *Canis familiaris*.

5.2.3.2 Canine database matching

Protein matching against *Canis familiaris* specific database did not detect matches with any degree of confidence. This may reflect the type of information contained in the database, much of which was derived from pathogens isolated from dogs.

5.2.3.3 Common host proteins

Digestive enzymes were easily detected in all samples, excluding sample 5. Peptidase and trypsin forms were present in samples 1 - 4 the majority of which were homologous to previously recorded digestive proteins from *C.familiaris*. Two samples contained trypsin proteins which were homologous to *C.familiaris* and *Bos taurus*, which may indicate that residual digestion enzyme used in sample preparation process remained in the sample, or that the peptide sequences from both species have identical homology.

Peptidase proteins detected included carboxypeptidase, aminopeptidase and endopeptidase. Amylase and sucrase-isomaltase proteins homologous to *C.familiaris* were present in samples 1 - 4, and galactose was detected in sample 1. Lipase was only detected in sample 2 and sample, 1, 2 and 3 all contained kinase enzymes. Mucin, detected in samples 1 - 4, was homologous to *C.familiaris* and *Homo sapiens*. Phosphatase was present in all samples, except for sample 2, occurring mainly as alkaline phosphatase with homology to *C.familiaris*. 
Immunoglobulins were present in samples 1 - 4, and apart from one which was homologous to rumen bacteria (*Prevotella sp.*), all matched peptides for *C.familiaris*. Overall, all samples analysed provided strong evidence of host immunoglobulins. Table 5.9 provides a description of different immunoglobulin types detected.

Hemoglobin detected in samples 2 and 4 was of avian homology not canine.

### 5.2.3.4 Proteins of interest

A specialized peptidase, kallikrein, was present in sample 3 and is known to be present in epithelial tissues and has a vasoactive role associated with inflammatory processes (Goettig *et al.*, 2010). It has been identified as a biomarker and linked to cancer (Borgono *et al.*, 2004) and allergic and skin lesions such as dermatitis.

Sample 1 contained 2 isoforms of a ‘Carcinoembryonic antigen related cell adhesion molecule’, which is a transmembrane cell adhesion protein first described in bile ducts of liver as a biliary glycoprotein, and subsequently confirmed as a cell-cell adhesion molecule located on leukocytes, epithelia, and endothelia (Oikawa *et al.*, 1992, Kuespert *et al.*, 2006, Ang and Nice, 2010).

Sample 1 contained lactotransferrin and also blood protein complement C3. Both were homologous to lactotransferrin for *C.familiaris* with 11 and 9 matching peptides, respectively.

Uncharacterized proteins were detected in all 5 samples, most of which showed homology with *C.familiaris*. In the case of sample 2, many of them showed matching homology to uncharacterized proteins of other species, including prokaryotes and other non-mammalian organisms.

### 5.2.3.5 Species composition of detected faecal proteins

The proteins matched to mass spectral data for each sample are displayed in piecharts below, which represent the species of origin.

The species composition of detected proteins are shown in Figures 5.5 - 5.8 and Tables 5.4 - 5.7.
Table 5.4 Sample 1 Host faecal proteome

<table>
<thead>
<tr>
<th>N</th>
<th>Protein Accession Number or DNA identifier</th>
<th>Protein Name</th>
<th>Species</th>
<th>Number of Matching Peptides (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>E2RH46</td>
<td>Uncharacterized protein</td>
<td>Canis familiaris</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>XP_856212.1</td>
<td>Amylase, alpha 2B; pancreatic precursor isoform 12</td>
<td>Canis familiaris</td>
<td>42</td>
</tr>
<tr>
<td>3</td>
<td>XP_849413.1</td>
<td>Immunoglobulin lambda-like polypeptide 1 precursor (Immunoglobulin-related 14.1 protein) (omega p)</td>
<td>Canis familiaris</td>
<td>34</td>
</tr>
<tr>
<td>4</td>
<td>XP_547299.2</td>
<td>Calcium activated chloride channel 1 precursor</td>
<td>Canis familiaris</td>
<td>29</td>
</tr>
<tr>
<td>5</td>
<td>XP_536228.2</td>
<td>Leucine aminopeptidase</td>
<td>Canis familiaris</td>
<td>19</td>
</tr>
<tr>
<td>6</td>
<td>XP_538947.2</td>
<td>Meprin A alpha-subunit precursor (Endopeptidase-2) (N-benzoyl-L-tyrosyl-P-amino-benzoic acid hydrolase alpha sub)</td>
<td>Canis familiaris</td>
<td>22</td>
</tr>
<tr>
<td>7</td>
<td>XP_534605.2</td>
<td>Intestinal alkaline phosphatase precursor (IAP)</td>
<td>Canis familiaris</td>
<td>13</td>
</tr>
<tr>
<td>8</td>
<td>E2RT38</td>
<td>Uncharacterized protein</td>
<td>Canis familiaris</td>
<td>15</td>
</tr>
<tr>
<td>9</td>
<td>XP_541903.2</td>
<td>Lactotransferrin isoform 1</td>
<td>Canis familiaris</td>
<td>11</td>
</tr>
<tr>
<td>10</td>
<td>E2R3E3</td>
<td>Uncharacterized protein</td>
<td>Canis familiaris</td>
<td>17</td>
</tr>
</tbody>
</table>
Figure 5.6 Species composition of detected proteins (95% confidence) in Sample 2

Data was sorted for protein matches specific to the host species, *C. familiaris*. The ten highest scoring (most confident matches) out of 120 total protein matches are displayed in Table 5.5.

Table 5.5  Sample 2 Host faecal proteome

<table>
<thead>
<tr>
<th>N</th>
<th>Protein Accession Number or DNA identifier</th>
<th>Protein Name</th>
<th>Species</th>
<th>Number of Matching Peptides (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>XP_859442.1</td>
<td>Bile-salt-activated lipase precursor (BAL) (Bile-salt-stimulated lipase) (BSSL) (Carboxyl ester lipase) sterol</td>
<td><em>Canis familiaris</em></td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>P19090</td>
<td>Colipase</td>
<td><em>Canis familiaris</em></td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>E2QWR0</td>
<td>Uncharacterized protein</td>
<td><em>Canis familiaris</em></td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>P49822</td>
<td>Serum albumin</td>
<td><em>Canis familiaris</em></td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>XP_856212.1</td>
<td>amylase, alpha 2B, pancreatic precursor isoform 12</td>
<td><em>Canis familiaris</em></td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>ENSCAFP00000018537 ENSCAFG00000012561 transcript:ENSCAF0000019984</td>
<td></td>
<td><em>Canis familiaris</em></td>
<td>7</td>
</tr>
<tr>
<td>7</td>
<td>XP_856525.1</td>
<td>Ig lambda chain C regions isoform 19</td>
<td><em>Canis familiaris</em></td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>XP_536228.2</td>
<td>Leucine aminopeptidase</td>
<td><em>Canis familiaris</em></td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>E2R3N3</td>
<td>Uncharacterized protein</td>
<td><em>Canis familiaris</em></td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>P06596</td>
<td>Phospholipase A2</td>
<td><em>Canis familiaris</em></td>
<td>4</td>
</tr>
</tbody>
</table>
Figure 5.7 Species composition of detected proteins (95% confidence) in Sample 3

Data was sorted for protein matches specific to the host species, *C. familiaris*. The ten highest scoring (most confident matches) out of 22 total protein matches are displayed in Table 5.6.

Table 5.6 Sample 3 Host faecal proteome

<table>
<thead>
<tr>
<th>N</th>
<th>Protein Accession Number or DNA identifier</th>
<th>Protein Name</th>
<th>Species</th>
<th>Number of Matching Peptides (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>XP_856212.1</td>
<td>Amylase, alpha 2B: pancreatic precursor isoform 12</td>
<td><em>Canis familiaris</em></td>
<td>27</td>
</tr>
<tr>
<td>2</td>
<td>XP_534605.2</td>
<td>Intestinal alkaline phosphatase precursor (IAP)</td>
<td><em>Canis familiaris</em></td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>Q29474</td>
<td>Kallikrein</td>
<td><em>Canis familiaris</em></td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>ENSCAF00000020087 3</td>
<td>transcript:ENSCAFT00000021631</td>
<td><em>Canis familiaris</em></td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>P49822</td>
<td>Serum albumin</td>
<td><em>Canis familiaris</em></td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>ENSCAF00000018537 1</td>
<td>Transcript:ENSCAFT00000019984</td>
<td><em>Canis familiaris</em></td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>Q8WN6</td>
<td>Superoxide dismutase (Cu-Zn)</td>
<td><em>Canis familiaris</em></td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>XP_545265.2</td>
<td>Sucrase-isomaltase, intestinal</td>
<td><em>Canis familiaris</em></td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>XP_859826.1</td>
<td>Aminopeptidase N (hAPN) (Alanyl aminopeptidase) (Microsomal aminopeptidase) (Aminopeptidase M) (gp150) (Myeloid</td>
<td><em>Canis familiaris</em></td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>E2RH46</td>
<td>Uncharacterized protein</td>
<td><em>Canis familiaris</em></td>
<td>2</td>
</tr>
</tbody>
</table>
Figure 5.8 Species composition of detected proteins (95% confidence) in Sample 4

The ten highest scoring (most confident matches) out of 30 total protein matches are displayed in Table 5.7

Table 5.7 Sample 4 Host faecal proteome

<table>
<thead>
<tr>
<th>N</th>
<th>Protein Accession Number or DNA identifier</th>
<th>Protein Name</th>
<th>Species</th>
<th>Number of matching Peptides (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>XP_865239.1</td>
<td>Caldecrin precursor (Chymotrypsin C) isoform 4</td>
<td>Canis familiaris</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>ENSCAFP0000002008 ENSCAFG00000023953</td>
<td>transcript:ENSCAFT00000021631</td>
<td>Canis familiaris</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>XP_848289.1</td>
<td>Pancreatic alpha-amylase precursor (PA) (1,4-alpha-D-glucan glucanohydrolase) isoform 1</td>
<td>Canis familiaris</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>E2RH46</td>
<td>Uncharacterized protein</td>
<td>Canis familiaris</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td>XP_534605.2</td>
<td>Intestinal alkaline phosphatase precursor (IAP)</td>
<td>Canis familiaris</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>XP_535370.1</td>
<td>Elastase-3B precursor (Elastase IIIb) (Protease E) isoform 1</td>
<td>Canis familiaris</td>
<td>9</td>
</tr>
<tr>
<td>7</td>
<td>XP_545265.2</td>
<td>Sucrase-isomaltase, intestinal</td>
<td>Canis familiaris</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>XP_855328.1</td>
<td>calcium activated chloride channel 4</td>
<td>Canis familiaris</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>P55261</td>
<td>CarboxypeptidaseB</td>
<td>Canis familiaris</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>A0FGS8</td>
<td>Anionic trypsinogen (Fragment)</td>
<td>Canis familiaris</td>
<td>9</td>
</tr>
</tbody>
</table>
Species composition of detected proteins (95% confidence) in Sample 5 were all *Canis familiaris* and the three proteins detected are shown in Table 5.8.

**Table 5.8 Sample 5 Host faecal proteome**

<table>
<thead>
<tr>
<th>N</th>
<th>Protein Score</th>
<th>Protein Name</th>
<th>Species</th>
<th>Number of matching Peptides (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.75</td>
<td>Uncharacterized protein (Fragment)</td>
<td><em>Canis familiaris</em></td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>ENSCAF00000027296 gene biotype: protein coding transcript</td>
<td><em>Canis lupus familiaris</em></td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>1.43</td>
<td>Alkaline phosphatase (Fragment)</td>
<td><em>Canis familiaris</em></td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 5.9 Immunoglobulins detected**

<table>
<thead>
<tr>
<th>Gene Access number</th>
<th>Protein Name</th>
<th>Matching Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>XP_862349.1</td>
<td>Immunoglobulin J chain isoform 3 [Canis familiaris]</td>
<td>2</td>
</tr>
<tr>
<td>XP_849413.1</td>
<td>Immunoglobulin lambda-like polypeptide 1 precursor (Immunoglobulin-related 14.1 protein) (Immunoglobulin omega p [Canis familiaris]</td>
<td>34</td>
</tr>
<tr>
<td>XP_543600.2</td>
<td>Immunoglobulin lambda-like polypeptide 1 precursor (Immunoglobulin-related 14.1 protein) (Immunoglobulin omega p [Canis familiaris]</td>
<td>26</td>
</tr>
<tr>
<td>XP_543515.2</td>
<td>Ig lambda chain V-I region BL2 precursor [Canis familiaris]</td>
<td>8</td>
</tr>
<tr>
<td>XP_855004.1</td>
<td>Ig lambda chain V-I region BL2 precursor [Canis familiaris]</td>
<td>6</td>
</tr>
<tr>
<td>XP_855010.1</td>
<td>Ig lambda chain V-I region BL2 precursor [Canis familiaris]</td>
<td>5</td>
</tr>
<tr>
<td>XP_848837.1</td>
<td>Ig heavy chain V-III region VH26 precursor [Canis familiaris]</td>
<td>3</td>
</tr>
<tr>
<td>XP_849174.1</td>
<td>Ig heavy chain V-III region VH26 precursor [Canis familiaris]</td>
<td>3</td>
</tr>
<tr>
<td>XP_848599.1</td>
<td>Ig heavy chain V-III region VH26 precursor [Canis familiaris]</td>
<td>3</td>
</tr>
<tr>
<td>XP_543520.2</td>
<td>Ig lambda chain V-III region LOI [Canis familiaris]</td>
<td>1</td>
</tr>
<tr>
<td>XP_848500.1</td>
<td>Ig heavy chain V-III region VH26 precursor [Canis familiaris]</td>
<td>13</td>
</tr>
<tr>
<td>D1PEJ7</td>
<td>Putative immunoreactive antigen PG33 [Prevotella copri]</td>
<td>5</td>
</tr>
<tr>
<td>XP_856525.1</td>
<td>Ig lambda chain C regions isoform 19 [Canis familiaris]</td>
<td>4</td>
</tr>
<tr>
<td>XP_855621.1</td>
<td>Immunoglobulin lambda-like polypeptide 1 precursor (Immunoglobulin-related 14.1 protein) (Immunoglobulin omega p [Canis familiaris]</td>
<td>6</td>
</tr>
<tr>
<td>XP_848877.1</td>
<td>Ig heavy chain V-III region VH26 precursor [Canis familiaris]</td>
<td>2</td>
</tr>
<tr>
<td>XP_543600.2</td>
<td>Immunoglobulin lambda-like polypeptide 1 precursor (Immunoglobulin-related 14.1 protein) (Immunoglobulin omega p [Canis familiaris]</td>
<td>14</td>
</tr>
<tr>
<td>XP_849413.1</td>
<td>Immunoglobulin lambda-like polypeptide 1 precursor (Immunoglobulin-related 14.1 protein) (Immunoglobulin omega p [Canis familiaris]</td>
<td>13</td>
</tr>
<tr>
<td>P01785</td>
<td>Ig heavy chain V region MOO [Canis familiaris]</td>
<td>4</td>
</tr>
<tr>
<td>XP_848749.1</td>
<td>Ig heavy chain V-III region VH26 precursor [Canis familiaris]</td>
<td>2</td>
</tr>
</tbody>
</table>

Different immunoglobulin proteins were detected across all samples and the confidence of identification is shown in Table 5.9 by number of matching peptides. All three structural types of immunoglobulin were detected, Joining, Variable and Constant,
and the number of matching peptides ranged from 1-34 indicating some immunoglobulin forms are in sufficient quantity to be detected in faecal matter.

5.4 Discussion

Spectra were analysed and matched to proteins (at high level of confidence) entries in the GiardiaDB database. The proteomic data discussed in this section examines these G. duodenalis proteins identified by mass spectrometry that are reported in Table 5.2. The results presented here include the proteins matched with the highest confidence and supported by multiple matching peptides.

5.4.1 Parasite proteome

The most important proteins are discussed with regard to the technical success of highly confident protein matching and significance of the potential applications for understanding the biology, biochemistry and disease pathogenesis of Giardia spp. infections. This section also includes details of the individual peptides and their significance in contributing to protein identification (as shown in Table 5.3) and also includes comments from comparative sequence alignment for individual proteins. Section 5.4.2 illustrates an overview of how the proteome map fits with the genomic data, and is discussed in relation to the genetic characterization results from Chapter 2.

5.4.1.1 Coiled coil proteins

Two coiled coil proteins were detected as G. duodenalis Assemblage B proteins, (C6LY87, C6LZ39). The highest protein score for all the proteins detected in this study was 4.5 for the coiled coil protein C6LZ39 GL50581_4068 matching an assemblage B gene sequence. Four matching peptides were each detected at 99% confidence level and no homologous sequence matches could be identified to any other organism by alignment to existing database entries. The four peptides were also detected with a high degree of confidence with a top ion score of 13, shown in Table 5.3. This signifies identification of some unique G. duodenalis peptides, HDMHLSLEQSSK, LSHSAQSVALLQEQVSQR, TEPSCQRKEGEQTILLDR,
Importantly, these peptides of coiled coil proteins were not matched to sequences in the *G. duodenalis* Assemblage A database, making them priority candidates for further phenotypic characterisation.

### 5.4.1.2 Hypothetical proteins

In total, seven different hypothetical *Giardia* spp. proteins were detected with unique sequences at more than 95% confidence, shown in Table 5.10. These are an important discovery because until now gene sequences have been predicted but evidence for protein expression has been absent. More work is required to fully describe and characterize these proteins.

The most confidently matched hypothetical protein (C6LS33) matched to sequences in the *G. duodenalis* Assemblage B database, with four matching peptides, although there was little other data available to inform the type of protein this might be. A second, high scoring hypothetical protein (C6LUY7) with three matching peptides has amino acid sequence similar to Lymphocryptovirus protein clusters and conserved viral glycoprotein domains. Related sequences matching to reproductive and respiratory type proteins were confirmed from existing database entries.

There was one hypothetical protein (A8BBE2, GL50803_117192) that was detected in two different samples. This may indicate this protein as a potential biomarker of active *Giardia* infection with a protein score of 2.0 and a highly confident matching peptide, KCGIQHSSRR. As the samples originated from symptomatic and asymptomatic dogs, with different genotypic of *Giardia* spp. infections, this peptide may be potentially useful as a biomarker for *Giardia* spp. infections in dogs. This hypothetical protein (A8BBE2) contains similar peptide sequences to proteins known to be associated with spindle pole ATP binding (GLP15_4271) in *G. duodenalis* isolate P15 (Assemblage E) Jerlstrom-Hultqvist *et al.* (2010) and ATP binding reverse transcriptase in *Monosiga brevicollis* (Iyer *et al*., 2008).

Two hypothetical proteins matched to Assemblage A and four matched proteins predicted for Assemblage B.
<table>
<thead>
<tr>
<th>Hypothetical protein</th>
<th>Gene Identifier</th>
<th>Genetic Assemblage</th>
<th>Protein identifier</th>
<th>Protein Clusters</th>
<th>Conserved domains</th>
<th>Related sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GL50803_117192</td>
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<td>A8BBE2</td>
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<td>None</td>
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</tr>
<tr>
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<td>A8B8B6</td>
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<td>None</td>
<td>2 Giardia protein sequences (230 related sequences) matching to RNA binding proteins and pre-RNA splicing factors (isolated from fungi and <em>Plasmodium</em> spp.)</td>
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<tr>
<td>3</td>
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<td>1</td>
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<td>Phage like glycoproteins</td>
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<tr>
<td>7</td>
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<td>B</td>
<td>C6LUY7</td>
<td>1</td>
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<td>Arterivirus G1, GS, M, N</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Epstein-Barr virus protein</td>
</tr>
</tbody>
</table>
5.4.1.3 Pyruvate flavodoxin oxidoreductase

The mass spectral data detected two different and overlapping peptides LGAGKNSVR (in pink) and NSVRLVAGGR (in yellow), shown in Figure 5.9. Both peptides detected in this study were matched to *Giardia intestinalis* (strain ATCC 50803 / WB clone C6 GL50803_114609) Protein E2RTZ1 and to *G. intestinalis* (strain ATCC 50581 / GS clone H7 GL50581_4147) Protein C6LZB6, for pyruvate flavodoxin oxidoreductase protein. The overall protein score of 1.6 is a confident protein match supported by several peptide identifications as shown in Tables 5.2 and 5.3. A comparison of amino acid sequences of the two proteins, using clustal omega from uniprot protein database, showed 96.3% identity for sequences from *G. duodenalis* Assemblage A and B, with 1207 identical positions and 40 similar positions. *G. duodenalis* strains WB and GS have homologous sequence for NSVRLVAGGR peptide. Proteomic data supports detection of this peptide as a biomarker of *Giardia* spp. infections. The amino acid sequence LGAGKNSVR was observed for *G. duodenalis* Protein E2RTZ1 (WB clone C6 GL50803_114609) an Assemblage A genotype. The genomic data for Assemblage B genotype (strain ATCC 50581 / GS clone H7 GL50581_4147) Protein C6LZB6, suggests that two point mutations (in green) may occur at amino acid residue positions 365 and 367 for the overlapping peptide, although the proteomic data in the work presented here supports only the peptide sequence shown in pink for LGAGKNSVR. These possible differences in amino acid sequences may constitute phenotypic differences between the *G. duodenalis* strains WB and GS or, that specific sequences for canine *Giardia* spp. isolates were detected in this study.

![Figure 5.9 Amino acid point mutations in PFOR genotypes of G. duodenalis (full sequence shown in Figure 5.4).](image-url)
5.4.1.4 Nek Kinase

Comparison of two peptide and amino acid sequences for different *G. duodenalis* genotypes show 92% homology between Assemblages A and B sequence data for Nek kinase detected in this study are C6LMX7 (GL50581_75) and A8BVL0 (GL50803_92498). Whilst the protein score assigned to Nek kinase was below the 95% confidence level, the detection of a highly confident peptide (99%), LSYDEIVR, was important in this protein identification in addition to the biological significance of high numbers of Nek kinases known to involved with *Giardia* spp. metabolism (Manning et al., 2011, Moniz et al., 2011, Smith et al., 2012). The peptide identified by proteomic analysis in this study, shown in Figure 5.1, is highlighted in yellow for amino acid positions 424 – 431. The red circles mark consecutive amino acid insertions or deletions, four or six amino acids in length. The yellow circles mark amino acid point mutations. The majority of the 30 indel (insertion or deletion) positions were amino acid residues of glutamine, the significance of this is not yet known. Further work examining protein-protein interactions may provide more insight, and in similar studies these types of interactions have identified protein kinases in parasite control mechanisms (Taylor et al., 2011).

5.4.1.5 Mismatch repair protein

The peptide KNSILNTLK was detected and matched with 99% confidence to database sequences for the identification of Mismatch repair protein. The peptide sequence matched through proteomic analysis in this study to both *G. duodenalis* Assemblages were A8B6C1 (GL50803_34058) Assemblage A and C6LTM7 (GL50581_2123) Assemblage B. The location of the peptide in the protein amino acid sequence is highlighted in yellow for amino acid positions 788 – 797 in Figure 5.3. This peptide covers a region of homologous sequence between the two *G. duodenalis* strains examined. Mismatch repair protein sequences showed more variation between genotypes than other proteins with 78.3% homology between two sequences, and a large number of point mutations are apparent. The large number of point mutations and
two indels occurring in other areas of this protein amino acid sequence warrant further investigation to confirm the nucleotide sequences predicted from genomic studies. One approach may be to use a proteogenomic approach to further validate protein identity by confirming that intragenic amino acid sequences of peptides match to the nucleotide coding sequences of genomic DNA (Nesvizhskii, 2014). Examination of exons and other non-coding regions of RNA transcripts have been successfully used to identify novel peptides and to quantify and confirm protein expression.

5.4.1.6 Axoneme Central Apparatus protein

The results of novel peptides detected for *G. duodenalis* axoneme central apparatus protein (A8BQ10) in this study confirms the presence of the protein predicted by gene sequence for *G. duodenalis* deposited to NCBI collection by (Morrison et al., 2007). Detection of this protein matched to both Assemblages A8BQ10 GL50803_16202 and C6LYT9 GL50581_3967. The protein score of 2.0 was considered an accurate identification supported by a highly confident match to the peptide EAAAWALGYIAR. Genomic data suggest that axoneme apparatus protein has a relatively conserved (99.8% homology), with only one amino acid difference between the 2 Assemblages examined. There were no differences in amino acid sequence in the peptide region identified using mass spectrometry in this study.

5.4.1.7 Histone Methyl transferase

This study detected histone methyltransferase MYST1 (GL50581_2825), an Assemblage B predicted protein (Manning et al., 2011). The protein score, shown in Table 5.2 was 2.0, whilst the peptide assigned to this protein was highly confident at 99% and with an ion score of 10, shown in Table 5.3. This unique identification of the peptide sequence SQLFNIR was not matched to assemblage A DNA sequence database. This proteomic data suggests that this is an interesting protein to study in more detail as a potential strain specific biomarker of *Giardia* spp. infection in dogs.
5.4.2 Giardia spp. assemblage specific proteins

Four proteins matched only to genomic *G. duodenalis* Assemblage A proteins. Three were hypothetical proteins (A8BBE2, A8B8B6, A8B278) and one was a translation protein tRNA/rRNA cytosine methylase (A8BA69).

Eight proteins matched only to genomic *G. duodenalis* Assemblage B proteins. Four were hypothetical proteins (C6LN73, C6LS33, C6LSL4, C6LYUY7).

Two were cytoskeletal Coiled Coil proteins (C6LY87, C6LZ39) Two were proteins associated with DNA replication Histone Methyltransferase (C6LVL9) and Signal Recognition Receptor protein (C6LN45).

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**Faecal proteome of Giardia spp.**

![Diagram](image.png)

Figure 5.10 Summary of *Giardia* spp. proteins detected in the canine faecal proteome

*Giardia* spp. proteins detected in the canine faecal proteome are summarized in Figure 5.10 according to genotype (Assemblage A or B). Four of the proteins detected were matched to entries in the *Giardia* database for both Assemblages A and B. These were Mismatch Repair protein (C6LTM7, A8B6C1), Pyruvate Flavodoxin Oxidoreductase...
(E2RTZ1, C6LZB6), Axoneme Central Apparatus protein (A8BQ10, C6LYT9) and Nek Kinase (C6LMX7, A8BVL0).

The ability to differentiate between genotypes for canine *Giardia* spp. infections is limited in the absence of Assemblage C and D genomic data. Many peptides matched proteins on both Assemblage A and Assemblage B databases. This does not indicate the presence of genotype specific proteins, but examination of the peptides showed differences in amino acid sequences for PFOR contributing to our understanding of *Giardia* spp. phenotypic expression.

The value of this preliminary proteomic study suggests that some proteins are commonly expressed, regardless of *Giardia* spp. genotype. Of particular interest are the *Giardia* spp. proteins, which appear to be genotype specific. These proteins are important to pursue further, and different aspects that could be explored. One would be to obtain genomic data for canine host specific *Giardia* spp. isolates and analyse the existing mass spectral data against new DNA sequences or RNA transcripts to search for confident protein matches and compare to the existing proteome map data. This may prove successful in validating phenotypic differences between different isolates. Additionally, from a clinical perspective, it would be useful to confirm protein expression levels according to infection status that is, in acute, chronic and post-infection stages.

### 5.4.3 Biology – introduction

Sixteen proteins of *G. duodenalis* origin were successfully extracted and identified from canine faecal material. They were diverse in structure and appeared to possess functions integral to cycling transitions between two morphological forms of *Giardia*, cysts and trophozoites, likely to have regulatory control functions.

#### 5.4.3.1 Cytoskeletal Protein Function

##### 5.4.3.1.1 Coiled coil proteins

Several unique peptide sequences were matched to *G. duodenalis* Assemblage B coiled coil protein gene sequences, (C6LY87, C6LZ39). The significance of the identification of these peptides and proteins by proteomic analysis is important.
Specific roles for coiled coil proteins include serving as domains for binding tubulin subunits in the microtubule surface lattice (Holberton et al., 1988). Coiled coil proteins are not unique to *Giardia* spp. having been identified in more than 65 eukaryotic organisms (Gerke and Moss, 2002).

### 5.4.3.1.2 Flagella

The roles of flagella are for trophozoite motility, fluid movement, signal mediation and protein trafficking by intraflagellar transport proteins. Flagella may play a key role in establishing infections and signaling responses, either immune or pathophysiological (Pazour et al., 2005) and are indirectly associated with trophozoite attachment to epithelial cells (House et al., 2011).

### 5.4.3.1.3 Involvement of median and basal bodies in *Giardia* trophozoite axoneme construction

Axonemes, are the microtubule structures, arranged circularly as nine doublets forming the core structure of flagella (Luck, 1984; Zhang et al., 2005). Comparative genomic research has also predicted a role for axoneme central apparatus proteins in flagella of other protozoan parasites such as *Leishmania* sp.(Ivens et al, 2005) and *T. Cruzi* (El-Sayed et al 2005). In a biological context the detection of highly confident peptides matching to axoneme central apparatus protein is not a commonly observed, in contrast to other proteins, such as enzymes detected in most eukaryotic organisms. The peptide sequence for axoneme central apparatus protein detected in this study occurs in single protein cluster NCBI database (ID 2432253).

Fifteen entries displayed in the publicly available entry for axoneme central apparatus protein cluster originate from only those species with protozoan or protist taxonomy, as shown in the phylogenetic alignment tree in Figure 5.11.
Key features that place the axoneme central apparatus protein within the large Thioredoxin-like protein Superfamily include a distinctive thioredoxin fold structure, with a redox active CXXC motif (Haque et al., 2012). They function as protein disulfide oxidoreductases, altering the redox state of target proteins via the reversible oxidation of their active dithiol site (Gilbert, 1997). Whilst axoneme structure is conserved across a number of different species (Zhang, 2005) research has demonstrated that central apparatus proteins are associated with the dynamic regulation of flagella movement (Dawson et al., 2007). Axoneme central apparatus protein participates in regulation of microtubule movement and interaction with dynein arms for energy generation in creation of flagella waveforms (Dawson, 2011a).

5.4.3.2 Regulatory protein function

Protein kinase enzymes play a critical role in cell metabolism of most living organisms, and all eukaryotic organisms, and kinases have been described and characterized into superfamilies based upon cellular location and function. There are many types of protein kinase, and Nek kinase is one of the serine/threonine protein kinases (Manning et al., 2002, Smith et al., 2012).
5.4.3.2.1 Nek kinase

Nek kinase GL50803_92498 detected in this study has been associated with *Giardia* caudal flagella signaling and association with adhesive disk and protein phosphatase 2A Manning *et al.* (2011) although the exact pathway is still to be determined, but functions as an ATP protein transphosphorylase. In the same study, this Nek 1 (GL50803_92498) kinase was also immunolocalised to anterior and posterio-lateral flagella, basal and median bodies.

The actions of Nek kinase in *G. duodenalis* trophozoites are multiple and repetitive, and integral to both ciliar and centrosomal processing (Parker *et al*., 2007, Davids *et al*., 2008, Lauwaet *et al*., 2011).

5.4.3.3 Genetic replication protein functions

5.4.3.3.1 Histone Methyl transferase

This study detected histone methyltransferase MYST1 (GL50581_2825), an assemblage B protein (Manning *et al*., 2011). This protein has also been reported as a protein of interest in the TDR database, [www.tdrtargets.org](http://www.tdrtargets.org), a chemogenomics resource for neglected tropical disease, (Magarinos *et al*., 2012).

Histone methyltransferase, a regulatory protein located in the nucleosome of eukaryotic organisms appears to operate both in isolation and in combination with other proteins. Cell cycling events involving transcription, replication, DNA repair, and apoptosis are all mediated by the action of histone methyltransferase.

5.4.3.3.2 Mismatch Repair Protein.

Mismatch repair proteins are an integral component of DNA replication and recombination to correct transcription errors through nucleotide insertions, deletions and repair of damaged or mis-incorporated DNA. Four key steps involve mismatch recognition, identification of newly synthesized strand, removal of mismatch segment, and gap repair by DNA polymerase. The specific DNA repair pathways which have been identified in *G. duodenalis* include base excision repair, nucleotide excision repair and genetic evidence for mismatch repair proteins identified in genomic data by
(Morrison et al., 2007). Repair of double-strand DNA breaks in *G. duodenalis* are predominantly performed by homologous recombination and some genes detected in other eukaryotes are also present to perform non-homologous end joining repair.

5.4.3.3.3 Signal recognition receptor particle protein

Signal recognition receptor particle protein contains ribonucleic acids (RNA) and is responsible for transport of membrane and secreted nascent proteins to the endoplasmic reticulum (Shan and Walter, 2005) by a mechanism known as co-translational protein targeting.

Results from this research identified a signal recognition receptor particle matching the core protein GL50581_145, also identified by Manning et al. (2011), although not discussed. The dependency on a signal-mediated pathway for molecular transport of proteins from the endoplasmic reticulum has been confirmed for soluble cyst cell wall proteins (Hehl et al., 2000). The ability to detect a signal recognition receptor particle from a water soluble fraction of *Giardia* spp. infected canine faecal material appears to support the concept that an external Golgi complex pathway exists for secretory protein sorting.

5.4.3 Disease and future considerations

The results shown in Tables 5.3 -5.7 confirm that proteins of *G. duodenalis* origin can be detected in canine faecal material and that highly confident matches to host *Canis familiaris* proteins were also achieved.

This study has illustrated two interesting aspects of *Giardia* spp. metabolism, firstly that the location of axoneme central apparatus protein within the thioredoxin superfamily may suggest that this protein could be involved with metabolic processes during replication. Secondly, the mutational changes to the amino acid residues are also apparent for the PFOR protein.

Greater understanding of synthesis and conformational structure of coiled coil proteins assists with identifying mechanisms of pathogenesis, such as in viral invasion mechanisms (Moutevelis and Woolfson, 2009, Reed et al., 2009). Potential therapeutic
intervention (Chan et al., 1998, Mason and Arndt, 2004) for G. duodenalis treatments may focus on coiled coil proteins. Additional information about Nek kinase is needed to overcome the poorly understood role of this protein family in G. duodenalis metabolism, and to document any unique profiles or structures in G. duodenalis isolates infecting canines. Understanding evolution and phylogenetic links to other parasites is important and applying this information should contribute to a better understanding of protein kinase functions and potential as drug targets.

Canine faecal protein analysis identified potential biomarker proteins for G. duodenalis infections, suggesting that proteomics may provide a useful tool for detection of canine host responses to Giardia spp. infections. The data will also be useful for directing the next steps of future research, seeking to answer questions concerning differentiation between G. duodenalis genotypes and inter-species transmission. In particular, the information can contribute to pinpointing therapeutic candidates or development of G. duodenalis diagnostics. In addition, this may improve our understanding of the infectious challenges to the host immune system and changes to gastro-intestinal tract environment and function.
Chapter 6

Lactoferrin levels in canine faecal samples

6.1 Introduction
Lactoferrin is a bioactive molecule secreted in milk involved with regulation of small intestinal development in neonates (Liao et al., 2012). *Giardia* spp. infections in dogs are considered to cause inflammation and one marker of intestinal inflammation is lactoferrin (Kane et al., 2003, Halliez and Buret, 2013).

6.1.1 Protein structure
Lactoferrin is a single chain glycoprotein, in a folded structure containing two loops, each capable of binding ferric iron (III) with a molecular mass of 80kDa. This glycoprotein belongs to the transferrin family of iron-transport proteins, and the features that distinguish lactoferrin from transferrin are based on the cellular location of each protein and the pH of enzyme activity. Lactoferrin operates within a wider pH range, capable of retaining iron molecules until the pH reduces to 3.0 or lower (Brock, 2002).

6.1.2 Cellular location
Lactoferrin is present in neutrophilic granules of secondary polymorphonuclear cells, which release the contents of the granules into body fluids including lacrimal fluid, saliva, mucus, bile and breast milk (Eckmann, 2003). Lactoferrin has been associated with anti-inflammatory, anti-bacterial, anti-parasitic and innate immune responses.

Neutrophils are the most abundant white cell type in blood (65%-75% of all leukocytes). They form the first response of the innate immune system to challenges by pathogenic microbes. Neutrophils originate from pluripotent haemopoietic progenitor cells of the bone marrow, where granules are synthesized. Three types of granules, azurophiic, specific and gelatinase are found inside neutrophils, and are responsible for phagocytic and host defence peptide mechanisms for destruction of pathogens. The contents of the granules, including lactoferrin are released to effect damage to the invading organism, by the enzymatic action of hydrolase, phosphatase or elastase or in primary
granules by anti-microbial peptides such as defensins or cathepsins in humans, although there is no evidence for defensins in canines (Shanahan et al., 2011).

6.1.3 Secretion in milk

Lactoferrin secretion in lacrimal fluid, saliva, mucus, bile and breast milk has been demonstrated in humans and bovines (Adlerova et al., 2008) although some reports suggest that canine milk and colostrum does not contain lactoferrin (Vorland, 1999, Masson and Heremans, 1971). Although work has now shown that relatively low levels of lactoferrin are secreted in canine milk (Berlov et al., 2007, Sinkora et al., 2007) Work has also shown the protective effects of cross species lactoferrin in humans (Ochoa et al., 2012). Pope et al. (2006) studied the effect of bovine lactoferrin supplements in dogs, and found no impact on the health indices recorded during the study. In contrast, Hellweg et al. (2008) reported that bovine lactoferrin included in the diet of Beagle dogs positively altered cellular immune responses and faecal microbial populations of healthy adult dogs. This included increased numbers of monocytes, T cells and cytotoxic T cells in the blood and the proliferative response of peripheral blood mononuclear cells.

6.1.4 Biological functions.

Unlike transferrin where a clear role in iron transport and homeostasis is established, no such role has been demonstrated for lactoferrin, although a wide range of functions have been proposed (Farnaud and Evans, 2003). It may play a role in iron absorption in the newborn infant, although whether it promotes or inhibits this process is controversial (Suzuki et al., 2005).

The principal roles known for transferrin involve homeostasis and iron transportation, whereas the entire repertoire of lactoferrin functions are still to be fully determined.

Lactoferricin, which has a highly basic N-terminal peptide, is the active peptide component of lactoferrin. This anti-microbial peptide acts by direct protein or peptide interaction with target molecules causing membrane disruption (Farnaud and Evans, 2003), and is suggested to have potential use as a therapeutic agent (Brock, 2012).
In addition to iron adsorption, other roles acknowledged for lactoferrin are associated with innate immune responses and differentiation of mucosal epithelia (Lönnerdal and Iyer, 1995). A range of lactoferrin biological functions have been reviewed including protein binding to cell membrane DNA and binding to nuclear DNA to activate transcription (Kanyshkova et al., 2001).

The role of lactoferrin as a feedback mechanism and control point for inflammatory cytokines is a key feature of its role as an anti-inflammatory, and the immunomodulation mechanism of increased neutrophil production and degranulation to reduce circulating interleukin-1 and tumour necrosis factor released from monocytes and macrophages (Vorland, 1999).

Numerous other functions including anti-inflammatory and immunomodulatory effects have been proposed for lactoferrin, including inhibition of tumour cell growth, and regulation of gene transcription (Legrand et al., 2005).

### 6.1.5 Lactoferrin receptors

The mammalian small intestine contains lactoferrin receptor sites. It has been suggested that serosal iron transport occurs by transferrin binding and that lactoferrin mediates iron transport across epithelial membranes in pigs (Liao et al., 2007, Kane et al., 2003). Lactoferrin receptors, in infants, specify conditions for proteolytic cleavage, when pH levels are slightly higher and iron binding capacity is optimal (Suzuki et al., 2005).

General receptors for lactoferrin and transferrin are non-species specific and binding is iron dependent (hololactoferrin), whereas specific lactoferrin binding at N terminal sites to receptors can occur in the absence of iron (apolactoferrin) (Kanyshkova et al., 2001).

### 6.1.6 Faecal lactoferrin detection

Gastro-intestinal tract physiology and function varies between species (Ajinomoto et al., 1999) suggesting that specialized adaptations may have evolved due to diet, gut microflora, host genetics or, physiological differences. Faecal material is known to contain lactoferrin, and is associated with inflammatory processes and has been used
as a biomarker for Inflammatory Bowel Disease (IBD) in humans (Walker et al., 2007). Lactoferrin has also been reported as an important biomarker distinguishing between IBD and IBS in humans (Sidhu et al., 2010) where elevated lactoferrin levels are indicative of clinically active IBD, although normative values have not been agreed by researchers. Walker et al. (2007) reported that elevated lactoferrin levels were observed in clinically well patients who later developed a flare up of symptoms. Faecal calprotectin, another inflammatory marker has been used as a biomarker for colon cancer (Roseth et al., 1997, Bunn et al., 2001). The stability of lactoferrin in faecal samples and the correlation with calprotectin has been recommended for non-invasive assessment of gastro-intestinal inflammation in humans (Lamb and Mansfield, 2011).

6.1.7 Antimicrobial peptides (AMP)

Recognition of antimicrobial peptides by prokaryotic organisms occurs by a two component system within bacterial cell membranes (Ho et al., 2012). These usually comprise highly conserved signalling pathways involving histidine kinase and an intracellular response regulator, such as BasR in E.coli (Nagasawa et al., 1993). Lactoferrin, whilst resistant to proteolytic degradation by trypsin, has been shown to contain a different bioactive terminal peptide in humans and bovines (Vorland, 1999, Bellamy et al., 1992). These antimicrobial peptides (lactoferricin) from humans (H) and bovines (B), products of pepsin hydrolysis, are extremely small (6 – 11 amino acids) containing tryptophan and basic residues within a disulphide loop, with arginine residues binding to lipopolysaccharide (LPS) sites of the cell membrane (Farnaud and Evans, 2003). Recognition and signalling pathways in amitochondriate protists such as Giardia spp. are unpublished and the importance of lactoferrin and other antimicrobial peptides in canines are undetermined.

6.1.8 Lactoferrin and Giardia spp. infections

G. duodenalis, similar to many other parasitic protozoa has an obligate requirement for iron to fulfil metabolic processes (Townson et al., 1994, Adam, 2001).
Metalloproteins have been demonstrated, *in vitro*, to be non-immune host defence factors, such as the N-terminal peptide of lactoferrin directed against *G. duodenalis* trophozoites (Turchany *et al.*, 1995). Lactoferrin is also known to be associated with protein binding in six pathogenic parasitic protozoan infections, providing the organism with a potential iron (Fe3+) source (Ortiz-Estrada *et al.*, 2012). Three other mechanisms for iron acquisition include proteolytic enzymes to cleave lactoferrin; interaction of reductase enzymes to convert lactoferric iron to ferrous form; and specialized compounds such as siderophore action in *Trichomonas foetus* or rhoptry proteins in *Toxoplasma gondii* (Ortiz-Estrada *et al.*, 2012). Lactoferrin as an iron source for *T foetus* is internalised via a receptor mediated endocytosis across a pH gradient to release the iron for metabolic processes (Tachezy *et al.*, 1996). Results from proteomic analysis of canine faecal samples in Chapter 5 identified lactoferrin in at least one sample, as a highly confident protein match.

### 6.1.9 Hypothesis

**That faecal lactoferrin is associated with parasite infection when clinical signs of infection are observed.**

A commercial antigen kit was used to determine if canine lactoferrin could be detected in faecal samples from *Giardia* spp. infected dogs. Determination of an association between lactoferrin levels, clinical signs of infection and presence of other parasites, or antigens was performed.
6.2 Methods
Canine faecal samples previously screened for protozoan parasite infections formed the basis of this experiment. Samples selected included those positive for *Giardia* spp. infections, with differing levels of cyst intensity from both symptomatic and asymptomatic dogs. The samples included those with discordant diagnostic results for microscopy and copro-antigen test and some samples that tested negative for *Giardia* spp. but positive for other parasites.

Canine faecal samples were tested for lactoferrin content using a commercially available lateral flow colorimetric immunochromatographic assay (Certest, Zaragoza, Spain) designed to detect lactoferrin in human faeces. The test did not detect lactoferrin in any of the samples tested and results are not reported here.

Twenty-three fresh or frozen canine faecal samples were brought to room temperature. The Lactoferrin Enzyme Linked Immunosorbent Assay (ELISA) was performed as instructed by the manufacturer of Lactoferrin ELISA (Bluegene™, Shanghai, China). Sterile water (300µl) was added to each solid faecal sample, mixed thoroughly and then centrifuged for 2 minutes at 1500g to sediment particulates. Aliquots of 100 µl were used to perform the assay. Each sample, standard and control was prepared in duplicate, adding 100µl of solution to well strips. Phosphate Buffered Saline was used as the blank control. Balance solution (10µl) was added to each well containing faecal sample supernatant. 50µl of enzyme conjugate was added to each well, except blank controls. The plate was placed on a vortex machine for 30 seconds to mix the contents thoroughly. The plate was sealed and incubated at 37°C for 1 hour and then wells were washed. The wells were refilled with wash solution and washed a total of five times. The plates were inverted and the wells emptied of contents by firmly tapping onto blotting paper to dry. To all the empty wells, including blank control well, 50 µl Substrate A and 50 µl Substrate B was added. The plate was sealed and incubated in the dark at 37°C.
After incubating for 15 minutes, 50 µl of stop solution was added to each well, including blank control wells to stop the reaction and the contents were mixed thoroughly. The optical density was immediately determined at 450nm using a microplate spectrophotometer reader. The results were interpreted by plotting standard concentrations x axis against the optical density (y axis) to produce a standard curve.
6.3 Results

Sample standards provided in the Canine Lactoferrin ELISA kit (Bluegene™, Shanghai, China) were included with the faecal samples as described in the methods, above. Results were used to construct the standard curve shown in Figure 6.1.

![Faecal lactoferrin standard curve (0-5000pg/ul)](image)

Figure 6.1 Faecal lactoferrin standard curve (0-5000pg/ul)

To calculate the concentration of lactoferrin in the faecal samples a linear log plot was produced, and the results for individual faecal samples were determined.

![Log plot of faecal lactoferrin concentration against optical density](image)

Figure 6.2 Log plot of faecal lactoferrin concentration against optical density
Table 6.1 Calculated concentration of lactoferrin content in canine faecal samples presented with *Giardia* spp. diagnostic test results

<table>
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Table Key: N (Negative), P (Positive) bracketed numbers (Cyst intensity)
Some of the samples tested using the canine faecal lactoferrin ELISA were excluded and are not shown here as the duplicate results were inconsistent, probably due to the length of time the samples had been kept frozen, resulting in loss of bioactivity.

6.3.1 Comparison of faecal lactoferrin content

Results are shown in Table 6.1

6.3.1.1 Highest levels (>5000pg/ml) of faecal lactoferrin

From 23 samples tested (Table 1), six samples (6/23, 26%) showed lactoferrin concentrations, which exceeded the limits of the test, greater than 5000pg/ml. Of these 6 samples, 4 (75%) were from dogs with clinical signs of gastro-intestinal dysfunction (loose stool form) and 2 (25%) were considered as asymptomatic infections. For the six faecal samples that had lactoferrin concentration >5000pg/ml four samples (75%) were positive for *Giardia* spp. cysts, and 2 (25%) were negative for *Giardia* spp. and one (8%) was positive for *Cystoisospora* sp. infection.

6.3.1.2 High level (≥2000pg/ml) of faecal lactoferrin

Five samples (5/23) gave a faecal Lactoferrin content greater than 2000pg/ml, all were confirmed positive for *Giardia* spp. cysts and two were also positive for *Cystoisospora* sp. infection.

6.3.1.3 Mid range level (>500 < 2000pg/ml) of faecal lactoferrin content

Nine canine faecal samples (39%) gave a result for faecal lactoferrin content greater than 500 but less than 2000pg/ml. Two of these samples (22%) contained no parasitic organisms detected by light microscopy, and only one was considered to be from symptomatic dogs.

6.3.1.4 Low levels (≤500pg/ml) of faecal Lactoferrin

In total, 13% of samples had a faecal Lactoferrin content less than 500pg/ml, and 1 from symptomatic and 2 from asymptomatic dogs. One *Giardia* spp. positive sample was also concurrently infected with *Sarcocystis* sp. with faecal lactoferrin content less than 500pg/ml.
6.3.2 Comparison of faecal lactoferrin content with detection of *Giardia* spp. antigen

All samples were also tested with *Giardia* spp. copro-antigen test 1. Ten samples gave a positive test result, and 13 were negative results. Of the 10 positive for the presence of *Giardia* spp. antigen 2 were symptomatic and 8 asymptomatic and all contained *Giardia* spp. cysts, confirmed by light microscopy.

Three samples (3/10, 33%) that tested positive with *Giardia* spp. copro-antigen test 1 also had faecal lactoferrin levels in excess of 2000pg/ml and 1/3 (33%) was symptomatic. In addition, two of these samples with high levels of faecal lactoferrin and considered to be symptomatic, were concurrently infected with *Cystoisospora* sp.

6.3.3 Comparison of faecal lactoferrin content with *Giardia* spp. genotype

The results indicate that all but one of the samples tested and genetically characterized were host specific *Giardia* spp. Assemblage C or D infections and that lactoferrin content ranged from 340 - >5000 pg/ml. The single faecal sample genotyped as Assemblage A tested for lactoferrin gave a result of 2000pg/ml.

6.4 Discussion

The results presented here demonstrate that faecal lactoferrin levels were detected in dogs infected with protozoan parasites, and the highest levels detected occurred in faecal samples containing *Giardia* cysts. Faecal lactoferrin level is not currently a parameter used by veterinarians to indicate clinical signs of parasite infections. The results from this study suggest that canine *Giardia* spp. infections may be associated with the presence of lactoferrin in faecal samples. Further validation is required, especially with uninfected dogs, and a possible indicative value of 300pg/ml threshold level. Confirming a threshold value holds merit as it is likely that lactoferrin is present in uninfected faecal samples. The ability to measure differences and elevated levels of faecal lactoferrin is important in the diagnosis of inflammation and to determine potential causes, such as parasite infections.
Pathogenic and physiological changes to intestinal enterocytes include disruption of tight junction proteins, ion hypersecretion, apoptosis, luminal fluid accumulation and disaccharide deficiencies (Cotton et al., 2011). Large amounts of faecal lactoferrin are suggestive of intestinal inflammation, with the host responses to infectious challenges inducing lactoferrin secretion as physiological mechanism for modulating immune responses to *Giardia* spp. infections (Legrand et al., 2005). Further work is required to confirm findings of this study. Such work may include host genetic traits to determine if the host is pre-disposed to *Giardia* spp. infections and lacks the ability to mount an effective response, as described for Irish Setters with granulocytopeny syndrome (Renshaw and Davis, 1979).

The results from proteomic analysis in Chapter 5 identified a range of faecal immunoglobulins and IgA has been shown to be present in canine faeces, at four times the level circulating in serum (Reynolds and Johnson, 1970). In a more recent study, no association was found upon comparison of immunoglobulin content in the serum and faeces of gerbils however, the timing of elevated levels of immunoglobulin secretion were related (Amorim et al., 2010). Reports in the literature suggest that ingestion of bovine lactoferrin by mice causes both innate and humoral responses (Debbabi et al., 1998). This study in dogs provides further evidence associating lactoferrin with immunoglobulin secretion in the gastro-intestinal tract.

Recent studies examining *G. duodenalis* infections in humans suggest that faecal lactoferrin levels reduce overtime and maybe associated with protection against severity of clinical signs of infection (Kohli et al., 2008). Brock (2012) refers to differences in host anti-parasitic mechanisms by the action of lactoferrin for mitigation of *Giardia* spp. parasite infections, rather than by iron deprivation. Recommendations for further work to establish the roles of lactoferrin in parasitic infections, as iron is known to be a nutritional requirement for *G. duodenalis* growth and replication have been recommended (Davids et al., 2011, Brock, 2012). Earlier studies seeking diagnostic methods for distinguishing between inflammatory and non-inflammatory
diarrheal infections used faecal lactoferrin as a marker of fecal leukocytes (Guerrant et al., 1992). The importance of lactoferrin described by Kanyshkova et al. (2001) as an acute phase protein is the use of this glycoprotein as a biomarker of IBD infections in humans (Lamb and Mansfield, 2011).

Whilst the results presented here are from only a small sample size, from a dog owner or veterinary practitioners perspective, addressing problems of diarrhoea in dogs is important. Due to the presence of asymptomatic infections this may require the combination of a rapid copro-antigen test for Giardia spp. with a faecal lactoferrin test, where a positive titre >2000pg/ml may indicate an anti-inflammatory response to protozoal parasite infections. Those samples positive for Cystoisospora spp. produced results indicating consistently high levels of faecal lactoferrin content, even when Giardia cysts were not observed. The results of this work confirm the approach of seeking routes to assess intestinal disturbances and further understand host-parasite interactions. Development of a biomarker panel may be more appropriate for confirming Giardia spp. infections in dogs.

Whilst the exact requirements for iron of this amitochondriate parasite, remain unclear, in contrast to T. foetus and T. vaginalis where differences in lactoferrin and transferrin requirements as an iron source have been attributed to physiological location with the host (Tachezy et al., 1996). Detection of lactoferrin is an indirect measure of inflammation, and greater understanding of parasite localization within the digestive tract could contribute to improved understanding of sites of inflammation and anti-inflammatory mechanisms. The bioactive lactoferrin glycoprotein regulates immune system responses to infectious challenge. Its structural confirmation also facilitates interaction with host cells, in addition to an ability to utilize antimicrobial properties within biofilms (Ammons and Copié, 2013). These factors are evidence of a potent anti-inflammatory role, thus the levels of lactoferrin present and its role in physiological processes inversely reflect the measure of inflammation. This study suggests that
further investigation into canine lactoferrin is justified, especially as the anti-microbial peptides found in canines are uniquely different to those found in humans.
Chapter 7

General discussion

This study, the first of its kind, has demonstrated the use of proteomic tools in detecting parasite proteins of a pathogenic protozoan from infected canine faecal samples, and has provided a greater understanding of the dynamics of Giardia spp. infections in dogs. In addition, detection of host proteins has enabled tentative comments to be made concerning host-parasite interactions and impacts of Giardia spp. infections on the canine host.

This chapter discusses the research findings in six sections, the first of which examines results concerning accurate detection of Giardia spp. infections in canine faecal samples, including the impact of Giardia spp. infections in dogs with reference to patency, polyparasitism and clinical signs of infection. The second section discusses the potential zoonotic transmission of Giardia spp. The overall findings from using proteomic tools for the phenotypic characterization of Giardia spp. infections in dogs is presented in section three. This section comments upon the methods and aspects of the unique faecal proteome as an interface between the canine host and this protozoan parasite and also reflects on the proteomic information obtained from faecal samples and their relevance to the cellular and molecular interactions occurring in the gastrointestinal tract.

The fourth section offers some considerations for the management of Giardia spp. infections in dogs, and the final sections includes comments on the limitations to study design and suggests areas for further research in the future.

7.1 Impact of Giardia spp. infections on dogs

Giardia spp. prevalence rates reported in this study were high, at 29-75% in litters of puppies, as shown in Table 2.3.2. If the accepted prevalence rate of 10-20% in dogs from other published reports is correct, and includes both symptomatic and
asymptomatic infections, this may continue to be an under-estimation if microscopy is used as the diagnostic standard (Palmer et al., 2008).

This study has provided further details of dog (C. familiaris) susceptibility to Giardia spp. infections and evidence that enteric protozoan infections can cause both clinical and subclinical signs of infection in naturally infected populations. The data supports and adds to findings by other researchers that immature dogs are susceptible to Giardia spp. and other enteric protozoan infections (Batchelor et al., 2008, Epe et al., 2010, Itoh et al., 2011). The pre-patent period of less than 17 days, the earliest recorded detection of patent Giardia spp. infection in puppies confirmed during this study, is similar to preliminary findings of Barutzki and Schaper (2013) who reported detection of Cystoisospora sp. and Giardia spp. in the fourth week of life. The data collected on faecal cyst intensity indicates that during the time of patent infection the numbers of excreted cysts can be high, which perpetuates infection and re-infection of littermates and the maternal dam. The high level of cyst excretion occurs especially at the weaning stage (40 - 50 days of age), when the quantity of maternal milk intake reduces, along with protective factors in milk such as immunoglobulins (Heddle and Rowley, 1975). This reduction in dietary intake of immunoglobulins and protective factors is accompanied by a reduction in immunoglobulin levels in nasal secretions of puppies (German et al., 1998, Schafer-Somi et al., 2005). Thus the potential for resisting infectious challenges dramatically changes in the post-weaned canine host. The significance of this window of opportunity for parasite infections is a key element in transmission routes and also likely to be similar for other mammalian hosts, including human infants.

Whilst the work here investigated Giardia spp. infections in neonatal puppies, it is relevant to other hosts of Giardia spp., such as young children. The long-term implications of sub-clinical infections have yet to be determined. Signs of Giardia infections such as poor appetite, low weight gain or general malaise have been reported in both dogs and children (Sackey et al., 2003, Leticia Eligio-Garcia et al.,
2005). In children there are reports of associated impairment of cognitive function in addition to the impact of overt clinical signs reducing attendance at school and thus contributing to reduced educational opportunities and barriers to achieving academic potential (Thompson, 2001, Berkman et al., 2002, Cotton et al., 2011, Halliez and Buret, 2013). One of the important results from this study, discussed in section 3.4.5 is the demonstration that neonatal infections are likely to be of maternal origin.

7.1.1 Diagnostic tools

In a clinical setting it is important for cases presenting with gastro-intestinal signs of infection to undergo diagnostic panel tests for parasites, particularly protozoan parasites Giardia spp. and coccidian infections, to eliminate these as a source of gastro-intestinal signs of infection.

This study therefore, focused on investigating the impact of Giardia spp. infections in dogs with a high risk of infection (Thompson, 2000) as explained more fully in chapter 2. A number of diagnostic techniques were compared to provide insight into the reliability and suitability of their use for identifying Giardia spp. infections in dogs and differentiating between host specific and potentially zoonotic infections.

In the absence of a validated gold standard diagnostic method for Giardia spp., results from this study indicate that initial diagnostic screening of canine faecal samples should include centrifugal flotation and examination by light microscopy to ensure that the presence of all parasitic organisms are recorded. Observation of Giardia cysts is the definitive confirmation of patent Giardia spp. infections. However, in instances when Giardia cysts are not observed by microscopy, sequential testing using copro-antigen or molecular methods can be used to accurately confirm Giardia spp. infection status, as performed in this study. However, the issue of intermittent cyst shedding (Lopez et al., 1980) remains unresolved, and best practice standards of collecting three consecutive samples may be impractical in many situations. Liquid or semi-liquid stools are difficult to collect, and two of the copro-antigen tests used in this study are both portable and practical for collection of samples using dropper or swab, easily
overcoming obstacles to traditional faecal sample collection. In situations with limited faecal material available diagnosis of *Giardia* spp. infections may be more conveniently confirmed using a copro-antigen test and PCR with DNA sequencing. Nevertheless, importantly, this study has demonstrated confirmation that *Giardia* spp. infections are not the exclusive cause of clinical signs. In cases exhibiting diarrhoea or mucus in the stool these clinical signs may not be caused solely by *Giardia* spp., with the potential for misdiagnosis. In addition, despite the fact that *Giardia* spp. infections may not be the cause of clinical signs of infection, two thirds of dogs tested were considered symptomatic and one third asymptomatic, based upon stool consistency. The data presented here conflicts with reports by Barutzki and Schaper (2003) suggesting that host specific *Giardia* spp. assemblages are associated with clinical signs of infection in dogs. This new information is in disagreement over the definitive cause of symptomatic cases of diarrhoea in dogs infected with enteric parasites. Limitations exist when *Giardia* spp. diagnosis relies solely on copro-antigen tests. The presence of antigen may not indicate active infection, and may overlook other parasitic infections. Results from this study suggest that copro-antigen tests may preferentially select for particular *Giardia* spp. genotypes or stages of infection. Whilst manufacturers of the copro-antigen tests report 95% sensitivity and 95% specificity, there is no indication in the test protocol of the population descriptors used to determine positive predictive value and negative predictive value, both of which are affected by prevalence rates. The importance of diagnostic validation for infectious disease is one that is equally important within veterinary and medical fields (Whitfield *et al*., 2005, Gardner and Greiner, 2006, TDR Diagnostics Evaluation Expert Panel, 2010).

Moreover, the importance of accurately observing and describing clinical and sub-clinical signs of infection are critical, both in terms of risks of infection, transmission and management strategies.

In conclusion, whilst a range of diagnostic tests are currently available for confirming *Giardia* spp. infections in canines none of these are capable of determining the
infection status, new, active, post-infection or recrudescent infections. This type of information, together with identifying markers of *Giardia* spp. infection are important and proteomic tools may provide the next phase of infection information.

### 7.1.2 Concurrent *Giardia* spp. infections and clinical signs of infection

Results from this study show that faecal coproscopy is important for detection of coccidian parasites, and the high prevalence rates are in concordance with results of others in Australia and elsewhere (Palmer et al., 2008, Itoh et al., 2011). Diagnostic tests for coccidian parasites currently rely mainly upon microscopy techniques, with some genetic descriptors using PCR and immuno-antigen based tests. Nonetheless, the fact that increased likelihood of clinical symptoms occurs with *Cystoisospora* spp. suggests that development of tests for the specific detection of *Cystoisospora* spp. should be addressed, as this is a common infection in pigs and poultry with multiple reports in dogs (Barutzki and Schaper, 2003, Itagaki *et al.*, 2005, Becker *et al.*, 2012, Mircean *et al.*, 2012).

The presence of *Sarcocystis* spp. in dogs from Western Australia may also be due to the geolocality. Earlier evidence has reported that climate regions of high moisture in south Western Australia produced high prevalence rates of *Sarcocystis* spp. in cattle, (Savini *et al.*, 1992). Early detailed descriptions and taxonomic classification of Coccidians were performed both in terms of morphology and their tissue localization (Levine and Ivens, 1965, Levine, 1973). Now, equipped with genetic information it is timely to reassess this information at a species level and combine it with genetic data, missing from these earlier studies to fully understand the impact of these parasite infections in dogs (Tenter *et al.*, 2002).

The presence of *Giardia* cysts in faeces indicates an active infection, but is the number of cysts important? What is the significance of the cyclic occurrence of cyst excretion? Studies in Colombian children divided cyst excretors into three groups (high, low or mixed), and although there was limited understanding of what the implications were these did highlight the need for multiple samples to confirm an infection. Importantly,
this study concluded that clinical symptoms of diarrhoea did not correlate with quantity of *Giardia* cysts or trophozoites excreted in faecal material (Danciger and Lopez, 1975).

The value of being able to grade levels of cyst excretion is an important reason for retaining microscopy as the standard for *Giardia* spp. diagnostics, at least until the significance of different excretion levels is understood. In addition, as this study has demonstrated, visualization of parasites by light microscopy provides information on other enteric parasites, many of which would otherwise remain undetected if tests relied solely on genetic or antigenic factors to confirm or rule out the presence of a *Giardia* spp. infection.

### 7.1.3 Significance of coccidian infections

This study has demonstrated differences in clinical signs of infection, between parasite co-infections and the different *Giardia* spp. genetic assemblages in some populations studied, such as the predominance of host specific assemblages. It is a preliminary investigation and there are difficulties in assessing phenotypic differences attributed to the presence or absence of co-infections. However, all the faecal samples used for the proteomic analysis were not infected with any parasites other than *Giardia* spp. Some questions raised in relation to concurrent parasite infections within the dynamics of host microbial interactions concern 1) whether interactions occur between different species and 2) whether the presence or absence of single or multiple infections are influencing phenotypic traits (Bradley and Jackson, 2008). The work presented here suggests that co-infections between different parasite species may involve competition. The timeline displaying temporal dynamics of patent parasite infections shows that some species, including *Giardia* spp. are present, then absent then reappear again. Similar observations reported in the literature concern parasitism of other organisms and the potential for manipulative parasites to induce phenotypic changes to favour transmission (Leung and Poulin, 2007, Lefèvre *et al.*, 2009, Thomas *et al.*, 2011).
7.2 Evidence for zoonotic transmission

The results of this study suggest that potentially zoonotic assemblages are infective to neonatal puppies if there is exposure, such as from the maternal dam or environment. Despite the regular sampling used throughout the sampling period, the constraints of pooled sample collection from litters of puppies meant that this study was unable to confirm if two different genetic assemblages were causing simultaneous infections.

From all the successfully sequenced DNA samples, only three distinct *Giardia* spp. genotypes were detected in this study, using multiple gene loci. This genetic information revealed predominantly host specific genotypes of *Giardia* assemblages C and D. In addition to these host specific infections DNA sequencing results also showed evidence of both assemblage A and mixed infections of *Giardia* spp.

Evidence for genetic descriptions of assemblage B in this study was lacking, but the presence of Assemblage A can be considered to be a potentially zoonotic infection.

7.2.1 Molecular genetics

PCR results and genotyping of *Giardia* spp. infections showed mainly host specific infections, although the results are not conclusive, which confirms similar reports by Berrilli *et al.* (2004), Upjohn *et al.* (2010) and Uehlinger *et al.* (2013).

The problems in obtaining consistent results for *Giardia* spp. genotyping of Assemblages A and B despite using multi-locus genotyping are not exclusive to this study (Caccio and Ryan, 2008, Wielinga *et al.*, 2011, Huey *et al.*, 2013). A number of strategies may be appropriate to resolving the complexities of definitive and consistent genotyping of *Giardia* spp. isolated from canines. These include reconsidering the use of additional gene loci or revisiting alternative loci, such as IGS (Hopkins *et al.*, 1999, Lee *et al.*, 2006). In addition, improved methods for DNA extraction, as recently highlighted by Uda-Shimoda *et al.* (2014) and for GDH loci (Nantavisai *et al.*, 2007). The efficiency of cyst lysis is an important step in molecular genetics and studies have indicated that Proteinase K cleaves the peptide bonds of aliphatic and aromatic amino
acids which compose the cyst cell wall, although attention to optimal conditions are important (Harris and Petry, 1999, Adamska et al., 2011). Moreover, consideration of cyst intensity is an important factor for genetic analysis of faecal material and a suitable threshold value may be important for obtaining consistent PCR results (Wielinga and Thompson, 2007). The promising results from proteomic mapping described in Chapter 5 have highlighted candidate proteins suitable for further investigation and to explore proteomics approaches for genetic characterisation with increased precision (Gupta et al., 2008, Ansong et al., 2008, Castellana and Bafna, 2010).

7.3 Phenotypic expression of Giardia spp. infections in canines

The proteomic approaches used in this study are unique in attempting to profile biological manifestation of parasitic disease. Studying the faecal proteome using mass spectrometry techniques is a non-invasive method and captured details of the physiology of a single animal at a particular time point. The proteomic approach used in this study sought to detect peptides rather than intact proteins. Using the database matching software it was possible to match peptide sequences to whole proteins.

7.3.1 Parasite faecal Proteome

This research has shown that whole faecal material infected with Giardia cysts can provide a useful window into understanding the biology of Giardia spp. lifecycle events. The benefits of this novel proteomic approach to examine infected faecal samples by following methods for mass spectrometry peptide detection described earlier in this thesis, has identified Giardia spp. proteins and peptides. Further work using these methods and also Giardia spp. purified cysts directly isolated from faecal material may extend this knowledge, especially in light of concerns over the validity of data obtained using cysts generated from in vitro cultures (Faghiri and Widmer, 2014).

Results of the parasite faecal proteome include proteins associated with RNA binding and pre-RNA splicing factors that are of particular interest and relevance in potential parasite infective mechanisms. Some Giardia spp. are known to be infected with Giardia lamblia virus (GLV) a specific double stranded (ds) RNA virus (Wang and
Wang, 1986, Tai et al., 1993). Viral infections of axenic cultures of *Giardia* spp. have been shown to occur in isolates from particular geographical areas, and are more frequently detected in cultures kept over long time periods, suggesting that a process of adaptation may occur (De Jonckheere and Gordts, 1987). Studies of two different axenic *Giardia* spp. cultures, one infected with GLV and a second uninfected isolate susceptible to GLV infection showed that an intermediary single strand RNA is present functioning as viral mRNA (Furfine et al., 1989). This virus (GLV) may have evolved to be a key transporter of genetic material, or alternatively the organism may induce expression of a prion (Riesner, 2003, Leiman et al., 2009). GLV may be one factor responsible for alteration of transcription processes causing nucleotide substitution at intrahost level. The critical importance of this type of selective amino acid changes initiated by virus or virus-like particles have been proposed in the context of lateral gene transfer mechanisms of RNA viruses and immune evasion strategies (Holmes, 2009). Single strand RNA is known to be a method of host cell penetration used by other organisms, such as plant nematodes or parvovirus (Parrish, 2010, Hewezi et al., 2012). This type of novel transfer of genetic material has been reported for *Mycoplasma* and *Rhizobium* species and is considered a transcriptional process unique to these organisms (Nakagawa et al., 2010, Liang et al., 2013). There is a possibility that there may be some similarities with *Giardia* spp. infections, with critical control points controlled by transcription factors necessary for initiating the transcription process. Recently three novel proteins have been implicated as regulatory transcription factors during encystation ARID/Bright-like Protein (Wang et al., 2007, Pan et al., 2009, Su et al., 2011).

One hypothetical protein identified in this study was related to RNA binding and pre-RNA splicing factors, which may be associated with *Giardia* spp. trophozoite replication, or alternatively may be a mechanism for attachment, possibly through a single stranded RNA mechanism. Single strand DNA viruses, such as parvovirus are reliant upon host DNA polymerase for replication (Holmes, 2009).
The factors that are associated with successful trophozoite attachment that causes infection remain only partially explained, but this new approach has presented some potential explanations. Whilst the understanding of the parasite’s biological system and unique attributes of *Giardia* spp. are evident in the cell wall structure, binuclear and tetranuclear cells and cell division processes, the processes of transcription and translation of genetic information are only slowly being elucidated. Genes for five enzymes associated with cell wall synthesis are induced at the transcriptional level (Lopez *et al.*, 2003) and further work is required to fully understand the role of transcriptional processes in gene organization and regulation (Teodorovic *et al.*, 2007, Padmaja *et al.*, 2010). Outcomes from this research have provided some insight into the types of molecular mechanisms that occur, previously existing only as predictions from genomic studies.

Detection of tRNA/rRNA cystosine methylase in this study and the significance of this enzyme’s involvement in replication mechanisms in *Giardia* spp. are yet to be fully determined. Bioinformatic analysis of *G. duodenalis* genome data has failed to identify protein arginine methyltransferase genes, despite their presence in other protozoans and eukaryotes (Krause *et al.*, 2007, Fisk and Read, 2011). In the absence of these proteins the regulatory mechanisms for cellular processing in *Giardia* spp. is uncertain and control of transcription, RNA processing, DNA replication and repair, and signal transduction have been suggested to operate under alternative systems, such as adenovirus transcription factor (Su *et al.*, 2011). The value of proteomic data greatly adds to the genomic dataset that has predicted the existence of genes but where confirmed protein expression remains absent.

7.3.1.1 *Giardia* spp. life cycle transition between cyst and trophozoite

Aspects of the *Giardia* spp. life cycle that remain poorly understood are signalling and stages of encystation in human and other host species. This includes the specific localization in the digestive tract for potential trophozoite attachment and the subsequent encystment processes. Franzen *et al.* (2009) propose that some *Giardia*
spp. genotypes infecting humans are lacking encystation specific genes thus affecting excretion numbers, the ability for detection and potential infectivity. This finding supports research which reported difficulties encountered with attempts at *in vitro* encystation and excystation (Lauwaet *et al.*, 2007, Ratner *et al.*, 2008). A comparative study recommended in vivo methods for *Giardia* spp. excystation as the method of choice (Isaac-Renton *et al.*, 1992). More recently, this was supported in findings from transcriptional profiling of *Giardia* spp. cysts lacking an overlap in results from *in vitro* and *in vivo* sources of cysts (Faghiri and Widmer, 2014). The approach taken in this study has been to examine molecular characteristics of *Giardia* spp. *in situ*. The canine faecal samples constitute the final stage of *in vivo* processes containing cysts, the infective form of this protozoan parasite. The results reported here complement the work of other scholars and advance the knowledge of *Giardia* spp. proteins.

### 7.3.2 Host faecal proteome (*Canis familiaris*)

A panel of host (canine) proteins identified in this study are involved with inflammation, cell adhesion and immunomodulation, and additional sampling and proteome profiling would further extend the current knowledge.

#### 7.3.2.1 Immune response proteins

The preliminary dataset (Table 5.8) reported in Chapter 5 confirms earlier work that canine faecal material does contain a vast array of immune proteins (Reynolds and Johnson, 1970). Further detailed investigations are required to determine the types and significance of the immune proteins detected.

Whilst this study did not collect data concerning the immune status of individual animals, such as circulating immunoglobulin levels or types of lymphocytes or macrophages, observations can be made about the age when parasite infections occurred and potential impacts on growth and development. Considering that samples were collected from puppies ingesting maternal milk as the sole nutrient source up until the weaning stage it is likely that protective factors are maternally derived. During this period microbial colonisation of the digestive tract commences whilst development and
maturation of the small intestine are induced by dietary content. Post-weaning, the combined effects of growth and dietary changes accompanied by a reduction in maternal antibodies stimulates immune function. Internal receptors serve to signal presence of ‘foreign’ xenobiotic agents such as parasites and other microflora, including viruses and other common canine infections. It is unknown if innate immune system reduces immune factors in response to single infectious challenges or in a synergistic mechanism.

Whilst ever increasing information has become available for improved understanding of the human and murine immune responses to injury and infectious disease as defence mechanisms. There remains limited knowledge for other common species, such as companion animals, including dogs (*C. familiaris*) (Linde *et al.*, 2008). The alpha defensins, anti-microbial proteins of the human and murine immune systems, are absent in cattle and dogs (Shanahan *et al.*, 2011). To date, the immune system of the dog is known to possess three different beta defensins, one cathelicidin (K9CATH) and a hormonal peptide canine hepcidin (Fry *et al.*, 2004, Sang *et al.*, 2005, Sang *et al.*, 2007) but no studies have yet identified any canine intestinal tract anti-microbial peptides (AMP).

In the context of *Giardia* spp. infection and its ability to infect a wide range of species, plus the presence of frequently occurring cross species infections, a detailed understanding of the host response mechanisms is essential. Further studies into anti-*Giardia* spp. effector mechanisms in humans suggested by Singer (2011) could also include a focus on species-specific host-parasite protein interactions.

Patil *et al.* (2004) provided examples of differences and relationships in the types of beta-defensins immune responses with evidence for cross-species groupings. His phylogenetic study demonstrated distinct evolutionary lineage of AMP between groups, one for dog and rodent specific peptides; another for primate and dog specific peptides; and a rodent specific and dog specific groups of defensins. This suggests
that host specific infectious weaponry may provide a link to understanding the reasons for differences between host specific and cross species infections.

Improved understanding of immune function responses, particularly secretion of immunoglobulin A and associated protein production by plasma cells and lymphocytes, such as lactoferrin may provide further information about the attachment and disease processes of *Giardia* spp. (Ordaz-Pichardo *et al.*, 2013). Antimicrobial peptides including lactoferricin B have been implicated in the depolarization of cell membranes in order to penetrate and target intracellular macromolecule synthesis (Ho *et al.*, 2012).

### 7.3.2.2 Host microbiome

Composition of the gut microflora directly affects host physiology (Zoetendal *et al.*, 2011). This study, whilst concentrating on *Giardia* spp. proteins also generated proteomic information concerning other non-parasite proteins, many matching to prokaryotic organisms consistent with normal faecal microbial composition, however further analysis of the data is warranted. Studies have demonstrated that molecular patterns of microbial and pathogenic organisms (MAMP /PAMP) invoke immune responses in colonic epithelial cells of canine hosts (Swerdlow *et al.*, 2006). Molecular mechanisms proposed for mammalian innate immune responses to MAMP /PAMP are suggested to occur through membrane bound Toll receptors and cytosolic nucleotide binding sites with distinctive leucine rich repeats (LRR) (Medzhitov, 2001, Inohara and Nunez, 2003, Akira *et al.*, 2006). These LRR are a unique feature of protozoan parasites, associated with immune responses (Boceta *et al.*, 2000, Mcguinness *et al.*, 2003, Kedzierski *et al.*, 2004a, Loftus *et al.*, 2005, Daher *et al.*, 2006) and form an integral component of *Giardia* spp. cell wall proteins (Lujan *et al.*, 1995, Luján *et al.*, 1997). Expression of IL-7 has shown to be induced in canine cell lines associated with LPS of bacterial organisms and glycosylphosphatidylinositol in protozoan parasites (Ropert *et al.*, 2001, Swerdlow *et al.*, 2006).
7.3.3 Host-parasite interactions

Results reported here for the *Giardia* spp. faecal proteome were developed during this study seeking to assess the association of clinical signs with *Giardia* infections.

Following preliminary results from proteomic analysis one specific protein was selected for further study. This protein, lactoferrin, was considered a potential indicator of pathogenic infection based upon detection as one component of the canine host faecal proteome. Reports in the literature suggest a high likelihood that inflammation is occurring as a consequence of parasitic infection. Mammalian host remediation of inflammation occurs through secretion of lactoferrin (Vorland, 1999, Farnaud and Evans, 2003). Whilst the results from this preliminary study are limited, the results are promising and follow up studies should be carried out. These should include a reference control group of mature well cared for dogs as a comparative negative control group, and triplicate confirmation of control animals being free from *Giardia* spp.

And other parasite infections using microscopy, antigen and genetic evidence. More importantly, the elevated lactoferrin levels detected in this study need further examination to distinguish the causes of any inflammatory processes originating from *Giardia* spp. infection or from the coccidian infections. The early exposure to intestinal parasites must affect the developing immune system possibly through tolerance or protective immune mechanisms, which may also be affected by different *Giardia* spp. genotypes (Emery et al., 2014).

7.3.3.1 Lactoferrin

Studies in humans have demonstrated the immunomodulatory effect of lactoferrin and its relationship with secretory IgA (slgA) in breastfed babies (Hogendorf et al., 2013). The immunomodulatory function of lactoferrin in maternal milk promotes t-cell proliferation, maturation and differentiation and is involved with B-cell maturation to antigen presenting cells (APC). In addition, iron binding by lactoferrin deprives pathogenic bacteria of an iron source reducing bacterial infections. Other studies also suggest innate immune processes are less likely to be affected by antibodies, and
rather that protective functions are conferred by oligosaccharides present in human breast milk as fucosyloligosaccharides (Newburg et al., 2004). Humoral immune responses to infection have also recently been shown to cause increased leukocyte production in human breast milk (Hassiotou et al., 2013). Maternal genotype confers variation in the glycoconjugate structures ultimately resulting in tissue wide modification of carbohydrate receptor sites. These genetic variations in carbohydrate receptor sites arise from polymorphic mutations, partially controlled by Lewis blood group genes in utero, in the developing foetus, and consequently provide protective functions to suckling neonates by impeding access to intestinal binding sites by pathogenic organisms such as Giardia spp. and bacteria (Newburg et al., 2004). Development of the canine immune system does not mirror that of Homo sapiens and further studies are warranted to determine the relationship between lactoferrin and immune processes.

7.3.3.2 Secretory IgA

One important immunoglobulin present in milk and secreted in the small intestine is IgA, where the secretory form is bound to a component to prevent proteolysis. Secretory IgA is considered to provide passive immune protection and its relationship with lactoferrin and lysozyme has been investigated in human subjects (Hennart et al., 2002). New research has shown that the structure and function of sIgA may vary between species (Woof and Russell, 2011). Lagomorphs, such as rabbits possess thirteen genes coding for alpha heavy chain constant region immunoglobulin A in contrast to the one or two genes found in humans, primates and other mammals (Woof and Russell, 2011). Four allelic variants have been detected in the structure of canine IgA (Peters et al., 2004). The significance of these genetic – phenotypic differences is unclear, although variation between different dog breeds is believed to affect humoral immune system responses (Tengvall et al., 2013).

7.3.3.3 Novel peptides

This study identified both structural and metabolic proteins. Whilst it is known that cytoskeleton components play a role in cell cycle transitions, some of the specific
Chapter 7 General Discussion

protein expression pathways occurring during excystation and encystation however remain undescribed. Consequently, this study is only able to suggest that the structural and metabolic proteins detected are associated with *Giardia* spp. cell cycle regulation. Evolution of eukaryotic membrane trafficking mechanisms has been described (Dacks and Field, 2007) including functions for specialized GTPases, SNARE and, Rab proteins, none of which were identified here. The possibility of a cytoplasmic IFT-independent mechanism for axoneme assembly and IFT-mediated assembly for membrane bound axoneme components of *Giardia* cytoskeleton (Dawson, 2011a) may be associated with the ESV protein packaging and transport mechanism previously described by Reiner *et al.* (1990), Marti and Hehl (2003) and Touz (2012). Movement of proteins from ESV to the peripheral vacuole has been illustrated and mobilisation of innate immune factors are also associated with the membrane and endoplasmic reticulum (Hayakawa *et al.*, 2004, Godfrey *et al.*, 2006, Faso and Hehl, 2011, Wampfler *et al.*, 2014).

Detailed molecular analysis of *Giardia* cell wall proteins have been published using Green Fluorescent Protein (GFP) (Hehl *et al.*, 2000). Fluorescent tagging of IFT flagellar proteins combined with measuring rates of protein turnover, as a function of flagellar length, can be performed with total internal reflection fluorescence (TIRF) microscopy (Engel *et al.*, 2009). This technique has also been used to investigate and describe *Giardia* spp. adhesion (House *et al.*, 2011). Holmberg and Nollen (2013) provide the methods and protocols for analysis of protein aggregates using Native Agarose Gel Electrophoresis (NAGE) with fluorescent labelling. Future proteomic work investigating *Giardia* proteins or genetics may contribute to advances in knowledge when combined with these techniques and other methods used for *Giardia* spp. cell wall protein analysis using species specific DNA probes (Graczyk *et al.*, 2003, Erlandsen *et al.*, 2005).

Fragments of bacterial flagella have been shown to be highly potent immunogens and studies using rats have demonstrated induced immune tolerance to flagellin proteins
(Parish et al., 1969). In cases of canine inflammatory bowel disease one risk factor identified is nucleotide polymorphisms affecting TLR 5 signalling. Hyper-responsiveness to flagellin has been reported for susceptible haplotypes contributing to clinical signs of IBD whilst TLR 5 sensing of flagellin may also be a key factor for maintaining homeostasis (Kathrani et al., 2012). Conversely, TLR 9 expressed on intestinal epithelium cell surfaces play a key signalling role for optimal responses to oral infection and vaccination mediated by the DNA of commensal organisms of the gut microflora (Hooper et al., 1999, Hall et al., 2008). It remains unclear if similar associations between signalling and immune responses are present in response to Giardia spp. flagellar protein fragments.

This study detected seven hypothetical Giardia spp. proteins and seven hypothetical proteins of host origin. These were highly confident results with multiple peptides detected. One of the hypothetical Giardia spp. proteins (C6LS33) was identified with a high protein score and 4 matching peptides, although information is absent for the protein domain or cluster and no other related sequences were reported in the databases. This indicates the detection of novel peptides therefore making this protein and matching peptides of high interest for further investigation. Some of the peptide sequences of other hypothetical Giardia spp. proteins showed similarity to related sequences for other pathogenic proteins, such as the secretion protein reported for Mycobacterium tuberculosis (Alonso et al., 2007, Gong et al., 2012). In addition, other examples of the peptides detected matched to proteins with conserved domains, such as Lymphocryptovirus that are also related to phage-like glycoproteins, viral glycoproteins or penetration protein (Kim et al., 2011). It is unknown if this type of Giardia spp. peptide or protein is implicated in attachment to epithelial cells in a similar manner to other bacteria or virus found in the reproductive or respiratory tract.

Results presented here suggesting that Giardia spp. may not be the sole cause of clinical signs of infection. Instead a hypothesis is proposed here that Giardia spp. may in fact be relatively benign parasites, forming part of the normal gastro-intestinal
ecosystem rather than an obligate pathogenic parasite. This status may alter under specific conditions when it becomes opportunistic and adhesion to host cells is achieved. The parasite utilises a range of pathogenic adaptations and mechanisms to survive and replicate at the expense of the host (Cray et al., 2013). The mechanisms proposed here may occur in isolation, simultaneously or sequentially, or as optional alternatives. This theory proposes that constituents of microbial flora may play an active role in facilitating *Giardia* infections either by enhancing attachment and persistence, or by diminishing innate immune responses and the disruption of transcription processes. Studies have shown that *G. duodenalis* does possess some mucin degrading enzymes, N-acetylglucosaminidase and N-acetylgalactosaminidase, but not the extensive range required for complete breakdown of carbohydrate chains as seen in Trichomonad species (Connaris and Greenwell, 1997, Ryan et al., 2011). This suggests that metabolism of these mucins may form nutrients for trophozoites or that other factors could be associated with digestion of mucins. This study detected more than one type of bacterial species capable of digesting the “protective” mucin layer, such as *Akkermansia muciniphilia* or similar species (Collado et al., 2007, Ouwerkerk et al., 2013). *Giardia* spp. secrete proteases capable of digesting collagen, and possibly other components of the extracellular matrix, such as elastin, fibronectin and laminin. These secreted proteases have been implicated in cell apoptosis and leakage at tight junctions. The activation of protease secretion may be a genotype specific genetic characteristic as shown by Koh et al. (2012) or may be a common feature across all genotypes but requiring stimulus or an initiation factor to induce molecular and pathogenic changes.

7.3.3.4 Differentiation between assemblages requires further validation.

The proteomic data frequently contained peptide sequences that matched to proteins of the *G. duodenalis* Assemblage B genome, with an overall greater identification of Assemblage B proteins than Assemblage A proteins.
Chapter 7 General Discussion

This proteomic data suggests that differential protein expression between *Giardia* assemblages may occur or that anomalies in genome coverage exist.

*G. duodenalis* Assemblage B generally eludes genetic detection, using traditional methods, and this finding at the level of protein output is of great interest. Many researchers are of the opinion that Assemblage B incorporates high variability and is a cause of clinical infections (Kohli *et al.*, 2008, Lebbad *et al.*, 2008, Minvielle *et al.*, 2008, Al-Mohammed, 2011).

Thus, this clearly demonstrates that whilst evidence of genetic descriptions for assemblage B in this study was lacking, the proteomic analysis obtained by spectral data matching to peptides within existing genomic databases portrays a different classification between infections based upon protein identity.

This could indicate that *Giardia* spp. genotypes in dogs, host specific assemblages have genetic sequences closely related to assemblage B genotypes, although these were not detected by PCR. Interestingly high prevalence rates of Assemblage B have been reported in faecal samples from humans in Western Australia (Yang *et al.*, 2010). A noteworthy trend of increasing detection of Assemblage B infections in humans has also been reported from Asian countries (Ajjampur *et al.*, 2009, Singh *et al.*, 2009, Boontanom *et al.*, 2011, Laishram *et al.*, 2012).

### 7.4 Management of *Giardia* spp. infections in canines

The significance of the high numbers of perceived asymptomatic infections found here and reported in epidemiology studies are yet to be fully understood. The data presented in Chapter 3 showed that earliest patent infections are occurring within the first three weeks of life. Studies into these rarely detected or reported asymptomatic infections within the wider population should become a higher priority over studies that solely report prevalence rates and do not take into consideration molecular epidemiology or transmission cycles. Research planning should consider the dynamics of *Giardia* spp. infections over a longitudinal time frame.
This study supports the observations concerning risk factors for *Giardia* spp. that communal housing and facility construction are important factors together with types of responses to detected infections, such as treatment or quarantine protocols (Ortuna and Castella, 2011). Improved routes for management of *Giardia* infections in dogs and reducing transmission to humans and other species should include education about responsible dog ownership (Macpherson, 2005, Palmer *et al.*, 2008).

Understanding the complexities of microbiota and host interactions is a key factor for tackling clinical signs of *Giardia* in dogs. Vaccination against *Giardia* is unlikely to be either cost-effective or appropriate if parasitic populations actually form a component of the natural ecosystem of the gastro-intestinal microflora. The consequences of using vaccination methods to eliminate *Giardia* spp. from the gut ecosystem may produce an unforeseen set of circumstances, particularly in relation to development of the innate immune system, digestion of nutrients and neurosignalling mechanisms.

### 7.5 Limitations to the design and implementation of this study

The use of faecal samples from canine subjects infected with *Giardia* spp. despite the lack of directly relevant genomic data for the canine *Giardia* spp. under study reduced the insights into phenotypic variation. The existing genomic data for *G. duodenalis* did however provide useful information on both the types of *Giardia* spp. proteins and differentiation between the two human genotypes. Collection of the evidence for *G. canis* infections (host specific Assemblages C and D) has progressed, and some insights into potential transmission routes have been explored.

Comparison of faecal proteome of symptomatic or asymptomatic cases of canine *Giardia* infection was beyond the scope of this study, whilst the definitive cause of signs of gastro-intestinal disorder were shown in this study not to be attributed solely to *Giardia* spp. parasite. For a thorough and comprehensive analysis of host proteomic data a broader genomic dataset would be beneficial, or data for specific breed types, such as Labrador retriever or miniature schnauzer as used in this study. For interpretation of immune protein detection the sex and tissue type is important, the
limitations of basing genomic datasets on a single dog breed, sex and tissue type have been reported elsewhere (Libert et al., 2010).

7.6 Future work

The discovery of unique peptide fragments by proteome mapping confirms the usefulness of this technology for further investigation into the potential zoonotic transmission of G. duodenalis. Embarking on new studies to examine unconventional routes for transmission is timely.

Further validation of the proposed biomarkers of inflammation may provide new insights into host-parasite interactions and the impacts of Giardia spp. on the host immune system.

This work has highlighted the importance of considering canine health from an ecosystem approach and consideration of the influence from other microorganisms on the pathogenic nature of Giardia spp. demands further investigation in canines and other host species.

7.7 Conclusion

The limitations of relying on a single method for Giardia spp. diagnosis have been identified in this study and shown that traditional methods, such as microscopy, are not inferior to technologically advanced methods for determining the causative factors of infection. Mass spectrometry results have, for the first time, illustrated a canine faecal proteome based upon peptide detection. The differential protein expression profile supports evidence for diverse Giardia spp. genotypes in canines. The canine faecal proteome has identified potential host responses to Giardia spp. infection and identified the value of faecal lactoferrin as a suitable biomarker candidate. The short pre-patent period in the neonatal canine reported here, together with temporal dynamics of cyst excretion are contributors to infectious transmission.
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### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AMP</td>
<td>Antimicrobial peptide</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>ATCC</td>
<td>Tissue Culture Collection</td>
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<tr>
<td>BasR</td>
<td>Bacterial Adaptive Response</td>
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<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CDS</td>
<td>Coding DNA sequence</td>
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<tr>
<td>CIE</td>
<td>Counter immune electrophoresis</td>
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<tr>
<td>CWP</td>
<td>Cell Wall Protein</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>ESCCAP</td>
<td>European Scientific Counsel Companion Animal Parasites</td>
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<tr>
<td>ESV</td>
<td>Encystation specific Secretory Vesicle</td>
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<tr>
<td>GALT</td>
<td>Gut Associated Lymphoid Tissue</td>
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<td>GDH</td>
<td>Glutamate Dehydrogenase</td>
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<td>GIT</td>
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<td>GlcNAc</td>
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<td>GSA</td>
<td>Giardia Specific Antigen</td>
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<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<td>Inflammatory Bowel Disease</td>
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<td>Immunofluorescent Antibody Test</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IGS</td>
<td>Intergenic Space</td>
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<td>KEGG</td>
<td>Kyoto Encyclopedia of Genes and Genomes</td>
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<tr>
<td>LCA</td>
<td>Least Common Ancestor</td>
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<td>LCMS</td>
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<td>LPS</td>
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<td>LRR</td>
<td>Leucine Rich Repeats</td>
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<td>MALDI</td>
<td>Matrix Assisted Laser Desorption Ionization</td>
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<td>MAMP</td>
<td>Microbe Associated Molecular Pattern</td>
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<td>MMMS</td>
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<td>mRNA</td>
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<td>MTOC</td>
<td>Microtubule Organizing Centre</td>
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<td>NAGE</td>
<td>Native Agarose Gel Electrophoresis</td>
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<td>NANA</td>
<td>N- acetylneuraminic (sialic) acid</td>
</tr>
<tr>
<td>NCBI</td>
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<tr>
<td>NPV</td>
<td>Negative Predictive value</td>
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<td>Polyacrylamide Gel Electrophoresis</td>
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<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
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<td>PBS</td>
<td>Phosphate Buffered saline</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PFOR</td>
<td>Pyruvate flavidoxin oxidoreductase</td>
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<td>PP2A</td>
<td>Protein phosphatase 2 A</td>
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<td>Positive Predictive Value</td>
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<td>PTM</td>
<td>Post-translational Modification</td>
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Appendices

Appendix 1

Prevalence and diagnostic data Chapters 2 and 3

2.1 Diagnostic test results for *Giardia* spp. infections in Labrador retriever breeding facilities in Victoria and WA

Canine faecal samples from Labrador retriever puppies and dogs in Victoria tested for *Giardia* spp. infection using copro-antigen test 1 produced 44 positive results as displayed in Table 2.1.1. Negative test results were obtained for 111 samples. True Positive (TP) indicates a positive test result for gold standard test and positive for a second diagnostic test (positive result for both tests). True negative (TN) indicates a negative test result for the gold standard test and a second diagnostic test (negative result for both tests). False Positive (FP) indicates a negative test result for gold standard test and positive test result for a second diagnostic test. False Negative (FN) indicates a positive test result for gold standard test and negative test result for a second diagnostic test.

Samples from WA tested with copro-antigen test 1 gave 12 positive results and 34 negative results.

Table 2.1.1 Diagnostic results for three different copro-antigen tests relative to the gold standard

<table>
<thead>
<tr>
<th>Sample source</th>
<th>Diagnostic test</th>
<th>N</th>
<th>TP</th>
<th>FP</th>
<th>FN</th>
<th>TN</th>
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<td>Copro-antigen test 1</td>
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<td>22</td>
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<td>Copro-antigen test 1</td>
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<td>1</td>
<td>3</td>
<td>1</td>
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<td>17</td>
<td>9</td>
<td>2</td>
<td>3</td>
<td>3</td>
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<tr>
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<td>4</td>
<td>10</td>
<td>3</td>
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<tr>
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<td>ELISA antigen test</td>
<td>26</td>
<td>9</td>
<td>0</td>
<td>13</td>
<td>4</td>
</tr>
</tbody>
</table>
2.2 *Giardia* spp. infections detected in canine faecal samples - diagnostic test results

Data collected from Western Australia during the first year (2011) were from pooled faecal samples from 2 litters of puppies aged 35-49 days old. In the second year, (2012) pooled faecal samples were collected from 1 litter of puppies aged 29-50 days old. During the third year (2013) individual samples were taken twice weekly (at 3-4 day intervals) from 1 litter of puppies aged 14 days – 57 days and individual faecal samples from the same litter of puppies aged 68-85 days old.

In 2012 canine faecal samples were collected at weekly intervals whilst the puppies were aged between 29 and 50 days old. Twenty-seven samples were tested for *Giardia* spp. infection, using copro-antigen test 1, and 4 positive test results were recorded. Three samples positive by microscopy for parasites were negative by copro-antigen test 1. Two of these samples were *Giardia* spp. co-infections with *Cystoisospora* spp.

In 2013 canine faecal samples from one litter of puppies were collected from individual puppies over consecutive weeks between the ages of 68-85 days old, a total of 20 samples. Eight positive test results were recorded using zinc sulphate flotation and light microscopy. All samples were also tested with copro-antigen test 1 giving two positive test results and six negative test results.

Table 2.2.1 *Giardia* spp. diagnostic data obtained using light microscopy gold standard

<table>
<thead>
<tr>
<th>Year</th>
<th>N</th>
<th>Positive</th>
<th>Negative</th>
<th>Age</th>
<th>PREVALENCE (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>39</td>
<td>29</td>
<td>10</td>
<td>35-49</td>
<td>73.7% (0.569-0.866)</td>
</tr>
<tr>
<td>2012</td>
<td>27</td>
<td>8</td>
<td>19</td>
<td>29-50</td>
<td>29.6% (0.138-0.502)</td>
</tr>
<tr>
<td>2013</td>
<td>20</td>
<td>15</td>
<td>5</td>
<td>68-85</td>
<td>75.0% (0.509-0.914)</td>
</tr>
<tr>
<td>2011-13</td>
<td>86</td>
<td>52</td>
<td>34</td>
<td>68-85</td>
<td>60.0% (0.489-0.705)</td>
</tr>
</tbody>
</table>

Table 2.2.2 Diagnostic test results for Copro-antigen test 1 relative to gold standard

<table>
<thead>
<tr>
<th>Litter</th>
<th>Year</th>
<th>N</th>
<th>TP</th>
<th>FP</th>
<th>FN</th>
<th>TN</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>2012</td>
<td>27</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>21</td>
</tr>
<tr>
<td>4</td>
<td>2013</td>
<td>20</td>
<td>8</td>
<td>0</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>3-4</td>
<td>2012-13</td>
<td>47</td>
<td>12</td>
<td>0</td>
<td>8</td>
<td>27</td>
</tr>
</tbody>
</table>

Table 2.2.3 Diagnostic test results using Copro-antigen Test 2 relative to the gold standard

<table>
<thead>
<tr>
<th>Group</th>
<th>Year</th>
<th>N</th>
<th>TP</th>
<th>FP</th>
<th>FN</th>
<th>TN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Litter 3</td>
<td>2012</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Litter 4</td>
<td>2013</td>
<td>14</td>
<td>8</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Litter 3-4</td>
<td>2012-13</td>
<td>17</td>
<td>9</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

Seventy-four samples in total from a shelter population and two Labrador retriever breeding facilities (WA and Victoria) were tested using ELISA copro-antigen plate assay test. Results are shown in Table 2.2.4
Table 2.2.4 Diagnostic test results for ELISA relative to the gold standard for all groups of dogs tested

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>TP</th>
<th>FP</th>
<th>FN</th>
<th>TN</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHELTER WA</td>
<td>26</td>
<td>1</td>
<td>7</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>LAB RET WA</td>
<td>26</td>
<td>9</td>
<td>0</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td>LAB RET VIC</td>
<td>22</td>
<td>5</td>
<td>4</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>ALL GROUPS TESTED</td>
<td>74</td>
<td>15</td>
<td>11</td>
<td>28</td>
<td>20</td>
</tr>
</tbody>
</table>

Twenty-seven of the canine faecal samples tested positive using ELISA. Eight samples were from shelter dogs, ten from Labrador retriever breeding facilities in WA and nine from Labrador retriever breeding facilities in Victoria.

2.3 Molecular genetics – comparison of polymerase chain reaction (PCR) with the light microscopy gold standard for Victorian dogs

**Year 1 (2011)**

Thirty-nine pooled faecal samples from two litters of puppies aged 35-49 days old were tested by PCR at the 18s gene locus. Fifteen samples were positive giving positive results for 38% of all samples tested (and 52% of all samples positive by light microscopy). From these positive samples 8 were successfully sequenced and genotyping showed 5 samples of mixed assemblage A/D, 2 samples were assemblage A and 1 sample was assemblage D. Further PCR testing at BG and GDH loci were unsuccessful except for one sample which was genotyped as assemblage D. This sample was consistent for all 3 loci.

**Year 2 (2012)**

Twenty-seven faecal samples were collected as pooled samples from one litter of puppies, aged 29-49 days old. One of four samples was considered asymptomatic and the other 3 symptomatic. 4 samples were positive by PCR at the 18s gene locus which is 15% of all samples tested or 40% of all samples confirmed positive by light microscopy. There were three samples containing *Giardia* cysts, confirmed by light microscopy, that did not successfully amplify, and one sample, which gave a positive PCR result but no *Giardia* spp. cysts were observed by microscopy. Sequencing of 4 positive samples was successful for 3 of them, and all were genotyped as Assemblage D. Different gene loci were used to determine genotype. All three were confirmed at the 18s gene locus, one at 18s locus only, one at beta giardin locus only and one at beta giardin and GDH loci.

**Year 3 (2013)**

20 faecal samples from individual puppies from this same litter of puppies were collected over consecutive weeks between the ages of 68-85 days old. 11 samples tested positive by PCR at 18s gene loci. That is 55% of all samples tested, or 73% of all light microscopy positive samples. Sequencing was carried out on all 11 samples, and 8 were successfully genotyped at the 18s gene locus. Three samples were genotyped at single locus, two at 2 loci, and one at 3 loci. One was genotyped as
assemblage A (18s) two were assemblage C (18s and GDH) and five were assemblage D (3 at 3 loci, 18s, BG, GDH) 1 at 2 loci (18s and GDH) and one at a single (18s) loci. One of the assemblage C samples and the assemblage A sample (genotyped at 18s locus) were positive for *Giardia* spp. by light microscopy but negative using Copro-antigen tests 1 and 2. PCR was unsuccessful in amplifying 6 samples (40%) that had been confirmed positive for *Giardia* cysts by light microscopy. Three samples positive by microscopy for parasites were negative by Copro-antigen test 1. Two samples were *Giardia* spp. co-infections with *Cystoisospora* spp. and two of these samples were also tested using Copro-antigen test 2; one gave a positive result and one gave a negative result. The sample which was positive with Copro-antigen test 2 was also positive by PCR at 18s genotype D. One sample was negative by both microscopy and Copro-antigen test 1, but positive using Copro-antigen test 2, this sample was also PCR positive at 18s, genotype D.

### 2.4 Molecular genetics - comparison of PCR test relative to gold standard light microscopy for a breeding population in Perth, Western Australia

Eighty-six canine faecal samples were tested for *Giardia* spp. over a three year period, 29 samples were positive for *Giardia* spp. although 23 samples negative using PCR were positive for *Giardia* spp. cysts by microscopy, as shown in Table 2.4.1.

Table 2.4.1 PCR Diagnostic test results relative to the gold standard

<table>
<thead>
<tr>
<th>Year</th>
<th>N</th>
<th>TP</th>
<th>FP</th>
<th>FN</th>
<th>TN</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>38</td>
<td>15</td>
<td>0</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>2012</td>
<td>27</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>2013</td>
<td>20</td>
<td>9</td>
<td>1</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>2011-13</td>
<td>85</td>
<td>27</td>
<td>2</td>
<td>22</td>
<td>34</td>
</tr>
</tbody>
</table>

### Appendix 2

#### 3.1 Cyst intensity

Table 3.1.1 Frequency of *Giardia* spp. infections, confirmed by detection of cysts using light microscopy, in pooled faecal samples from multiple litters of Labrador retriever puppies

<table>
<thead>
<tr>
<th>AGE (days)</th>
<th><em>Giardia</em> spp. cyst intensity</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low +1</td>
<td>Moderate +3</td>
</tr>
<tr>
<td>17</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>26</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>28</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>36</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>35</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>42</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>45</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>67</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>30</td>
<td>4</td>
</tr>
</tbody>
</table>
Table 3.1.2 Frequency of *Giardia* spp. infections, confirmed by detection of cysts using light microscopy, in pooled faecal samples from Labrador retriever puppies in WA (2013)

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Low</th>
<th>Moderate</th>
<th>High</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>68</td>
<td>+1</td>
<td>+2</td>
<td>.+3</td>
<td>+4</td>
</tr>
<tr>
<td>72</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>73</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>75</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>76</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>83</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>84</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>85</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>86</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>9</td>
<td>1</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 3.1.3 Intensity of infection (cyst) from different Labrador retriever breeding populations

<table>
<thead>
<tr>
<th>Location</th>
<th>Year</th>
<th>Low</th>
<th>Moderate</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>WA</td>
<td>2011</td>
<td>24/28 (85%)</td>
<td>3/28 (11%)</td>
<td>1/28 (6%)</td>
</tr>
<tr>
<td>WA</td>
<td>2012</td>
<td>2/6 (33%)</td>
<td>2/6 (33%)</td>
<td>2/6 (33%)</td>
</tr>
<tr>
<td>WA</td>
<td>2013</td>
<td>9/19 (47%)</td>
<td>6/19 (32%)</td>
<td>4/19 (21%)</td>
</tr>
<tr>
<td>VIC</td>
<td>2011-13</td>
<td>30/37 (81%)</td>
<td>4/37 (11%)</td>
<td>3/37 (8%)</td>
</tr>
</tbody>
</table>

Table 3.1.4 Frequency of parasite detection in faecal samples from Labrador retriever puppies in WA (2013) confirmed positive by microscopy for parasite infections

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Sarcocystis sp.</th>
<th>Cystoisospora sp.</th>
<th>Toxocara canis</th>
<th>Giardia spp.</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>32</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>52</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>56</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>1</td>
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<tr>
<td>68</td>
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<tr>
<td>72</td>
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<td>73</td>
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<td>4</td>
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<tr>
<td>75</td>
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<td>76</td>
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<tr>
<td>85</td>
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<td>3</td>
<td>4</td>
</tr>
<tr>
<td>86</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TOTAL</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>7</td>
<td>8</td>
</tr>
</tbody>
</table>
Appendices

Table 3.1.5 Frequency of parasite detection in pooled faecal samples from Labrador retriever puppies (2013) in a Victorian breeding facility

<table>
<thead>
<tr>
<th>Parasite species</th>
<th>Labrador Retriever (Victoria)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency</td>
<td>Percentage</td>
<td></td>
</tr>
<tr>
<td>Sarcocystis sp.</td>
<td>11</td>
<td>13.6</td>
<td></td>
</tr>
<tr>
<td>Cystoisospora sp.</td>
<td>26</td>
<td>32.1</td>
<td></td>
</tr>
<tr>
<td>Toxocara canis</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Giardia spp.</td>
<td>30</td>
<td>37.0</td>
<td></td>
</tr>
<tr>
<td><strong>Giardia spp. co-infections</strong></td>
<td>14</td>
<td>17.3</td>
<td></td>
</tr>
<tr>
<td><strong>Total infected</strong></td>
<td>81</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total negative</strong></td>
<td>74</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total tested</strong></td>
<td>155</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.2 Faecal parasites infection and association with clinical signs of infection

Table 3.1.6 Endoparasite infections in faecal samples from Labrador retriever dogs in two breeding facilities

<table>
<thead>
<tr>
<th></th>
<th>2011</th>
<th>2012</th>
<th>2013</th>
<th>TOTAL Perth</th>
<th>TOTAL Victoria</th>
<th>ALL Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td>38</td>
<td>27</td>
<td>20</td>
<td>85</td>
<td>155</td>
<td>240</td>
</tr>
<tr>
<td><strong>Sarcocystis spp. (only)</strong></td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td><strong>Cystoisospora spp. (only)</strong></td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>12</td>
<td>26</td>
<td>38</td>
</tr>
<tr>
<td><strong>Giardia spp. (only)</strong></td>
<td>27</td>
<td>3</td>
<td>7</td>
<td>37</td>
<td>30</td>
<td>67</td>
</tr>
<tr>
<td><strong>Giardia spp. co-infections</strong></td>
<td>1</td>
<td>5</td>
<td>8*</td>
<td>14</td>
<td>14</td>
<td>28</td>
</tr>
<tr>
<td><strong>No parasitic organisms</strong></td>
<td>10</td>
<td>7</td>
<td>3</td>
<td>20</td>
<td>74</td>
<td>94</td>
</tr>
</tbody>
</table>

*Figures marked with an asterisk were co-infections with *Giardia* spp. and *Toxocara canis*. *Giardia* co-infections were recorded as a separate category, addition of figures reported for *Giardia* spp. and *Giardia* spp. co-infections gives the total number of samples infected with *Giardia* spp.

Table 3.1.7 Enteric parasite infections and clinical signs in Labrador retriever dogs

<table>
<thead>
<tr>
<th>Parasite species</th>
<th>Number (N)</th>
<th>Symptomatic</th>
<th>Asymptomatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Total tested) N</td>
<td>240</td>
<td>122</td>
<td>118</td>
</tr>
<tr>
<td><em>Sarcocystis</em> spp.</td>
<td>13</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td><em>Cystoisospora</em> spp.</td>
<td>38</td>
<td>29</td>
<td>9</td>
</tr>
<tr>
<td><em>Giardia</em> spp.</td>
<td>67</td>
<td>38</td>
<td>29</td>
</tr>
<tr>
<td><em>Giardia</em> co-infections</td>
<td>28</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>No parasitic organisms</td>
<td>94</td>
<td>36</td>
<td>58</td>
</tr>
</tbody>
</table>
Appendix 3

Proteomic data (Chapters 4 and 5)

Table 4.11 Protein matches to spectral data for Sample 2 Host faecal proteome

<table>
<thead>
<tr>
<th>N</th>
<th>Protein Accession Number or DNA identifier</th>
<th>Protein name</th>
<th>Species</th>
<th>Number of matching peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>E2QS94</td>
<td>Uncharacterized protein</td>
<td>Canis familiaris</td>
<td>3</td>
</tr>
<tr>
<td>34</td>
<td>P06857</td>
<td>Pancreatic lipase-related protein 1</td>
<td>Canis familiaris</td>
<td>2</td>
</tr>
<tr>
<td>40</td>
<td>E2R6T0</td>
<td>Uncharacterized protein</td>
<td>Canis familiaris</td>
<td>2</td>
</tr>
<tr>
<td>50</td>
<td>XP_540774.2</td>
<td>Mucin-2 precursor or (Intestinal mucin 2)</td>
<td>Canis familiaris</td>
<td>2</td>
</tr>
<tr>
<td>55</td>
<td>XP_862298.1</td>
<td>hypothetical protein XP_857205 isof orm 4</td>
<td>Canis familiaris</td>
<td>2</td>
</tr>
<tr>
<td>60</td>
<td>XP_535770.2</td>
<td>Mucin-13 precursor (Down-regulated in colon cancer 1)</td>
<td>Canis familiaris</td>
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4.2 Spectral evidence for identification of *G. duodenalis* proteins

*Figure 4.2.1* Annotated ms spectra for peptides matched to *G. duodenalis* proteins
Protein1 Hypothetical protein A8BBE2
Spectra 3.1.9.22.5 matching to *Giardia* database at 72.8% confidence level

Figure 4.2.2 Annotated ms spectra for peptide 1 matched to database entries
Protein 1 Hypothetical protein A8BBE2
Hypothetical protein matched to Giardia database (A&B) at 99% confidence level

Figure 4.3.1 Annotated ms spectra for peptides matched to G. duodenalis proteins
Protein 2 Hypothetical protein C6LSL4

Spectra 1.5.39.1 peptide 1 matching to Giardia database (A&B) at 99% confidence level

Figure 4.3.2 Annotated ms spectra for peptide 1 Hypothetical protein C6LSL4
Spectra 2.5.25.4 for peptide 2 matching to *Giardia* database at 21.6%

Spectra 2.5.25.4 for peptide 2 poorly matching to Universal database

Figure 4.3.3 Annotated ms spectra for peptide 2 matched to database entries
Hypothetical protein C6LSL4
Figure 4.4.1 Annotated ms spectra for peptides matched to *G. duodenalis* proteins
Protein 3 Axoneme central apparatus protein A8BQ10

Spectra 1.5.60.1 matched to *Giardia* database (A&B) at 99% confidence level

Figure 4.4.2 Annotated ms spectra for peptides matched to peptide 1
Protein 3 Axoneme central apparatus protein A8BQ10
Pyruvate–flavodoxin oxidoreductase protein E2RTZ1 matched to *Giardia* database at 95% confidence level with 6 supporting peptides

Figure 4.5.1 Annotated ms spectra for peptides matched to *G. duodenalis* proteins

Protein 4 Pyruvate flavodoxin oxidoreductase protein E2RTZ1
Spectra 2.6.53.3 matched to *Giardia* database at 97.9%

Spectra 2.6.53.3 poorly matched to Universal database

Figure 4.5.2 Annotated ms spectra for peptides matched to peptide 1
Protein 4 Pyruvate flavodoxin oxidoreductase protein E2RTZ1
Figure 4.5.3 Annotated ms spectra for peptide 2
Protein 4 Pyruvate flavodoxin oxidoreductase protein E2RTZ1

Spectra 1.9.46.6 peptide 2 matched to Giardia database at 14.5%

Spectra 1.9.46.6, peptide 2, matched to Universal database at 21.2% confidence level
Figure 4.6.1 Annotated ms spectra for peptides matched to *G. duodenalis* proteins
Protein 5 Mismatch repair protein C6LTM7
Coiled coil protein C6LY87 matched to *Giardia* DB at 95% confidence level with 7 supporting peptides

Figure 4.7.1 Annotated ms spectra for peptides matched to *G. duodenalis* proteins
Protein 6 Coiled coil protein C6LY87

Spectra 2.6.41.2 matching to *Giardia* database at 99% confidence level

Spectra 2.6.41.2 poorly matching to Universal database

Figure 4.7.2 Annotated ms spectra for peptide 1 coil protein C6LY87
Hypothetical protein C6LS33 matched to *Giardia* database at 99% confidence level with many supporting peptides

Figure 4.8.1 Annotated ms spectra for peptides matched to *G. duodenalis* proteins
Protein 7 Hypothetical protein C6LS33
Spectra 2.2.7.63.7, peptide 1, matched to Giardia database at 99% confidence level

Spectra 2.2.7.63.7, peptide 1, poorly matched to Universe database

Figure 4.8.2 Annotated ms spectra for peptide 1 Hypothetical protein C6LS33
Spectra 3.2.9.54.5, peptide 2, matched to *Giardia* database at 99% confidence level

Spectra 3.2.9.54.5, peptide 2, matched to Universal database at equivalent confidence level

Figure 4.8.3 Annotated ms spectra for peptide 2 matched to database entries
Hypothetical protein C6LS33
Spectra 3.1.9.54.20, peptide 3, matching to *Giardia* database at 99% confidence level

Spectra 3.1.9.54.20, peptide 3, matching to Universal database at equivalent confidence level

Figure 4.8.4 Annotated ms spectra for peptide 3 Hypothetical protein C6LS33
Figure 4.8.5 Annotated ms spectra for peptide 4 Hypothetical protein C6LS33
Histone methyltransferase protein matched to *Giardia* database (A&B) at 95% confidence level

Figure 4.9.1 Annotated ms spectra for peptides matched to *G. duodenalis* proteins
Protein 8 Histone methyltransferase protein C6LVL9

Spectra 2.1.6.83.13 matched to *Giardia* database (A&B) at 99% confidence level

Spectra 2.1.6.83.13 poorly matched to Universal database

Figure 4.9.2. Annotated ms spectra for peptide 1smatched to database entries
Histone methyltransferase protein C6LVL9
Coiled coil protein C6LZ39 matched to *Giardia* database at 99% confidence level with 26 supporting peptides

Figure 4.10.1 Annotated ms spectra for peptides matched to *G. duodenalis* proteins
Protein 9 Coiled coil protein C6LZ39
Spectra 3.1.9.49.3 peptide 1, matched to *Giardia* database at 99% confidence level

Figure 4.10.2 Annotated ms spectra for peptide 1 matched to database entries
Protein 9 Coiled coil protein C6LZ39

Spectra 3.1.9.49.3, peptide 1, poorly matched to Universal database
Spectra 4.2.6.74.8, peptide 2, matched to *Giardia* database at 99% confidence level.

Figure 4.10.3 Annotated ms spectra for peptide 2 matched to *G. duodenalis* proteins
Protein 9 Coiled coil protein C6LZ39
Spectra 2.1.7.104.1, peptide 3, matched to *Giardia* database at 99% confidence level

Spectra 2.1.7.104.1, peptide 3, poorly matched to Universal database

Figure 4.10.4 Annotated ms spectra for peptide 3 matched to database entries
Protein 9 Coiled coil protein C6LZ39
Spectra 2.1.7.93.7, peptide 4, matched to *Giardia* database at 99% confidence level

Spectra 2.1.7.93.7, peptide 4, matched to Universal database at equivalent confidence level

Figure 4.10.5 Annotated ms spectra for peptide 4 matched to database entries
Protein 9 Coiled coil protein C6LZ39
Signal recognition receptor protein C6LN45 matched to *Giardia* database (A&B) at 99% confidence level with 6 supporting peptides

Spectra 4.1.6.55.20 matched to *Giardia* database at 99% confidence level

Spectra 4.1.6.55.20 poorly matching to Universal database

Figure 4.11 Annotated ms spectra for peptides matched to *G. duodenalis* proteins
Protein 10 Signal recognition receptor protein C6LN45
Hypothetical protein A8B278 matched to *Giardia* database (A&B) at 99% confidence level

Figure 4.12 Annotated ms spectra for peptides matched to *G. duodenalis* proteins
Protein 11 Hypothetical protein A8B278
Spectra 4.1.6.71.7 matched to *Giardia* database at 99% confidence level

Spectra 4.1.6.71.7 poorly matched to Universal database

Figure 4.12.1 Annotated ms spectra for peptide 1 matched to database entries
Protein 11 Hypothetical protein A8B278
Figure 4.13 Annotated ms spectra for peptides matched to *G. duodenalis* proteins
Protein 12 Nek kinase protein C6LMX7

Spectra 1.2.6.81.2 matching to *Giardia* database (A&B) at 99% confidence level

Ludwig spectra 1.2.6.81.2 poorly matching to Universal database

Figure 4.13.1 Annotated ms spectra for peptide 1 matched to database entries
Protein 12 Nek kinase protein C6LMX7