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MOLECULAR EPIDEMIOLOGY OF *GIARDIA DUODENALIS*

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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DECLARATION

I, Weerapol Taweenan, declare that the work in this thesis is original, was carried out solely by myself or with due acknowledgements. It has not been submitted in any form for another degree or professional qualification.

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ABSTRACT

Giardia duodenalis is a parasitic protozoan that affects the gastrointestinal tract, causing abdominal disorders of various animals and humans. To date, *G. duodenalis* has been genotypically divided into seven groups (assemblages), namely A to G, found in different host ranges. Whilst assemblages C to G are specific genotypes affecting restricted animal hosts, assemblages A and B parasitise both humans and a number of animal species, and have been considered as having zoonotic potential. The main objective of the current study was to investigate the molecular epidemiology of *G. duodenalis* in animals and humans in the UK. The current study also evaluated multilocus genotyping and determined the protein changes between assemblages A and B.

Faecal samples from pet animals (252 diarrhoeic dogs and 60 cats) that had been microscopically confirmed as *G. duodenalis* positive, were analysed by PCR using ssu rRNA as a marker. In dogs, 200 samples were amplified by PCR and 126 samples were successfully sequenced. Assemblage A was detected in two (2%) samples, while dog assemblages C and D were detected in 51 (40%) and 56 (44%) samples, respectively. Mixed assemblage C+D was found in 17 samples (14%). In cats, 58 samples were successfully amplified by PCR and 39 samples were sequenced. Assemblage A was found in four samples (10%), F in 32 samples (82%), mixed F+C in two samples (5%) and mixed F+D in one sample (3%). In both cat and dog studies, there was no correlation between gender, age, or breed, and the genotypes found.

In farm animals, 384 faecal samples from clinical cases without *Giardia* positive confirmation were collected from various animal species, including cattle (285), sheep (69) and pigs (30). Out of 141 PCR positive samples, 102 were selected and successfully sequenced. In cattle, out of 93 PCR positive results, 63 samples were successfully sequenced and genotyped as 16 assemblage A (25%), one mixed assemblage A+C (2%), one mixed assemblage A+D (2%), six mixed assemblage A+E (10%), two assemblage C (3%), four assemblage D (6%), and 33 assemblage E (52%). In sheep, out of 26 samples sequenced, assemblage E was identified in 20 samples (77%), whilst assemblage A was found in five samples (19%), with one mixed assemblage A+D (4%). Gender, age and breed of cattle were not related to the genotypes detected in cattle and sheep. In pigs, 18/30 (60%) samples had positive PCR results and 13 samples were sequenced. In total, one assemblage A (8%), two mixed assemblage A+C (15%), two assemblage C (15%), one mixed

assemblage C+D (8%), three assemblage D (23%), two assemblage E (15%) and two assemblage F (15%), were identified.

In human patients, 66/71 (93%) diarrhoeic samples were PCR positive. Out of 66 samples, 60 (91%) were successfully sequenced. Assemblage A was found in 17 samples (28%) and assemblage B was detected in 43 samples (72%). There was no correlation between gender or history of travelling outside the UK, and the assemblages found. However, a significant linear trend for increased isolation of assemblage A in older subjects ($p=0.0497$) was detected.

Multilocus genotyping was performed using the β-giardin (*bg*) and glutamate dehydrogenase (*gdh*) genes, combined with findings from the ssu rRNA analysis. The results confirmed the presence of the categorised assemblages, and could discriminate at the sub-assemblage level, with some polymorphisms detected. Microsatellite markers were also evaluated in this study, although they failed to amplify clinical isolates.

Proteomics analysis investigated the differential expression of *G. duodenalis* trophozoite proteins between assemblage A and assemblage B, using 2D-SDS PAGE, with differential in gel electrophoresis (DIGE) technology, and mass spectrometry. The results identified nine proteins, with five proteins increasing in assemblage A and four proteins increasing in assemblage B ($p\leq0.001$).

In conclusion, the current project presented the general picture of the molecular epidemiology of *G. duodenalis* in animals and humans in the UK. Further perspectives have been discussed.

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Chapter 1 Introduction

1.1 Biology of *Giardia*

Giardia is a flagellated unicellular parasitic protozoan found in the gastrointestinal tract in both humans and a variety of animals. The parasite, along with other multi-flagellates, is grouped in the Class Zoomastigophora and the Order Diplomonadida (Thompson 2004). This eukaryotic parasite has two forms. The first form, trophozoite, is approximately 15 μm × 8 μm in size and is motile, propelled by its flagella: four pairs in the caudal third of the body. When detected by light microscopy, the pear-shaped parasite appears as a "human smiling face", due to its bi-nuclei (forming "eyes"), the axonemes passing longitudinally between nuclei (forming the "nose") and the median body passing transversely (forming the "mouth") (Figure 1.1). *Giardia* also has a ventral adhesive disk made of microtubules. The trophozoite form mainly dwells in the host intestinal lumen, attaching to the mucosal epithelium (Barr 1998). The second form, cyst, measures 12 μm × 7 μm, and is oval-shaped. The *Giardia* cyst is responsible for transmission to another host, and can survive for several months under wet and cold conditions, although it is usually susceptible to desiccation in hot and dry environments (Barr 1998).

1.2 Life cycle and transmission

Giardia has a direct life cycle that begins when the host ingests contaminated food or water containing infective cysts. The excystation occurs in the proximal small intestine before two binucleated trophozoites are released from each cyst. The trophozoite multiplies asexually by longitudinal binary fission and attaches to the brush border of the host mucosal epithelium of the small intestine via its ventral adhesive disks. The distribution of trophozoites within the intestine varies with host and diet (Barr 1998). For example, in ruminants, *Giardia* trophozoites are found throughout the small intestine, and particularly on the surface of the jejunum after excystation (O'Handley et al. 2001). As multiplication continues, some trophozoites, which encyst, are passed in the faeces. Usually, only the cyst form is passed in the stool, but trophozoites may be detected in severe cases of diarrhoea. Cysts, which are resistant to environmental conditions, are then ready to be transmitted to the new host (Figure 1.1). The prepatent period of Giardiasis varies according to host. For example, in the dog, it ranges from 5–12 days (Barr 1998), while in cats, it ranges from 5–16 days (Kirkpatrick and Farrell 1984). In humans, the prepatent period can be approximately 14 days, but is usually less than 3 weeks (Jokipii and Jokipii 1977).

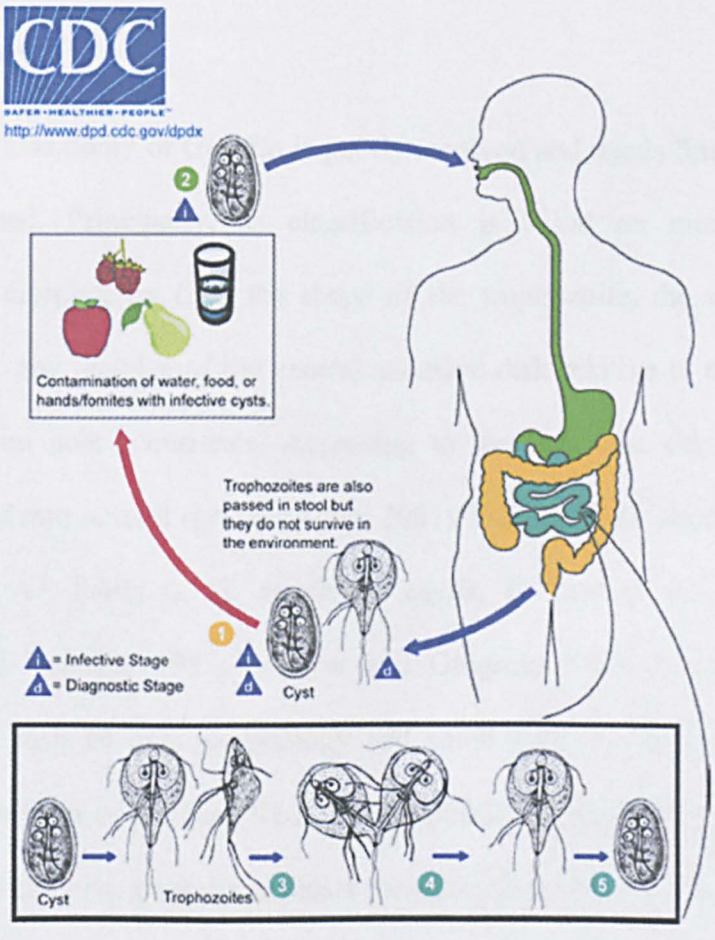


Figure 1.1 The life cycle of *Giardia duodenalis* (1). The cysts are hardy and can survive for several months in cold water. Infection occurs by the ingestion of cysts in contaminated water, food, or by the faecal–oral route (2). In the small intestine, excystation releases trophozoites (each cyst produces two trophozoites) (3). Trophozoites multiply by longitudinal binary fission, remaining in the lumen of the proximal small bowel where they can be free or attached to the mucosa by a ventral sucking disk (4). Encystation occurs when the parasites transit toward the colon. The cyst is the stage found most commonly in non-diarrhoeal faeces (5). (Picture taken from http://www.dpd.cdc.gov/dpdx/HTML/Giardiasis.htm)

1.3 Taxonomy

At present, the taxonomy of *Giardia* is poorly resolved and needs further study to be fully defined. Principally, its classification is based on microscopic or ultrastructural morphology (i.e., the shape of the trophozoite, the shape of the median bodies, and the size of the ventral adhesive disk relative to the length of the cell), and on host occurrence. According to these criteria, *Giardia* can be initially divided into several species (Adam 2001)(Table 1.1), *G. duodenalis* (syn. *G. intestinalis*, *G. lamblia*), *G. muris*, *G. agilis*, *G. ardeae* and *G. psittaci* (Erlandsen and Bemrick 1987; Sogayar and Gregorio 1989; Erlandsen et al. 1990). On the basis of cyst morphology and small subunit rRNA analysis, *G. microti* has also been added (van Keulen et al. 2002). Amongst these species, *G. duodenalis* can colonise a variety of hosts including humans, livestock, domestic pets and other mammals. *G. muris* and *G. microti* are found in rodents, whilst *G. agilis* is found in amphibians. *G. ardeae* and *G. psittaci* are avian parasites (Thompson 2000; Monis and Thompson 2003). Due to its broad host range, including humans, and its supposed zoonotic source, *G. duodenalis* is an interesting pathogen, and the study of its molecular biology and genotype is of notable importance.

Table 1.1 *Giardia* species in various hosts

Species	Hosts	Morphology		Molecular data
		Light microscopy	Electron microscopy	
G. duodenalis (syn. *G. intestinalis*, *G. lamblia*)	Most mammals including humans, livestock, pets	Pear shaped; one or two transverse, claw-shaped median bodies		Clade with multiple genotypes
G. muris	Rodents	Short and rounded; small rounded median body		Distant from *G. duodenalis*
G. microti	Voles and muskrats	Same as *G. duodenalis*	Cysts contain two trophozoites with mature ventral disks	Similar to *G. duodenalis* genotypes
G. psittaci	Psittacine birds	Same as *G. duodenalis*	Incomplete ventrolateral flange, no marginal groove	NA
G. ardeae	Herons	Same as *G. duodenalis*	Ventral disk and caudal flagellum similar to *G. muris*	Closer to *G. duodenalis* than to *G. muris*
G. agilis	Amphilbians	Long and slender; teardrop shaped median body		NA

NA=not available

1.4 Pathogenesis and clinical findings

Giardia is one of several parasites that cause various clinical symptoms in the host, such as diarrhoea, flatulence, greasy stools, stomach cramps and nausea, which can lead to weight loss and dehydration (Barr 1998; Adam 2001). Giardiasis does not produce emesis or fever, but its effects can be either acute or

chronic. Importantly, the effects of *Giardia* infection have also been investigated in immunocompromised individuals (Angarano et al. 1997). A study of Giardiasis in HIV+ patients revealed that Giardiasis was not a major cause of enteritis, but it is often observed amongst AIDS patients, particularly in the most advanced stage of disease (Angarano et al. 1997). In some studies of Giardiasis, the parasite was shown to increase the epithelial permeability that leads to an inflammatory response and both digestive and absorptive changes (Buret et al. 2002; Scott et al. 2002). This increased intestinal permeability may result in the uptake of antigens that produce allergic reactions (Scott et al. 2002).

1.5 Host–pathogen interaction

The host–pathogen interaction between *Giardia* spp. and the infected host has been described by Stevens (1982). Once *Giardia* infects the host, the parasite can cause malabsorption of nutrients through the host epithelium by directly affecting the epithelial surface, secreting toxins and physically altering the epithelium directly. The parasite competes with the host for nutrients and induces inflammation of the intestine. Moreover, the parasite may promote infection by other organisms (Stevens 1982). An *in vitro* study by Gupta et al (1989) demonstrated destruction of polymorphonuclear leukocytes by *Giardia.* With prior treatment with the anti-*Giardia* serum, pathogen agglutination was observed and the cytotoxic capacity of the parasite was reduced (Gupta et al. 1989). Kamda and Singer (2009) found that the parasite actively interfered with the host innate immunity and led to an immune response without a severe inflammatory reaction.

1.6 Diagnosis

The diagnosis of Giardiasis is conventionally based on detecting parasitic cysts or trophozoites in clinical samples, since clinical signs and laboratory tests are not pathognomonic (Barr 1998). The direct faecal smear is an effective preliminary technique, particularly in symptomatic clinical cases in which trophozoites can be detected. A drop of Lugol's iodine can aid diagnosis, helping to visualise *Giardia* morphology, although a negative result cannot rule out a positive diagnosis (Barr 1998). If a direct smear does not provide enough sensitivity, the floatation technique using concentrated zinc sulphate can be performed. Other sensitive diagnostic tools include the commercial enzyme-linked immunosorbent assay (ELISA), detecting *Giardia* antigen (Barr 1998), and the direct immunofluorescent test, using fluorescent-labelled monoclonal antibodies to detect *Giardia* cysts in faeces (Barr 1998). For epidemiological studies, PCR can provide high sensitivity and specificity, but it is not used routinely for diagnosis in the laboratory due to the high cost (Thompson et al. 2008b).

1.7 Treatment and control

Regardless of whether a Giardiasis case is symptomatic or asymptomatic, treatment is recommended because of the possible zoonotic risk (Thompson et al. 2008b). There are a number of drugs that have been use to treat Giardiasis in both humans and animals.

Metronidazole and furazolidone have been used to inhibit *in vitro Giardia* cyst differentiation (Hausen et al. 2006). In cats, metronidazole 25 mg/kg orally, twice a day for 7 days, was used effectively without toxic drug side effects such as neurologic disorders, lethargy, anorexia, vomiting and diarrhoea (Scorza and Lappin 2004). However, when metronidazole was given at high doses in cats, neurotoxicosis was observed (Caylor and Cassimatis 2001). In one study, metronidazole and mebendazole were compared for the treatment of clinical Giardiasis in children (Sadjjadi et al. 2001). Mebendazole (200 mg given three times a day for 5 days), was able to successfully treat 86% (43/50) of patients while metronidazole (given 15 mg/kg/day given in three divided doses for 7 days) was highly efficient, successfully treating 90% (45/50) of patients (Sadjjadi et al. 2001).

Albendazole has been found to be an easy, safe and effective treatment for Giardiasis in humans (Alizadeh et al. 2006). It was originally used as an anthelmintic, but giving 400 mg of albendazole twice a day for 3 days to treat Giardiasis yielded a 70% effective response (Baqai et al. 2001).

In cases of metronidazole-resistant and albendazole-resistant Giardiasis, 1.5 g nitazoxanide twice a day for 30 days was able to successfully treat Giardiasis in patients with AIDS (Abboud et al. 2001). In children, 7.5 mg/kg of nitazoxanide given twice a day for 3 days was used for the treatment of Giardiasis (Escobedo et al. 2008). A single 50 mg/kg dose of tinidazole was also effective against Giardiasis (Escobedo et al. 2008).

In calves, albendazole and fenbendazole have been found to have significant efficacy. Giving fenbendazole at 5 mg/kg per day for 3 days or 0.8 mg/kg per day for 6 days, is efficacious for the treatment of Giardiasis in calves (O'Handley and Olson 2006). Albendazole given at 20 mg/kg per day for 3 days also effectively treats bovine Giardiasis (O'Handley and Olson 2006).

The basic purpose of treatment for Giardiasis is to reduce the excretion of cysts in the faeces. In theory, to control infection of *G. duodenalis*, animal husbandry is vital, and should include cleaning the animal environment, i.e. kennel or cattery. The animal cages should be steam or chemical cleaned using disinfectants. Using drugs to treat animals once they have shown infection, and also cleaning cysts from the animal coats, can prevent reintroduction of the parasite. As the *Giardia* cyst is very susceptible to dry conditions, allowing animal cages to dry for several days before being populated can efficiently reduce the chance of infection (Barr 1998).

A commercial *Giardia* vaccine (GiardiaVax^{TM}, Fort Dodge Animal Health, Overland Park, Kansas, USA) is available for pet companion animals. The vaccine was found to reduce the development of diarrhoea in vaccinated animals compared to a non-vaccinated group, and vaccinated animals retained body weight whereas non-vaccinated animals lost body weight. The duration of cyst shedding and the number of cysts shed were also reduced by the vaccine (Olson et al. 2000). However, a vaccine for Giardiasis is not available for other animals or humans.

1.8 Genotyping of *G. duodenalis*

G. duodenalis is considered as a species complex, and it can be discriminated into seven assemblages by the use of molecular genotyping tools (Meloni et al. 1995; Monis et al. 2003). These tools include isoenzyme electrophoresis (allozyme analysis) and sequencing analysis of target genes at different loci, such as glutamate dehydrogenase (*gdh*) (Read et al. 2004; Bertrand et al. 2005), triose phosphate isomerase (*tpi*) (Amar et al. 2002; Bertrand et al. 2005), small subunit ribosomal RNA (ssu rRNA) (Hopkins et al. 1997; Sulaiman et al. 2003), elongation factor 1-alpha (*ef1-α*) (Traub et al. 2004), and β-giardin (*bg*) (Trout et al. 2003; Lalle et al. 2005a).

By means of isoenzyme electrophoresis, *G. duodenalis* can be phylogenetically divided into assemblages A, B, C, D, E, F and G (Monis et al. 2003). The hosts of assemblage A are humans and other primates, dogs, cats, livestock, rodents and other wild mammals (Thompson and Monis 2004). Assemblage B is isolated from humans and other primates and dogs, whereas assemblages C and D are considered to be specific to dogs. Assemblage E affects cattle and other hoofed livestock, whilst cats are parasitised by host-specific assemblage F. Assemblage G is found in rodents (Thompson 2004) (Table 1.2).

Table 1. 2 Genotype and host range within *Giardia duodenalis*

Genotype/Assemblage	Host range
A	Humans, livestock, cats, dogs, beavers, guinea pigs, slow loris
B	Humans, slow loris, chinchillas, dogs, beavers, rats, siamang
C, D	Dogs
E	Cattle, sheep, pigs
F	Cats
G	Rats

1.9 Molecular epidemiology

During the past decade, a number of studies have investigated the molecular epidemiology of *G. duodenalis* in various hosts using a variety of molecular markers.

For instance, in Central and Southern Australia the *G. duodenalis* genotypes C and D were first described in dogs. These assemblages, which were distinct from previously described assemblages A and B, were identified by allozyme analysis and microscopic morphology (Monis et al. 1998). This was the first study to describe the *G. duodenalis* genotypes in dogs. However, allozyme analysis requires the propagation of trophozoites from axenic cultures or experimentally infected mice, hence it is not standard practice.

In Japan, a study investigated the genotypes of *Giardia* in dogs that were captured in Osaka city or reared in an animal shop in Kanazawa city. By sequencing the

gdh gene and restriction fragment length polymorphisms (RFLPs), assemblage D was identified (Abe et al. 2003). Although the study was performed in only four dogs, it was able to confirm the identification of assemblage D, originally described in the Australian study (Monis et al. 1998).

Later, the same authors identified assemblage A in two asymptomatic patients and assemblage B in a diarrhoeal HIV+ patient using ssu rRNA and *gdh* gene sequencing (Abe et al. 2005). This Japanese investigation was one of the early studies of *G. duodenalis* genotypes in humans, although only a few samples were included. In addition, another study in Japan investigated the *G. duodenalis* genotype in 24 dogs (from households and breeding kennels), three pet cats, five dairy calves, and three wild monkeys. By sequencing the *gdh* gene, the study found assemblages A, C, D or A+D in dogs, F in cats, A or E in calves, and B in monkeys (Itagaki et al. 2005). This was the first demonstration of *G. duodenalis* in calves, cats and wild monkeys, and used larger numbers of samples than previous studies in Japan. The study also reported mixed infection of assemblages A and D, suggesting that dogs were infected with both genotypes.

Another case report in Japan identified assemblage B in a female patient and assemblage E in a calf using *gdh* as a marker (Matsubayashi et al. 2005). The patient had diarrhoea, while the calf was asymptomatic when samples were collected. Although *G. duodenalis* genotypes were identified in these samples, a correlation between the assemblages detected and the presence of clinical signs could not be made because of the low number of samples collected.

An investigation of the molecular epidemiology of *G. duodenalis* was carried out in human patients in Italy, based on sequencing the β-giardin and ssu rRNA genes. In this study, 30 clinical patient samples were genotyped as 24 assemblage A and six assemblage B (Caccio et al. 2002). The study attempted to clarify the diversity of *G. duodenalis* genotypes in patients with diarrhoeal symptoms using a large number of samples. However, by using β-giardin as a marker, diversity at sub-assemblage level was detected and only symptomatic samples were investigated.

Another study in Italy investigated *Giardia* genotyping in multiple animals. Seventeen isolates from dogs, one from a cat, and three from dairy calves, were characterised by ssu rRNA gene sequencing. The results revealed that 76.5% of dog isolates had the dog-specific genotypes (assemblages C, D and the mixed assemblage C+D), whereas 23.5% yielded the zoonotic genotypes (assemblage A and the mixed assemblage A+C). The sample from the cat showed genotype A, and the hoofed-livestock genotype E was detected in all calves. The results implied that the zoonotic potential of these farm animals was not likely to be of significant importance (Berrilli et al. 2004). This research was based on ssu rRNA gene sequencing, which was a conservative approach, and could discriminate *G. duodenalis* at the assemblage level. The study was also the first to investigate the molecular genotypes of *G. duodenalis* in a range of animals in Italy although more samples may be required to confirm these findings. Notably, the study identified the mixed infection of assemblages.

In Mexico, cysts of *G. duodenalis* were collected from children with symptomatic Giardiasis, and from dogs, and were analysed by amplification of the *bg* gene. The study showed that sub-assemblage A1 was found in the majority of human samples, with other samples having sub-assemblage A3. In puppies, both sub-assemblage A1 and A3 were detected, but not the canine-specific genotypes C and D (Lalle et al. 2005a). The study demonstrated the absence of assemblage B in humans, and canine-specific genotypes in dogs. This implies that geographic location may affect the distribution of *G. duodenalis* genotypes.

The same authors also investigated the molecular characteristics of *G. duodenalis* in Italy. A number of faecal samples from humans (n=37) and animals (n=46), including dogs, cat and calves, were analysed by sequencing at the β-giardin locus. Assemblages A and B were both found in humans, whereas calves carried assemblages A, B or E. A single cat sample presented assemblage F, and dog isolates displayed genotype A, C or D. By genotyping at the β-giardin locus, the genetic heterogeneity among human and animal isolates was detected, and sub-assemblages were designated: Assemblage A comprising 8 sub-genotypes, B comprising 6 sub-genotypes, D comprising 2 sub-genotypes, and E comprising 3 sub-genotypes. Five of these sub-genotypes (A1, A2, A3, A4 and B3), were related to the infections of humans, dogs, and calves, which implied the role of these animals as a reservoir for zoonotic infection (Lalle et al. 2005b). This study constructively investigated the molecular characterisation of *G. duodenalis* in a range of hosts. However, the research was performed in Italy so, again,

geographic locality might be an influencing factor. Also, as sub-genotypes were newly designated, more studied samples together with the use of additional markers, may be needed to clarify the diversity of *G. duodenalis* genetic characterisation.

In farm animals, a number of studies have been performed in many countries. In Alberta, Canada, the prevalence and genotyping of *G. duodenalis* was investigated in beef calves (nine farms with 495 faecal samples examined). The prevalence of *G. duodenalis* ranged from 7–60% across the individual farms. By sequencing the ssu rRNA locus, 41 of the 42 isolates were found to have the hoofed livestock genotype E, with one isolate showing assemblage A. The results implied that transmission of *Giardia* genotype E amongst animals is typical in this area, and that zoonotic infection rarely presents (Appelbee et al. 2003). The study demonstrated the prevalence and molecular epidemiology of *G. duodenalis* in beef farms, with genotype E occurring commonly in these areas.

In another study in Canada, the molecular epidemiology of *G. duodenalis* was investigated in healthy, non-lactating, Holstein Friesian cows of the Atlantic Veterinary College (AVC) bovine teaching herd (Uehlinger et al. 2006). Of 14 samples sequenced, assemblage A was identified in 43% (6/14), while assemblage E was identified in 57% (8/14) (Uehlinger et al. 2006). The study, which was performed in 30 animals, showed that there could be a risk of transmission of *G. duodenalis*, as the assemblage with zoonotic potential was detected in up to 43% of cases.

The molecular characterisation of *G. duodenalis* was also investigated in a study in eastern Ontario, Canada (Coklin et al. 2007). A total of 143 samples from healthy calves, heifers and cows were examined, and the prevalence of *G. duodenalis* was 42% across all animals. In total, assemblage E was detected 17.5% (25/143) of animals, whereas assemblage B was detected in 24.5% (35/143) of the samples tested (Coklin et al. 2007).

The first two studies in Canada, described above, detected assemblage A but not B, whereas the latter study detected assemblage B but not A. This suggests that even within the same country, different areas could have a different distribution of *G. duodenalis* molecular epidemiology.

In most studies, assemblage E was found to be a common genotype in farm animals. So far, this assemblage does not seem to have zoonotic potential for human Giardiasis, and there is no evidence of these hoofed hosts playing a role as a reservoir for human transmission (Olson et al. 2004). Nevertheless, studies have not always identified assemblage E in cattle. A number of studies in New Zealand identified only the two zoonotic genotypes, with no detection of assemblage E. In one study, research was carried out in two regions in North Island (Manawatu and Waikato), finding assemblage A in 73% of samples, and assemblage B in 27% of samples (Hunt et al. 2000). A second study, also performed in the Waikato region, identified assemblage A in 55% and assemblage B in 45% of samples (Learmonth et al. 2003). Consistent with these findings, a recent investigation of *G. duodenalis* molecular genotyping in the South Island of New Zealand found only

assemblage A (88%) and assemblage B (12%), while assemblage E was not detected (Winkworth et al. 2008).

The studies in New Zealand suggested that geographic location may be a factor affecting the molecular epidemiology of *G. duodenalis*.

The molecular characterisation of *G. duodenalis* has also been studied in other animals. For instance, *Giardia* isolates from horses in the USA and Australia were genetically characterised. Phylogenetic results placed horse isolate genotypes into assemblages A1, A2 and B4 of *G. duodenalis*. The existence of A1 and A2 isolates in horses implies a high risk of zoonotic transmission to humans (Traub et al. 2005). Moreover, by sequencing ssu rRNA and *ef1-α*, a novel *Giardia* genotype has been isolated from faeces collected from a quenda (*Isoodon obesulus*) – a rat-like mammal from the southwest of Western Australia (Adams et al. 2004).

1.10 Main research objectives

In a recent UK study of the prevalence of gastrointestinal parasites in symptomatic dogs, in which all samples were subjected to commercial laboratory testing, *Giardia* spp was predominant (380/4526 samples) (Batchelor et al. 2008). In another UK study of human patients based on PCR-RFLP of the *tpi* gene, the assemblages of human *G. duodenalis* were discriminated into genotypes A and B, with further sub-genotypes (groups A1 and A2) designated. Of the whole 33

samples sequenced, nine were classified as assemblage A group 2, 21 as assemblage B, and three as a mixture of assemblage A group 2 and assemblage B (Amar et al. 2002).

As shown by the literature review above, *G. duodenalis* is important in both humans and a variety of animals, and further investigation of its molecular epidemiology is necessary. Furthermore, to date, the molecular characterisation of *G. duodenalis* has not been studied in animals in the UK. Consequently, the current Ph.D. study aimed to investigate the epidemiological characterisation of *G. duodenalis* in animals and human patients in the UK, based on a large number of samples. In addition, using different molecular markers, the current study was also performed in human samples with large amount of samples. In summary, the research project encompassed the following elements:

- The conventional ssu rRNA gene was used as a marker for genotyping *G. duodenalis* isolates in dog and cat samples that had been confirmed as *Giardia* positive by light microscopy
- Genotype analysis was carried out in farm animals, such as cattle, sheep and pigs, to investigate the distribution of *G. duodenalis* genotypes. All these samples were from symptomatic animals, but the presence of *G. duodenalis* had not been confirmed
- The investigation was also performed in symptomatic human patients, whose faecal samples were confirmed as *G. duodenalis* positive

- Multilocus genotyping was used to discriminate sub-genotypes of *G. duodenalis*
- Proteomics analysis was used to preliminarily identify the difference in expressed proteins between two genotypes

The current study was carried out with the cooperation of many related organisations. Faecal samples were obtained from different sources, including dog and cat faeces collected by the commercial diagnostic laboratory (NationWide Laboratories), various farm animal faeces collected by the Veterinary Laboratories Agency (VLA), and human faeces collected by the Health Protection Agency (HPA). As the collection of samples was performed through these establishments, the opportunity to collect samples from both symptomatic and asymptomatic cases was limited. For example, only dog, cat and human samples were known to be *G. duodenalis* positive, whilst all farm animals showed clinical signs only, without the confirmation of *Giardia* infection.

Chapter 2 Molecular epidemiology of *G. duodenalis* in domestic dogs and cats

2.1 Introduction

Parasitic infection is a worldwide problem in pet dogs and cats. These animals are affected by a variety of parasites including arthropods, helminths and protozoa, all of which have an important impact on animal health status (Thompson 2004). The gastrointestinal tract is a common site of parasitic infestation and many studies have surveyed the impact of the gastrointestinal parasites in dogs and cats (Thompson 2004; Claerebout et al. 2009). Some of these parasites have become important to public health, given their potentially zoonotic capability. For instance, the roundworm of dogs, *Toxocara canis*, can cause not only emaciation in dogs but also visceral larval migrans in young children (Martinez-Barbabosa et al. 2008). Further, the canine hookworm, *Ancylostoma* spp., that causes diarrhoea and anaemia in dogs, can induce cutaneous larval migrans in humans (Hendrix et al. 1996; Malgor et al. 1996). *Cryptosporidium* spp., an enteric protozoan parasite that infects a wide range of vertebrates including humans, can also be potentially zoonotic, particularly *Cryptosporidium canis* (Fayer et al. 2001; Xiao et al. 2004). The prevalence of these parasites varies widely depending on the age of the host and living conditions (Oliveira-Sequeira et al. 2002; Ponce-Macotela et al. 2005; Miro et al. 2007; Claerebout et al. 2009).

G. duodenalis is one of the most prevalent protozoa in dogs, and infection occurs via ingestion of food or water contaminated with cysts. After ingestion, two trophozoites are released from each cyst, which then multiply and attach to the wall of the small intestine. Reproduction and invasion of the villous epithelium of the small intestine leads to diarrhoea and malabsorption. It is likely that puppies are infected more frequently than adult dogs (Batchelor et al. 2008; Claerebout et al. 2009). The main clinical sign seen with *Giardia* infection is diarrhoea, although most infections are asymptomatic (Thompson 2004). Acute diarrhoea tends to occur in very young puppies shortly after infection whilst, in older dogs, diarrhoea may be acute and short lived, intermittent or chronic. Faeces are often pale, malodorous and steatorrheic due to malabsorption. Other clinical signs can include anorexia, loss of weight, and fatigue. The most definitive method for diagnosis is detection of *Giardia* cysts or trophozoites in faeces or samples taken from the intestinal tract, since clinical signs are not specific (Barr 1998; Itoh et al. 2005; Claerebout et al. 2009). An ELISA has also been developed to diagnose *Giardia* infection, as the procedure can produce 100% accurate results, and may be performed by a single technician in a short period (Marshall et al. 1997; Itoh et al. 2006).

In many studies of the prevalence of gastrointestinal parasitic infections in dogs, *Giardia* was one of the most common protozoan parasites found. For example, in Australia, *Giardia* was detected more frequently (22.1%) than other helminth parasites, and most often parasitised puppies less than 6 months of age, dogs

living in households with more than one dog, and dogs from refuges (Bugg et al. 1999). In Brazil, a study investigated the prevalence of *Giardia* and *Cryptosporidium* in dogs living in different conditions (Mundim et al. 2007). Faecal samples were collected from 89 stray dogs living in shelters, 199 dogs from commercial kennels and 122 household dogs. *Giardia* was found in 119 of 410 faecal samples (29%), while *Cryptosporidium* spp. was detected in 6 of 433 samples (1.4%). *Giardia* was most frequently found in kennel dogs (49.7%), while *Cryptosporidium* was more commonly detected in dogs from shelters (2.2%). *Giardia* was also identified more often in young animals (<1 year old), than in adult dogs (≥1 year old) (Mundim et al. 2007). In Belgium, 1159 faecal samples were collected from 451 household dogs, 357 kennel dogs and 351 dogs with gastrointestinal disorders. In household dogs, *Giardia* was the most prevalent parasite (9.3%), followed by *Toxocara canis* (4.4%). A high proportion of kennel dogs were infected with *Giardia* (43.9%), followed by *T. canis* (26.3%) and *Cystoisospora* (syn. *Isospora*) spp. (8.8%); *Giardia* spp. was the most common parasite in dogs with diarrhoea (18.1%), followed by *Cystoisospora* (8.8%) and *T. canis* (7.4%). Age prevalence studies showed that puppies were the most infected dog age group (Claerebout et al. 2009). In Italy, a recent study of *Giardia* infection in kennel dogs found an overall prevalence of 20.5% in canine faecal samples, with the highest prevalence associated with the largest kennel density, younger animals, and diarrhoeic dogs (Scaramozzino et al. 2009). These surveys demonstrate that *G. duodenalis* is a very common parasite in companion pet animals and has a high prevalence in young animals.

In the UK, a large study examined the prevalence of endoparasites in the faeces of dogs with signs of gastrointestinal disease. Samples were submitted to a commercial diagnostic laboratory for bacteriological and parasitological examination over a two-year period (between December 8th 2003 and December 7th 2005). *Giardia* was isolated from 380/4526 symptomatic dogs (8.4%), *Isospora canis* from 232/4526 dogs (5.1%), *T. canis* from 63/4526 dogs (1.4%) and *Cryptosporidium* from 29/4526 dogs (0.6%). As with many other studies, the prevalence in dogs <1 year of age was significantly greater than the prevalence in dogs ≥1 year of age for each parasite (Batchelor et al. 2008). This investigation provides an example of the prevalence of *G. duodenalis* in clinical diarrhoeal dogs in the UK.

As is clear from the multitude of studies described above, the reported prevalence of *Giardia* infection in dogs is affected by many factors, including age, living conditions, animal density, nutritional and immune status, and the methods used to diagnose the infection. However, the significance of such findings, in terms of both animal and public health, are difficult to gauge in light of the fact that many different assemblages are known to exist within this species complex (see below). Therefore, molecular investigation of *Giardia* is essential in order to provide a proper understanding (Scaramozzino et al. 2009).

As described in the previous chapter, *Giardia duodenalis* is considered to be a species complex, and assemblages C and D are mainly found in dogs (Berrilli et al. 2004; Barutzki et al. 2007; Souza et al. 2007). However, assemblages A and B,

which have been considered as the zoonotic genotypes, have also been identified in dogs in many studies (Read et al. 2002; Berrilli et al. 2004; Itagaki et al. 2005; Inpankaew et al. 2007; Leonhard et al. 2007). The identification of these genotypes in dogs appears to vary in different studies and geographic areas. For example, two studies in Brazil detected only assemblage A (Volotao et al. 2007), or assemblage A and assemblage B (Traub et al. 2004), with no detection of the canine genotypes. Given the fact that some studies have shown that dogs can be potential reservoirs harbouring the zoonotic *Giardia* genotypes (Volotao et al. 2007; Thompson et al. 2008b; Scaramozzino et al. 2009), it is tremendously important and valuable to investigate the molecular genotyping of this organism.

In cats, the prevalence of *Giardia* has been reported in a number of studies. In the Netherlands, faecal and hair samples were collected from healthy household cats in Dutch veterinary practices. The samples were analysed by microscopy, ELISA and PCR, and *Giardia* was identified in 14% of the (60) samples (Overgaauw et al. 2009). In Australia, a national study reported that *Giardia* prevalence in cats was 2% (n=1,063) (Palmer et al. 2008a). Another study in Australia revealed that, of 21 *Giardia* positive samples from cats, eight were successfully sequenced at the β-giardin locus: seven were assemblage F, and one was assemblage D, which is uncommon (Palmer et al. 2008b). In Japan, faecal samples were collected from 600 household cats and examined for the *Giardia* antigen using a commercial ELISA kit (Itoh et al. 2006). The *G. duodenalis* antigen was detected in 40% of the faecal specimens (Itoh et al. 2006). In addition, a prevalence study of parasitic

infections was conducted in 263 faecal samples from cats in central New York State in which *Campylobacter* (0.8%), *Salmonella* (0.8%), *Toxocara cati* (33.0%) and *Giardia* (7.3%) were identified (Spain et al. 2001).

According to molecular epidemiology, cats are considered to harbour assemblage F, the feline-specific genotype, and also the wide-range host assemblages A and B (Read et al. 2004; Souza et al. 2007). Although assemblage F was generally identified in cats in most studies (Itagaki et al. 2005; Fayer et al. 2006; Santin et al. 2006; Souza et al. 2007), assemblage A was reported as a single genotype detected in one study in Italy (Papini et al. 2007). Therefore, cats may also be of importance to public health as a source of the zoonotic genotype of *Giardia* infection.

The aim of the research in this chapter is to determine the molecular genotypes of *G. duodenalis* in symptomatic domestic dogs and cats in the UK using ssu rRNA genotyping analysis. All faecal samples used were collected by veterinary surgeons to aid in the diagnosis of gastrointestinal disease, and were submitted to a commercial diagnostic laboratory in the UK. It must be highlighted that all samples were confirmed as positive for *Giardia* (by identifying cysts or trophozoites by light microscopy).

2.2 Materials and methods

2.2.1 Preparation of positive control of genomic Giardia DNA

Trophozoites from the *Giardia* isolate WB C6 were utilised for preparing positive reference genomic DNA of *G. duodenalis*. To extract DNA, the GenomicPrep Cell and Tissue DNA Isolation Kit® (Amersham Pharmacia Biotech) was used according to the guidelines of the manufacturer. To disrupt the *Giardia* cell wall, a 4×10^8 trophozoite pellet was added to 600 µl cell lysis solution and incubated at 37 °C for 1 hr. RNA was removed by adding 3 µl RNase A solution, followed by incubation at 37 °C for 1 hr. To precipitate protein, 200 µl protein precipitation solution was added, before centrifugation at 1300 ×*g* for 3 min to tighten the protein pellet. For DNA precipitation, the supernatant containing the DNA was transferred into a new 1.5 ml microcentrifuge tube containing 600 µl of 100% isopropanol, and the protein pellet was discarded. The sample was then mixed, by inverting gently 50 times, until the white thread of DNA formed a visible clump. The sample was then centrifuged, at 1300 ×*g* for 1 min, and DNA was packed as a small white pellet at the bottom of the tube. The supernatant was poured away, and the tube was drained on clean absorbent paper. To wash the DNA pellet, 600 µl of 70% ethanol was added to the tube, which was inverted several times and centrifuged at 1300 ×*g* for 1 min. The ethanol supernatant was then removed without dislodging the DNA pellet. The tube was again drained on clean absorbent paper and allowed to air-dry for 30 min. Finally, DNA was rehydrated

by adding 100 µl DNA hydration solution and left overnight at room temperature. The extracted DNA was analysed by agarose gel electrophoresis at 100V and 120 mA for 45 min and identified with ultraviolet light after staining with 1% ethidium bromide.

2.2.2 Origins of dog and cat samples

Diarrhoeic faecal dog and cat samples were collected from all parts of the UK by veterinary surgeons, and submitted to a commercial diagnostic laboratory (NationWide Laboratories, Poulton-le-Fylde, United Kingdom) between October 2006 and March 2008. This laboratory was accredited for diagnostic faecal analysis (reference ISO17025, United Kingdom Accreditation Service UKAS, http://www.ukas.org). Samples were examined under the light microscope for inspecting *Giardia* cysts or trophozoites. For each dog and cat, computerised laboratory records were checked for age, breed, gender and date of sampling (Appendix 1 and 2). Having been microscopically confirmed, a total of 252 *Giardia*-positive samples from dogs and 60 samples from cats were transported, by courier and on ice, to the Department of Veterinary Preclinical Sciences, Faculty of Veterinary Sciences, University of Liverpool. All samples were stored at 4°C before further molecular analysis.

2.2.3 DNA isolation

Giardia genomic DNA was isolated from faecal samples using the QIAamp® DNA Stool Mini Kit (QIAGEN, UK), according to the manufacturer's instructions, with only minor modification. Approximately 180–220 g of each faecal sample was placed into a 2 ml microcentrifuge tube, and 1.4 ml of stool lysis buffer (buffer ASL) was added. The tube was vortexed continuously for 1 min until the faecal sample was thoroughly homogenised, and the suspension was then heated for 10 min at 70 °C. After vortexing for 15 seconds, the sample was centrifuged at 1300 ×*g* for 1 min to pellet faecal particles. Next, 1.2 ml of supernatant was pipetted into a new 2 ml microtube and the pellet was discarded. A tablet of InhibitEX® was added to the sample to adsorb DNA-degrading substances and PCR inhibitors, before vortexing immediately until the tablet was completely suspended. The sample was then incubated for 1 min at room temperature to allow inhibitors to adsorb to the InhibitEX® matrix, before centrifuging at 1300 ×*g* for 3 min to pellet the inhibitor bound to the InhibitEX® matrix. Subsequently, 1.2 ml of supernatant was pipetted into a new 1.5 ml microcentrifuge tube, and the pellet was removed, before centrifugation at 1300 ×*g* for another 3 min. To eliminate proteins completely, 15 µl proteinase K, 200 µl supernatant from the previous step, and 200 µl Buffer AL were added to a new 1.5 ml microcentrifuge tube, vortexed briefly and incubated at 70 °C for 10 min. To precipitate DNA, 200 µl of absolute ethanol (96–100%) was added to the sample followed by vortexing briefly to mix all components. The complete lysate was

then applied to the QIAamp® spin column, without moistening the rim, before being centrifuged at 1300 ×*g* for 1 min. The spin column was then placed into a new 2 ml collection tube, and the tube containing the filtrate was discarded. To wash the DNA bound to the silica membrane of spin column, 500 µl buffer AW1 (washing buffer) was added and the spin column was centrifuged at 1300 ×*g* for 1 min, before being placed into a new 2 ml collection tube, and discarding the filtrate collection tube. The final wash was carried out by adding 500 µl washing buffer AW2 (washing buffer) to the column, followed by centrifugation at full speed for 3 min. To completely remove the residual washing buffer AW2, the spin column was centrifuged for another 1 min with a new 2 ml collection tube. To elute DNA, the spin column was transferred to a new, labelled 1.5 ml microcentrifuge tube and then 100 µl Buffer AE (elution buffer) was pipetted directly onto the QIAamp® membrane. The spin column was incubated at room temperature for 1 min before being centrifuged at full speed for 1 min. The eluted genomic DNA was stored at -20 °C.

2.2.4 Polymerase chain reaction (PCR) amplification

The 292-293 bp fragment of ssu (18S) rRNA gene was amplified using the forward primer RH11 (5′- CAT CCG GTC GAT CCT GCC -3′) and the reverse primer RH4 (5′- AGT CGA ACC CTG ATT CTC CGC CAG G -3′) (Hopkins et al. 1997). The PCR mix consisted of 1× buffer containing 18 mM Tris-HCL, 4.4 mM $(NH_4)_2SO_4$, 1.8 mM $MgCl_2$, 452 µg BSA, 1.76 µM EDTA, 400 µM of each

dNTP, 10 pmol of each primer, 2.5 units of *Taq* DNA polymerase (QIAGEN, UK), and 1–5 µl of purified DNA in a final volume of 20 µl. The reactions were performed as follows: after an initial denaturation step of 2 min at 96°C, a set of 35 cycles was run, each consisting of 20 sec at 96°C, 20 sec at 59° and 30 sec at 72°C, followed by a final extension of 7 min at 72°C in a DNA Engine Dyad® Peltier Thermal Cycler. To determine the amplified fragments, 5 µl of PCR products were electrophoresed in a 1% agarose gel containing ethidium bromide at 100V, 120 mA for 45 min, and then examined in an ultraviolet light imaging chamber. The PCR positive samples were purified using QIAquick® PCR Purification Kit (QIAGEN, UK) according to the manufacturer's instruction.

Purified PCR products were sent to either the DNA Sequencing Core service, Molecular Biology Support Unit, Cardiff University, Cardiff, UK, or to GeneService, Nottingham, UK; each product was sequenced in both directions using either primer RH11 or RH4. Computer software was used to interpret (freeware BioEdit) and align (Clustal W) the DNA sequences (Thompson et al. 1994). The reference assemblages used for sequence alignment were obtained from GenBank (accession number AF199446 for assemblage A, AF199447 for assemblage B, AF199449 for assemblage C, AF199443 for assemblage D, AF199448 for assemblage E and AF199444 for assemblage F).

2.2.5 Statistical analyses

Statistical analyses were performed with a computer software programme Stats Direct, version 2.6.2 (Stats Direct Ltd. Altrincham, UK). Continuous data were first assessed for normality with a Shapiro-Wilk test. Parametric tests were used when data were normally distributed, and included logistic regression. Non-parametric statistical tests were used when data were not found to be of normal distribution, and included Fisher's exact test and the Kruskal-Wallis test. The level of statistical significance was set at $p<0.05$.

2.3 Results

2.3.1 Preparation of genomic Giardia DNA

Genomic DNA from *G. duodenalis* trophozoites was successfully extracted (Figure 2.1), and no gradation or sheering of the DNA was seen. The DNA was stored in a -20°C freezer, and subsequently used as a positive DNA control for all experiments.

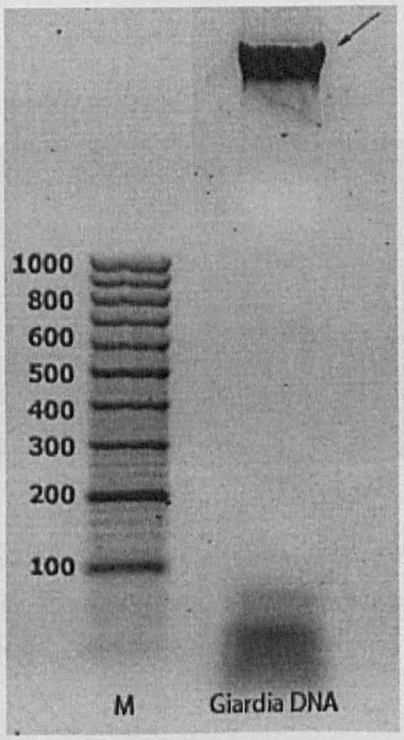


Figure 2.1 *Giardia duodenalis* genomic DNA (arrow) extracted from *Giardia* trophozoites visualised on 1% agarose gel stained with ethidium bromide. M lane is the marker (bp).

2.3.2 Sequence analysis of ssu rRNA gene

2.3.2.1 Sequence analysis of *ssu r*RNA gene in domestic dogs

All of the 252 canine faecal samples that had been identified as positive for *Giardia* by light microscopy were used for extraction of DNA. Of these, 200 samples were successfully amplified by PCR at the ssu rRNA locus, while only 126 samples were selected and successfully sequenced (Figure 2.2). The multiple alignments of some sequences using the software BioEdit and ClustalW are shown in Figure 2.3.

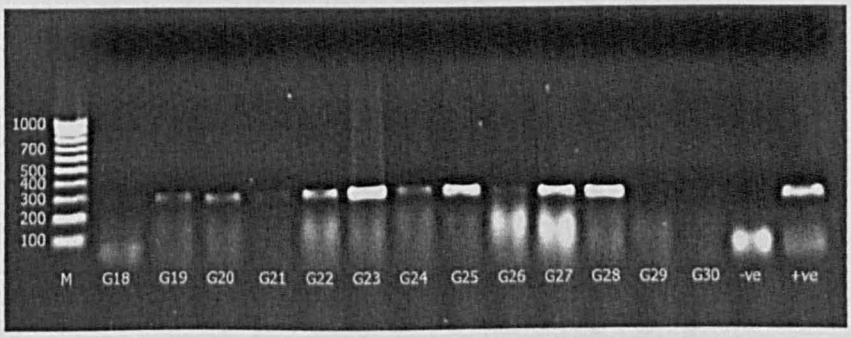


Figure 2.2 Electrophoresis results of some canine samples after PCR amplification of ssu rRNA gene. The molecular weight marker (M), in base pairs (bp), is shown on the left. Negative (-ve) and positive control (+ve) are on the right.

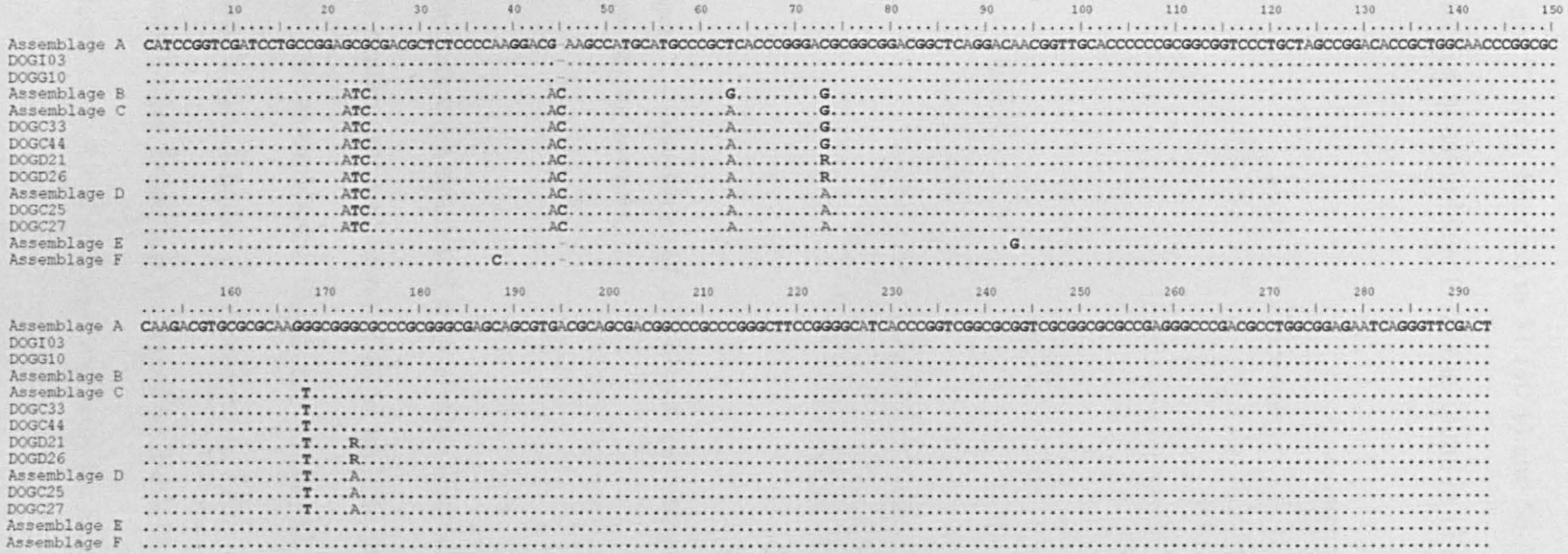


Figure 2.3 Multiple alignments of the sequences from some dog samples using the software BioEdit and ClustalW. Samples numbered DOGI03 and DOGG10 are assemblage A while samples numbered DOGC33 and DOGC44 are assemblage C. Samples numbered DOGC25 and DOGC27 are assemblage D. Samples numbered DOGD21 and DOGD26 display a mix of assemblage C and D. Note that R in the alignment indicates strong peaks of both G and A.

Assemblage A was detected in two (2%) samples, while dog-specific assemblages C and D were detected in 51 (40%) and 56 (44%) samples, respectively. Mixed infection of assemblage C+D was found in 17 samples (14%) (Figure 2.4).

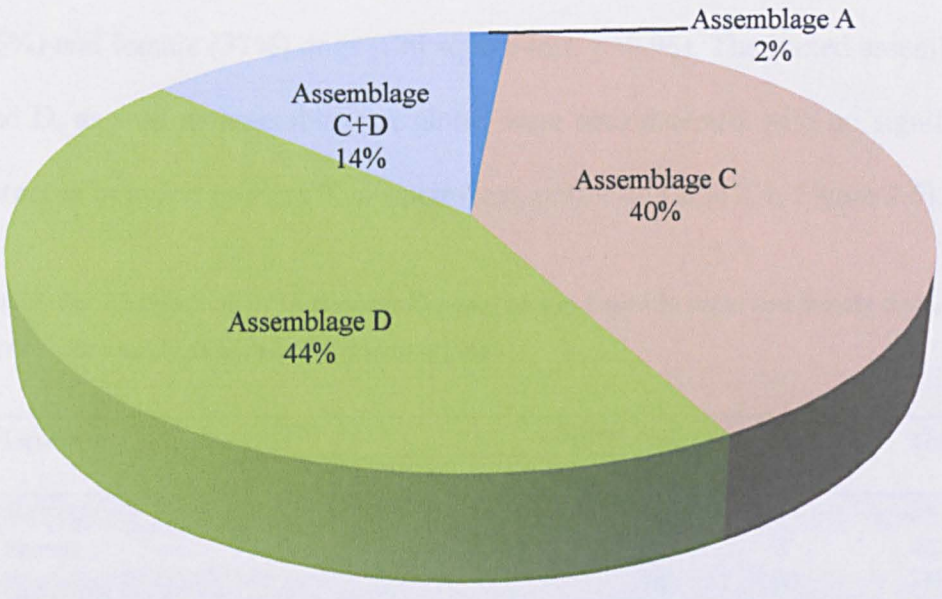


Figure 2. 4 Distribution of *G. duodenalis* assemblages by sequence analysis of ssu rRNA gene from 126 dog samples.

The data were examined to determine whether there was any correlation between the gender, age and breed of dogs, and the genotypes identified. Data on gender and the assemblages found are displayed in Table 2.1. Data on age and assemblages detected are displayed in Table 2.2. Data on the breed of dogs and assemblages found are displayed in Table 2.3. Not all data on gender, age and breeds of dogs and cats were available for interpretation.

Regarding dog gender, although the frequency of isolation of assemblage C in female dogs (43.5%) was slightly higher than in male dogs (40%), the differences were not significant (Chi square test, p=0.52). Similarly, there was no significant difference between the frequency of detection of assemblage D between male (44.6%) and female (37%) dogs (Chi square test, p=0.96). The mixed assemblage C and D, as well as assemblage A alone, were also detected with no significant differences between genders (Chi square test, p=0.99) (Table 2.1, Figure 2.5).

Table 2.1 The distribution of *G. duodenalis* assemblages found in male and female dogs, using sequence analysis of ssu rRNA genotyping

Gender	Assemblage				Total
	A	C	D	C+D	
Male	1	26	29	9	65
Female	1	20	17	8	46
Unknown	0	5	10	0	15
Total	2	51	56	17	126

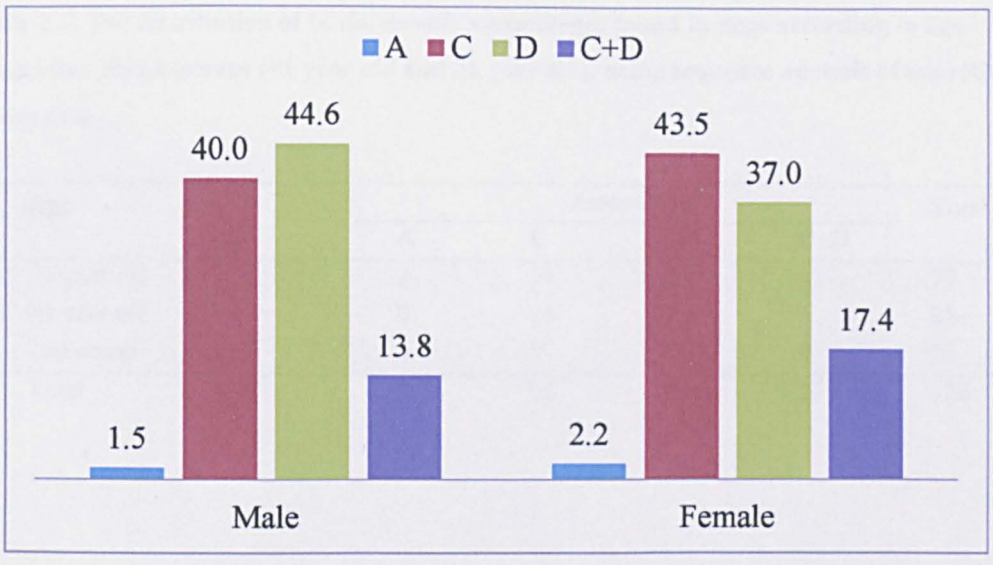


Figure 2. 5 The percentage of *G. duodenalis* assemblages found in male (n=65) and female (n=46) dogs by sequence analysis of ssu rRNA genotyping. All data were from symptomatic dogs excluding unknown.

Regarding age, assemblage D was found as often in dogs under one year of age as in dogs over one year (42.9% in both), and there was also no significant difference (Fisher's exact test, p=0.82) in the frequency of isolation of assemblage C between the age groups (dogs ≥1 year old, 46.4%; dogs <1 year old, 40.3%), and this was also the case for the mixed assemblage C+D. Assemblage A was not identified in dogs ≥1 year old, but was detected occasionally (2.6%) in younger dogs; however, there was no statistical difference detected (Fisher's exact test, p=0.54) (Table 2.2, Figure 2.6).

Table 2. 2 The distribution of *G. duodenalis* assemblages found in dogs according to age categorised into 2 groups (<1 year old and ≥1 year old), using sequence analysis of ssu rRNA genotyping

Age	Assemblage				Total
	A	C	D	C+D	
<1 year old	2	31	33	11	77
≥1 year old	0	13	12	3	28
Unknown	0	7	11	3	21
Total	2	51	56	17	126

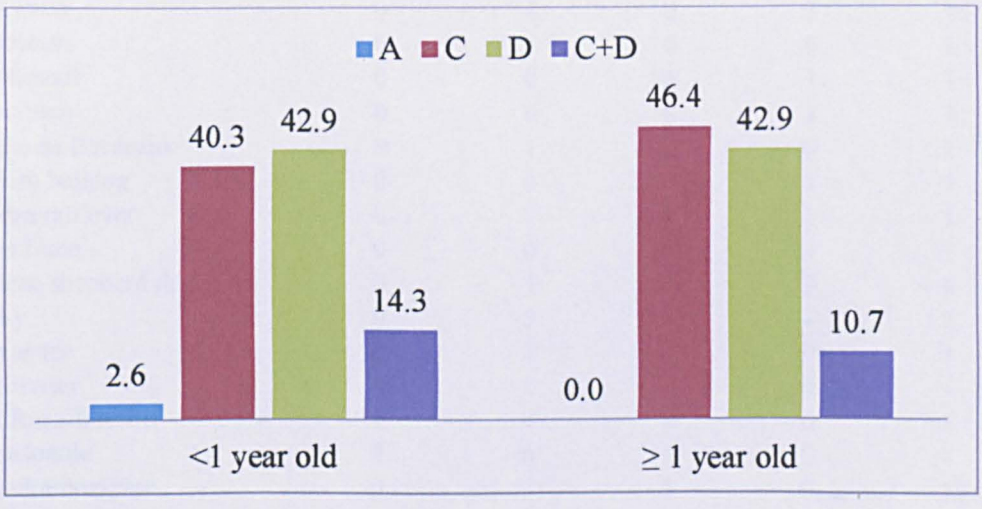


Figure 2. 6 The percentage of *G. duodenalis* assemblages found in each age group of dogs (<1 year old, n=77; ≥1 year old, n=28) by sequence analysis of ssu rRNA genotyping. All data were from symptomatic dogs excluding unknowns.

As the number of each breed of dogs was limited, statistical analysis between breeds was not calculated. Assemblages found in each breed are shown in Table 2.3.

Table 2. 3 The distribution of *G. duodenalis* assemblages found in different breeds of dog using sequence analysis of ssu rRNA genotyping

Breed	Assemblage				Total
	A	C	D	C+D	
Afghan hound	0	0	1	0	1
Beagle	0	2	3	1	6
Bichon Frise	0	1	0	0	1
Border collie	0	7	4	0	11
Border terrier	0	0	1	0	1
Boxer	0	0	1	0	1
Briard	0	0	1	0	1
Bull mastiff	0	1	0	0	1
Chihuahua	0	1	0	0	1
Cavalier King Charles spaniel	0	3	4	1	8
Cockapoo	0	1	0	0	1
Cocker spaniel	0	5	0	1	6
Crossbred	0	4	9	3	16
Dalmatian	0	1	0	0	1
Deerhound	0	0	0	1	1
Doberman	0	0	0	1	1
Dogue de Bordeaux	0	1	0	0	1
English bulldog	0	0	0	1	1
Golden retriever	0	0	1	0	1
Great Dane	0	0	0	1	1
German shepherd dog	0	1	3	2	6
Husky	0	0	1	1	2
Irish setter	0	1	0	0	1
Irish terrier	0	0	1	0	1
Jack Russell terrier	1	0	2	0	3
Labradoodle	0	0	1	0	1
Labrador retriever	0	7	5	0	12
Lakeland terrier	0	1	0	0	1
Lurcher	0	1	1	0	2
Mastiff	0	1	0	0	1
Rottweiler	0	1	0	0	1
Samoyed	0	0	1	0	1
Staffordshire bull terrier	0	0	1	1	2
Shih Tzu	0	1	0	0	1
Springer spaniel	1	1	5	1	8
Weimaraner	0	1	1	0	2
West Highland white terrier	0	3	3	2	8
Yorkshire terrier	0	1	1	0	2
Unknown	0	4	5	0	9
Total	2	51	56	17	126

2.3.2.2 Sequence analysis of ssu rRNA gene in domestic cats

All of the 60 faecal cat samples that had been identified as positive for *Giardia* by light microscopy were used for extraction of DNA. Of these, 58 samples were successfully amplified by PCR at the ssu rRNA locus, but only 39 samples were selected and sequenced. The assemblages found in cats were: assemblage A in four samples (10%), assemblage F in 32 samples (82%), the mixed infection of assemblage F+C in two samples (5%), and the mixed infection of assemblage F+D in one sample (3%) (Figure 2.7).

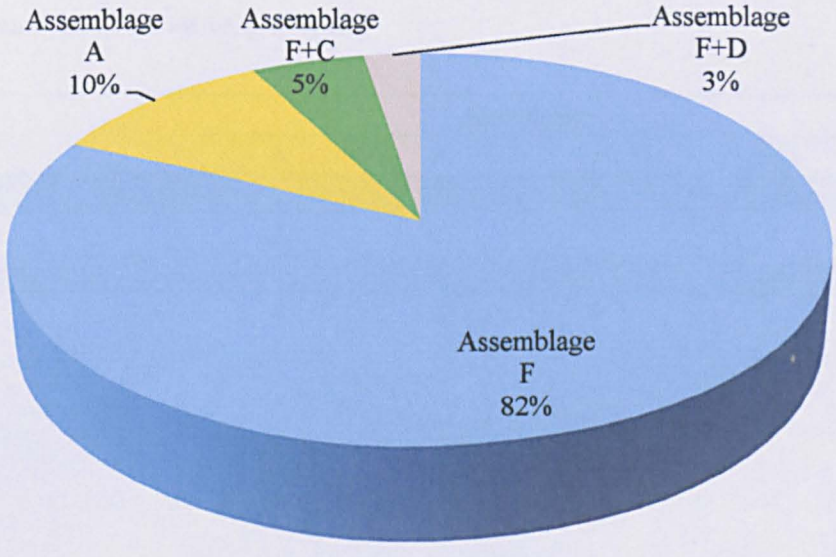


Figure 2.7 Distribution of *G. duodenalis* assemblages by sequence analysis of ssu rRNA gene from 39 cat samples.

There was no significant difference in the frequency of isolation of assemblage F between male (80%) and female (71%) cats (Fisher's exact test, p=0.49). Further, the frequency of isolation of assemblage A was found more frequently in female (14%) than in male (13%) cats, but it was not significantly different (Fisher's exact test, p=0.49). Interestingly, the dog-specific genotypes were identified in feline samples in this study. For example, the mixed assemblage F+D was detected in male (7%) cats, while the mixed assemblage F and C was identified in female cats (14%) (Table 2.4, Figure 2.8).

Table 2.4 The distribution of *G. duodenalis* assemblages found in male and female cats by sequence analysis of ssu rRNA genotyping

Gender	Assemblage				Total
	A	F	F+C	F+D	
Male	2	12	0	1	15
Female	2	10	2	0	14
Unknown	0	10	0	0	10
	4	32	2	1	39

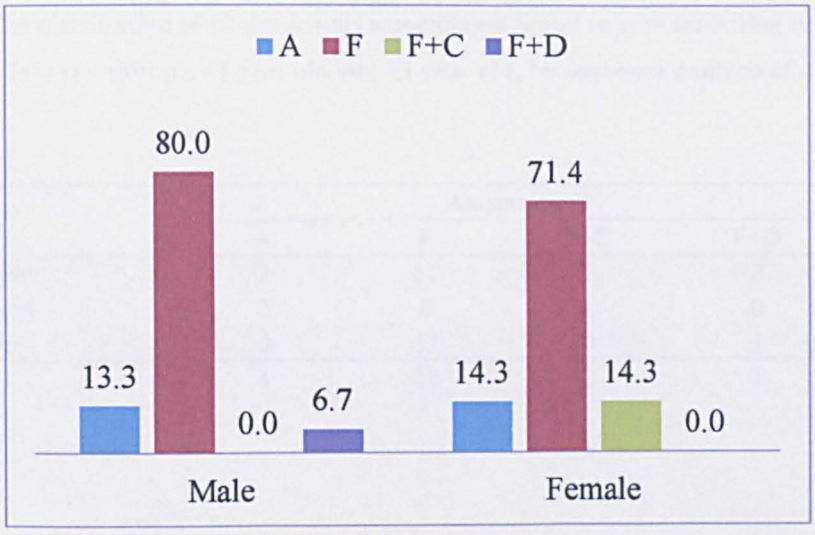


Figure 2.8 The percentage of *G. duodenalis* assemblages found in male (n=15) and female cats (n=14) by sequence analysis of ssu rRNA genotyping. Data were from symptomatic cats excluding unknowns.

The percentage of *G. duodenalis* assemblages found in two age groups of cats, <1 year old and ≥1 year old, were assessed. The feline genotype F was found in both groups, with no significant differences (Chi square test, p=0.94): 78.6% in <1 year old cats and 72.7% in ≥1 year old cats. Assemblage A occurred more frequently in ≥1 year old cats (18.2%) than <1 year old cats (14.3%), but the difference was not significant. The mixed assemblage F and C was detected in 7.1% of <1 year old cats and 9.1% of ≥1 year old cats, with no significant differences between groups (Chi square test, p=0.94) (Table 2.5, Figure 2.9).

Table 2.5 The distribution of *G. duodenalis* assemblages found in cats according to age, categorised into two groups, <1 year old and ≥1 year old, by sequence analysis of ssu rRNA genotyping

Age	Assemblage				Total
	A	F	F+C	F+D	
<1 year old	2	11	1	0	14
≥1 year old	2	8	1	0	11
Unknown	0	13	0	1	14
	4	32	2	1	39

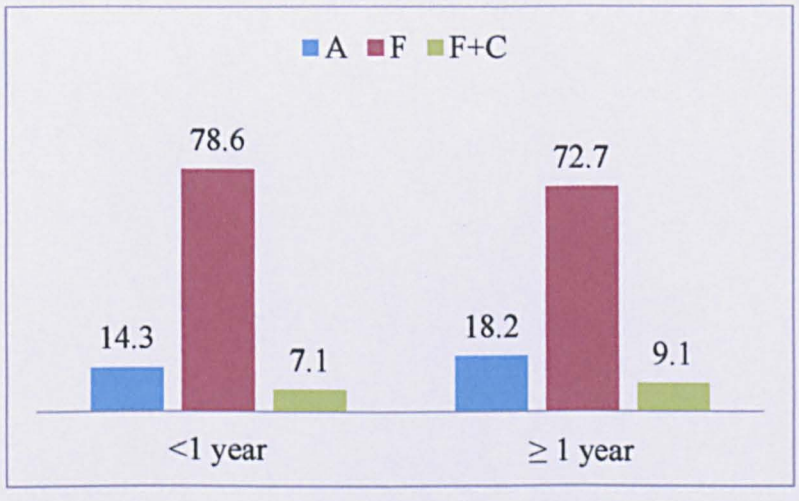


Figure 2.9 The percentage of *G. duodenalis* assemblages found in two different age groups of cats, <1 year old (n=14) and ≥1 year old (n=11), by sequence analysis of ssu rRNA genotyping. Data were from symptomatic cats excluding unknowns.

Due to the limited number of samples, cat breeds were grouped into pedigree vs. non-pedigree for statistical analysis. There was no significant effect of breed on the presence of any of the *Giardia* assemblages (Fisher's exact test, p=0.15) (Table 2.6).

Table 2. 6 The distribution of *G. duodenalis* assemblages found in different breeds of cats by sequence analysis of ssu rRNA genotyping

Breed	Assemblage				Total
	A	F	F+C	F+D	
Bengal	2	4	0	0	6
British blue	0	1	0	0	1
Domestic Longhair	0	1	0	0	1
Domestic Shorthair	1	23	1	1	26
Persian	0	1	0	0	1
Siamese	0	0	1	0	1
Unknown	1	2	0	0	3
	4	32	2	1	39

2.4 Discussion

In the current study, all faecal samples were from dogs and cats with clinical signs of diarrhoea, collected at the Nationwide Laboratories, before being examined for gastrointestinal parasites under the light microscope. Samples with *Giardia* cysts were marked as positive, then genotyped at the Faculty of Veterinary Science, University of Liverpool. All samples underwent PCR amplification at the ssu rRNA locus. In dogs, although all 252 samples were tested, only 200 samples (79.4%) exhibited positive results. There are several possible explanations as to why some "positive" *Giardia* samples failed to be amplified by PCR. These include an inadequate amount of faeces, the distribution of *Giardia* cysts in faeces, the quantity of *Giardia* cysts in faeces, and the degradation of *Giardia* DNA in faeces.

At the beginning of the present research, all faecal samples from dogs, cats and farm animals arrived at approximately the same time. Therefore, it was necessary to store all samples in the freezer, as the DNA extraction from this large number of samples could not be completed in a short period. Later, *Giardia* DNA was extracted from many of these frozen samples and PCR was performed, but these samples yielded poor results, giving a negative PCR outcome, compared to the fresh samples or those stored in the fridge. The degradation of *Giardia* cyst DNA at very low temperatures could be one reason why the amount of DNA obtained was not sufficient in some samples. From this point onwards, all samples were stored in the fridge before undergoing extraction of the parasite genomic DNA.

In the 126 dog samples sequenced, assemblage D was the predominant genotype in this study (44%). This corresponded to the findings of other studies reporting assemblage D in dogs at up to 55% in Germany (Barutzki et al. 2007), 60% in Hungary (Szénási et al. 2007), and 74% in Brazil (Souza et al. 2007). Although the occurrence of genotype D found in this study was lower than in other studies, it was higher than the other genotypes in the present study. Assemblage C, another dog-specific genotype, was also common, being identified in 51 samples (40%) in the current investigation. These results are consistent with many other studies reporting that assemblage C and assemblage D are the most common genotypes in dogs (Monis and Andrews 1998; Sulaiman et al. 2003; Berrilli et al. 2004; Barutzki et al. 2007). Assemblage A, which has a broad range of hosts and is considered to be a high potential zoonotic genotype (Monis et al. 2003; Thompson 2004), was found in two samples (2%) in the present study. This may suggest that dogs play a less important role as a carrier for human Giardiasis in the UK.

In the current report, the mixed infection of assemblage C+D was identified in 17 dog samples (14%) and this was not surprising since many studies had demonstrated mixed infections in dogs, including assemblage A+C (Berrilli et al. 2004; Leonhard et al. 2007), C+D (Berrilli et al. 2004; Barutzki et al. 2007; Szénási et al. 2007), A+D (Itagaki et al. 2005; Lalle et al. 2005b; Barutzki et al. 2007; Inpankaew et al. 2007), and A+B (Lalle et al. 2005a; Inpankaew et al. 2007). These results may vary considerably, depending on geographic locality,

experimental design, and the nature of specimens collected. The current study implies that dogs can be infected with different assemblages. Also, infection of assemblage A in dogs may be from humans or from dogs, although further investigation is needed in this aspect.

Of the 60 cat faecal samples included in the current study, 58 were successfully amplified by PCR (97%) and, of these, 39 samples were successfully genotyped. In line with many other studies, assemblage F was detected in most samples (82%), while assemblage A with zoonotic potential, was identified in four samples (10%). In Brazil, Souza et al. (2007) investigated the molecular identification of *G. duodenalis* in 18 cats and detected both assemblage F (58%) and A (42. In addition, studies of the molecular prevalence of *G. duodenalis* in Japan (Itagaki et al. 2005), Colombia (Santin et al. 2006), and the USA (Fayer et al. 2006), found 100% infection with assemblage F in the studied cats (n=3, 3 and 8, respectively). However, these studies were small in terms of sample size compared with the current study. In contrast to the observed predominance of assemblage F described above, another study in Italy found that all *G. duodenalis* isolates from ten stray and owned cats were classified as assemblage A by sequencing ssu rRNA (Papini et al. 2007). These studies and the current research suggest that assemblage F is common in cats. Further to this, the current work demonstrates that the zoonotic genotype of *G. duodenalis* is also present in cats in the UK.

The current study also detected mixed infection of assemblage F and the canine-specific assemblages C and D in cats. The mixed infection of assemblage F+C was found in two samples (5%) and assemblage F+D in one sample (3%). These results are interesting because they support other studies that report the identification of dog assemblages in cats. For example, in Australia, Read et al (2004) identified assemblages A (6/18), B (2/18), C (2/18), D (7/18) and E (1/18) in cats using glutamate dehydrogenase as a marker. It was noted that assemblage F was not detected in the study. Furthermore, another study in Australia reported one assemblage D, and seven assemblage F in cats (Palmer et al. 2008b). These reports and the current study suggest that cats can be infected with the dog-specific genotypes of *G. duodenalis*. However, how the cats are infected is unknown.

The overall prevalence of *Giardia* infection in domestic dogs and cats was not determined in the current study, since only diarrhoeic samples found to be *Giardia* positive by microscopic examination, were used. However, the results demonstrate that *G. duodenalis* is one of the intestinal parasites that is likely to be associated with the cause of diarrhoea in dogs and cats. It should be noted that no healthy dogs and cats were analysed in the present study, and that samples were not examined for other parasites.

Assemblage A was detected in the current research, and these pets were consequently considered as reservoirs for zoonotic genotypes of *G. duodenalis*. However, the molecular characterisation of *G. duodenalis* in healthy dogs and cats

might provide a different view. For this reason, more work is needed to investigate the role that pet animals play in zoonotic infections, particularly in terms of the transmission dynamics of *Giardia* and the level of transmission between pets and humans. The relationship between susceptible humans and pets should be focussed upon in order to prevent and control the infection that can occur in both pets and owners.

Chapter 3 Molecular epidemiology of *G. duodenalis* in farm animals

3.1 Introduction

In farm animals, infection with *Giardia duodenalis* can cause gastrointestinal disorders, which is particularly problematic in young livestock (Ruest et al. 1997). *Giardia* infection is of public health significance because of the possibility of zoonotic transmission (O'Handley et al. 2000). Several studies have reported *G. duodenalis* colonisation of farm animal species, including cattle, sheep, goats and pigs (Fayer et al. 2000; O'Handley et al. 2000; Bomfim et al. 2005; Santin et al. 2007; Trout et al. 2007; Trout et al. 2008; Armson et al. 2009). In cattle, *G. duodenalis* has been identified as the aetiological agent in cases of diarrhoea, either alone or in combination with other enteric pathogens (O'Handley et al. 1999; Huetink et al. 2001). For example, concurrent infection of *Giardia* spp. and *Cryptosporidium* spp. was found to be a primary cause of diarrhoea in calves <30 days of age, and *Giardia* spp. infection alone was associated with diarrhoea in calves >30 days of age (O'Handley et al. 1999). *G. duodenalis* generally infected calves aged between 5 and 10 weeks, but could also affect calves as young as 4 days (Xiao and Herd 1994). Peak shedding of *G. duodenalis* cysts occurred between the ages of 2 and 5 weeks, but could remain at a high level until 7 weeks of age (Xiao and Herd 1994). The cumulative infection rate was 73–100% in some herds of calves studied (Xiao and Herd 1994; Uehlinger et al. 2006).

The prevalence of *G. duodenalis* infection in cattle varies between studies. A survey of *G. duodenalis* infection in 14 farms in the USA reported a prevalence varying from 9–93% (average 40%) in 407 pre-weaned dairy calves (Trout et al. 2004), 20–80% (average 52%) in 456 post-weaned calves (Trout et al. 2005), 11–75% (average 36%) in heifers (Trout et al. 2006), and 3–64% (average 27%) in adult dairy cows (Trout et al. 2007). A small study in Maryland USA assessed healthy, asymptomatic, post-weaned cattle and mature cattle, and found prevalences of 17% (4/23) and 10% (2/19), respectively (Fayer et al. 2000).

A longitudinal study was performed in Western Australia to determine the spread of naturally acquired *G. duodenalis* in dairy cattle. Faecal samples were collected weekly from birth to weaning. It was found that calves rapidly acquired infections from 4–7 weeks of age, and *G. duodenalis* was detected in 89% of sampled calves (Becher et al. 2004). A study by O'Handley et al. (2000) investigated Holstein calves located on a commercial dairy near Lethbridge, Alta, Canada, and on two commercial dairies near Perth, Western Australia. The prevalence of *G. duodenalis* infection at these two different locations was similar, 58% (21/36) and 57% (16/28), respectively. Finally, a study of *G. duodenalis* in 379 healthy adult cattle aged between 3 and 13 years from 60 dairy farms in Galicia (NW Spain), revealed a prevalence of 26.6% (Castro-Hermida et al. 2007). Thus, although the prevalence of *G. duodenalis* infection varies noticeably between localities, it is likely that the infection rate is higher in young livestock, particularly in pre-weaned and post-weaned calves, than in heifers and adult cattle.

According to molecular genotypes, *G. duodenalis* assemblage E is the most commonly reported assemblage in cattle (Appelbee et al. 2003; Becher et al. 2004; Trout et al. 2007; Geurden et al. 2008b), although the zoonotic assemblages A and B have also been reported in many studies (Lalle et al. 2005b; Trout et al. 2005; Trout et al. 2006; Uehlinger et al. 2006; Coklin et al. 2007; Mendonca et al. 2007). The detection of assemblages A and B in cattle has particular public health significance, since these genotypes are considered to have zoonotic potential.

A number of studies have reported the prevalence of *G. duodenalis* infection in sheep (Giangaspero et al. 2005; Castro-Hermida et al. 2007; Santin et al. 2007; Yang et al. 2009). For instance, in a Spanish study of *G. duodenalis* prevalence in 38 herds of healthy sheep, 86 of 446 faecal samples (19.2%) tested positive (Castro-Hermida et al. 2007). Faecal samples from 325 native sheep from 20 farms in the Abruzzo region of Italy have also been examined, and *G. duodenalis* cysts were detected in five animals (1.5%) from two farms, with a mean isolation rate of 450 cysts/g (Giangaspero et al. 2005). As with cattle, assemblage E, the 'hoofed livestock' genotype, is the most commonly reported *G. duodenalis* genotype isolated in sheep (Castro-Hermida et al. 2007; Santin et al. 2007; Yang et al. 2009), although the zoonotic genotype assemblages A (Giangaspero et al. 2005; Ryan et al. 2005) and B (Castro-Hermida et al. 2007) have also been isolated.

There are a limited number of studies investigating *G. duodenalis* infection in pigs. The affected animals are usually asymptomatic, and the parasites are mainly

localised on the surface of intestinal crypts (Koudela et al. 1991). A Danish study demonstrated that the prevalence of *G. duodenalis* infection varies amongst different age groups, with 84% of weaned pigs, 22% of nursing piglets and 18% of sows affected (Maddox-Hyttel et al. 2006). As with other farm animal species, molecular characterisation of *G. duodenalis* genotypes in pigs has identified the livestock-specific assemblage E more frequently than other assemblages (Langkjaer et al. 2007; Armson et al. 2009).

Giardiasis has also been studied in several other farm animals. In goats, *G. duodenalis* infection is usually asymptomatic, and its prevalence has been investigated infrequently. From the work conducted, young animals appear to be more commonly affected than adults, and clinical signs are similar to those described above, usually comprising diarrhoea and weight loss (Ruiz et al. 2008). In a Spanish study, the prevalence of *G. duodenalis* was reported to be 42% in young goat kids, and all isolates displayed the 'hoofed livestock' assemblage E (Ruiz et al. 2008). In Belgium, prevalence of *G. duodenalis* has been studied in both lambs and goat kids, and the overall isolation rates were 26% and 36%, respectively. Again, assemblage E was a major genotype found in both species, although the zoonotic assemblage A was also identified (Geurden et al. 2008c). Furthermore, *G. duodenalis* was found in unweaned llamas and alpacas with diarrhoea (Cebra et al. 2003), and molecular characterisation of isolates from three alpacas in Maryland, USA, revealed the presence of assemblage A (Trout et al. 2008).

The current study was performed in collaboration with the Veterinary Laboratories Agency (VLA), Preston, UK, which provided faecal specimens from all parts of the UK. The aim of this chapter is to investigate the molecular characterisation of *G. duodenalis* isolates from farm animals, either with clinical signs of infection, or taken *post mortem* from carcasses after slaughter. As such, this represents the first large scale investigation of *G. duodenalis* genotypes in the UK, and highlights the potential public health significance of this problem.

3.2 Materials and methods

3.2.1 Origin of faecal samples

Between June 2007 and June 2008, 384 faecal samples were collected from farm animals, including cattle (285), sheep (69) and pigs (30). Samples were collected by veterinarians in several parts of the UK, taken directly from the animal rectum, from the carcass, or as a pooled faecal sample from the animals' environment. After collection, samples were submitted to the Veterinary Laboratories Agency (VLA), Barton Hall, Garstang Road, Barton, Preston, Lancashire, PR3 5HE. Most animals showed clinical signs such as diarrhoea or emaciation. Each animal was recorded for gender, age and breed (Appendix 3). Faecal samples were placed in individual plastic, screw cap specimen containers, stored in an insulated box and transported in iced parcels to the Department of Veterinary Preclinical Sciences, Faculty of Veterinary Science, University of Liverpool, Liverpool, L69 7ZJ. Upon arrival, samples were stored at 4°C before being analysed.

3.2.2 Detection of Giardia duodenalis by microscopic examination

Microscopic examinations were performed with the intention of confirming the presence of *Giardia* cysts, and as such were not intended to be statistically analysed. A selection of cattle faecal samples were randomly picked, and the presence of *G. duodenalis* cysts was determined by a conventional floatation method. Saturated saline solution (20 ml) was added to approximately 5 g of faeces in a clean plastic container, and the mixture was strained through three layers of gauze into a new plastic container. A 15 ml tube was then filled to the brim with the filtrate, a coverslip was placed on top, and *G. duodenalis* cysts were allowed to float and stick to the coverslip for 15–20 min. Thereafter, the coverslip was transferred to a glass slide and examined using light microscopy at 40x magnification.

3.2.3 DNA isolation and Polymerase chain reaction (PCR) amplification

All 401 samples underwent genomic *G. duodenalis* DNA extraction using the QIAamp® DNA Stool Mini Kit (QIAGEN, UK). The procedures were identical to those previously described in Chapter 2. All samples were analysed by PCR, amplifying the small subunit rRNA gene using forward primer RH11 and reverse primer RH4. The PCR conditions were established as previously described in Chapter 2. Amplicons were purified using QIAquick® PCR Purification Kit (QIAGEN, UK), according to the manufacturer's instructions, before submission to the DNA Sequencing Core service, Cardiff University, Cardiff, or GeneService,

Nottingham. Sequencing was undertaken in both directions using either primer RH11 or RH4. The DNA sequences were interpreted (BioEdit, freeware) and aligned (Clustal W) with computer software (Thompson et al. 1994). The reference assemblages used for sequence alignment were obtained from GenBank (accession number AF199446 for assemblage A, AF199447 for assemblage B, AF199449 for assemblage C, AF199443 for assemblage D, AF199448 for assemblage E and AF199444 for assemblage F).

3.2.4 Statistical analyses

Statistical analyses were performed with Stats Direct software, version 2.6.2 (Stats Direct Ltd. Altrincham, UK). The prevalence of *G. duodenalis* in different age groups of cattle was tested with a Chi-square test for trend. The level of statistical significance was set at $p<0.05$.

3.3 Results

3.3.1 Demonstration of G. duodenalis cysts

A representative selection of samples was tested with conventional flotation procedures to confirm the presence of *G. duodenalis* cysts. Two samples were confirmed to be positive (Figure 3.1). This result was not statistically analysed.

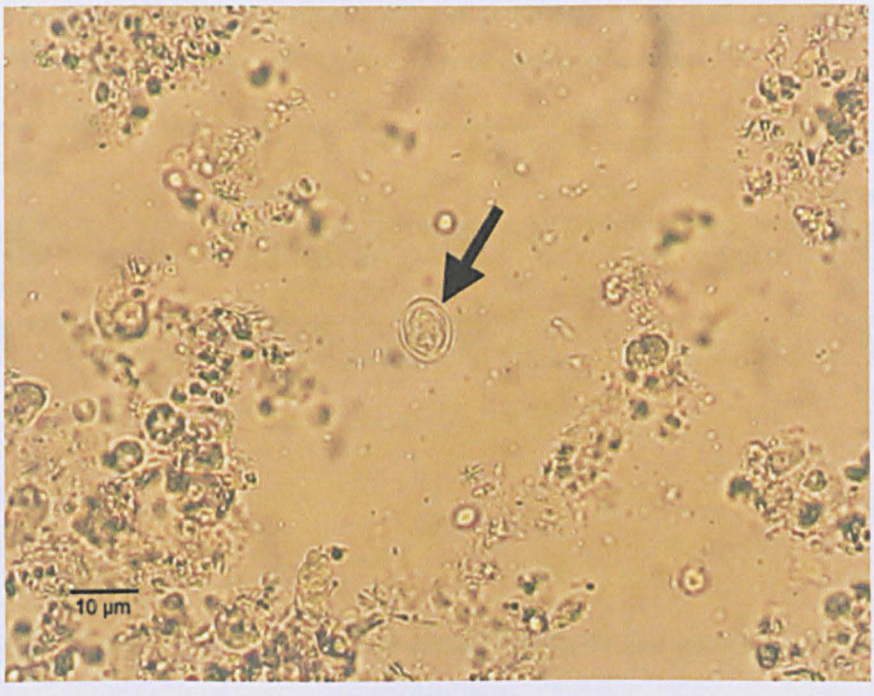


Figure 3.1 *G. duodenalis* cysts identified from two separate bovine faecal samples in the present study (arrow). The cyst was obtained by a faecal floatation technique, as described in the methods section, and examined using light microscopy.

3.3.2 PCR amplification of ssu rRNA gene

Of the 384 faecal samples tested, 141 revealed PCR positive results: 93/285 (33%) from cattle, 30/69 (44%) from sheep and 18/30 (60%) from pigs. Of the 141 PCR positive samples, 102 were selected and successfully sequenced (Table 3.1).

Table 3.1 Number of samples on which PCR was performed, number of positive results, and number of successfully sequenced samples for each species of animal

Animals	No. of PCR tests performed	No. of PCR positive samples	No. of samples sequenced
Cattle	285	93	63
Sheep	69	30	26
Pigs	30	18	13
	384	141	102

Table 3.2 The distribution of *G. duodenalis* assemblages in studied species of animals

Animals	Assemblage									Total
	A	A+C	A+D	A+E	C	C+D	D	E	F	
Cattle	16	1	1	6	2	0	4	33	0	63
Sheep	5	0	1	0	0	0	0	20	0	26
Pigs	1	2	0	0	2	1	3	2	2	13
	22	3	2	6	4	1	7	55	2	102

3.3.2.1 Sequence analysis in cattle

Of the 93 PCR positive results for cattle, 63 samples were selected and successfully sequenced (Table 3.1). The molecular characterisation of *G. duodenalis* consisted of 16 assemblage A (25%), one mixed assemblage A+C (2%), one mixed assemblage A+D (2%), six mixed assemblage A+E (10%), two assemblage C (3%), four assemblage D (6%), and 33 assemblage E (52%) (Table 3.2).

Cattle were categorised into four age groups: neonatal (0–2 weeks), pre-weaned (2–6 weeks), post-weaned (6 weeks–1 year) and adult (>1 year). By PCR amplifying ssu rRNA, *G. duodenalis* was detected in 9/31 (29%) neonatal calves, 28/60 (47%) pre-weaned calves, 8/25 (32%) post-weaned calves, and 31/139 (22%) adult cattle (Figure 3.2). A significant linear trend for decreasing prevalence with increasing age was identified in this study (Chi-square test for trend, p=0.014). *G. duodenalis* was identified in 17/30 (57%) cattle of unknown age, which were not included in the statistical analysis.

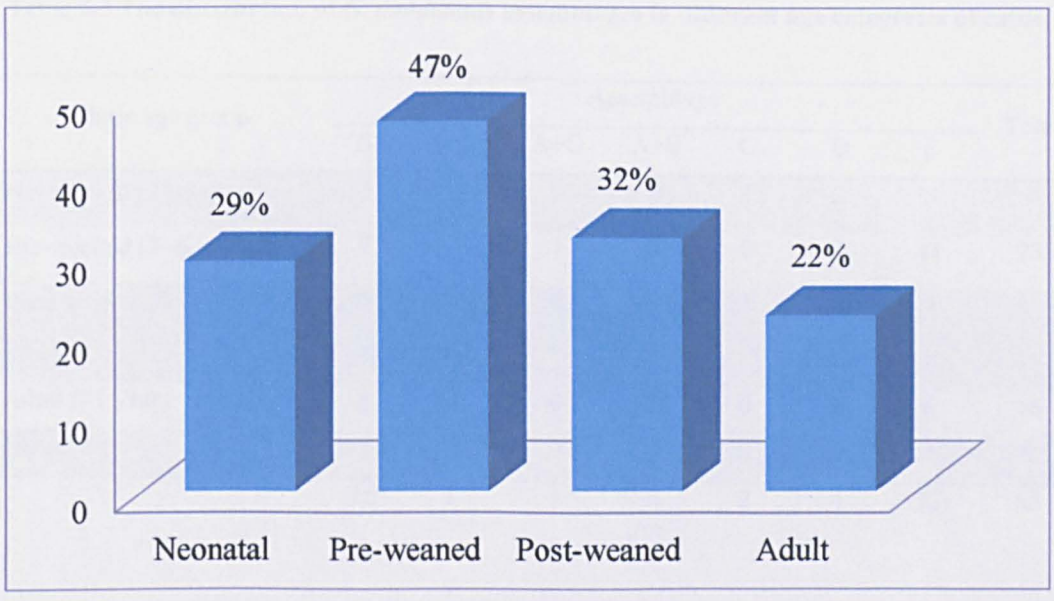


Figure 3.2 Percentage of *G. duodenalis* PCR positive samples in different age groups of cattle: neonatal (0–2 weeks), pre-weaned (2–6 weeks), post-weaned (6 weeks–1 year), and adult (>1 year).

The distribution of *G. duodenalis* assemblages in different age groups of cattle is presented in Table 3.3. Assemblage E was the most frequently identified assemblage in all age groups. Moreover, mixed infections were found in pre-weaned, post-weaned and adult animals. The zoonotic assemblage A was present in all age groups.

Table 3.3 The distribution of *G. duodenalis* assemblages in different age categories of cattle

Cattle age group	Assemblage							Total
	A	A+C	A+D	A+E	C	D	E	
Neonatal (0–2 weeks)	3	0	0	0	1	0	5	9
Pre-weaned (2–6 weeks)	7	1	1	2	0	1	11	23
Post-weaned (6 weeks–1 year)	0	0	0	1	1	0	4	6
Adult (>1 year)	5	0	0	3	0	2	6	16
Unknown	1	0	0	0	0	1	7	9
	16	1	1	6	2	4	33	63

3.3.2.2 Sequence analysis in sheep

Of 30 PCR positive results in sheep, 26 samples were selected and successfully sequenced (Table 3.1). Assemblage E was detected in 20 samples (77%), whereas the zoonotic assemblage A was only found in 5 samples (19%). The mixed assemblage A+D was detected in one sample (4%) (Table 3.2).

Sheep were divided into four age groups: neonatal (0–7 days), pre-weaned (1 week–3 months), post-weaned (3 months–1 year) and adult (>1 year). PCR analysis in each group revealed 2/3 (67%) positive results in neonatal animals, 12/26 (46%) in pre-weaned, 5/13 (38%) in post-weaned, and 8/23 (35%) in adult sheep (Figure 3.3). *G. duodenalis* was detected in 3/4 sheep of unknown age. There was no significant linear trend for *Giardia* detection and age of sheep (Chi-square test for trend, p=0.263).

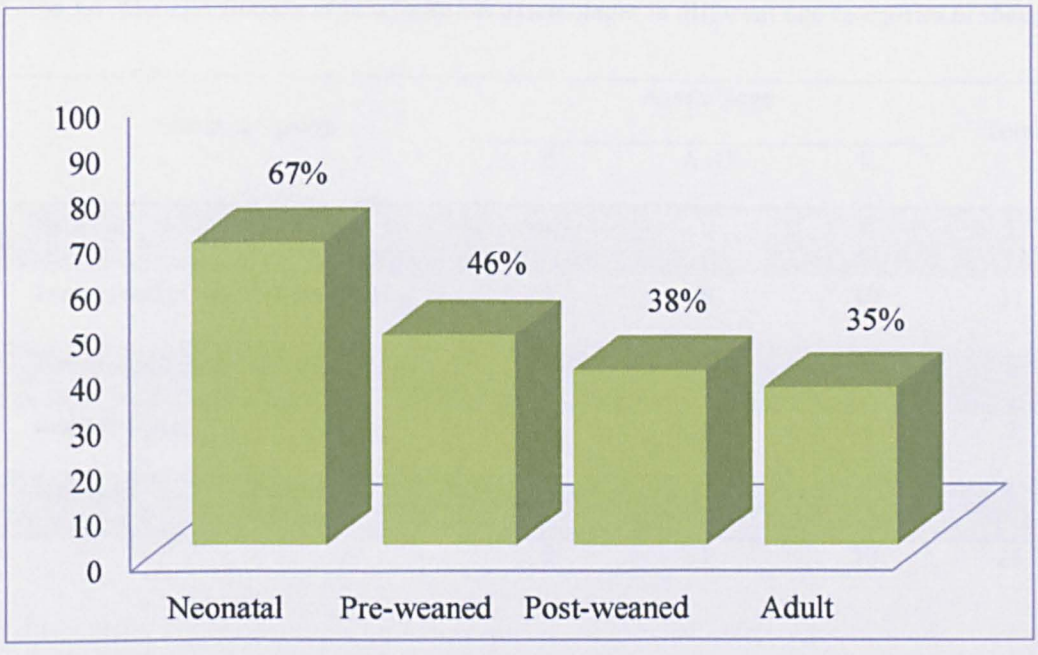


Figure 3.3 Percentage of *G. duodenalis* PCR positive samples in different age groups of sheep: neonatal (0–7 days), pre-weaned (1 week–3 months), post-weaned (3 months–1 year), and adults (>1 year).

The distribution of *G. duodenalis* assemblages was investigated in the different age groups of sheep. As with cattle, assemblage E was the most frequently identified assemblage in all groups (except for neonatal, sample size 1). Interestingly, one mixed infection of assemblage A and D was found in one post-weaned sheep (Table 3.4).

Table 3.4 The distribution of *G. duodenalis* assemblages in different age categories of sheep

Sheep age group	Assemblage			Total
	A	A+D	E	
Neonatal (0–7 days)	1	0	0	1
Pre-weaned (1 week–3 months)	1	0	10	11
Post-weaned (3–12 moths)	0	1	5	6
Adult (>1 year)	3	0	4	7
Unknown	0	0	1	1
	5	1	20	26

3.3.2.3 Sequence analysis in pigs

In pigs, 18/30 (60%) samples were positive for *G. duodenalis*. Of the 18 PCR positive results, 13 samples were selected and successfully sequenced (Table 3.1). Of these 13 animals, one assemblage A (8%), two mixed assemblage A+C (15%), two assemblage C (15%), one mixed assemblage C+D (8%), three assemblage D (23%), two assemblage E (15%), and two assemblage F (15%) were identified (Table 3.2). Interestingly, the dog-specific (C, D) and cat-specific (F) genotypes were identified in pigs. Unlike with cattle and sheep, pigs were not categorised by age due to insufficient data, and statistical analysis was not performed.

3.4 Discussion

The current study is the largest UK study ever conducted to determine the prevalence and molecular genotype of *G. duodenalis* in farm animals. However, it should be noted that this study did not consider healthy animals. All samples were collected from clinical or symptomatic cases that had not previously been examined for the presence of *G. duodenalis*. Overall, the PCR analysis yielded 144 positive results from 401 UK samples submitted to the VLA. The results support those in other studies and confirm that the prevalence of *G. duodenalis* infection in farm animals is considerable; for example, the prevalence of *G. duodenalis* in cattle was 33%. However, the true prevalence may be over-estimated since samples were only collected from symptomatic animals. Nonetheless, results are similar to those previously reported, for example, in Canada, the overall prevalence of *G. duodenalis* infection in calves, heifers and adult cattle was 42% in 143 faecal samples investigated (Coklin et al. 2007). Among four age groups of cattle in the current work, the pre-weaned (47%) and post-weaned calves (32%) had higher prevalence than in adults (22%). This result is in accordance with the following studies which suggest that young calves and heifers are the groups with the highest prevalences. A study in the Netherlands revealed that shedding of *G. duodenalis* cysts was found in all age groups, but peaked in calves aged 4–5 months (54.5%) (Huetink et al. 2001). The mean age at which *G. duodenalis* cysts were first detected was 31.5 ± 4.1 days (O'Handley et al. 1999). A longitudinal study in Maryland, USA, of *G. duodenalis* genotypes in

dairy cows from birth to 2 years of age found the highest prevalence (61%) in pre-weaned calves (<8 weeks old). The prevalence in post-weaned calves (3–12 months old) and heifers (12–24 months old) was 32% and 11% respectively (Santin et al. 2009).

In the present study, assemblage E was the most frequently identified *G. duodenalis* genotype in cattle, (33/63 samples; 52%). Mixed assemblage A+E was identified in 6/63 samples (10%). The zoonotic assemblage A was identified in 16 samples (25%), and was also present mixed with other assemblages (A+C 2%, A+D 2%, and A+E 10%). The presence of assemblage E, considered as the 'hoofed livestock' genotype, has been demonstrated in numerous previous studies. In Western Canada (Alta.), 8/10 calves were infected with assemblage E and 2/10 with assemblage A, while in Western Australia (Perth), 4/5 calves were parasitised with *G. duodenalis* assemblage E, and 1/5 with assemblage A (O'Handley et al. 2000). In Alberta, Canada, an overall prevalence of 34% was identified in beef calves, with assemblage E detected in 41/42 (98%) and assemblage A in 1/42 (2%) (Appelbee et al. 2003). In Japan, *G. duodenalis* genotypes were identified in 5 dairy calves, and consisted of 4/5 assemblage E and 1/5 assemblage A (Itagaki et al. 2005). In Belgium, a molecular epidemiological study was conducted on dairy and beef farms to estimate the prevalence of different *G. duodenalis* assemblages in calves younger than 10 weeks of age; assemblage E was identified in 54/101, assemblage A in 16/101 and the mixed assemblage A+E in 31/101 samples (Geurden et al. 2008a). In addition,

two cattle samples from three farms in Georgia, USA, were characterised as the mixed infection assemblage A+E and, out of 58 samples sequenced, 8 assemblage A and 48 assemblage E were identified (Feng et al. 2008). In Brazil, five samples from naturally-infected cattle were analysed for *G. duodenalis* molecular characterisation; assemblage E was detected in four samples and assemblage A in one sample (Souza et al. 2007). The current research used clinical samples to confirm that assemblage E was predominant in cattle in the UK.

In the current study, assemblage B was not detected in cattle samples. Assemblage B is mainly found in humans but can be found in other mammals (Thompson 2000). In contrast, a study in Canada identified that the infection of assemblage B (35/60) in cattle was higher than assemblage E (25/60) (Coklin et al. 2007), and in the Waikato Region of New Zealand, assemblage A was detected in 26 faecal samples from dairy cattle and assemblage B in 22 samples (n=48), while assemblage E was not found (Learmonth et al. 2003). Again in New Zealand, the molecular characterisation of *G. duodenalis* isolates from 40 calves was investigated and no assemblage E was detected, but assemblage A (88%) and assemblage B (12%) were reported (Winkworth et al. 2008). In Italy, molecular characterisation was performed in 24 cattle. Analysing at the beta-giardin locus, assemblage A (n=12) and assemblage B (n=5) were frequently found whereas the host-specific assemblage E was rarely detected (n=3). The mixed infections of assemblage A+B (n=2) and A+E (n=2) were also reported (Lalle et al. 2005b). Given that assemblage B was not found in the current work, it appears that the

distribution of *G. duodenalis* genotypes might be affected by geographical location.

In the present study, the canine specific assemblages C and D were detected in two (3%) and four cattle samples (6%), respectively. This finding is interesting given that it is the first reported incidence of the canine-specific assemblage found in cattle. Conversely, assemblage E is known to be artiodactyl host-specific, but has been reported in a human isolate in Egypt (Foronda et al. 2008). The current study implies that cross infection of *G. duodenalis* genotypes among animals could potentially occur.

The prevalence of *G. duodenalis* in sheep in the current study was 44%. Nevertheless, this may be an over-estimate of the true prevalence in the UK since only symptomatic animals were tested. Even though the infection was highest in neonates, with a lower prevalence in adult sheep, this was not significant. In Western Australia, 500 sheep faecal samples were screened for the presence of *G. duodenalis*, finding a prevalence of 44% (Ryan et al. 2005). Furthermore, a total of 477 faecal samples from pre-weaned sheep from 5 different farms in the south-west of Western Australia were screened for the presence of *Cryptosporidium* and *Giardia* using PCR, finding an overall prevalence of 11% (Yang et al. 2009). In Belgium, the prevalence of *G. duodenalis* infection in lambs was 26% (35/137). In Maryland, USA, the prevalence of *G. duodenalis* in ewes and lambs was 12% and 4%, respectively (Santin et al. 2007). The results presented here indicate that *G. duodenalis* is a common parasite found in sheep with diarrhoea.

The genetic sequences of amplified ssu rRNA fragments were determined in 26 sheep. Due to the limited number of samples sequenced, statistical analysis was not conducted among sheep groups. However, it was clear that the artiodactyl host-specific genotype E was the principal genotype. The only potentially zoonotic genotype detected was assemblage A, with one mixed assemblage A+D, whilst assemblage B was not identified. In Maryland, USA, of 14 sheep samples tested, assemblage E was detected in 13 samples and assemblage A in one sample (Santin et al. 2007). In Western Australia, a study indicated that sheep may not be an important zoonotic reservoir for *G. duodenalis*, since assemblage A was detected in 11/46 and assemblage E in 33/46 (Ryan et al. 2005). Another investigation in Australia reported that *G. duodenalis* genotype E was predominantly found (36/53), with assemblage A detected in five positive isolates, and mixed assemblage A+E infection found in 11 samples (Yang et al. 2009). In Spain, the molecular characterisation of *G. duodenalis* in healthy adult domestic sheep identified genotype E in 11 sheep samples and assemblage A in one sample (Castro-Hermida et al. 2007). Moreover, assemblage E was identified in all 14 sheep in a Mexican study (Di Giovanni et al. 2006). However, a study in central Italy identified that all five sheep samples sequenced were of assemblage A, and the hoofed genotype was not seen (Giangaspero et al. 2005).

The overall prevalence of *G. duodenalis* infection in pigs was 60% (18/30) in the current study. However, pigs were not sub-divided into different age groups due to insufficient data. In Canada, a study was undertaken to investigate the

prevalence of *G. duodenalis* infection in farm animals, including pigs. Two hundred and thirty-six pig samples were examined and the prevalence was 9% (Olson et al. 1997). In Denmark, the prevalence of *G. duodenalis* in three different age groups of Danish pigs was investigated. In sows, nursing piglets and weaner pigs, the prevalence was 18%, 22% and 84%, respectively (Maddox-Hyttel et al. 2006). Diarrhoea in pigs can occur for several reasons, but the current study suggests that Giardiasis may be a causal factor, and that *G. duodenalis* is present in pigs in the UK.

Of the pig samples that were positive for *G. duodenalis*, 13 were genotyped. The assemblages found in pigs comprised of the zoonotic assemblage A, both canine specific genotypes C and D, the hoofed livestock genotype E, and the cat-specific assemblage F. The current results were similar to those of a study of the prevalence and molecular genotyping of *G. duodenalis* in pigs in Australia (Armson et al. 2009), which identified the cat-specific assemblage F in one post-weaned pig. Additionally, assemblage D was demonstrated in two pigs in Denmark (Langkjaer et al. 2007). Along with these studies, the current work confirmed that pigs can be infected with several genotypes of *G. duodenalis*, not only the hoofed-livestock specific assemblage E.

In conclusion, the prevalence and genotypic characterisation of *G. duodenalis* isolates from a variety of farm animal species was determined, although the true prevalence may be somewhat lower than that obtained, given that only symptomatic animals were tested. Nevertheless, the current results imply a

number of interesting points. Firstly, assuming a causal association with clinical signs, *G. duodenalis* infection was likely to be harmful to animal health. Secondly, as reported in a number of previous studies, a zoonotic potential is likely given that *G. duodenalis* assemblage A was identified in many host species. Therefore, the transmission of *G. duodenalis* cyst from animals to humans is possible, either by direct contact, or from contaminated food or water. Thirdly, the absence of another zoonotic genotype, assemblage B, suggested that geographical location might be one of the factors affecting the distribution of *G. duodenalis* genotypes, which would explain the variation seen between the studies described above. Fourthly, although the study was limited only to animals with clinical signs submitted to the VLA, this survey was the largest scale investigation of *G. duodenalis* genotyping in animals and humans in the UK. The results suggest that further research is required in terms of epidemiology. Lastly, mixed infections in this study implied that, in accordance with other studies above, cross infection between hosts is possible.

Chapter 4 Molecular epidemiology of *G. duodenalis* in humans

4.1 Introduction

Giardia duodenalis is one of a number of gastroenteric organisms that cause gastroenteritis in humans and other mammals. It is of particular significance in immuno-compromised patients, for example those with HIV infection (AIDS). Both food-borne and water-borne transmission of Giardiasis has been described. *Giardia duodenalis* is also considered to be a zoonotic disease (WHO 1979). In many developing countries within Asia, Africa and Latin America, human symptomatic Giardiasis has an estimated incidence of 500,000 cases per year (Thompson et al. 2000). However, there is still some debate about whether *Giardia* can be transferred from animals to humans under natural conditions. The clinical signs of Giardiasis in humans vary greatly, from severe diarrhoea to asymptomatic infection (Astiazaran-Garcia et al. 2000).

Giardiasis is particularly significant in young children, individuals who have not previously been exposed to the parasite, and individuals who have travelled to endemic areas. Patients can show clinical signs including malabsorption, intermittent or persistent diarrhoea, and loss of weight. Retarded growth may be observed in young children or infants (Farthing 1997). In general, *Giardia* rarely causes morbidity in humans, but various symptoms may be detectable depending

on host factors and the severity of infection (Farthing 1997). In children undergoing esophagogastroduodenoscopy, Giardiasis can cause chronic mucosal inflammation, frequently of eosinophilic nature (Koot et al. 2009).

The highest prevalence of *Giardia* in humans is found in children. A retrospective study of *Giardia* surveillance in Scotland over the 16-year period, 1988–2003, was recently undertaken by Health Protection Scotland (HPS). The study found that between 1988 and 1998, the annual number of *Giardia* infections remained constant at approximately 300–400, whereas from 1999 the number of reports decreased, and in 2003 only 192 cases were reported to the HPS. During the period studied, young children between 0–4 years were the most affected, while a second peak occurred in individuals between the ages of 20–39 years (Pollock et al. 2005).

According to molecular genotyping based on a number of molecular markers, assemblage A and assemblage B have been reported as the most common genotypes found in humans. The two assemblages can be further divided into sub-genotypes. Assemblage A consists of two distinct sub-groups, AI and AII. AI comprises a mixture of intimately related human and animal isolates, and has been focused on as a zoonotic sub-group, whilst AII is considered to be a purely human isolate. Assemblage B consists of two sub-groups, BIII and BIV, of which the latter appears to be human-specific (Thompson 2004).

A number of studies have mentioned the correlation between *Giardia* assemblage and symptomatology in humans. In Turkey, the genotyping of 56 *Giardia* human

isolates, from symptomatic and asymptomatic patients, was investigated (Aydin et al. 2004). Both stool and duodenal aspirate samples were used in the study. A total of 44 stool specimens (20 from symptomatic and 24 from asymptomatic individuals) and 12 duodenal aspirates (6 from symptomatic and 6 from asymptomatic individuals) were microscopically examined. In stool specimens, assemblage A was found in 17/20 (85%) symptomatic patients and assemblage B was found in 22/24 (92%) asymptomatic patients. Of the 12 duodenal aspirate samples, 5/6 (83%) symptomatic cases displayed assemblage A and 1/6 (17%) displayed assemblage B, whilst 5/6 (83%) asymptomatic cases displayed assemblage B and 1/6 (17%) displayed assemblage A (Aydin et al. 2004).

In Bangladesh, samples were taken from patients with diarrhoea at the hospital of the International Centre for Diarrhoeal Disease Research. Of all 322 samples studied, assemblage B was the most prevalent genotype, but in symptomatic cases, assemblage A was more prevalent than assemblage B (Haque et al. 2005). Moreover, a longitudinal study of day-care centres in Western Australia reported that children infected with *Giardia* isolates from assemblage A were 26 times more likely to have diarrhoea than children with assemblage B (Read et al. 2002).

In the UK, a molecular characterisation of *G. duodenalis* has been performed (Amar et al. 2002). Faecal samples from patients with diarrhoea, in which *Giardia* cysts had been detected by clinical microbiology laboratories using conventional techniques, were collected at the Food Safety Microbiology Laboratory, Public Health Laboratory Service, London. The samples were collected from patients in

England and Wales with sporadic cases of Giardiasis diagnosed between September 1995 and March 2000, and from individuals involved in a nursery outbreak of Giardiasis that occurred in North Wales during April 2000 (Amar et al. 2002). In this study, the *tpi* gene was amplified with a set of PCR primers specific to assemblages A and B. Assemblage B was found to be the predominant assemblage in patients with symptomatic Giardiasis, although the research was performed in a relatively small group of patients (Amar et al. 2002).

In the current study, the main analysis was based on molecular genotyping of *G. duodenalis* DNA sequences amplified by PCR using ssu rRNA as a marker. All faecal samples were collected from symptomatic patients who had been confirmed as positive for *Giardia* infections, using microscopic examination or ELISA, by the laboratories at Manchester Royal Infirmary and the Royal Preston Hospital. The current project was in collaboration with authors from the Health Protection Agency, UK. The aim of the study was to investigate the molecular genotypes of *G. duodenalis* in symptomatic patients in the UK.

4.2 Materials and methods

4.2.1 Origin of human samples

From January 2008 to August 2009, human faecal samples were collected from diarrhoeic patients whose faeces were submitted to the laboratories at Manchester Royal Infirmary and the Royal Preston Hospital. The gender, age and history of travelling of patients was recorded (Appendix 4). This research was in collaboration with The Health Protection Agency, Cumbria & Lancashire Health Protection Unit, 5 Albert Edward House, 1st Floor, Room 14, The Pavilions, Ashton-on-Ribble, Preston PR2 2YB, and Lancashire Teaching Hospitals NHS Foundation Trust, Cumbria and Lancashire Health Protection Unit, 1st Floor, York House, Ackhurst Business Park, Foxhole Road, Chorley, PR7 1NY. Only faecal samples confirmed to be *Giardia duodenalis* positive by ELISA and microscopic examination were transferred to the Faculty of Veterinary Sciences, University of Liverpool, for further analysis in the current study. All faecal samples were stored at 4°C before being analysed.

4.2.2 DNA isolation and polymerase chain reaction (PCR) amplification

G. duodenalis genomic DNA was extracted from the human faecal samples using a QIAamp® DNA Stool Mini Kit (QIAGEN, UK), following instructions from the manufacturer's manual, as described in the previous chapter. Briefly, faecal

samples were lysed to release *Giardia* genomic DNA, then unwanted materials were absorbed using a QIAGEN InhibitEX® tablet. Proteins were eliminated with proteinase K, and DNA was precipitated with absolute ethanol before being eluted through a QIAamp spin column. The eluted *Giardia* DNA was collected and then stored at -20°C before further analysis.

PCR was performed as described in Chapter 2, section 2.2. Briefly, the 292–293 bp fragment of ssu rRNA was amplified using forward primer RH11 and reverse primer RH4 (Hopkins et al. 1997). The PCR conditions were as follows: 2 min initial reaction at 96°C, followed by 35 cycles at 96°C for 20 sec, 59°C for 20 sec, 72°C for 30 sec, and a final extension at 72°C for 7 min. The amplicons were visualised on an ethidium bromide stained agarose gel under ultraviolet light, and purified using a QIAquick® PCR Purification Kit (QIAGEN, UK).

The purified amplicons were sent to Geneservice, Nottingham, where they were sequenced in both directions using either primer RH11 or RH4. The sequences were interpreted with the freeware software, BioEdit, and aligned with Clustal W (Thompson et al. 1994). The following reference assemblage sequences used for sequence alignment were obtained from GenBank; accession number AF199446 for assemblage A, AF199447 for assemblage B, AF199449 for assemblage C, AF199443 for assemblage D, AF199448 for assemblage E and AF199444 for assemblage F.

4.2.3 Statistical analyses

In order to analyse the relationship between gender, age or history of travelling and assemblage, statistical analyses were performed with Stats Direct software, version 2.6.2 (Stats Direct Ltd. Altrincham, UK). The age data were categorised into four groups, i.e., ≤20 years, >20 to ≤40 years, >40 to ≤60 years, and >60 years. For travel history, subjects were categorised according to whether or not they had travelled abroad in the past 3 months. Proportions of assemblage A and B isolates were compared with either the Chi-square test for trend (age data) or Fisher's exact test (gender and travel data). The level of statistical significance was set at $p<0.05$.

4.3 Results

4.3.1 Sequence analysis of ssu rRNA gene

The 292–293 bp fragments of the ssu rRNA locus of 71 DNA samples were amplified with PCR (Figure 4.1). Although all 71 samples were confirmed to contain *Giardia* cysts by microscopic examination, only 66 samples yielded positive results (93%). Out of 66 samples, 60 (90.9%) were selected and successfully sequenced and genotyped (Table 4.1). The results of the sequencing analysis completely matched the GenBank references described in section 4.2.2. Assemblage A was found in 17 samples (28%) and assemblage B was found in 43

samples (72%) (Figure 4.2). Other host-specific genotypes were not identified in the current investigation.

Table 4.1 Genotyping results of 60 human isolates by sequence analysis of the ssu rRNA

Isolate	Original ID	Assemblage	Isolate	Original ID	Assemblage
HM001	P08.0401874.W	B	HM033	220733	B
HM002	P08.0403199.D	A	HM034	222390	B
HM003	P08.0403800.N	B	HM035	220123	A
HM004	P08.0404164.Y	A	HM036	220750	B
HM005	P08.0404415.M	B	HM037	221704	B
HM006	P08.0405071.S	A	HM038	221752	B
HM007	P80.0405729.B	B	HM039	222722	B
HM008	P08.0405942.N	A	HM040	222658	B
HM009	P08.0406166.H	A	HM041	223076	A
HM010	P08.0407075.N	B	HM042	223112	B
HM011	P08.0408324.J	B	HM043	223489	B
HM012	P08.0408632.D	B	HM046	223872	B
HM013	P08.0401212.Y	B	HM047	224461	B
HM015	P08.0401990.S	B	HM048	224342	A
HM016	P08.0403118.M	B	HM049	225397	A
HM017	P08.0403617.W	B	HM050	225834	B
HM018	P08.0403855.Q	B	HM051	225941	B
HM019	P08.0404298.Q	B	HM053	225977	B
HM020	P08.0404989.A	B	HM054	225981	A
HM021	P08.0405504.P	B	HM055	226453	B
HM023	P08.0405983.Y	B	HM056	226478	A
HM024	P08.0406965.R	B	HM059	226943	A
HM025	P08.0407237.N	B	HM060	227104	B
HM026	P08.0408255.P	A	HM061	226962	B
HM027	P08.0408375.P	A	HM062	227460	B
HM028	411630	B	HM064	228065	A
HM029	411016	B	HM065	228094	A
HM030	412422	B	HM066	228166	B
HM031	412185	B	HM070	228835	A
HM032	412512	B	HM071	228930	B

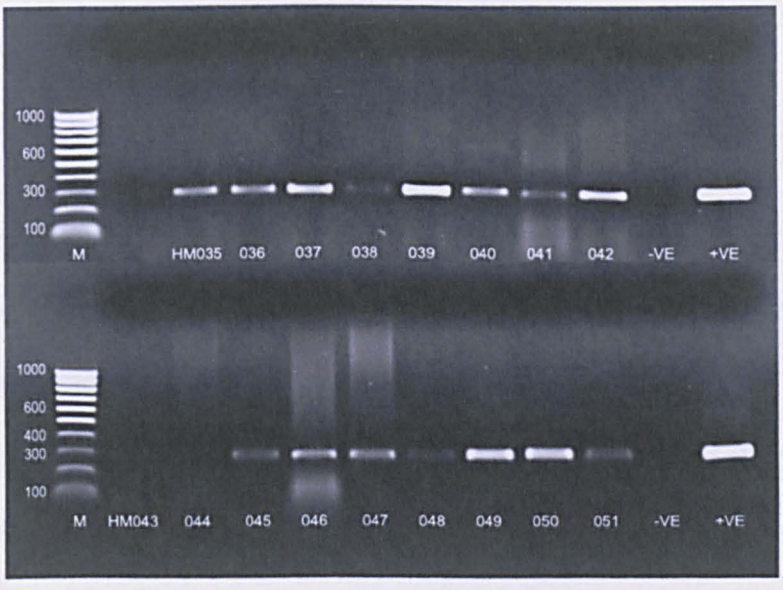


Figure 4.1 Electrophoretic separation of ssu rRNA PCR products (292–293 bp) of *G. duodenalis* from human faecal sample isolates HM035 to HM051. Lane M is the 100-bp marker with the negative (-VE) and positive (+VE) controls located in the lanes on the right.

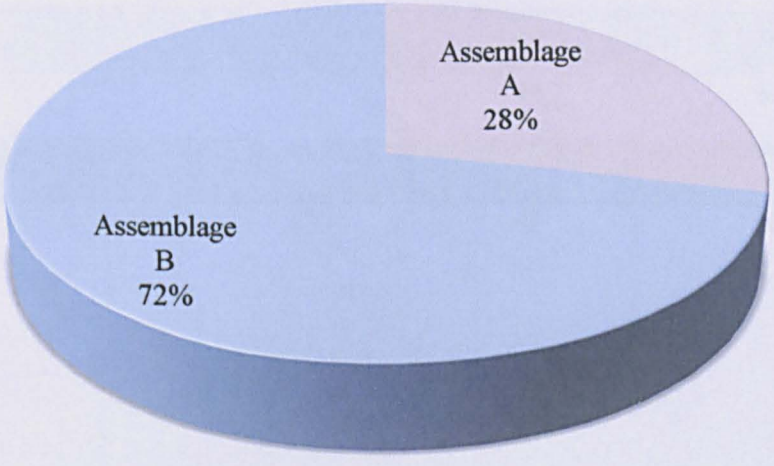


Figure 4.2 Distribution of *G. duodenalis* assemblages by sequence analysis of ssu rRNA gene from 60 human samples. Assemblage A was found in 17 samples (28%) whilst assemblage B was detected in 43 samples (72%).

4.3.2 The correlation between gender, age, or history of travelling, and G. duodenalis assemblages

In the current study, both male and female patients were found to be infected with assemblage B more frequently than assemblage A. Of 35 samples from males, nine (26%) were infected with assemblage A and 26 (74%) with assemblage B. Of 21 samples from females, assemblage A was detected in seven (33%) and assemblage B in 14 (67%). More males were found to be infected with *G. duodenalis* than females. However, there was no significant difference between male and female subjects in the proportion of samples that were positive for the two assemblages (A and B) (Fisher's exact test, p=0.56) (Table 4.2, Figure 4.3).

Table 4.2 The distribution of *G. duodenalis* assemblages according to gender

Gender	Assemblage		Total
	A	B	
Male	9	26	35
Female	7	14	21
Unknown	1	3	4
	17	43	60

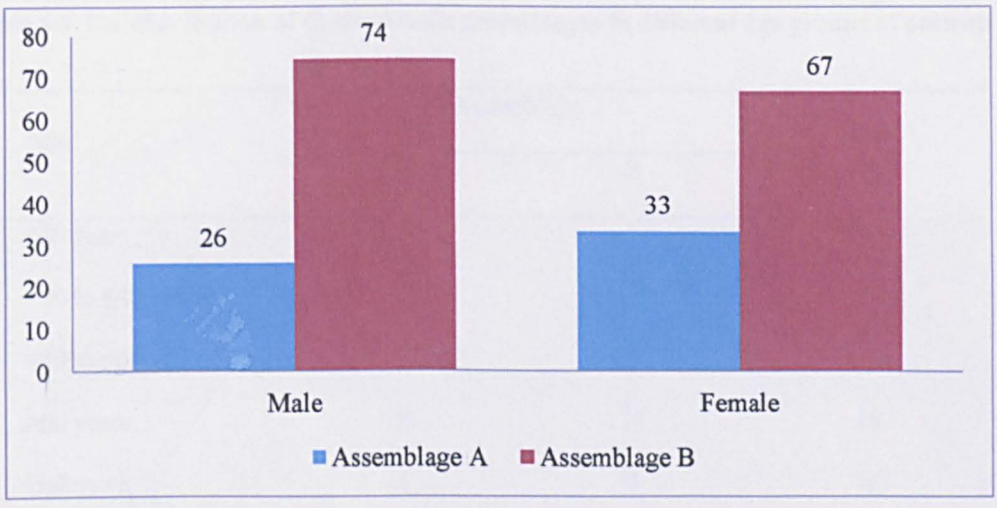


Figure 4.3 The percentage of assemblages of *G. duodenalis* found in male and female patients (n=56). Samples from patients of unknown gender were excluded.

Regarding age, patients were divided into 4 groups; ≤20 years, >20 to ≤40 years, >40 to ≤60 years, and >60 years. Assemblage B was detected more frequently than assemblage A in all age groups. Interestingly, age was significantly associated with the type of *Giardia* assemblage isolated, with a significant linear trend for increasing isolation of assemblage A in older subjects (Chi-square test for trend, p=0.0497) (Table 4.3, Figure 4.4).

Table 4.3 The distribution of *G. duodenalis* assemblages in different age groups of patients

Age	Assemblage		Total
	A	B	
≤20 years	1	5	9
>20 to ≤40 years	3	16	19
>40 to ≤60 years	4	9	3
>60 years	8	10	18
Unknown	1	3	4
	17	43	60

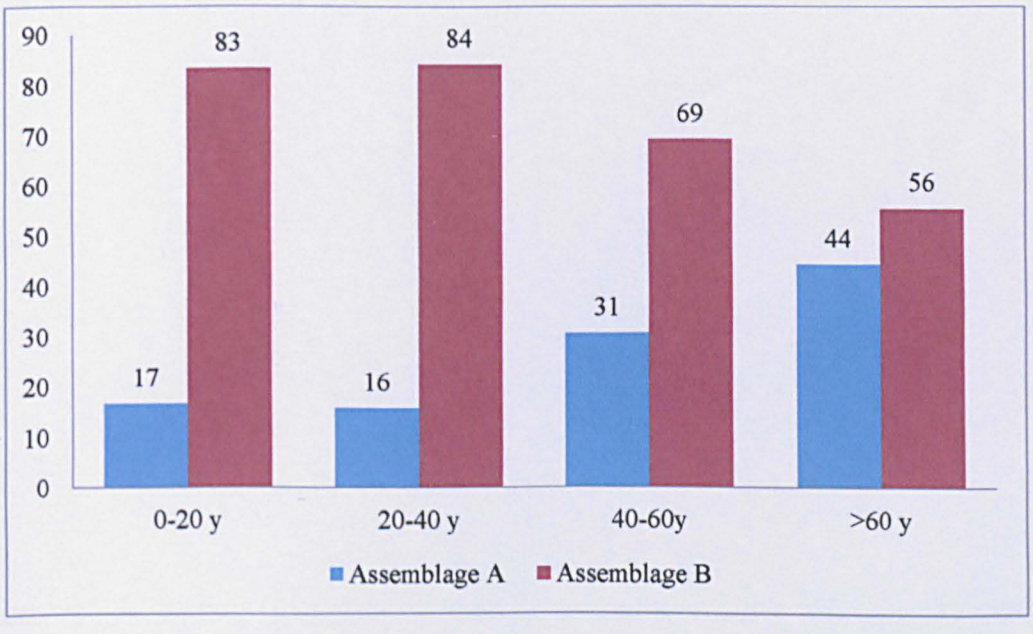


Figure 4.4 The percentage of assemblages of *G. duodenalis* found in different age groups of patients. Samples from patients of unknown age were excluded (n=56).

Both patients that had travelled outside the UK and those that had not were infected with assemblage B more frequently than assemblage A. Of 11 patients with a history of travelling, two were infected with assemblage A (one had travelled to Spain and one to Nepal), and nine were infected with assemblage B (two had travelled to Egypt, one to France, one to Pakistan then to Dubai, one to the United Arab Emirates, and four to India). Patients who did not leave the UK for 3 months prior to the study harboured assemblage B more than assemblage A. However, there was no significant difference in the proportion of samples positive for the two assemblages (A and B) between subjects who had travelled overseas and those who had not (Fisher's exact test, p=0.35) (Table 4.4, Figure 4.5).

Table 4.4 The distribution of *G. duodenalis* assemblages in patients with different travel histories

Travel outside the UK	Assemblage		Total
	A	B	
Yes	2	9	11
No	12	23	35
Unknown	3	11	14
	17	43	60

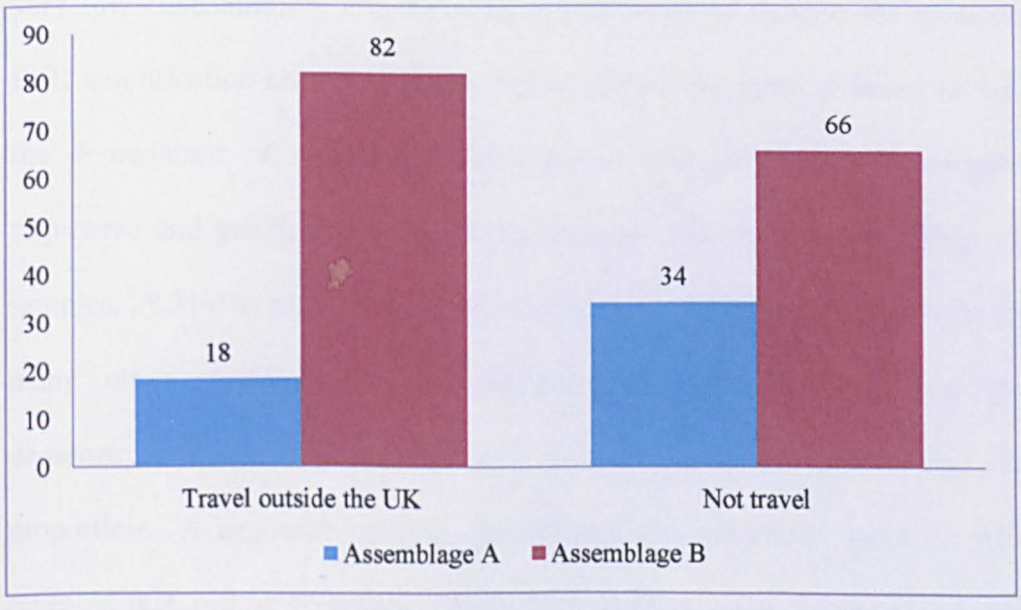


Figure 4.5 The percentage of assemblages of *G. duodenalis* found in two groups of patients with or without a history of travelling outside the UK (n=46). Samples from patients with an unknown travel history were excluded.

4.4 Discussion

Although all faecal samples were confirmed as *Giardia* positive by means of microscopic examination and ELISA, PCR amplification of ssu rRNA fragments was not wholly successful. This was also the case in a study in France by Bertland et al. (2005), who found that despite all 26 human faecal samples being confirmed as positive for *Giardia* in the hospital laboratory, only 21 (81%) and 25 (96%) samples were successfully amplified at the glutamate dehydrogenase gene and the triose phosphate gene, respectively. In the current study, as some faecal samples were collected in very small volumes, the DNA extraction may have yielded a very low concentration. Other potential hypotheses to explain the unsuccessful PCR amplification are a low concentration of *Giardia* cysts in faecal samples or the degradation of organisms. Nevertheless, 60 samples were successfully sequenced and genotyped at the ssu rRNA gene. The ratio of assemblage A (17 samples, 28.3%) to assemblage B (43 samples, 71.7%) was comparable to that in many other studies that used different molecular tools. Several studies demonstrate that assemblage A and assemblage B are present in various proportions. A sequence analysis (genotyping the ssu rRNA gene) in Albania revealed that, out of 50 samples studied, 10 isolates corresponded to assemblage A (20%) and 12 isolates to assemblage B (24%) (Berrilli et al. 2006). In Australia, using ssu rRNA PCR analysis, 36 isolates from 23 children showed assemblage A in 30.4% (7/23) and assemblage B in 69.6% (16/23) (Read et al. 2002). In the Netherlands, a study on Dutch patients identifying *G. duodenalis* genotypes using

ssu rRNA analysis, showed assemblage A in 35% (34/98) and assemblage B in 65% (64/98) (van der Giessen et al. 2006). Assemblage B was also found to be dominant in an investigation in Malaysia in which, of 42 samples sequenced, assemblage B was detected in 98% and assemblage A in 2% (Mohammed Mahdy et al. 2009). In 108 Spanish patients, assemblage A was detected in 40%, assemblage B was detected in 56%, and a mixed infection of both assemblages was detected in four samples (Sahagun et al. 2008). In France, Bertrand et al. (2005) compared two target genes for detection and genotyping of *Giardia* human faecal samples by PCR and PCR-RFLP. These authors found that based on *tpi* and *gdh* genotyping analyses, 36% (9/25) were assemblage A whereas 64% (16/25) were assemblage B. However, some studies found that assemblage A was predominant. In Italy, a study using both ssu rRNA and *bg* PCR-RFLP molecular markers, found that 24/30 patients had assemblage A and 6/30 had assemblage B (Caccio et al. 2002). In Peru, a study of the prevalence and genotyping of *G. duodenalis* in 845 children found an overall prevalence of 23.8%. They characterised 16 samples, of which 10 were identified as assemblage A and six as assemblage B (Perez Cordon et al. 2008). Moreover, studies in Brazil (n=62) (Volotao et al. 2007) and Mexico (n=8) (Lalle et al. 2005a) found that, by genotyping at *bg*, all isolates were classified as assemblage A.

Although the results of the current study were not comparable to the epidemiological data from asymptomatic cases, as all faecal samples were collected from diarrhoeic patients, it was noted that assemblage B infection was

predominant in symptomatic Giardiasis. In addition, the current study was similar to a study by Amar et al (2002) who investigated *G. duodenalis* in human patients with symptomatic Giardiasis in the UK and, by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the *tpi* gene, found that of 33 samples from patients with sporadic cases sequenced, nine (27%) displayed assemblage A2, 21 (64%) contained assemblage B, and three (9%) contained a mixture of assemblage A2 and B. Samples from individuals involved in a nursery outbreak displayed 21 assemblage B isolates out of 24 samples studied (Amar et al. 2002).

A number of previous studies have investigated clinical Giardiasis. For example, in a study in Ethiopia which used *bg* as a genetic marker, 12/13 (92%) symptomatic cases (nausea, abdominal pain and diarrhoea) harboured assemblage B while assemblage A was associated with 18/31 (58%) symptomatic cases. Assemblage F was also detected in one case in this study (Gelanew et al. 2007). Homan and Mank (2001) found that among 18 Dutch patients, assemblage A was detected in mild, intermittent diarrhoea, whereas assemblage B was identified in severe, actual/persistent diarrhoeic cases.

However, some studies provide evidence linking *Giardia* genotypes and clinical signs in humans. In Peru, all samples from diarrhoeic children had assemblage A, while normal stools harboured assemblage B (Perez Cordon et al. 2008). In Spain, *G. duodenalis* genotype AII was frequently found in diarrhoeic children, particularly those <5 years of age. In contrast, assemblage B was detected more frequently in asymptomatic cases (Sahagun et al. 2008).

Statistical analyses were performed to investigate the correlation between gender, age or history of travelling and *G. duodenalis* genotypes found in the present study. There was no significant difference in the correlation between assemblages detected and gender (p=0.56), although assemblage B was the most common genotype in both males and females. This result differed from a study in Malaysia by Mohammed Mahdy et al. (2009), who found a strong correlation between assemblage B and clinical symptoms among female patients. Also, there was no significant difference between assemblage B and symptomatic and asymptomatic males (Mohammed Mahdy et al. 2009). Interestingly, in the present study, it was found that assemblage A was likely to infect older people with symptomatic Giardiasis, given that a significant linear increase was detected in older subjects (p=0.0497). Mohammed Mahdy et al. (2009) found that there was no significant correlation between assemblage B among age groups (≤12 years and >12 years). Further, a study in Ethiopia revealed that despite assemblage B being significantly related to symptomatic cases, there was no correlation between the presence of clinical symptoms and age, gender or geographic location of patients (Gelanew et al. 2007). In addition, a history of travel had no correlation to the assemblages detected in the current study. However, this suggested that both genotypes may infect patients whether individuals had travelled overseas or not, as no significant difference was detected (p=0.35).

In conclusion, the current study demonstrated the prevalence of common *G. duodenalis* assemblages A and B in symptomatic patients in the UK, without

mixed infection or any other genotypes. The proportions of assemblage A and B found in the current work agrees with some previous studies, while disagreeing with others, suggesting that geographic location may be one factor that influences distribution. Ideally, the present research would have collected samples from patients both with and without clinical symptoms to compare the distribution of *G. duodenalis* genotypes. However, obtaining faecal samples from healthy patients is difficult.

The next chapter presents the analysis of multilocus genotyping of *G. Duodenalis*, to determine which sub-genotypes are predominant in British patients.

Chapter 5 Multilocus genotyping of *G. duodenalis*

5.1 Introduction

In order to study the molecular epidemiology of *Giardia duodenalis*, molecular characterisation, using several genetic loci as markers, has been widely used. PCR amplified genetic markers, including small subunit ribosomal RNA (ssu rRNA or 18S rRNA), β-giardin (*bg*), glutamate dehydrogenase (*gdh*), elongation factor 1-alpha (*ef1-α*), and triose phosphate (*tpi*), can clarify the differences between *Giardia* assemblages (Caccio et al. 2005). Initially, assemblages A and B were categorised into sub-assemblages, namely A1, A2, B3 and B4, by iso-enzyme analysis (Monis et al. 2003). However, routine use of this technique is not practical due to the *in vitro* and *in vivo* propagation of *Giardia* (Caccio et al. 2008), therefore molecular genotyping based on genetic loci has been applied in most clinical or environmental cases.

Many studies of the molecular epidemiology of *Giardia duodenalis* have been based on single markers. The ssu rRNA locus has been widely used as a marker, as it is a conserved gene that is specific to *Giardia*, but it can only discriminate seven conventional genotypes – namely assemblages A to G – as described in the previous chapters. Additionally, many studies have used other genes, such as *bg* or *gdh*, which can further discriminate *G. duodenalis* genotypes into a number of sub-genotypes. In humans, the *bg* gene was used as a molecular marker to

investigate the genetic characterisation of *G. duodenalis* in Brazil. It revealed that, of the 62 cases of assemblage A found, 60 were distinguished as A1, and two as A2 (Volotao et al. 2007). In Italy, a study by Lalle et al (2005b) identified a number of sub-genotypes within assemblages A (8 sub-genotypes), B (6 sub-genotypes), D (2 sub-genotypes), and E (3 sub-genotypes). Five of these sub-genotypes, namely A1, A2, A3, A4 and B3, were found to be associated with infections of humans, dogs and calves. By amplifying the *bg* gene, the same authors also identified A1 and A3 in human (15 A1, two A3) and dog (four A1, one A3) samples, in Mexico (Lalle et al. 2005a).

The *gdh* gene has also been widely used for studying *Giardia* molecular characterisation (Read et al. 2004; Lalle et al. 2005b). By using this gene, *G. duodenalis* has been discriminated into a number of sub-assemblages. A study on samples collected from several municipalities within the state of Sao Paulo, Brazil, found assemblage A2 in 29 patients, and assemblage B in 8 patients (Souza et al. 2007). In many studies, in order to genetically characterise the diversity within *G. duodenalis* genotypes, a combination of several markers have been utilised, i.e., ssu rRNA and *gdh* (Read et al. 2004; van der Giessen et al. 2006; Thompson et al. 2008a; Yang et al. 2010), ssu rRNA and *bg* (Castro-Hermida et al. 2008; Geurden et al. 2008c), or ssu rRNA, *gdh* and *bg* together (Caccio et al. 2008; Lalle et al. 2009; Abe et al. 2010).

Microsatellites are simple, tandemly repeated DNA sequences found in the genomes of organisms. A microsatellite usually consists of one (mono-), two (di-),

three (tri-), or four (tetra-) nucleotide sequences that are repeated 10 to 100 times. Repeats that occur more than 10 to 15 times without interruption tend to be polymorphic; the larger the number of repeats, the higher the probability of polymorphism. The number of repeats at particular locus is highly polymorphic between individuals of the same species, which makes microsatellites tremendously useful for genetic analysis (Barker 2002). Many studies have exploited microsatellites as markers for epidemiology (Anderson et al. 2000; Jamonneau et al. 2002; Mallon et al. 2003b). However, there has been controversy regarding how to define the number of repeats in a microsatellite. It has been reasonably suggested that a microsatellite should consist of short segments of 2–6 nucleotides repeated in more or less uniform tracts up to ~102 nucleotides long. In contrast, a minisatellite has been defined as moderately repeated 10–100 nucleotide segments, forming more or less uniform tracts of 102–105 nucleotides in length (Chambers and MacAvoy 2000).

A preliminary study of microsatellites was performed in *Leishmania*. Three groups of microsatellites were found on heterozygous chromosomes of the same strains in an uncertain distribution. The sequences of these microsatellites were $(CA)_n$, $(CT)_n$, and $(GCA)_n$ (Rossi et al. 1994). Another study in *Leishmania* identified a sequence of at least four tandem repeats on one chromosome. That sequence was characterised as a repetitive microsatellite and GC-rich simple repeat DNA, from the nuclear genome of New World *Leishmania* (Rodriguez et al. 1997).

Microsatellites were also developed as molecular markers for trypanosomes. The $(CA)_{(n)}$ microsatellite markers of the genomic sequence of a species-specific antigen of *Trypanosoma vivax* have been identified. No cross amplification with other trypanosome species was detected (Morlais et al. 2001). Indeed, the microsatellites in *Trypanosoma cruzi* proved to be stable across 70 generations of the clone in culture, including two major genetic groups of the subspecies (Macedo et al. 2001). The microsatellite was also utilised to identify two monoclonal *Trypanosoma cruzi* strains (Be-62 and Be-78) (Cruz et al. 2006).

The use of highly polymorphic microsatellites in *Cryptosporidium* has been described. Fourteen microsatellite loci were identified from *C. parvum* DNA sequences, with A, AT and AAT repeats reflecting the high AT content of the *C. parvum* genome. At least three microsatellites were defined in a sample of 19 isolates, with the majority of isolates displaying a unique multilocus fingerprint (Feng et al. 2000). In another study, the microsatellite could trace and discriminate between anthroponotic and zoonotic transmission, as well as identify the source of infection (Caccio and Pozio 2001).

In the Netherlands, a study of the epidemiology of *Cryptosporidium* and *Giardia* infection was investigated in a dairy farm. Microsatellite analysis revealed two different subtypes (C3 and C1) of *Cryptosporidium parvum* calf strains, and two genotypes of *Giardia*. The results indicate that cow-to-calf and indirect calf-to-calf transmission are both important routes for acquiring *Cryptosporidium* infection (Huetink et al. 2001). Subsequently, 180 *Cryptosporidium* strains from

humans and cattle in Aberdeenshire were analysed using three minisatellite and four microsatellite markers, defining 38 multilocus genotypes. The results demonstrated that one group of human isolates was clonal, whereas the second group, common to both humans and animals, exhibited a panmictic population structure (Mallon et al. 2003a). Following a previous study, using 7 micro- and minisatellite markers, the isolates were collected over a period of 19 months from three different geographical locations within Scotland, and three different host species. Forty-eight multilocus genotypes within the Type 2 *C. parvum* isolates were identified, with no evidence of geographic or temporal sub-structuring of the populations (Mallon et al. 2003b).

The epidemiology of *Plasmodium falciparum* was investigated using length variation at 12 microsatellite loci in 465 infections collected from nine locations worldwide. The results revealed remarkable differences in parasite population structure in different locations (Anderson et al. 2000). Additionally, some authors have applied microsatellite markers and genetic maps to gene mapping, parasite typing, and studies of the parasite population changes of *Plasmodium falciparum* (Ferdig and Su 2000). Microsatellite analysis has shown the population structure of *Plasmodium falciparum* to be rather panmictic in high-infectivity regions, whilst principally clonal in regions of low infectivity (Razakandrainibe et al. 2005).

The current study took a multilocus genotyping (MLG) approach using β-giardin and glutamate dehydrogenase as molecular markers, in order to further

characterise *G. duodenalis* isolates for which assemblages were already classified by ssu rRNA marker genotyping (Chapters 2 and 4), and to evaluate novel microsatellite markers for genotyping in *G. duodenalis*.

5.2 Materials and methods

5.2.1 Source of samples

For multilocus genotyping, all 60 human samples that had been successfully sequenced and genotyped by ssu rRNA analysis (Chapter 2), were used in this experiment. For animal samples, only 17 dog and nine cat samples, selected on the basis of the strength of their bands on ssu rRNA PCR, underwent analysis. As this investigation was performed at almost the end of the research project, sample selection was affected by time constraints. The origins of all samples have been described in Chapter 2, section 2.2, and Chapter 4, section 4.2.

5.2.2 PCR amplification of β-giardin

Nested PCR amplification was performed at the *bg* locus. In the primary PCR reaction, to amplify a 753 bp fragment, the forward primer G7 (5′-AAG CCC GAC GAC CTC ACC CGC AGT GC-3′) and the reverse primer G759 (5′-GAG GCC GCC CTG GAT CTT CGA GAC GAC-3′) were used, as previously described (Caccio et al. 2002). The PCR mix consisted of 1× buffer containing 18 mM Tris-HCL, 4.4 mM $(NH_4)_2SO_4$, 1.8 mM $MgCl_2$, 452 μg BSA, 1.76 μM EDTA, 400 μM of each dNTP, 10 pmol of each primer, 2.5 units of *Taq* DNA polymerase (QIAGEN, UK), and 5 μl of purified DNA in a final volume of 20 μl. The reactions were performed as follows: after an initial hot start of 5 min at

94°C, a set of 35 cycles was run, each consisting of 30 sec at 94°C, 30 sec at 65° and 60 sec at 72°C, followed by a final extension of 7 min at 72°C. Then, 1 µl of PCR product was used as a DNA template in the secondary reaction, to amplify a 511 bp fragment using the forward primer 5′-GAA CGA GAT CGA GGT CCG-3′ and the reverse primer 5′-CTC GAC GAG CTT CGT GTT-3′ (Lalle et al. 2005b). The PCR was performed as follows: after an initial denaturation step of 15 min at 95°C, a set of 35 cycles was run, each consisting of 30 sec at 95°C, 30 sec at 55°C and 60 sec at 72°C, followed by a final extension of 7 min at 72°C. Both reactions were carried out in a DNA Engine Dyad® Peltier Thermal Cycler. The DNA template for the positive control used throughout the experiment was from *G. duodenalis* axenic strain WB C6, previously genotyped as assemblage A, sub-group A1. The amplified products were visualised on 1% agarose gels stained with ethidium bromide, before being purified using the QIAquick® PCR Purification Kit (QIAGEN, UK), and sequenced by Geneservice, UK, in both directions using the secondary reaction forward and reverse primers described above. Using BioEdit and Clustal W software, the sequences of *bg* were aligned against the assemblage references, downloaded from GenBank, as displayed in Table 5.1. The phylogenetic analysis was performed using the neighbour-joining component of MEGA software version 4 (Tamura et al. 2007).

5.2.3 PCR amplification of glutamate dehydrogenase

A fragment of approximately 432 bp of the *gdh* gene was amplified using a semi-nested PCR protocol with slight modification (Read et al. 2004). For the primary reaction, the external forward primer GDHeF (5′-GTT RTC CTT GCA CAT CTC C-3′) and reverse primer GDHiR (5′-GTT RTC CTT GCA CAT CTC C-3′), were used. The PCR components were identical to those described in the *bg* section, and the reaction was run as follows: initial hot start at 95°C for 15 min, a set of 50 cycles consisting of denaturation for 45 sec at 94°C, annealing for 45 sec at 60°C, and elongation for 45 sec at 72°C, followed by a final extension step for 10 min at 72°C. The secondary reaction used 1 µl PCR product from the first reaction as a template, using the internal forward primer GDHiF (5′-CAG TAC AAC TCY GCT CTC GG-3′), and reverse primer GDHiR. The PCR mix and reaction conditions were similar to those used for the primary reaction, except the annealing temperature was 45°C for 45 sec. The purified PCR products were sequenced in both directions using primers GDHiF and GDHiR at Geneservice, UK. Finally, the *gdh* sequences were aligned against the reference sequences downloaded from GenBank (Table 5.1) using BioEdit and Clustal W software. The phylogenetic analysis was performed using the neighbour-joining component of the MEGA software version 4 (Tamura et al. 2007).

Table 5.1 Reference sequences from GenBank used in this study

Isolate	GenBank accession no.	Gene	Assemblage	References
Portland-1	M36728	*bg*	A1	(Holberton et al. 1988)
KC8	AY072723	*bg*	A2	(Caccio et al. 2002)
ISSGF7	AY072724	*bg*	A3	(Caccio et al. 2002)
A44	AY545642	*bg*	A4	(Lalle et al. 2005b)
GD83	AY545643	*bg*	A5	(Lalle et al. 2005b)
GD37	AY545644	*bg*	A6	(Lalle et al. 2005b)
GD115	AY545645	*bg*	A7	(Lalle et al. 2005b)
A14	AY545649	*bg*	A8	(Lalle et al. 2005b)
Nij-5	AY072725	*bg*	B1	(Caccio et al. 2002)
LD-18	AY072726	*bg*	B2	(Caccio et al. 2002)
BAH8	AY072727	*bg*	B3	(Caccio et al. 2002)
ISSGF-4	AY072728	*bg*	B4	(Caccio et al. 2002)
A82	AY647265	*bg*	B5	(Lalle et al. 2005b)
A88	AY647266	*bg*	B6	(Lalle et al. 2005b)
GL5	FJ971438	*bg*	B*	(Kosuwin et al. 2010)
GL279	FJ971466	*bg*	B*	(Kosuwin et al. 2010)
ISSGd167	EU637579	*bg*	B*	(Caccio et al. 2008)
A29	AY545646	*bg*	C	(Lalle et al. 2005b)
A21	AY545647	*bg*	D1	(Lalle et al. 2005b)
A27	AY545648	*bg*	D2	(Lalle et al. 2005b)
A101	AY647264	*bg*	F	(Lalle et al. 2005b)
Swecat013	EU769219	*bg*	F*	(Lebbad et al. 2010)
Ad-1	L40509	*gdh*	A1	(Monis et al. 1996)
Ad-2	L40510	*gdh*	A2	(Monis et al. 1996)
BAH-12	AF069059	*gdh*	B3	(Monis et al. 1999)
AD-7	L40508	*gdh*	B4	(Monis et al. 1996)
AD-141	U60984	*gdh*	C	(Monis et al. 1998)
AD-148	U60986	*gdh*	D	(Monis et al. 1998)
AD-23	AF069057	*gdh*	F	(Monis et al. 1999)

*The definite sub-assemblage has not been categorised

5.2.4 Evaluation of microsatellite marker

5.2.4.1 Determining microsatellite sequences

Giardia databases were acquired from the NCBI website (http://www.ncbi.nlm.nih.gov). The sequences were then inspected for tandem repeat sequences (microsatellites) using the *Tandem Repeats Finder* programme (Benson 1999). The selected microsatellite sequences were used for designing primers via the web-based interface application from the Primer3 website (http://primer3.sourceforge.net/) (Table 5.2). The designed primers were synthesised by Genosys Sigma-Aldrich, UK.

5.2.4.2 Evaluation of microsatellite primers

To determine primer efficiency, all primers were used to amplify fragments of microsatellites. Using the optimum annealing temperature supplied by the manufacturer, the sets of primers MC01, MC03, MC05 and MC06 (comprising forward and reverse primers), were applied under the following PCR conditions: denaturation step of 94°C for 5 min, 35 cycles at 94°C for 45 sec, 50°C for 45 sec, 72°C for 1 min, and a final extension step of 72°C for 10 min. For primers MC02, MC04, MC07, MN01 and MN02, the PCR conditions were: denaturation at 96°C for 2 min, 35 cycles at 96°C for 20 sec, 59°C for 20 sec, and 72°C for 30 sec and a final cycle of 72°C for 7 min. The PCR components were identical to those described in section 5.2.2, using 5 µl of isolate WB C6 as a DNA template for the

positive control. Primers that successfully amplified microsatellite fragments were selected and used in the following reactions on clinical samples of *G. duodenalis* isolates. The DNA templates (5 µl) used in the PCR reactions for each assemblage were isolates CATG18 (A), HM015 (B), DOGC12 (C), DOGC14 (D), BV529 (E), CATC28 (F), and the control isolate WB C6 (A). The PCR products of each reaction were electrophoresed on 1% ethidium bromide-stained agarose gels and visualised by UV light.

Table 5. 2 The repeat regions of the microsatellites and designed primers

Repeat sequence	Type of region	Primer	Primer sequence	Predicted PCR product size (bp)
$(CACCA)_6$·CA	microsatellite	Forward MC01F Reverse MC01R	5′ - GCCTTTGGTGCTCAGTTTGT - 3′ 5′- CCATCAACCTGAACTGTACAACA - 3′	240
		Forward MC02F Reverse MC02R	5′ - GAATATCCCTGCCTTTGGTG - 3′ 5′ - CCATCAACCTGAACTGTACAACA - 3′	250
$(CT)_{18}$·C	microsatellite	Forward MC03F Reverse MC03R	5′ - CACGCAGCCCATCACTACTA - 3′ 5′ - GTTTCCTTTTTCCGTCAGGA - 3′	182
		Forward MC04F Reverse MC04R	5′ - GAGCTGTGTAGCCACCAACA - 3′ 5′ - GTTTCCTTTTTCCGTCAGGA - 3′	249
$(GTT)_9$·G	microsatellite	Forward MC05F Reverse MC05R	5′ - CGACGCTCTTGCTCTTCTTT - 3′ 5′- CTCCTACACGTCAGCAGCAG - 3′	223
$(CAA)_{10}$	microsatellite	Forward MC06F Reverse MC06R	5′ - CTCCGCCCTCCTATTCTTCT - 3′ 5′ - AGCTTCTTACGGCATCCTGA - 3′	198
$(TTG)_{10}$	microsatellite	Forward MC07F Reverse MC07R	5′ - AGCTTCTTACGGCATCCTGA - 3′ 5′ - AGCGGATAGGTTCACACGAC - 3′	242
$(TGTA)_6$·T	microsatellite	Forward MC08F Reverse MC08R	5′ - TAGGTAGTTGCGTGCCTCCT - 3′ 5′ - GTTGGGCATTGGGTTTAATG - 3′	207
$(GGTAATGGTA)_{73}$	minisatellite	Forward MN01F Reverse MN01R	5′ - AGTTCAAGCCTCACCACAGG - 3′ 5′ - CCCACAGGTTTCACTTTGTC - 3′	790
$(GGGTGATAGA)_3$·GGGTGATAG	minisatellite	Forward MN02F Reverse MN02R	5′ - GGGAAGGCGTGACTTAGGTA - 3′ 5′ - TTGGCTGCTGTGCATATCTC - 3′	219

5.3. Results

5.3.1 Sequence analysis of the β-giardin gene from human isolates

Of the 60 human samples sequenced and genotyped using the ssu rRNA gene, only 11 were successfully sequenced for the *bg* gene: four assemblage A, and seven assemblage B. Other samples were successfully amplified by PCR, but appeared faded on agarose gel electrophoresis, and multiple bands were not successfully sequenced (Figure 5.1).

In the *bg* gene analysis, the positive control was obviously identified as assemblage A1, whilst the investigated samples gave results that corresponded to the ssu rRNA analysis (Chapter 4). Of the four assemblage A samples identified, analysis of the 511 bp fragment of the *bg* gene detected assemblage A3 in three samples, and assemblage A2 in one sample (Figure 5.2, Appendix 5).

All samples classified as assemblage B by analysis of ssu rRNA were aligned with all reference sub-genotypes. None of the seven assemblage B samples identified were identical to the representative reference sequences. In the current study, the isolates HM015, HM017, HM046 and HM049 were identical to each other, but all had the same three single nucleotide polymorphisms (SNPs) which differed from the published isolate ISSGd167 (GenBank accession number EU637579; Table 5.3) (Caccio et al. 2008). The human isolate HM001 had three SNPs that differed from assemblage B3 (99% similarity), whilst the isolate

HM031 was classified as assemblage B3 with mixed infection with assemblage B4. The human isolate HM062 was 99% identical to assemblage B3 with two SNPs, but was 100% identical to isolate GL5 (GenBank Accession number FJ971438) and isolate GL279 (GenBank Accession number 971466) (Figure 5.3, Table 5.3, Appendix 6).

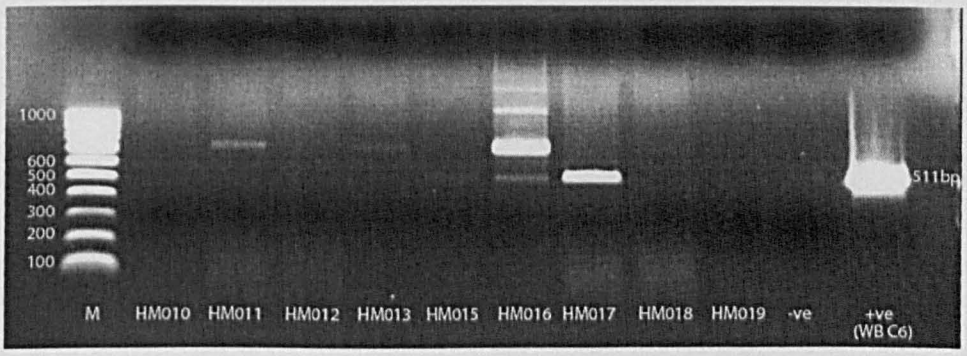


Figure 5.1 Electrophoresis results of human samples after PCR amplification of the *bg* gene. A 511 bp fragment of the *bg* gene was amplified. Lane M is the molecular marker (bp). The positive and negative controls are in the lanes on the right.

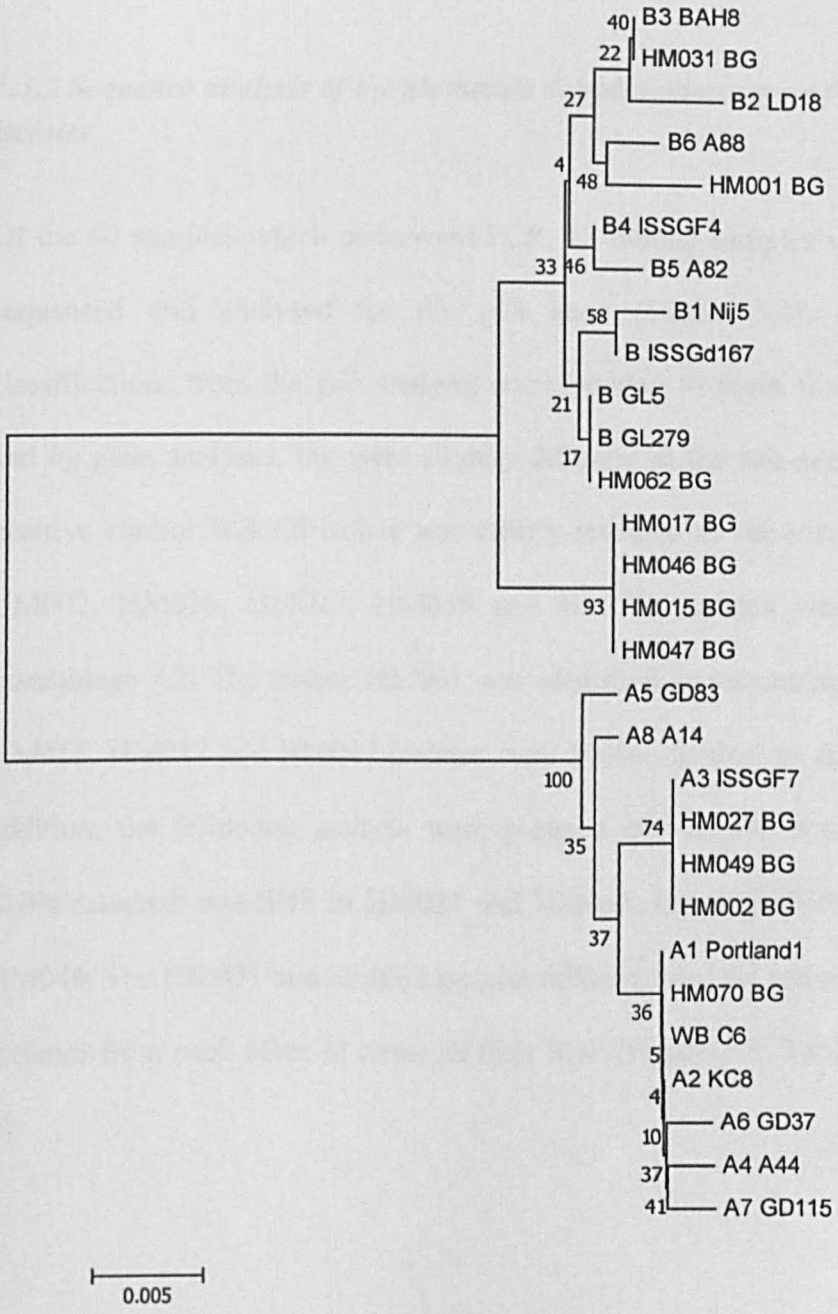


Figure 5.2 Phylogenic relationship of *G. duodenalis* human samples inferred by neighbour-joining analysis of the β-giardin nucleotide sequences. Only bootstrap values >90 are indicated.

5.3.2 Sequence analysis of the glutamate dehydrogenase gene from human isolates

Of the 60 samples which underwent PCR, 13 human samples were successfully sequenced and analysed for the *gdh* gene (Figure 5.4). The assemblage classifications from the *gdh* analysis corresponded to those from the ssu rRNA and *bg* gene analyses, but were slightly different at the sub-genotype level. The positive control WB C6 isolate was clearly revealed as sub-assemblage A1. The HM002, HM026, HM027, HM049 and HM070 isolates were genotyped as assemblage A2. The isolate HM001 was identified as assemblage B3, whilst the HM015, HM017 and HM047 isolates were 100% identical to assemblage B4. In addition, the following isolates were grouped into assemblage B4, with some SNPs detected: one SNP in HM031 and HM062, and four SNPs in HM028 and HM046. The HM031 and HM062 isolates differed from the reference B4, but also differed from each other in terms of their SNP (Figure 5.5, Table 5.3, Appendix 7).

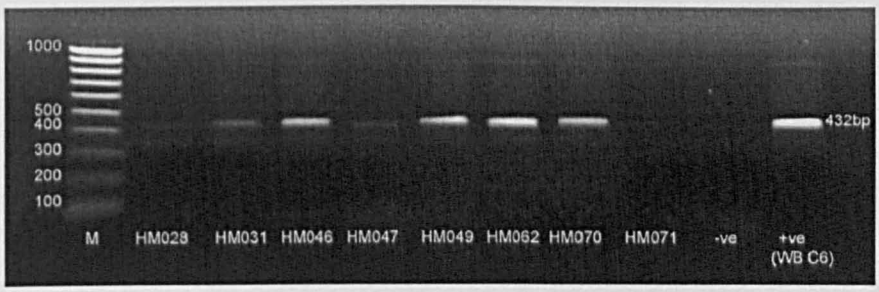


Figure 5.3 Electrophoresis results of human samples after PCR amplification of the *gdh* gene. A 432 bp fragment of the *gdh* gene was amplified. Lane M is the molecular marker (bp). The positive and negative controls in the lanes on the right.

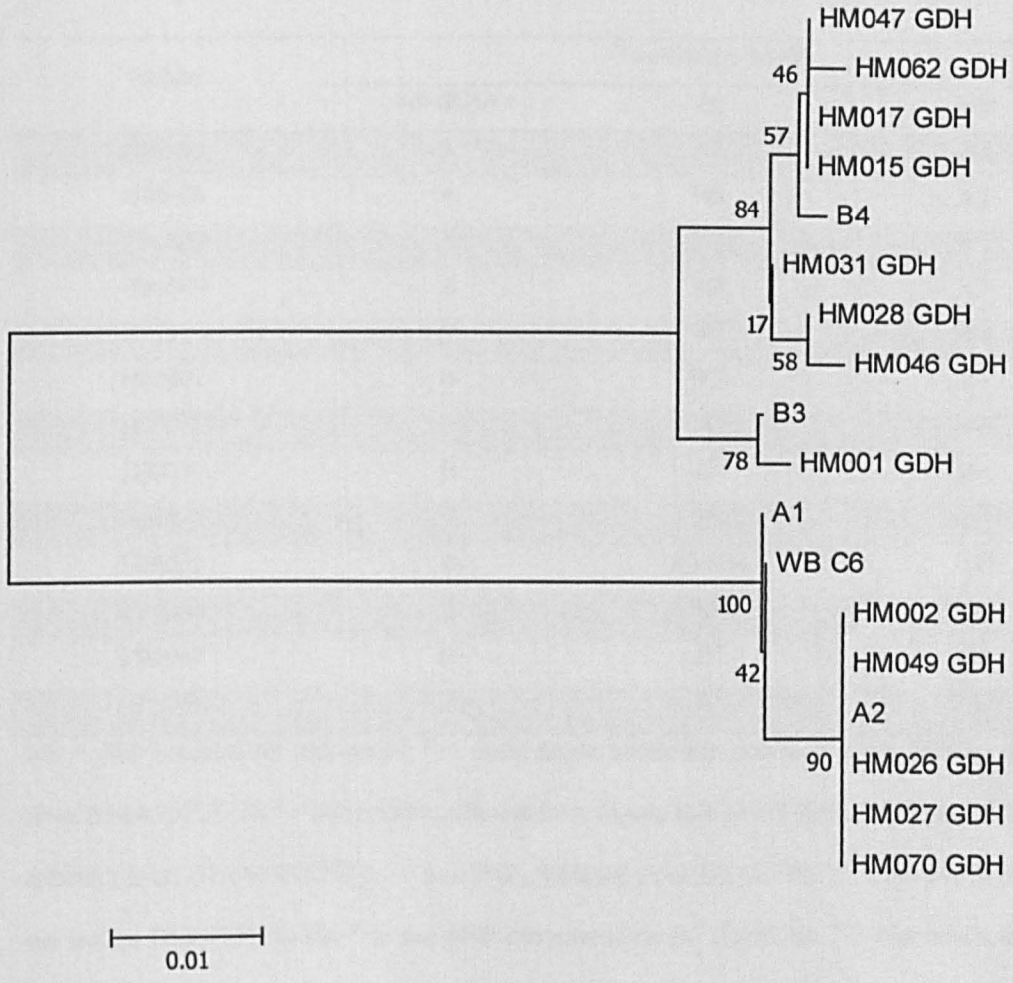


Figure 5.4 Phylogenic relationship of *G. duodenalis* human samples inferred by neighbour-joining analysis of the glutamate dehydrogenase nucleotide sequences. Only bootstrap values >90 are indicated.

Table 5. 3 Genotypes of *G. duodenalis* isolates from human samples examined in the current study by sequence analysis of the ssu rRNA, *bg* and *gdh* genes

Isolate	Genotyping results		
	ssu rRNA	*bg*	*gdh*
HM002	A	A3	A2
HM026	A	NS	A2
HM027	A	A3	A2
HM049	A	A3	A2
HM070	A	A2	A2
HM001	B	B3[a]	B3
HM015	B	B[b]	B4
HM017	B	B[b]	B4
HM028	B	NS	B4[e]
HM031	B	B3+B4	B4[d]
HM046	B	B[b]	B4[c]
HM047	B	B[b]	B4
HM 062	B	B[c]	B4[d]

NS = Not successfully sequenced; [a] = three single nucleotide polymorphisms (SNPs) different from B3 (AF072727); [b] = three SNPs different from isolate ISSGd167 (EU637579) and four SNPs different from B1 (AY072725); [c] = two SNPs different from B3 (AY072727) but 100% match to the isolate GL5 (FJ971438); [d] = one SNP different from B4 (L40508); [e] = four SNPs different from B4

5.3.3 Sequence analysis of the β-giardin gene from dog and cat isolates

In total, 13 dog isolates were successfully amplified and sequenced for the *bg* gene (Figure 5.5).

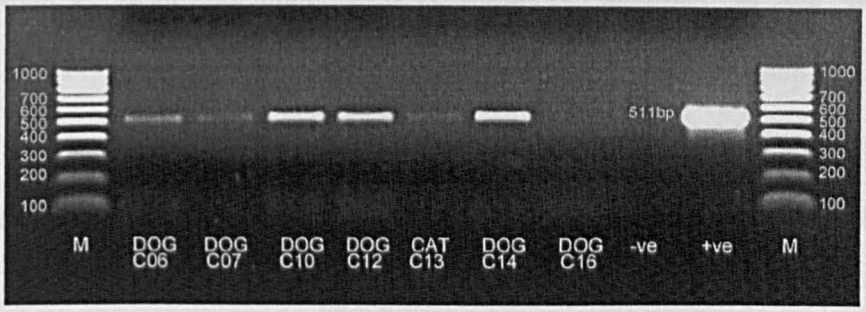


Figure 5.5 Electrophoresis results of dog and cat samples after PCR amplification of the *bg* gene. A 511 bp fragment of the *bg* gene was amplified. Lane M is the molecular marker (bp). The positive and negative controls are in the lanes on the right.

All sequences agreed with previous ssu rRNA analysis (Chapter 2). Only isolate DOGD11 was completely identical to the reference assemblage C, while the isolate DOGC14 was 99% identical (one SNP). Heterogeneous sequences were observed (nucleotides identified as Y, K, S, R, M) in some samples, and mixed infections of assemblages C and D1 were detected in isolates DOGC05 and DOGG08 (Figure 5.7). Samples previously classified as assemblage D by ssu rRNA analysis were identified as assemblage D1 in the current study. However, the isolate DOGD07 had five SNPs, and the isolate DOGC02 was found to be a

mixed infection of assemblage C and D1 (one SNP from assemblage D1) (Figure 5.6, Table 5.4, Appendix 8).

Four cat samples were successfully amplified and sequenced at the *bg* gene. In accordance with the ssu rRNA genotyping, all cat isolates were identified as assemblage F, although none were completely identical to the reference assemblage F. The isolates CATC28, CATC48 and CATD19 displayed 100% identity to the reference isolate Swecat013, whilst the isolate CATC13 contained five SNPs that differed from assemblage F (AY647264) and two SNPs that differed from the isolate Swecat013 (Figure 5.6, Table 5.4, Appendix 8).

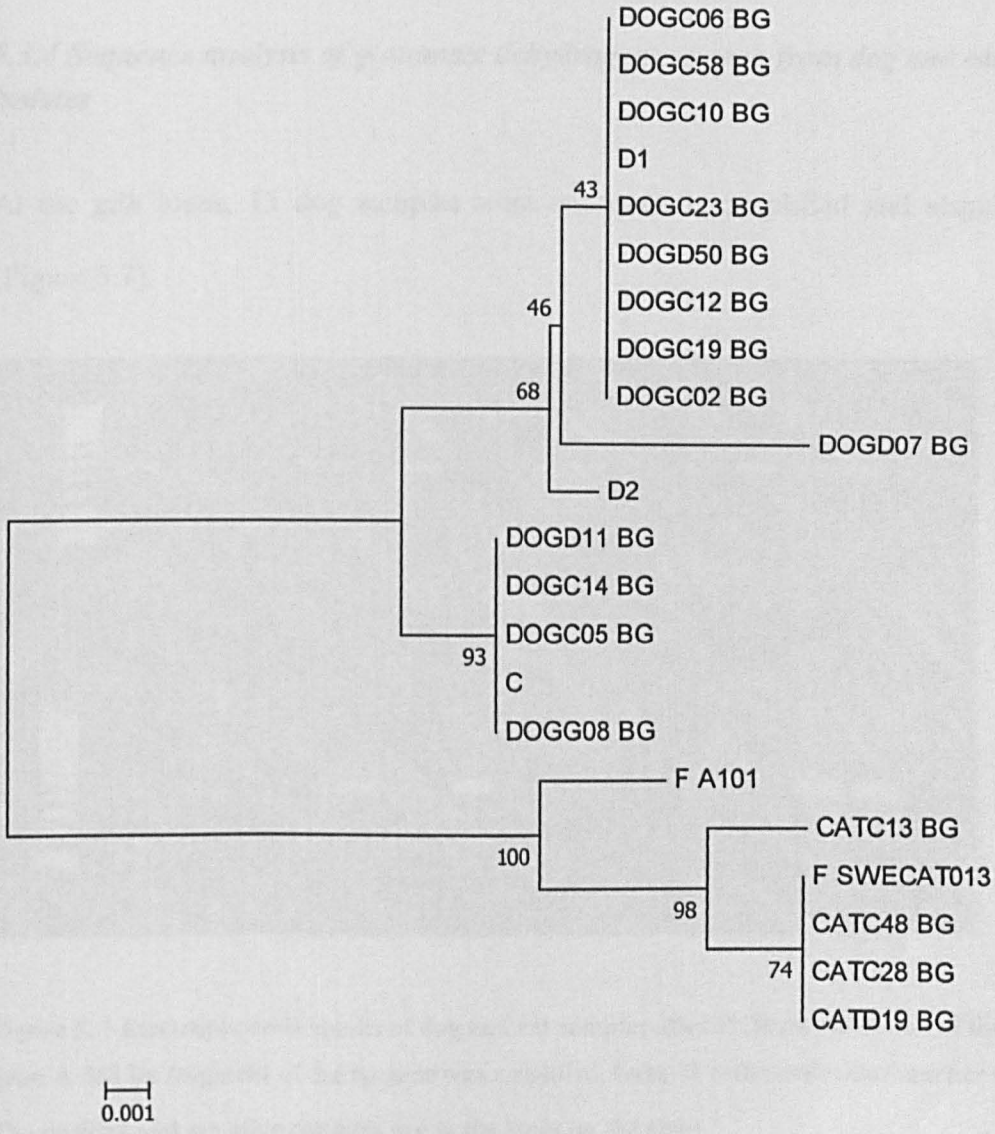


Figure 5.6 Phylogenic relationship of *G. duodenalis* dog and cat samples inferred by neighbour-joining analysis of the β-giardin nucleotide sequences. Only bootstrap values >90 are indicated.

5.3.4 Sequence analysis of glutamate dehydrogenase gene from dog and cat isolates

At the *gdh* locus, 13 dog samples were successfully amplified and sequenced (Figure 5.7).

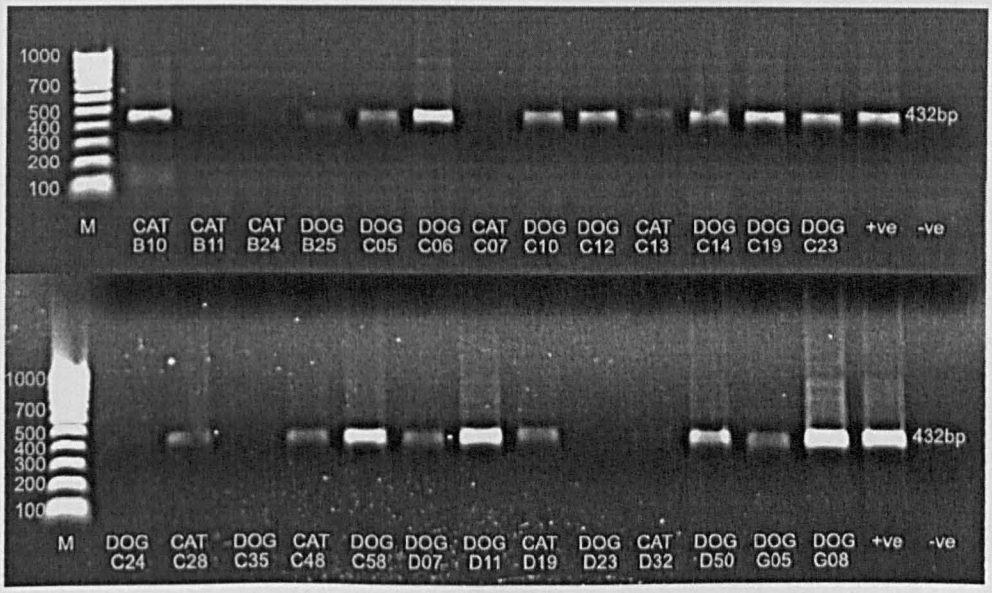


Figure 5. 7 Electrophoresis results of dog and cat samples after PCR amplification of the *gdh* gene. A 432 bp fragment of the *bg* gene was amplified. Lane M is the molecular marker (bp). The positive and negative controls are in the lanes on the right.

Of these, five samples were classified as assemblage C and eight as assemblage D. The assemblages genotyped in all 13 samples corresponded to those obtained by sequencing the ssu rRNA and *bg* genes. Within assemblage C, only isolate DOGC14 had one SNP, whilst all other isolates identified as assemblage C were 100% identical. For assemblage D, only isolate DOGC19 and DOGC23 were

100% identical to the reference sequence, whereas the following isolates contained a number of SNPs: one in DOGD07, DOGD50 and DOGC06, and two in DOGC58, DOGC10 and DOGC12 (Figure 5.8, Table 5.4, Appendix 9).

All five cat samples were successfully amplified and sequenced. By genotyping at the *gdh* locus, all isolates were recognised as assemblage F, corresponding to those previously analysed by ssu rRNA and *bg* genotyping. No SNPs were detected in any assemblage F identified (Figure 5.8, Table 5.4, Appendix 9).

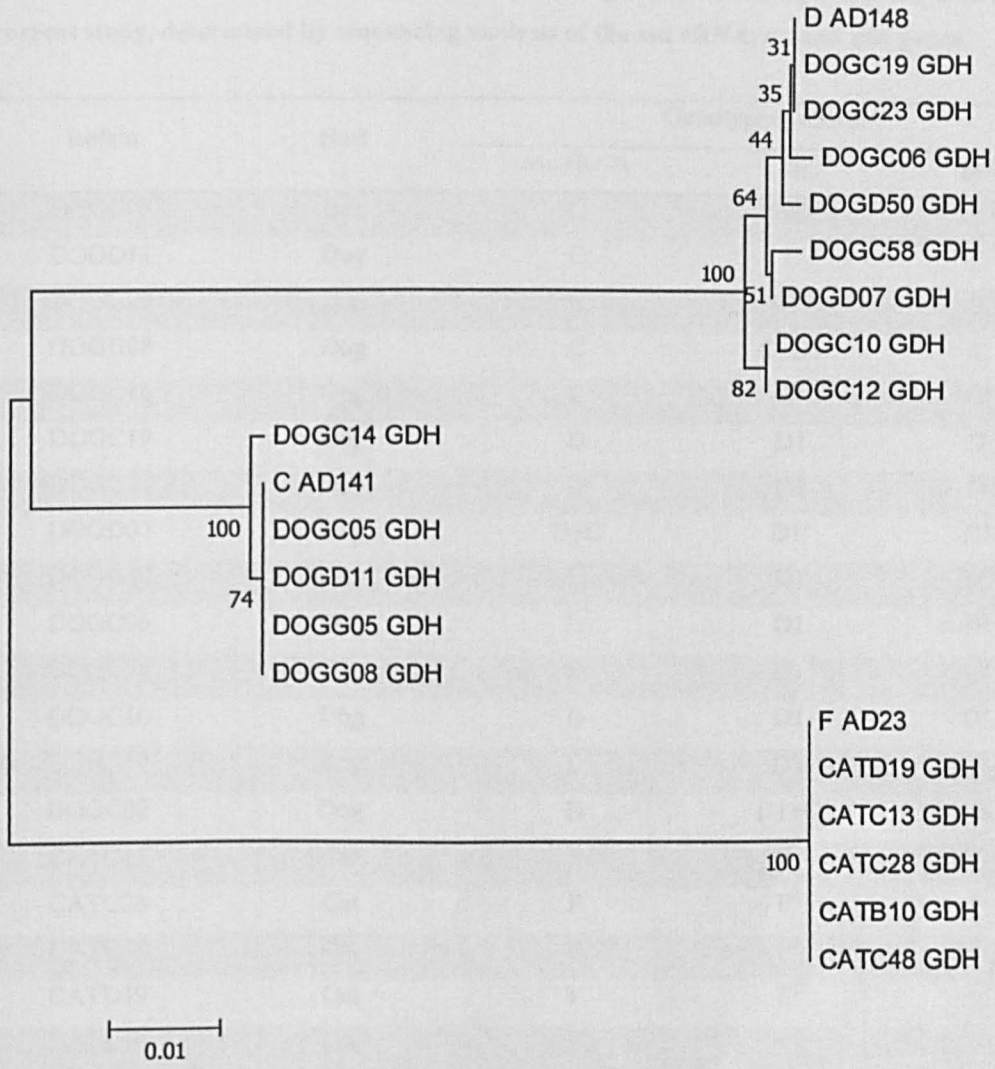


Figure 5.8 Phylogenic relationship of *G. duodenalis* dog and cat samples inferred by neighbour-joining analysis of the glutamate dehydrogenase nucleotide sequences. Only bootstrap values >90 are indicated.

Table 5.4 Genotypes of *G. duodenalis* isolates from dog and cat samples examined in the current study, determined by sequencing analysis of the ssu rRNA, *bg* and *gdh* genes

Isolate	Host	Genotyping results		
		ssu rRNA	*bg*	*gdh*
DOGC05	Dog	C	C+D1	C
DOGD11	Dog	C	C	C
DOGG05	Dog	C	NS	C
DOGG08	Dog	C	C+D1	C
DOGC14	Dog	C	C[a]	C[a]
DOGC19	Dog	D	D1	D
DOGC23	Dog	D	D1	D
DOGD07	Dog	D+C	D1[c]	D[a]
DOGD50	Dog	D	D1	D[a]
DOGC06	Dog	D	D1	D[a]
DOGC58	Dog	D	D1	D[b]
DOGC10	Dog	D	D1	D[b]
DOGC12	Dog	D	D1	D[b]
DOGC02	Dog	D	D1+C	NS
CATC13	Cat	F	F[e]	F
CATC28	Cat	F	F[d]	F
CATC48	Cat	F	F[d]	F
CATD19	Cat	F	F[d]	F
CATB10	Cat	F	NS	F

NS = Not sequenced; [a] = one SNP different from reference representative sequence; [b] = two SNPs different from reference representative sequence; [c] = five SNPs different from reference D1; [d] = five SNPs different from F (AY647264) but identical to isolate Swecat013 (EU769219); [e] = five SNPs different from F (AY647264), and two SNPs different from isolate Swecat013

5.3.5 Analysis of microsatellite markers

Of the microsatellite primer sets tested, primers MC05, MC06 and MC07 amplified fragments of 223 bp, 198 bp and 242 bp, respectively, whereas the primers MC01, MC02, MC03, MC04, MC08, MN01 and MN02 gave neither noticeable bands nor predicted results.

Subsequently, the primer sets MC05, MC06 and MC07 were used to amplify the clinical samples as described in section 5.2.4.2. Unfortunately, no positive results were detected, apart from the positive control, WB C6 isolate. Therefore, the microsatellite markers could not be analysed and compared with *bg* or *gdh* in the current study.

5.4 Discussion

The current study was a multilocus genotyping study of *G. duodenalis* in humans, dogs and cats, using three widely used genetic markers. Using the small subunit ribosomal RNA (ssu rRNA) gene as a marker, *Giardia* assemblages were clearly distinguished (Chapters 2–4). This gene is widely used for screening for *G. duodenalis*, and enables molecular genotyping at the assemblage level. By genotyping with *bg* and *gdh*, *G. duodenalis* can be distinguished to sub-assemblage level. In this study, a level of polymorphism was detected.

In the present study, all human isolates classified as assemblage A displayed 100% matches to the reference sequences, as shown by sequencing both *bg* and *gdh*. However, by sequencing *gdh*, all instances of assemblage A were typed as A2 whereas, by sequencing *bg*, only isolate HM070 was classified as A2 whilst isolates HM002, HM027 and HM049 were identical to the reference A3 (Figure 5.2). These results differed from those of a study by Lalle et al (2005b) who used *bg* as a genetic marker and could discriminate polymorphisms in assemblage A that formed eight sub-groups (A1–A7 in human isolates and A8 in an animal isolate). By analysis of *bg* using PCR-RFLP, a mixed infection between assemblage A2 and A3 was detected in a clinical sample in one study (Caccio et al. 2002). In the current study, at the *gdh* gene, all samples belonging to assemblage A showed the sub-genotype A2, which is associated only with human infection (Thompson 2000).

For assemblage B, the current study revealed high heterogeneity compared to the assemblage A isolates, which corresponded with previously published studies (Caccio et al. 2008; Lebbad et al. 2008). At the *bg* gene, isolate HM001 was likely to be classified as assemblage B3, similar to the result from typing *gdh*, but differed by three single nucleotide substitutions from the reference isolate BAH8 (Figure 5.3). The current multilocus genotyping (MLG) analysis suggested that the isolate HM001 may belong to assemblage B3.

At the *gdh* gene, isolates HM015, HM017 and HM047 were all typed as assemblage B4 but, at the *bg* gene, these isolates had four SNPs that differed from the reference isolate Nij5 (AY072725). It should be noted that the reference *bg* sequences used in this study were those submitted to GenBank by Caccio et al (2002) who classified isolate Nij5 as B1 (AY072725), and isolate LD18 as B2 (AY072726). In contrast, other studies, including Caccio et al, defined isolate Nij5 (AY072725) as B4, and isolate LD18 as B3 (AY072726) (Caccio et al. 2008; Lebbad et al. 2010). Therefore, there were differences in the classification of the same isolates from different studies. In this study, based on the analysis of ssu rRNA, *bg* and *gdh* genes, isolates HM015, HM017 and HM047 could be classified as assemblage B4, with a number of polymorphisms.

By typing at the *gdh* gene, isolates HM028 and HM046 had four SNPs that differed from assemblage B4. By typing at the *bg* gene, isolate HM028 was not successfully sequenced, but HM046 was 100% identical to HM015, HM017 and HM047 (Tale 5.3), therefore both isolates could be classified as B4.

By typing at the *bg* gene, a heterogeneous sequence (the presence of two overlapping nucleotide peaks at specific positions) was found in isolate HM031, which showed characteristics of both the reference sequences B3 and B4 (see Appendix 6). Therefore, this isolate was classified as a mixed infection of B3 and B4 – corresponding to a previous MLG study that observed SNPs between sub-assemblages (Caccio et al. 2008). At the *gdh* gene, isolate HM031 was 99% identical to assemblage B4 but showed heterogeneity and one SNP, therefore on the basis of typing three genes, this isolate was classified as infected with *G. duodenalis* assemblages B3 and B4. Lastly, isolate HM062 provided interesting results as it was 99% identical to assemblage B3, and 100% identical to isolate GL5 (GenBank Accession number FJ971438) and isolate GL279 (GenBank Accession number 971466) (Kosuwin et al. 2010). This sequence (isolate HM062) could be suggested as new sub-assemblage.

In dogs, the overall assemblages typed by the *bg* and *gdh* genes agreed with those classified by ssu rRNA analysis, with some mixed infections detected. By genotyping using *bg*, isolate DOGD11 was found to be completely identical to the reference assemblage C, and isolate DOGC14 revealed a slight polymorphism, whereas isolates DOGC05 and DOGG08 showed mixed infections of assemblages C and D1. In contrast, by typing using *gdh*, an absolute 100% match was detected in four isolates, with one showing a single nucleotide substitution (isolate DOGC14), and no mixed infections identified. This implied that typing at the *bg*

locus was probably able to detect a higher level of diversity compared to typing at the *gdh* locus, particularly in mixed infection cases.

By sequencing *bg*, most of the isolates that were previously classified as assemblage D by ssu rRNA analysis, showed 100% similarity to the reference sequence D1, except one isolate that displayed five SNPs (isolate DOGD07) and two isolates that showed mixed infections (DOGC05 and DOGG08). In the current study, instead of using a single reference assemblage D from isolate Ad-148 (U60906) (Monis et al. 1998; van der Giessen et al. 2006), sequences from isolate A21 (AY545647) and A27 (AY545648) (Lalle et al. 2005a) were downloaded from GenBank as representatives for sub-genotypes D1 and D2, although the sequences from isolate Ad-148 and isolate A21 were identical. No assemblage D2 was detected in this study by genotyping at *bg*. By genotyping assemblage D at *gdh*, only two isolates showed 100% similarity to the reference sequence, whilst three isolates had one SNP, and three isolates contained two SNPs. These results revealed that polymorphisms were detected at a higher rate in assemblage D when typing at *gdh*, compared to *bg*.

By genotyping at *bg*, all cat isolates were classified as assemblage F, agreeing with the results from the ssu rRNA analysis. However, three of the four isolates contained five SNPs compared to the reference sequence (isolate A101, GenBank number AY647264), but were 100% identical to isolate Swecat013 (GenBank number EU769219). Interestingly, one cat isolate had five single nucleotide substitutions from the reference assemblage F, and two SNPs compared to isolate

Swecat013. Polymorphisms were also identified in assemblage F by genotyping at the *bg* locus. By typing at the *gdh* locus, all five cat isolates exhibited a 100% match to the reference assemblage, with no polymorphisms detected. Taking into account genotyping at both genes, results in the current study were comparable to the study by Lebbad et al (2010), who found a high degree of polymorphism in cat isolates, naming the suggested novel assemblages "F new I" for isolate Swecat013, and "F new II" for the other three isolates by typing *bg* sequences. These authors also demonstrated these new sequences of assemblage F by typing using the *gdh* gene (Lebbad et al. 2010).

In the analysis of microsatellite markers, although microsatellite primers MC05, MC06 and MC07 could successfully amplify the fragments of *G. duodenalis* at the correct sizes in the positive control, they could not amplify the clinical samples that had a high DNA concentration and had been successfully sequenced by ssu rRNA analysis. It should be noted that the positive result detected in this study was from isolate WB C6 (assemblage A1), which was the *in vitro* culture and may be different from those samples collected from clinical cases. Ideally it would be helpful to have primers designed from each assemblage to optimise PCR conditions, allowing use of the correct annealing temperature for each assemblage.

Microsatellites have been shown to be useful for the investigation of molecular characterisation in other organisms. For example, microsatellites discriminated *Cryptosporidium parvum* genotype H into two sub-genotypes and genotype C into

four sub-genotypes, all of which varied in their characterisation of expansions or contractions of the microsatellite repeats (Caccio et al. 2000). In the Central Africa sub-region, seven microsatellite markers were used to analyse the population genetic structure of *Trypanosoma brucei gambiense* isolates, and found a low level of genetic polymorphisms within these regional isolates (Simo et al. 2010). As microsatellites have never been developed in *G. duodenalis* before, but have been demonstrated as effective in several previous studies, further development of microsatellite makers should be of interest.

Optimising PCR conditions by gradient PCR of microsatellite markers was also performed in the current study, but a number of unspecific bands or faded bands were observed (data not shown). Additionally, due to time constraints, fully working, specific and effective PCR for microsatellite markers could not be established. It is important that newly designed microsatellite primers are generated from available *G. duodenalis* sequences and investigated. The optimisation of PCR conditions, including PCR components, annealing temperature, and running time, is also essential. Eventually, more reference DNA samples should be tested in order to evaluate the microsatellite primers.

In conclusion, multilocus genotyping is useful for the study of molecular characterisation in *G. duodenalis*. The MLG technique could be used to investigate parasitic sub-groups at a detailed level, which would help to better understand the role of *G. duodenalis* zoonotic transmission, particularly with regard to assemblages A and B. Thus, further extensive analysis of sub-

assemblages A and B in both humans and animals is needed. Moreover, a high number of polymorphisms were identified in this study, and a broad diversity of *G. duodenalis* genetic characterisation was demonstrated. This may bring difficulties in classifying the taxonomy of *G. duodenalis* assemblages. The heterogeneity detected in this study suggests that mixed infections or mutations within parasitic isolates can occur in clinical samples.

Chapter 6 *Giardia duodenalis* proteomics

6.1 Introduction

Proteins are part of all living organisms and are produced in every cell. The study of proteins is called proteomics, and can lead to an understanding of differences in protein expression under different conditions. These changes may be important for medical or veterinary science. For example, in pathogen biology, proteomics can help us better understand host-pathogen relationships or roles of proteins in invading host cells in some apicomplexans (Wiersma et al. 2004; Holder and Veigel 2009; Wastling et al. 2009).

Typically, the experimental techniques used in proteomics separate proteins according to a particular physical property, and subsequently identify them by mass spectrometry. Amongst the more commonly used methods for the initial separation of proteins are gel-based separation techniques. For example, one-dimensional polyacrylamide gel electrophoresis (1D-PAGE) separates proteins in one dimension by their molecular mass. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) further aids the visualisation of proteins by first separating them by their iso-electric point (p*I*) in the first dimension (isoelectric focusing), and then separating them according to their mass or size on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. Another technique used to separate proteins is high performance liquid

chromatography (HPLC) using a column that retains (and thereby separates) proteins according to their physical or chemical properties prior to mass spectrometry. Combining the 2D gel technique with HPLC and mass spectrometry (and then comparing the results against a genomic database) identifies the proteins with better accuracy than either technique alone.

The proteomics of *Giardia* have been studied for several years, with the purpose of identifying protein expression, structural proteins, and the location of proteins from different life cycle stages (Smith et al. 1982; Capon et al. 1989; Steuart et al. 2008). For instance, the structure and protein composition of the *Giardia* attachment disk have been published, and the disk SF-assemblin-like protein (SALP-1) has also been characterised (Palm et al. 2005). In other studies, the leucine-rich polypeptides, cyst wall protein 1 (CWP1) and cyst wall protein 2 (CWP2), were found to be the main components up-regulated during the encystation stage, and transported through the encystation-specific vesicles (ESV) (Lujan et al. 1995; Mowatt et al. 1995).

As described in the previous chapters, *G. duodenalis* assemblage A and B affect a broad range of hosts and are considered as potentially zoonotic genotypes, whilst the other assemblages are likely to be host-specific. Cultures of the A and B genotypes are now available; therefore both assemblages can be studied in detail. As the *G. duodenalis* assemblage A genome has been completed (Morrison et al. 2007), and is accessible through GenBank or GiardiaDB (http://www.giardiadb.org/giardiadb/) (Aurrecoechea et al. 2009), an increasing

number of papers on *G. duodenalis* proteomics are being published. However, although many authors have investigated many aspects of *G. duodenalis* proteins, these studies have been based on different isolations, geographical regions, hosts and individuals. Using 2D-PAGE, Steuart et al (2008) identified an assemblage A-specific protein of human infective *G. duodenalis*, alpha 2 giardin – a known *G. duodenalis* protein (Palm et al. 2003). Although several protein spots were also detected uniquely in assemblage B in this study, they were not identified, as no genomic database was available for assemblage B at that time (Steuart et al. 2008). Following recent updates, assemblage B genomic data is now accessible on GiardiaDB (Aurrecoechea et al. 2009).

The aim of the current study was to identify the differential expression of *G. duodenalis* trophozoite proteins between assemblage A and assemblage B using 2D-SDS PAGE, with differential in gel electrophoresis (DIGE) technology, and mass spectrometry.

6.2 Materials and methods

6.2.1 Preparation of Giardia samples

Cultures of *G. duodenalis* assemblage A (WB C6) and assemblage B (GS-M-83-H7) were kindly provided by Prof. Andrew Hemphill at the Institute für Parasitologie at the University of Bern, Switzerland. Both genotypes were grown under anaerobic conditions in 10 ml culture tubes (Nunc, Roskilde, Denmark) containing TYI-S-33 medium (Keister 1983). Each *Giardia* assemblage was prepared in 4 replicates consisting of $2.5x10^7$ trophozoites per tube. Frozen *Giardia* samples were transported to the Department of Veterinary Preclinical Sciences, the Faculty of Veterinary Science, University of Liverpool, UK, in a sealed dry-ice safety box before being processed as detailed below.

6.2.2 Protein lysis and preparation

Four replicates of *Giardia* assemblage A, namely A1, A2, A3 and A4, and four replicates of assemblage B, namely B1, B2, B3 and B4, were lysed using cell lysis buffer consisting of 8 M urea, 30 mM Tris, 4% (w/v) CHAPS, complete mini protease inhibitor, and DNase, pH 8.5 (on ice). The protein concentration was measured using a BSA-Coomassie assay (Bio-Rad) and samples were adjusted to the same concentration (equal to the lowest measured concentration obtained) and stored frozen until required.

6.2.3 CyDye™ Minimal Labelling and gel allocation

Each *Giardia* replicate sample (A1–A4, B1–B4) was labelled with either Cy3 or Cy5 (Table 6.1). Labelling was performed according to the CyDye™ minimal labelling protocol (GE Healthcare) with a ratio of 400 pmol dye:50 μg protein (30 min on ice, in the dark). 6.25 μg unlabelled protein from each replicate was pooled and labelled with Cy2 as an internal standard reference. The reaction was quenched by adding 1 mM lysine, on ice, for 10 min. Labelled samples were stored frozen at -20°C until required. Prior to experimentation, labelled samples were randomly allocated to one of four gels, with each gel assigned protein from assemblage A, assemblage B, and the internal standard (Table 6.1).

Table 6.1 Gel identification and protein samples labelled with Cy2, Cy3 and Cy5. A and B refer to assemblage A and B, respectively, with numbering (A1, A2, etc.) referring to the biological replicate. STD is the internal standard pooled protein. An unlabelled preparative gel stained with Sypro Ruby was run on gel 5

Gel Id	Cy3	Cy5	Cy2	Sypro
1	A1	B4	STD	-
2	A2	B3	STD	-
3	B1	A3	STD	-
4	B2	A4	STD	-
5	-	-	-	Preparative

6.2.4 First dimension electrophoresis – isoelectric focusing

First dimension electrophoresis required the use of four 24 cm pH3-10 NL Immobiline DryStrips (GE Healthcare), which were rehydrated overnight with 450 µl of rehydration solution (8 M urea, 2% (w/v) CHAPS, 0.5% (v/v) IPG buffer, 20 mM DTT and trace bromophenol). Following this, 50 µg of labelled protein from each sample type (A1–A4, B1–B4, and the internal standard) were combined and made up to a final volume of 100 µl with sample buffer (maximum volume permitted for cup-loading method) and loaded on to the strips using the cup-loading technique. Samples were run overnight on an Ettan IPGphor II (GE Healthcare) using the isoelectric focusing protocol outlined in Table 6.2.

Table 6.2 Isoelectric focusing protocol. Total time was approximately 15 hours. In the constant steps, Voltage was maintained at the level indicated, whilst in the gradient steps, Voltage was increased to the level specified over the time specified. Duration of individual steps was either determined by a fixed length of time (hours) or the completion of a level of Volt hours

Step	Voltage	Constant/Gradient	Duration
1	500	Constant	1 hour
2	1000	Gradient	5200 Volt hours
3	10000	Gradient	3 hours
4	10000	Constant	4 hours

6.2.5 Second dimension Electrophoresis – SDS PAGE

For second dimension electrophoresis, the four experimental 12.5 % (w/v) acrylamide gels were cast simultaneously using the Ettan DALT gel caster (GE Healthcare) and allowed to polymerise overnight. The DryStrips were equilibrated for 15 min in equilibration solution (50 mM Tris, 6 M Urea, 30% (v/v) glycerol, 2% (w/v) SDS, trace bromophenol blue, pH 8.8), initially containing 0.5% (w/v) dithiothreitol (DTT), before a further 15 min in equilibration solution containing 4.5% (w/v) iodoacetamide (IAA), at room temperature. The strips were then applied to the top edge of the gels and sealed into place with 0.5% (w/v) agarose sealing solution and bromophenol. All four gels were run in parallel using the Ettan DALTsix (GE Healthcare) (8 W per gel, 20°C), until the bromophenol dye front reached the end of the gel (approximately 4.5 hr).

6.2.6 Image acquisition and analysis

Gel cassettes were scanned using an Ettan DIGE Imager (GE Healthcare) using the Ettan Imager software provided, and following the manufacturer's standard protocol. Images were scanned at each of the three wavelengths corresponding to the Cy2, Cy3 and Cy5 dyes, and were then imported into DeCyder™ software (GE Healthcare) with spot detection and matching algorithms applied. Statistical

analysis was performed to compare the abundance of individual protein spots, relative to the internal standard, and p-values were analysed using Student's *t*-test.

6.2.7 Spot picking and tryptic digestion

The selected protein spots were introduced as a spot list (x, y coordinates in tab delimited .txt file) into the Spot Picker robot (GE Healthcare), which automatically excised the spots from the gel and transferred them to 96-well plates. The following spot picking parameters (Table 6.3), optimised for bind silane treated glass, were used to minimise the loss of gel plugs.

Table 6.3 Spot picking parameters used in the Spot Picker

Parameter	Amount
Prefill volume	100 µl
Aspirate volume	50 µl
Aspirate flow rate	20 ml.min^{-1}
Dispense volume	150 µl
Dispense flow rate	30 ml.min^{-1}
Jazz (side-to-side oscillation)	1.1 mm

The gel plugs in the 96-well plate were transferred to 0.5 ml microcentrifuge tubes, ready for tryptic digestion. Plugs were destained in 25 mM ammonium bicarbonate and 50% (v/v) acetonitrile (ACN) for 10 min at 37°C. The destaining solution was replaced by 100% (v/v) ACN for 10 min at 37°C, and the solvent

removed. Plugs were allowed to air dry for 10 min. Following this, 10 µl of trypsin solution (25 µg trypsin in 250 µl of 50 mM acetic acid, diluted 1 in 10 with 25 mM ammonium bicarbonate) was added, and incubated for 1 hr at 37 °C before adding 10 µl 25 mM ammonium bicarbonate for an overnight incubation at 37 °C. The reaction was stopped by adding 2 µl of 2.6 M formic acid.

6.2.8 Determination of proteins by tandem mass spectrometry and database search

The tryptically digested proteins were clarified from residual particulate matter by ultracentrifugation before being transferred to a 96-well plate for separation by HPLC (Dionex u3000 HPLC using a c18 reversed phase chromatography column). The eluted peptides were then passed through a PicoTip and ionised at 1.8 kV before being analysed by mass spectrometry. The frequency of ms/ms scans over time was assessed using BioWorks Browser (Thermo Scientific) and areas not containing good quality data were excluded from the subsequent generation of .DTA files using Sequest.

The .DTA files were merged into a single file using a batch command "merge.pl" – a perl program converting .DTA into the Mascot generic format (.MGF file), which could be used for analysis in the Mascot search engine. The database search was carried out using the Mascot web-based interface ms/ms browser accessed from the in-house server. The parameters used for searching are described in

Table 6.4. The criteria set to select positive proteins from the search were a Mascot score of at least 50, and the presence of b or y ion series of at least 70%.

Table 6.4 Parameters used in the Mascot search engine to identify the picked proteins

Parameter	Setting
Database	*Giardia duodenalis* assemblage A and B
Fixed modifications	Carbamidomethylation (C)
Variable modifications	Oxidation (M)
Enzyme	Trypsin
Maximum missed cleavages	1
Peptide charge	1+, 2+ and 3+
Peptide tolerance	± 2.0 Da
MS/MS tolerance	± 0.8 Da
Data format	Mascot generic
Instrument	ESI-TRAP

6.3 Results

6.3.1 2D-DIGE of G. duodenalis assemblage A and assemblage B

In order to investigate protein changes between the two assemblages (A and B), 2D-DIGE was performed. Figure 6.1 displays the representative Gel 1, with a different wavelength for each CyDye™. Cy2 represents the internal standard whilst Cy3 and Cy5 display protein spots from assemblage A and B, respectively. An overlay of all channels is presented in the false colour composite.

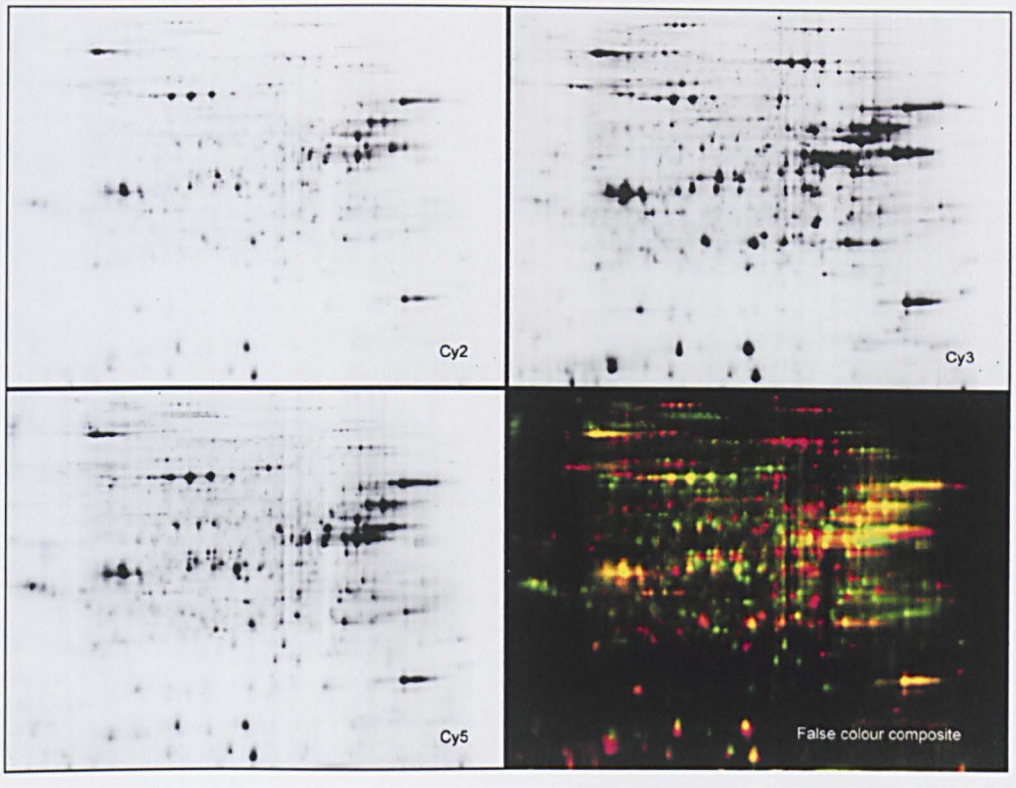


Figure 6.1 The 2D separation of Gel 1 with different wavelengths of CyDye™. *G. duodenalis* assemblage A, assemblage B and the internal standard (pooled proteins from both assemblages) are stained with Cy3, Cy5 and Cy2, respectively. The overlay of all channels is displayed as a false colour composite.

6.3.2 Identification of proteins

To select the protein spots from the preparative gel, the following criteria were set in the DeCyder™ software: proteins present in ≥10 spot maps, proteins with Student's *t*-test value ≤0.001 and average ratio ≥10 or ≤-10, and for proteins in the pick spot map, an internal standard volume of ≥ 2×10^5 and ≤1×10^8. As filtered by the above criteria, 19 spots were selected for protein spot picking (Table 6.5). Other criteria were set to determine number of protein spots selected by the DeCyder™ software (Table 6.6).

Table 6.5 The spots picked by DeCyder™ software before being analysed by tandem mass spectrometry. The selection criteria were as follows: proteins present in ≥10 spot maps, proteins with Student's *t*-test value ≤0.001 and average ratio ≥10 or ≤-10, and for proteins in pick spot map, an internal standard volume of ≥ 2×10^5 and ≤1×10^8

Position	Spot number	Appearance	*t*-test	Spot Vol.	Average Ratio
1	241	13 (13) , P2, P1	8.50E-05	300566	22.3
2	242	13 (13) , P2, P1	5.70E-05	277431	28.03
3	247	13 (13) , P2, P1	0.00036	214353	12.51
4	290	13 (13) , P2, P1	3.50E-06	273301	19.56
5	297	13 (13) , P2, P1	3.70E-05	286722	14.12
6	462	13 (13) , P2, P1	2.00E-05	388763	11.1
7	566	13 (13) , P2, P1	0.00061	383050	-10.28
8	592	12 (13) , P1	6.10E-06	826408	-23.34
9	605	13 (13) , P2, P1	7.80E-05	1611976	-40.17
10	611	13 (13) , P2, P1	0.00011	1654030	-57.67
11	714	13 (13) , P2, P1	6.00E-06	724221	-10.19
12	877	13 (13) , P2, P1	1.20E-05	567313	-17.4
13	918	13 (13) , P2, P1	9.00E-05	283398	-12.67
14	941	13 (13) , P2, P1	8.60E-06	471354	11.87
15	942	13 (13) , P2, P1	3.70E-04	1365279	26.62
16	1130	13 (13) , P2, P1	1.70E-06	451861	18.64
17	1576	13 (13) , P2, P1	2.10E-06	684288	14.04
18	1711	13 (13) , P2, P1	1.20E-06	12627149	15.29
19	3614	13 (13) , P2, P1	4.60E-05	1429503	-32.29

Table 6.6 The number of spots selected by DeCyder™ software with various criteria. All were restricted to proteins present in ≥10 spot maps and the volume of proteins mapped in the internal standard was set to ≥2×10^5 and ≤1×10^8.

Selected proteins with average ratio:	Student's *t*-test p-value		
	≤0.05	≤0.01	≤0.001
≥10 or ≤-10	19	19	19
≥5 or ≤-5	63	92	54
≥2 or ≤-2	192	180	130

Only 17 spots were successfully processed with tandem mass spectrometry. Spot number 592 was not picked due to inadequate appearance, while spot number 462 had a mass spectrometer error. After searching with Mascot, the protein with the highest score was selected from each spot. In total, nine proteins were identified (Table 6.7).

Table 6.7 Proteins with highest score from each spot identified by the Mascot search engine

Spot no.	Protein name	Protein ID	Location	Mass	Length	score	Average Ratio
241	Protein 21.1	GL50581_3278	ACGJ01002726:123268-125628(-)	87735	786	402	22.3
242	Protein 21.1	GL50581_3278	ACGJ01002726:123268-125628(-)	87735	786	78	28.03
247	Protein 21.1	GL50581_3278	ACGJ01002726:123268-125628(-)	87735	786	602	12.51
290	Protein 21.1	GL50581_3278	ACGJ01002726:123268-125628(-)	87735	786	285	19.56
297	Protein 21.1	GL50581_3278	ACGJ01002726:123268-125628(-)	87735	786	111	14.12
566	Arginine deiminase	GL50803_112103	CH991767:992691-994433(+)	65003	580	1045	-10.28
605	Arginine deiminase	GL50803_112103	CH991767:992691-994433(+)	65003	580	1095	-40.17
611	Arginine deiminase	GL50803_112103	CH991767:992691-994433(+)	65003	580	1047	-57.67
714	Malic enzyme	GL50803_14285	CH991767:761437-763110(+)	62431	557	474	-10.19
877	Aminoacyl-histidine dipeptidase	GL50803_15832	CH991767:1175570-1177144(-)	57899	524	666	-17.4
918	NIMA related Kinase (NEK)	GL50803_15409	CH991769:163707-165254(-)	57771	515	1238	-12.67
941	NADH oxidase lateral transfer candidate	GL50581_2357	ACGJ01002302:7124-8500(+)	51056	458	516	11.87
942	NADH oxidase lateral transfer candidate	GL50581_2357	ACGJ01002302:7124-8500(+)	51056	458	787	26.62
1130	Aminoacyl-histidine dipeptidase	GL50581_2286	ACGJ01002289:186-1715(+)	56458	509	356	18.64
1576	NADH oxidase lateral transfer candidate	GL50581_2357	ACGJ01002302:7124-8500(+)	51056	458	404	14.04
1711	Hypothetical protein	GL50803_104250	CH991779:161723-162094(+)	14004	123	352	15.29
3614	Hypothetical protein	GL50803_3910	CH991768:740427-740798(+)	13943	123	209	-32.29

Of the nine detected protein changes, arginine deiminase (ADI), malic enzyme, aminoacyl-histidine dipeptidase GL50803_15832, NIMA-Related Kinase (NEK) and the hypothetical protein GL50803_3910 increased in *G. duodenalis* trophozoite assemblage A, whilst protein 21.1, NADH oxidase lateral transfer candidate, aminoacyl-histidine dipeptidase GL50581_2286 and the hypothetical protein GL50803_104250 increased in assemblage B (Figure 6.2).

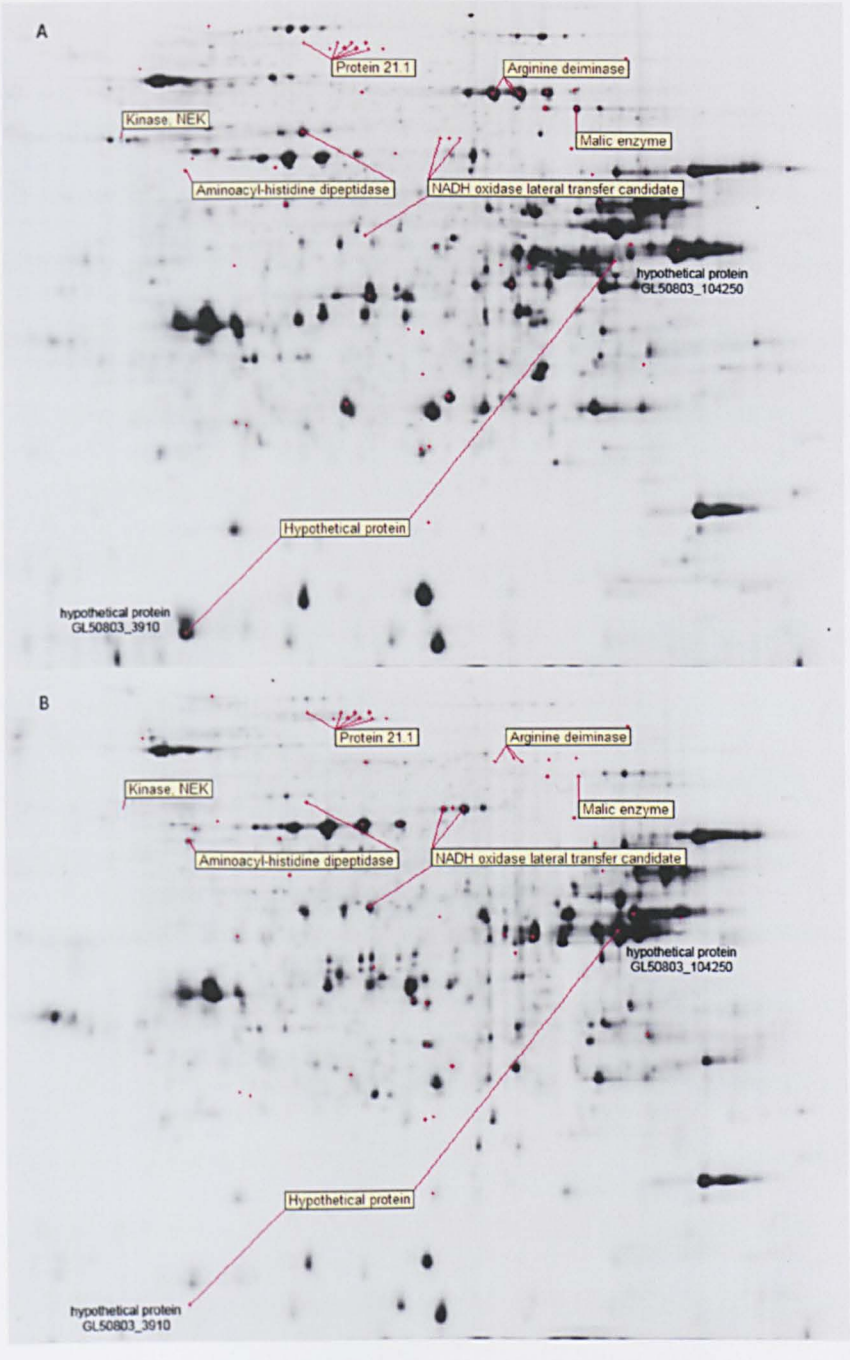


Figure 6.2 Protein spots detected by 2D-DIGE. *G. duodenalis* assemblage A was stained with Cy3 (A), and *G. duodenalis* assemblage B was stained with Cy5 (B).

6.4 Discussion

This study revealed some obvious differentially expressed proteins between *G. duodenalis* assemblage A and B. Using DeCyder™ software, 19 spots of proteins were selected for analysis. In each spot analysed, the top scoring protein hit (as scored by Mascot) was selected. In assemblage A, arginine deiminase (ADI) was found to be up-regulated. Known as a metabolic enzyme, ADI is believed to be used by *G. duodenalis* to produce energy from free L-arginine under anaerobic conditions (Touz et al. 2008). The enzyme also functions as a peptidylarginine deiminase, converting protein-bound arginine into citrulline, and has been successfully purified (Knodler et al. 1997). Additionally, ADI in *G. duodenalis* binds to and citrullinates the arginine in the conserved CRGKA tail of variant-specific surface proteins (VSPs) in particular, affecting both antigenic switching and antibody-mediated cell death. During encystation, ADI translocates from the cytoplasm to the nuclei and has been shown to play a regulatory role in the expression of encystation-specific genes (Touz et al. 2008). In *Giardia*, only one VSP, from a pool of approximately 250 genes present in the genome, is expressed on the surface of trophozoites at any point in time (Morrison et al. 2007). In relation to VSPs, the up-regulated expression of ADI in assemblage A suggests that the capability of this genotype to infect a very broad range of hosts should be further investigated.

Expressed in both genotypes, malic enzyme, as known as malate dehydrogenase (MDH), was identified as increasing in assemblage A. This enzyme is related to

metabolism and has been sequenced previously (Sanchez et al. 1996; Roger et al. 1999). Both assemblages expressed aminoacyl-histidine dipeptidase (GL50803_15832), with a score of 666 in assemblage A and 413 in assemblage B. A study by Morrison et al has described this enzyme and others in *Giardia* (Morrison et al. 2007).

NEK, a cell cycle related protein, was also found up-regulated in assemblage A in the current study. With relevance to this, Kim et al (2009) investigated protein expression changes during encystment in trophozoites, encysting trophozoites and cysts. Twenty protein spots up-regulated during encystation were investigated using Matrix-assisted laser desorption/ionisation (MALDI) in ToF (time-of-flight) mass spectrometry with 14 individual proteins identified. These included cytoskeletal proteins (beta giardin, beta tubulin and alpha-1 giardin), metabolic enzymes (ornithine carbamoyl transferase and vacuolar ATPase catalytic subunit A), heat shock proteins (HSP-70 and HSP-90 alpha) and NEK. It was observed that HSP-70 and HSP-90 both increased in trophozoites, whilst beta tubulin, NEK and vacuolar ATPase all showed no significant increase in gene expression (Kim et al. 2009). On the other hand, in assemblage B, protein 21.1 (unknown function), NADH oxidase lateral transfer candidate, aminoacyl-histidine dipeptidase (GL50581_2286) appeared to increase in expression. The dipeptidase is known as an enzyme with broad substrate specificity and enables organisms to utilise cysteinylglycine as a cysteine source (Suzuki et al. 2001).

The present study also indicated that two different hypothetical proteins were highly expressed in each assemblage. The hypothetical GL50803_3910 protein was up-regulated in assemblage A and the hypothetical GL50803_104250 protein was up-regulated in assemblage B. However, the functions of these proteins are unknown. As seen in this study, there were still more protein expressions were not identified.

Regarding the comparison of the two genotypes, a study by Franzen et al (2009) has recently compared the draft genome sequencing of *G. duodenalis* assemblage B isolate GS to assemblage A isolate WB. The two genomes displayed 77% nucleotide and 78% amino acid identity in protein coding regions. Comparative analyses identified 28 unique GS and 3 unique WB protein coding genes, and the VSP repertoires of the two isolates were entirely different. The authors also suggested that the assemblages could be two different species (Franzen et al. 2009). The Franzen et al study confirmed *G. duodenalis* as a species complex comprising a variety of genomes between assemblages, which could make its taxonomic category more complicated.

In conclusion, although nine proteins were identified in the current study, further investigations of these proteins are needed. For instance, there are several more sensitive quantitative proteomics platforms that could be employed to extend our knowledge of *G. duodenalis* proteins. Examples include the ^{18}O-labeling technique (Miyagi and Rao 2007), absolute quantification (termed AQUA) of proteins (Gerber et al. 2003), isotope-coded affinity tags (ICATs) (Gygi et al.

1999), and metabolic labelling of proteins (Beynon and Pratt 2005). Moreover, further studies could use bioinformatics tools to predict the functions of the nine proteins identified and infer the relationships between them using pathway analysis software such as Ingenuity Pathway Analysis (IPA) (http://www.ingenuity.com/) or Pathway studio (http://www.ariadnegenomics.com/). Finally, to identity specific functions and locate proteins based on their ability to bind to specific antibodies, the western blot technique could be applied.

Chapter 7 Summary and forward perspectives

In the current research, molecular characterisation of *Giardia duodenalis* was performed in human and animal samples obtained from different sources. This was the first large-scale study of *G. duodenalis* genotype distribution in the UK. The aim of the study, which was achieved, was to genetically characterise *G. duodenalis* in dogs, cats, cattle, sheep, pigs and humans using small subunit ribosomal RNA as a PCR marker. Samples were collected from different sources under different conditions. Dog and cat faecal samples were obtained from a commercial laboratory and were confirmed to be *Giardia* positive by microscopy. Human samples were obtained from diarrhoeic hospital patients infected with Giardiasis. Whilst domestic pet and human samples were from symptomatic Giardiasis cases, samples from farm animals consisted of diarrhoeic faeces without confirmed presence of *G. duodenalis* or other parasites. Any negative results in the farm animal analyses were not interpreted, as these animals were not infected with *G. duodenalis*.

The second chapter described the molecular genotyping of *G. duodenalis* in dogs and cats. As all dog and cat samples were previously identified as *Giardia* positive by conventional microscopic diagnosis, the actual prevalence might be higher than estimated here, so the overall prevalence of *G. duodenalis* in dogs and cats in the UK could not be determined. Unsurprisingly, both of the canine-specific genotypes C and D were detected in a high proportion of animals.

Assemblages A and B, considered as zoonotic genotypes due to their capability to infect both humans and animals, were rarely found (A) or not detected (B) in this study. Consequently, dogs may not be an important source of zoonotic genotypes. The gender, age and breed of dogs were not related to the *G. duodenalis* genotypes found.

Whilst most cats in the present study carried the feline-specific genotype assemblage F, the zoonotic genotype A was detected in some isolates. Although this study was not a true survey of Giardiasis in dogs and cats, it provided a perspective on the distribution of *Giardia* genotypes in domestic pets in the UK, given that dogs carry canine-specific assemblages and cats carry a feline-specific assemblage. The mixed infections found in dogs and cats in the current study indicate that the animals in question have ingested sources contaminated either by different assemblages or by a heterogeneous mixture of *G. duodenalis*.

In chapter 3, *G. duodenalis* assemblages were genotyped in clinical samples from cattle, sheep and pigs. Similar to other studies in many parts of the world, the current study demonstrated that assemblage E was the predominant genotype in cattle and sheep. Although farm animals have been reported to carry the zoonotic assemblages A and B (Lalle et al. 2005b; Trout et al. 2005; Winkworth et al. 2008), assemblage B was not identified in this work, whereas assemblage A was detected either alone or in mixed infection with the 'hoofed livestock' specific genotype E. This suggested that the distribution of *G. duodenalis* genotypes in the UK might be affected by geographical location.

Interestingly, the dog-specific assemblages C and D were detected in cattle, sheep (assemblage D only) and pigs, and the cat-specific assemblage F was detected in pigs. This may imply cross-transmission between different hosts of *G. duodenalis*, confirming the complexity of this organism. Consequently, the role of these animals as reservoir hosts of Giardiasis for potential human infection may need further investigation.

This study was carried out with only a single molecular marker, the ssu rRNA, and therefore confirmation of the presence of these canine and feline genotypes at other loci or by other techniques may be required. The present study verified that farm animals can act as a potential zoonotic source, since the zoonotic genotypes were detected. Farm animals can be affected by Giardiasis as a primary infection or in combination with other pathogens (O'Handley et al. 1999). In the present study, no parasites other than *G. duodenalis* were examined in the farm animal samples, therefore it could not be concluded that *G. duodenalis* was the etiological pathogen for the animal symptoms.

Taking into account the results from the pet and farm animal analyses, the current research revealed that assemblage A, the zoonotic potential genotype, is present in animals in the UK. Even though this genotype was detected at a low rate, it should be suspected as the source of human Giardiasis.

In chapter 4, as all humans samples had been confirmed as positive for the parasite, the study was defined as the molecular characterisation of *G. duodenalis* in symptomatic cases. Assemblages A and B, the only genotypes known to infect

humans (Thompson 2004), were clearly detected in this study, with no incidences of any other assemblages. Whilst assemblage B (72%) was found more frequently than assemblage A (28%), the infection rates were not significantly different. No correlation was found between *G. duodenalis* genotype and patient gender or history of travelling. However, there was a significant linear trend for an increased isolation of assemblage A in older subjects (Chi-square test for trend, p=0.0497). This result was based on patients with diarrheic symptoms in the UK.

Many studies have demonstrated various proportions of infection rates with assemblage A and B in symptomatic cases of Giardiasis. For example, a study in the Netherlands detected assemblage B in 65% of patients and assemblage A in 35% of patients (van der Giessen et al. 2006). In a study in Mexico, all 19 patients harboured assemblage A (Ponce-Macotela et al. 2002), and in Bangladesh, all 29 diarrhoeic patients were infected with assemblage A (Haque et al. 2005). A study in Peru found that 19 patients showing diarrhoea carried assemblage A, whilst six asymptomatic cases were infected with assemblage B (Perez Cordon et al. 2008). These surveys imply that the distribution of *G. duodenalis* genotypes can vary according to geographical location. The human faecal samples in the current study were collected from patients in the UK, and it can be concluded that both assemblage A and B are common in the UK. Nonetheless, further study is required. Ideally, samples should be collected from healthy people throughout the UK.

In chapter 5, multilocus genotyping was performed. By PCR genotyping at several target genes, the *G. duodenalis* assemblage can be classified using a single marker, and the sub-assemblage level can be identified by genotyping at specific markers, for example the *bg* gene. However, combining these markers is a more precise way to study the molecular genetic diversity of *G. duodenalis*, since this parasite is known to be a complex species. In this study, the *bg*, *gdh* and ssu rRNA genes were selected to analyse human and pet samples. Using the multilocus genotyping approach, the current study identified sub-genotypes of *G. duodenalis* in humans and animals. A high level of polymorphism was detected, confirming that *G. duodenalis* is a complex species. Some isolates could not be classified to the exact sub-assemblages, which could suggest that these groups are novel sub-genotypes, particularly assemblage B in humans.

The multilocus genotyping of *G. duodenalis* could be improved in future research by adding more molecular markers, i.e., *ef1-α*, *tpi* and microsatellites. Due to time limitations, the current study analysed only a few samples from pet animals and no samples from farm animals, so future work should also include a greater number of samples. Furthermore, it would be valuable to collect faecal samples from humans and their pets living in the same locality, to investigate the zoonotic potential of this parasite.

Regarding microsatellite markers, although they successfully amplified the positive control sample (assemblage A1 isolate WB C6) in this study, they failed to amplify all clinical isolates tested. These results could suggest that new designs

of microsatellite markers and optimal PCR conditions are needed. Additionally, the sequences of assemblage B and E are now available from http://www.giardiadb.org, and the newly designed primers for these genotypes should be used. If possible, future work using microsatellites should aim to define new primers based on available sequences of assemblages A, B and E, before optimising PCR conditions in terms of annealing temperature, PCR components and reaction times. The optimised PCR conditions with newly designed microsatellite primers should be employed in clinical samples so that the exact assemblages can be confirmed.

Regarding the proteomic study of *G. duodenalis*, as assemblage A and B have been successfully cultured and their genomic databases have been published and are accessible from GiardiaDB (http://www.giardiadb.org) (Aurrecoechea et al. 2009), the current study was established to make a preliminary assessment of the difference in protein expression between the two genotypes. Using 2D-PAGE and DIGE, nine proteins were identified, with five proteins increasing in assemblage A and four proteins increasing in assemblage B. As this study is represent preliminary research, further comparative protein experiments may be needed. Recently, assemblage E has been made available by GiardiaDB (accessible from http://www.giardiadb.org). The study of proteins found to be predominantly increased in the non-zoonotic genotype, i.e., assemblage E, as compared to the zoonotic genotypes, i.e., assemblage A and B, may help in understanding how each assemblage infects the host.

In overall conclusion, the current research revealed information about the distribution of *Giardia* genotypes in both humans and animals. However, it was not representative of the true prevalence of Giardiasis, since all samples were collected from symptomatic cases, and thus may reveal a higher infection rate than a study of normal samples. The genotyping in this study was based on ssu rRNA, which has been widely used to discriminate *Giardia* from other parasites and can classify *G. duodenalis* assemblages. To better understand the molecular genetics of *G. duodenalis*, a combination of techniques may be required, for instance, MLG, or proteomics analysis. In the current study, genotyping using the *bg* and *gdh* loci was not completely successful in all samples, although all were successfully sequenced using the ssu rRNA gene. Some samples were difficult to amplify and yielded a very low concentration of PCR products, which could not be successfully sequenced. Hence the PCR components, the PCR reaction conditions and the quality of DNA samples may affect the results. Moreover, the genetic diversity of *G. duodenalis* may also influence the outcome of a PCR assay at a particular marker, producing a variety of results. Future work should focus on increasing the sample sizes in both normal cases and symptomatic samples, and developing new molecular markers, particularly microsatellites. Multilocus genotyping could be performed with more markers, for example, *tpi*, and finally, the evaluation of *G. duodenalis* proteomics may also be necessary.

Appendix

Appendix 1 Spreadsheet of dog samples

Date	Sample ID	Genotype	NWL No	Date_RCVD	Breed	Sex	Yrs	Mths	PostCode	History
20/02/2008	G10	A	790945	06/12/2007	JRT	F		5	DN	BLOOD GLU 6.5MMOL/L POOR DOER V QUIET SOME BLOOD IN MOTIONS TODAY - D
07/05/2008	I03	A	819872	14/02/2008	Springer spaniel	M		4	S2	
08/03/2007	B35	C	611549	11/10/2006	WHWT	F		3	SK	
09/03/2007	C05	C	654550	24/01/2007	crossbred	M		4	M2	
13/03/2007	C14	C	653242	22/01/2007	CKCS	M	2		LS	VOMITING AND DIARR
13/03/2007	C15	C	652851	20/01/2007	Cocker spaniel	F		2	NE	
14/03/2007	C17	C	645242	04/01/2007	Irish setter	M		5	YO	
14/03/2007	C22	C	642548	23/12/2006	Yorkshire terrier	MN	12		CF	N/H D
14/03/2007	C24	C	647178	09/01/2007	crossbred	M	1	6	LS	
28/03/2007	C33	C	641994	22/12/2006	CKCS	F		3	OX	
04/04/2007	C44	C	638803	14/12/2006	Cocker spaniel	MN	1		NN	
02/11/2007	D11	C	674336	09/03/2007	Labrador retriever	F		3	BB	
02/11/2007	D29	C	666777	21/02/2007	Lurcher	MN	1		WD	RESCUE DOG,CHRONIC DIARR,NO BLOOD,V ONLYFEW TIMES BUT NOT IN LAST WEEK
02/11/2007	D06	C	676860	15/03/2007	Weimaraner	F		8	SK	SEE HIST
02/11/2007	D08	C	675014	12/03/2007	Labrador retriever	M		3	SK	N/H
16/11/2007	E21	C	751541	08/09/2007	Chihuahua	F		3	B7	
16/11/2007	E23	C	751822	10/09/2007	Labrador retriever	M		3	CH	
16/11/2007	E24	C	756559	20/09/2007	border collie		1	6	PR	
16/11/2007	E32	C	745907	29/08/2007	Shih Tzu	F			FY	
31/01/2008	F10	C	737706	08/08/2007		MN			BH	
31/01/2008	F11	C	744684	24/08/2007	mastiff	F			PR	NH
31/01/2008	F12	C	740856	15/08/2007	Bull mastiff	F		5	WA	
31/01/2008	F14	C	737246	07/08/2007	Bichon Frise	FN		3	CW	PROFUSE WATERY AND BLOODY DIARRHOEA
31/01/2008	F17	C	728392	17/07/2007	border collie	MN	11	8	SK	TEMP 103F, CONT DYSCHEZIA + MUCOID HAEMORHAGIC, DIARR ++. D
31/01/2008	F19	C	730344	20/07/2007	Rottweiler	M	1		M4	NO HIST MON
31/01/2008	F02	C	728594	18/07/2007	crossbred	M		10	B9	
31/01/2008	F09	C	732826	27/07/2007				6	S2	
20/02/2008	G13	C	772097	26/10/2007	Cocker spaniel	FN	9	2	PR	
22/02/2008	G16	C	779187	13/11/2007		MN			WR	
22/02/2008	G19	C	774489	01/11/2007	CKCS	M		2	GU	
20/02/2008	G02	C	774448	01/11/2007	Labrador retriever	M		3	CT	
22/02/2008	G20	C	784600	24/11/2007	GSD	FN			WD	SH D
22/02/2008	G26	C	789783	04/12/2007	border collie	F		4	OX	
22/02/2008	G27	C	749618	23/10/2007	border collie	F		11	DA	
20/02/2008	G05	C	774172	31/10/2007	Cocker spaniel	F		3	WF	
20/02/2008	G08	C	770137	23/10/2007	border collie	M		3	PR	
30/04/2008	H10	C	827487	28/02/2008	Dalmatian	M		6		SH Previous numbers 0808133957, 080811880
30/04/2008	H15	C	834295	14/03/2008	Labrador retriever	M	6		LL	CHRONIC RECURRING D+
30/04/2008	H18	C	832145	10/03/2008					BL	
02/05/2008	H23	C	814515	02/02/2008	Lakeland terrier	M	2	6	GL	
02/05/2008	H31	C	811361	26/01/2008	WHWT	M		3	WA	

02/05/2008	H33	C	816818	07/02/2008	border collie	FN	1	3	CW	
09/05/2008	H39	C	785671	08/02/2008	Cockapoo	M	3	6	SW	
09/05/2008	H41	C	819875	14/02/2008	Beagle	F		5	NN	
09/05/2008	H42	C	821903	15/02/2008	WHWT			3	M2	
09/05/2008	H44	C	818360	11/02/2008	Labrador retriever	F		4	WA	
07/05/2008	I01	C	821873	15/02/2008	border collie	M		4	OL	
02/05/2008	K23	C	777225	07/11/2007	Beagle			3	SK	
02/05/2008	K31	C	798076	22/12/2007	Springer spaniel	M		5	SK	
02/05/2008	K32	C	780984	16/11/2007	Labrador retriever	M		8	N1	
02/05/2008	K33	C	803924	10/01/2008	Dogue de Bordeaux	F		3	SK	
02/05/2008	K34	C	804570	11/01/2008	crossbred	M			BL	
02/05/2008	K35	C	785362	14/01/2008	Cocker spaniel	F		6	KT	D
02/11/2007	D21	CD	684204	03/04/2007	SBT	F	1	1	PR	
02/11/2007	D26	CD	688143	13/04/2007	Husky	M		3	M4	
02/11/2007	D07	CD	677719	17/03/2007	GSD	M		8	GU	SH
16/11/2007	E25	CD	746198	30/08/2007	GSD	F		6	DL	SH
31/01/2008	F13	CD	730329	20/07/2007	Cocker spaniel	F			WA	
31/01/2008	F16	CD	740608	15/08/2007	Dobermann	F		5	SK	
20/02/2008	G11	CD	770577	24/10/2007	Springer spaniel	M	10		DL	
20/02/2008	G12	CD	772459	27/10/2007	WHWT	M			SN	
22/02/2008	G14	CD	780697	15/11/2007	Great Dane	M		3	PR	
22/02/2008	G22	CD	765427	12/10/2007	Deerhound	F		5	NR	
22/02/2008	G24	CD	785043	06/12/2007	crossbred	F		3	ME	D
20/02/2008	G03	CD	770700	24/10/2007	WHWT	F		6	BB	
20/02/2008	G04	CD	792014	08/12/2007	Beagle	F			S0	
20/02/2008	G06	CD	771446	25/10/2007	crossbred	M	2			NH
20/02/2008	G07	CD	786162	26/11/2007	crossbred	MN		3	FY	
20/02/2008	G09	CD	791221	06/12/2007	English bulldog	M		3	CW	
30/04/2008	H17	CD	834588	14/03/2008	CKCS	M		2	M4	
26/02/2007	B06	D	622183	08/11/2006	Irish terrier	M		7	OX	
26/02/2007	B12	D	618673	31/10/2006	WHWT	F		2	FY	
01/03/2007	B14	D	617170	27/10/2006	Samoyed	MN	2		LS	
07/03/2007	B25	D	630781	17/10/2006	Border terrier				SK	NH
08/03/2007	B29	D	617045	26/10/2006	Springer spaniel	M		3	BB	
09/03/2007	B41	D	616541	25/10/2006	crossbred	M		2	FY	
09/03/2007	C02	D	652631	20/01/2007	border collie	M		3	WD	
09/03/2007	C06	D	652791	20/01/2007	beagle	M		3	NP	NH D
13/03/2007	C09	D	652321	19/01/2007	Weimaraner	FE		4	WD	SEEH
13/03/2007	C10	D	652267	19/01/2007					TF	
13/03/2007	C12	D	652269	19/01/2007					TF	
14/03/2007	C19	D	647711	10/01/2007	crossbred			2	BH	SEE PREV M
14/03/2007	C23	D	645239	04/01/2007	Labrador retriever	M		2	ML	
14/03/2007	C25	D	643781	29/12/2006	border collie	F		5	SK	
14/03/2007	C27	D	643506	29/12/2006	crossbred	M	9	4	NP	
28/03/2007	C31	D	643797	29/12/2006		F			LS	
28/03/2007	C35	D	646945	09/01/2007	Golden retriever	M	1	2	BH	SEE PREV. M
04/04/2007	C45	D	638767	14/12/2006	Labrador retriever	M		2	NR	

11/04/2007	C58	D	629728	24/11/2006	Labrador retriever	F	10	6	SK	
11/04/2007	C60	D	635773	07/12/2006		M			WR	
02/11/2007	D23	D	686545	11/04/2007	Lurcher	F	2		ST	
02/11/2007	D25	D	686757	11/04/2007	GSD				BL	
02/11/2007	D28	D	664546	15/02/2007	crossbred	MN	1		TF	
02/11/2007	D37	D	663529	13/02/2007	CKCS	M		5	FY	
02/11/2007	D04	D	675233	13/03/2007	crossbred	F	1	1	WR	
02/11/2007	D43	D	678483	20/03/2007	CKCS	M		3	L3	
02/11/2007	D44	D	674225	09/03/2007	Beagle	F		3	CH	
02/11/2007	D05	D	677891	19/03/2007	WHWT	F		3	CA	
02/11/2007	D50	D	680945	26/03/2007	crossbred	M		2	WA	
02/11/2007	E12	D	753636	13/09/2007	Springer spaniel	M	2		ML	
16/11/2007	E20	D	755895	19/09/2007	Labrador retriever	M		4	SN	
16/11/2007	E37	D	754376	14/09/2007	GSD				PR	
31/01/2008	F15	D	737714	08/08/2007	Springer spaniel	MN		10	CW	
31/01/2008	F08	D	735284	02/08/2007	CKCS	M		3	M3	
22/02/2008	G15	D	772106	26/10/2007	crossbred	M	2		L6	
22/02/2008	G28	D	790663	05/12/2007	Boxer	M		3	FY	URGENT + FREQ D+ OCCASIONAL BLOOD + MUCOUS
30/04/2008	H01	D	831015	07/03/2008	border collie			2	PE	
30/04/2008	H12	D	834497	14/03/2008	briard	F	2	6	LL	
30/04/2008	H13	D	832143	10/03/2008					BL	
30/04/2008	H14	D	834315	14/03/2008	border collie	F		3	SN	
30/04/2008	H16	D	832146	10/03/2008	SBT	F		6	M2	
30/04/2008	H02	D	830909	07/03/2008	WHWT	F		3	S0	
02/05/2008	H26	D	813430	31/01/2008	Springer spaniel	M	2		WR	
02/05/2008	H32	D	785550	29/01/2008	Labradoodle	M		9	SE	SH
30/04/2008	H04	D	830787	06/03/2008	Labrador retriever	F		3		NH
09/05/2008	H40	D	824787	22/02/2008	Beagle	M		5	WA	
09/05/2008	H43	D	825275	23/02/2008	crossbred				BN	
30/04/2008	H06	D	785993	05/03/2008	Springer spaniel	F		3	ME	D
30/04/2008	H08	D	829334	04/03/2008	Yorkshire terrier				RM	
30/04/2008	H09	D	826755	27/02/2008	crossbred	M	3		EX	
07/05/2008	I02	D	816947	07/02/2008	CKCS	M		3	SK	
02/05/2008	K19	D	777727	08/11/2007	GSD	F		2	PR	
02/05/2008	K24	D	780901	16/11/2007	JRT	M		3	WD	DIARR OS OTHER DOG HAS CAMPYLOBACTER
02/05/2008	K25	D	780902	16/11/2007	JRT	M		3	WD	
02/05/2008	K29	D	781127	16/11/2007	Afghan hound	M		3	LL	
02/05/2008	K30	D	782010	19/11/2007	Husky	F			FY	

Appendix 2 Spreadsheet of cat samples

Date	Sample ID	Genotype	NWL No	Date_RCVD	Breed	Sex	Yrs	Mths	PostCode	History
07/03/2007	B20	A	611457	11/10/2006		M	10		B6	
22/02/2008	G18	A	775914	05/11/2007	DSH	FN	2		L3	
02/05/2008	H24	A	810922	25/01/2008	Bengal	F		6	CW	
02/05/2008	K36	A	802187	08/01/2008	Bengal	MN		4	NN	
26/02/2007	B10	F	617034	26/10/2006		F		4	WA	
26/02/2007	B11	F	619447	01/11/2006	DSH	FN			SK	
07/03/2007	B24	F	612654	13/10/2006	DSH				B6	
07/03/2007	B26	F	612654	13/10/2006	DSH				B6	
09/03/2007	B37	F	613282	18/10/2006	Persian	ME	1	5	GL	NH
09/03/2007	C04	F	652264	19/01/2007	DSH				M2	
13/03/2007	C07	F	652861	20/01/2007	DSH	MN	11		NG	
13/03/2007	C13	F	651427	17/01/2007					M4	
28/03/2007	C28	F	645009	04/01/2007	Bengal			6	S0	
28/03/2007	C32	F	640730	19/12/2006	Bengal	MN		7	M1	
04/04/2007	C48	F	650032	15/01/2007	DSH	F			M4	BLOOD IN D++ FOR 4-5 DAYS
02/11/2007	D01	F	677450	16/03/2007	Bengal	F	6		OL	
02/11/2007	D19	F	685248	05/04/2007	DSH	F		9	WD	
02/11/2007	D32	F	664186	14/02/2007	DSH	MN	1	6	PR	
02/11/2007	D40	F	659063	05/02/2007	DSH					NH
02/11/2007	D41	F	659064	05/02/2007	DSH					NH
02/11/2007	E01	F	758730	26/09/2007	DSH	F			IV	
02/11/2007	E11	F	762906	05/10/2007	DSH	M		5	WA	
02/11/2007	E13	F	759537	27/09/2007	DLH				FY	
16/11/2007	E26	F	750405	06/09/2007	DSH	F		4	YO	
16/11/2007	E30	F	745143	28/08/2007	DSH				CA	
16/11/2007	E35	F	751559	08/09/2007	DSH	F			YO	
16/11/2007	E39	F	749142	19/09/2007	DSH	M		5	SE	SH DIAG
02/11/2007	E09	F	757263	21/09/2007	DSH	M		3	PR	
22/02/2008	G21	F	772081	26/10/2007	DSH	M		5	L3	
22/02/2008	G23	F	788864	01/12/2007	Bengal	M		6	S0	
30/04/2008	H11	F	827762	28/02/2008	DSH	F	11		FY	SH D
02/05/2008	H25	F	811072	25/01/2008	DSH	M	1	6	WA	
02/05/2008	H28	F	812768	30/01/2008	DSH	FN	11		GL	SH
02/05/2008	H35	F	807083	17/01/2008	British blue	M		7	NN	
07/05/2008	I04	F	819906	14/02/2008	DSH	M	6		BS	
02/05/2008	K26	F	798088	22/12/2007	DSH				DG	
31/01/2008	F01	FC	744713	24/08/2007	Siamese	FN	1	3	BL	
31/01/2008	F06	FC	744442	24/08/2007	DSH	F		3	DH	
31/01/2008	F04	FD	730512	21/07/2007	DSH	M			NG	

Appendix 3 Spreadsheet of farm animal samples

Submission Ref	Genotype	County	Species	Breed	Sex	Age Detail	Age indicator	Age category	Presenting sign	Presenting sign 2	Diagnosis Summary (Full Text)
17-C0006-09-07	A	Lancashire	Cattle	Limousin_x	Female	5.00	Weeks	Unknown	Diarrhoea	None	235 Rotavirus infection.
17-C0011-12-07	A	Cheshire	Cattle	Holstein_friesian	Female	0.00	None	Adult	Diarrhoea	None	962 Diagnosis not reached - digest.
17-C0033-12-07	A	North Yorkshire	Cattle	Limousin	Male	3.50	Years	Adult	Diarrhoea	Wasting	144 Johne's Disease.
17-C0116-12-07	A	North Yorkshire	Cattle	Fr	Female	3.00	Weeks	Prewean	Diarrhoea	Recumbt	162 Salmonellosis dt S. Dublin.
17-C0119-12-07	A	Derbyshire	Cattle	Holstein	Female	0.00	None	Adult	Diarrhoea	None	962 Diagnosis not reached - digest.
17-C0189-12-07	A	Lancashire	Cattle	Fr	Female	6.00	Days	Neonatal	Fnddead	None	235 Rotavirus infection. 318 Cryptosporidiosis. 522 Hypogammagloblinaemia.
17-C0207-12-07	A	Cumbria	Cattle	Charolais_x	Male	9.00	Days	Prewean	Diarrhoea	None	962 Diagnosis not reached - digest.
17-C0006-02-08	A	Lancashire	Cattle	Holstein_friesian	Female	0.00	None	Adult	Malaise	Milkdrop	371 Fasciolosis.
17-C0206-04-08	A	Lancashire	Cattle	Limousin_x	Male	0.00	None	Mixed	Diarrhoea	Respir	201 Mucosal disease. 235 Rotavirus disease. 238 Coronavirus infection: neonata. 248 BVD Viraemia.
17-C0218-04-08	A	Lancashire	Cattle	Aberdeen_angus	Male	0.00	None	Neonatal	Repro	Malaise	123 Colisepticaemia. 522 Hypogammaglobulinaemia.
17-C0268-04-08	A	Lancashire	Cattle	Limousin	Female	3.00	Weeks	Prewean	Fnddead	None	318 Cryptosporidiosis.
17-C0266-04-08	A	Lancashire	Cattle	Simmental	Male	2.00	Weeks	Prewean	Fnddead	None	318 Cryptosporidiosis. 853 Skeletal defects NOS.
17-C0316-04-08	A	Lancashire	Cattle		Mixed	10.00	Days	Prewean	Diarrhoea	None	318 Cryptosporidiosis.
17-C0317-04-08	A	North Yorkshire	Cattle		Male	6.00	Weeks	Unknown	Nervous	None	983 Diagnosis not listed - respira.
17-C0353-04-08	A	Lancashire	Cattle	Holstein_friesian	Male	0.00	None	Neonatal	Malaise	None	674 Intestinal torsion.
17-C0385-04-08	A	North Yorkshire	Cattle	Highlands	Female	0.00	None	Adult	Fnddead	None	999 Diagnosis not reached.
17-C0232-12-07	AC	Lancashire	Cattle	Holstein_friesian	Mixed	0.00	None	Prewean	Diarrhoea	None	235 Rotavirus disease.
17-C0047-12-07	AD	Lancashire	Cattle	Holstein_friesian	Mixed	2.00	Weeks	Prewean	Malaise	Fnddead	235 Rotavirus disease.
17-C0126-09-07	AE	Greater Manchester	Cattle	Holstein_friesian	Unknown	8.00	Weeks	Postwean	Unknown	None	962 Diagnosis not reached - digest.
17-C0093-11-07	AE	Lancashire	Cattle	Fr	Female	0.00	None	Adult	Milkdrop	Wasting	961 Diagnosis not reached - system.
17-C0143-12-07	AE	Lancashire	Cattle	Holstein	Female	4.00	Years	Adult	Na	None	
17-C0011-	AE	Lancashire	Cattle	Holstein_x	Male	2.00	Weeks	Prewean	Diarrhoea	None	235 Rotavirus disease. 318 Cryptosporidiosis.

02-08											
17-C0316-04-08	AE	Lancashire	Cattle		Mixed	10.00	Days	Prewean	Diarrhoea	None	962 Diagnosis not reached - digest.
17-C0375-04-08	AE	Lancashire	Cattle	Holstein	Female	7.00	Years	Adult	Fnddead	None	982 Diagnosis not listed - digesti.
17-C0045-04-08	C	Lancashire	Cattle	Limousin_x	Castrate	13.00	Months	Postwean	Recumbt	Malaise	966 Diagnosis not reached - nervou.
17-C0186-04-08	C	Lancashire	Cattle	Holstein_friesian	Female	10.00	Days	Neonatal	Malaise	Fnddead	238 Coronavirus disease. 522 Hypogammaglobulinaemia.
17-C0038-12-07	D	Cheshire	Cattle	Belgian_blue_x	Female	0.00	None	Prewean	Diarrhoea	None	962 Diagnosis not reached - digest.
17-C0101-12-07	D	Cheshire	Cattle	Fr	Female	6.00	Years	Adult	Diarrhoea	Milkdrop	144 Johne's Disease.
17-C0121-12-07	D	Lancashire	Cattle	Holstein_friesian	Unknown	0.00	None	Mixed	Malaise	Diarrhoea	318 Cryptosporidiosis.
17-C0137-12-07	D	North Yorkshire	Cattle	Holstein_friesian	Female	0.00	None	Adult	Wasting	None	961 Diagnosis not reached - system.
17-C0046-09-07	E	Lancashire	Cattle	Charolais	Male	11.00	Weeks	Postwean	Respir	None	746 Pneumonia dt M bovis.
17-C0049-09-07	E	Greater Manchester	Cattle	Holstein_friesian	Female	12.00	Weeks	Postwean	Diarrhoea	None	962 Diagnosis not reached - digest.
17-C0154-10-07	E	Lancashire	Cattle	Holstein	Female	0.00	None	Adult	Milkdrop	None	963 Diagnosis not reached - respir.
17-C0090-11-07	E	North Yorkshire	Cattle	Holstein	Female	0.00	None	Adult	Respir	Milkdrop	963 Diagnosis not reached - respir.
17-C0074-11-07	E	Cheshire	Cattle	Holstein	Female	0.00	None	Mixed	Na		pooled wec <50
17-C0074-11-07	E	Cheshire	Cattle	Holstein	Female	0.00	None	Mixed	Na		pooled wec 50
17-C0060-12-07	E	North Yorkshire	Cattle	Holstein_friesian	Female	0.00	None	Neonatal	Diarrhoea	None	127 Colibacillosis EPEC/VTEC. 235 Rotavirus disease.
17-C0077-12-07	E	Lancashire	Cattle	Holstein_friesian	Female	0.00	None	Mixed	Diarrhoea	None	371 Fasciolosis.
17-C0121-12-07	E	Lancashire	Cattle	Holstein_friesian	Unknown	0.00	None	Mixed	Malaise	Diarrhoea	318 Cryptosporidiosis.
17-C0130-12-07	E	Lancashire	Cattle	Holstein_friesian	Mixed	4.00	Weeks	Prewean	Malaise	Diarrhoea	235 Rotavirus infection.
17-C0223-12-07	E	Lancashire	Cattle	Simmental	Male	14.00	Days	Prewean	Diarrhoea	Respir	235 Rotavirus disease. 318 Cryptosporidiosis.
17-C0231-12-07	E	Lancashire	Cattle	Zza_cattle_mixed	Unknown	0.00	None	Prewean	Diarrhoea	Fnddead	162 Salmonellosis dt S. Dublin.
17-C0231-12-07	E	Lancashire	Cattle	Zza_cattle_mixed	Unknown	0.00	None	Prewean	Diarrhoea	Fnddead	661 Abomasal ulceration.
17-C0305-12-07	E	North Yorkshire	Cattle	Holstein_friesian	Female	6.00	Years	Adult	Diarrhoea	Wasting	962 Diagnosis not reached - digest.
17-C0043-02-08	E	Lancashire	Cattle	Fr	Unknown	0.00	None	Adult	Diarrhoea	Wasting	144 Johne's Disease.
17-C0021-04-08	E	Staffordshire	Cattle		Mixed	5.00	Days	Neonatal	Diarrhoea	None	318 Cryptospridiosis

17-C0165-04-08	E	Lancashire	Cattle	Holstein_friesian	Female	6.00	Weeks	Prewean	Fnddead	None	657 Bloat.
17-C0193-04-08	E	Lancashire	Cattle	Fr	Female	5.00	Months	Adult	Diarrhoea	None	962 Diagnosis not reached - digest.
17-C0030-05-08	E	Merseyside	Cattle	Aberdeen_angus	Unknown	0.00	None	Mixed	Na		wec <50 , 1000 oocysts
17-C0030-05-08	E	Merseyside	Cattle	Aberdeen_angus	Unknown	0.00	None	Mixed	Na		wec <50 , 300 oocysts
17-C0030-05-08	E	Merseyside	Cattle	Aberdeen_angus	Unknown	0.00	None	Mixed	Na		wec <50 , 1200 oocysts
17-C0066-05-08	E	Lancashire	Cattle		Female	0.00	None	Prewean	Unknown	None	318 Cryptosporidiosis.
17-C0053-05-08	E	Lancashire	Cattle		Mixed	0.00	None	Neonatal	Diarrhoea	None	318 Cryptosporidiosis.
17-C0141-05-08	E	Channel Islands or Isle of Man	Cattle	Limousin	Male	4.00	Years	Adult	Diarrhoea	Lame	962 Diagnosis not reached - digest.
17-C0150-05-08	E	Lancashire	Cattle	Holstein_friesian	Male	4.00	Weeks	Prewean	Malaise	Diarrhoea	318 Cryptosporidiosis. 643 Meningitis/encephalitis NOS. 705 Navel Ill/Joint Ill.
17-C0153-05-08	E	Lancashire	Cattle	Holstein_friesian	Female	0.00	None	Neonatal	Diarrhoea	None	962 Diagnosis not reached - digest.
17-C0153-05-08	E	Lancashire	Cattle	Holstein_friesian	Female	0.00	None	Neonatal	Diarrhoea	None	962 Diagnosis not reached - digest.
17-C0091-06-08	E	Lancashire	Cattle	Holstein_friesian	Female	10.00	Days	Prewean	Recumbt	Diarrhoea	162 Salmonellosis dt S. Dublin. 318 Cryptosporidiosis. 522 Hypogammaglobulinaemia.
17-C0137-06-08	E	Lancashire	Cattle	Limousin_x	Male	3.00	Weeks	Prewean	Fnddead	None	962 Diagnosis not reached - digest.
17-C0138-06-08	E	Lancashire	Cattle	Limousin_x	None	5.00	Weeks	Prewean	Diarrhoea	None	962 Diagnosis not reached - digest.
17-C0212-06-08	E	Lancashire	Cattle		Mixed	3.00	Months	Prewean	Fnddead	None	417 Poisoning dt lead.
17-C0250-06-08	E	Lancashire	Cattle	Simmental_x	Mixed	6.00	Weeks	Postwean	Fnddead	None	628 Portocaval thromboembolism. 705 Navel Ill/Joint Ill.
17-C0250-06-08	E	Lancashire	Cattle	Simmental_x	Mixed	6.00	Weeks	Postwean	Fnddead	None	628 Portocaval thromboembolism. 705 Navel Ill/Joint Ill.
17-P0125-09-07	A	Unknown	Pig			0.00		X-na	X-na		
17-P0098-09-07	AC	Unknown	Pig			0.00	None	Unknown	X-na		
17-P0123-09-07	AC	Unknown	Pig		None	0.00	None	X-na	X-na		
17-P0096-09-07	C	Unknown	Pig		None	0.00	None	X-na	X-na		
17-P0031-04-08	C	Lancashire	Pig		Mixed	18.00	Weeks	Postwean	Fnddead	None	172 Streptococcus suis. 610 Myopathy NOS.
17-P0095-09-07	CD	Unknown	Pig			0.00		X-na	X-na		
17-P0083-09-07	D	Unknown	Pig			0.00		X-na	X-na		

17-P0097-09-07	D	Unknown	Pig		None	0.00	None	X-na	X-na		
17-P0159-09-07	D	Unknown	Pig		None	0.00	None	X-na	X-na	None	
17-P0064-09-07	E	Unknown	Pig			0.00		X-na	X-na		
17-P0124-09-07	E	Unknown	Pig		None	0.00	None	X-na	X-na		
17-P0232-04-08	F	Lancashire	Pig		Mixed	14.00	Weeks	Postwean	Lame	Respir	157 Pneumonia dt Pasteurella multo. 160 Salmonellosis dt Salmonella NO. 240 PRRS porcine reproductive and . 712 PMWS - Postweaning multisystem.
17-P0232-04-08	F	Lancashire	Pig		Mixed	14.00	Weeks	Postwean	Lame	Respir	157 Pneumonia dt Pasteurella multo. 160 Salmonellosis dt Salmonella NO. 240 PRRS porcine reproductive and . 712 PMWS - Postweaning multisystem.
17-S0231-04-08	A	North Yorkshire	Sheep		Male	6.00	Weeks	Prewean	Fnddead	None	118 Cl. perfringens D disease.
17-S0304-04-08	A	Lancashire	Sheep	Zzc_sheep_unspecified	Female	0.00	None	Adult	Fnddead	None	511 Hypocalcaemia. 740 Pneumonia NOS. 984 Diagnosis not listed - urinary.
17-S0304-04-08	A	Lancashire	Sheep	Zzc_sheep_unspecified	Female	0.00	None	Adult	Fnddead	None	511 Hypocalcaemia. 740 Pneumonia NOS. 984 Diagnosis not listed - urinary.
17-S0333-04-08	A	Greater Manchester	Sheep		Female	2.00	Days	Neonatal	Nervous	None	118 Cl. perfringens D disease.
17-S0184-06-08	A	Unknown	Sheep	Other_sheep	Male	0.00	None	Adult	Diarrhoea	Wasting	144 Johne's Disease.
17-S0105-12-07	AD	Lancashire	Sheep	Swaledale	Female	8.00	Months	Postwean	Fnddead	None	181 Pasteurella trehalosi septicae.
17-S0368-08-07	E	Lancashire	Sheep	Blue_faced_leicester	Female	16.00	Months	Adult	Recumbt	None	643 Meningitis/encephalitis NOS. 986 Diagnosis not listed - nervous.
17-S0042-09-07	E	Lancashire	Sheep		Male	0.00	None	Postwean	Fnddead	None	544 Trauma/Fracture.
17-S0057-09-07	E	Lancashire	Sheep		Mixed	3.00	Months	Postwean	Diarrhoea	Malaise	962 Diagnosis not reached - digest.
17-S0136-09-07	E	Lancashire	Sheep	Beltex_sheep_x	Female	0.00	None	Unknown	Recumbt	None	118 Cl. perfringens D infection.
17-S0028-11-07	E	Lancashire	Sheep	Dorset_x	None	6.00	Months	Unknown	Malaise	None	322 Parasitic gastroenteritis (PGE. 512 Pine/Cobalt deficiency.
17-S0060-11-07	E	Lancashire	Sheep		Female	7.50	Months	Postwean	Wasting	None	322 Parasitic gastroenteritis (PGE. 513 Hypocupraemia/Hypocuprosis. 519 Hyposelenaemia.
17-S0022-12-07	E	Lancashire	Sheep	Swaledale	Female	7.00	Months	Postwean	Diarrhoea	None	322 Parasitic gastroenteritis (PGE.
17-S0240-12-07	E	Cheshire	Sheep	Devon_long_wool	Mixed	1.00	Years	Adult	Na		
17-S0040-02-08	E	Lancashire	Sheep	Beltex_sheep	Female	0.00	None	Adult	Fnddead	None	373 Chronic fasciolosis.
17-S0136-02-08	E	Greater Manchester	Sheep	Mule	Female	2.00	Years	Adult	Malaise	Wasting	373 Chronic fasciolosis.
17-S0203-	E	Lancashire	Sheep		Unknown	1.00	Months	Prewean	Malaise	Diarrhoea	962 Diagnosis not reached - digest.

04-08											
17-S0203-04-08	E	Lancashire	Sheep		Unknown	1.00	Months	Prewean	Malaise	Diarrhoea	318 Cryptosporidiosis.
17-S0022-05-08	E	Lancashire	Sheep	Texel_x	Unknown	8.00	Weeks	Prewean	Fnddead	None	169 Pneumonia dt Mycoplasma ovipne. 740 Pneumonia NOS.
17-S0022-05-08	E	Lancashire	Sheep	Texel_x	Unknown	8.00	Weeks	Prewean	Fnddead	None	169 Pneumonia dt Mycoplasma ovipne. 740 Pneumonia NOS.
17-S0024-05-08	E	Lancashire	Sheep	Jacob	Male	3.00	Months	Prewean	Fnddead	None	312 Coccidiosis.
17-S0024-05-08	E	Lancashire	Sheep	Jacob	Male	3.00	Months	Prewean	Fnddead	None	312 Coccidiosis
17-S0235-05-08	E	Lancashire	Sheep		Mixed	2.00	Months	Prewean	Diarrhoea	None	337 PGE - Nematodirosis.
17-S0253-05-08	E	Lancashire	Sheep	Mule-x	None	2.00	Months	Prewean	Other	None	322 PGE NOS. 337 PGE - Nematodirosis. 785 Nephrosis.
17-S0253-05-08	E	Lancashire	Sheep	Mule-x	None	2.00	Months	Prewean	Other	None	322 PGE NOS. 337 PGE - Nematodirosis. 785 Nephrosis.
17-S0291-05-08	E	Lancashire	Sheep		Unknown	8.00	Weeks	Prewean	Malaise	None	337 PGE - Nematodirosis. 322 PGE NOS

Appendix 4 Spreadsheet of human samples

Spl no.	Hpa id	Genotype	Spec_id	Date sample collected	Age years	Sex	Travel outside the uk	Country visited 1	Country visited 2	Country visited 3	Travel in UK	Place visited 1	Place visited 2	Place visited 3
HM001	P08.0401874.W	B	P080401874	21-Feb-08	55	F	Yes	India			No			
HM002	P08.0403199.D	A	P080403199	31-Mar-08	40	F	No				No			
HM003	P08.0403800.N	B	P080403800	14-Apr-08	35	M	No							
HM004	P08.0404164.Y	A	P080404164	25-Apr-08	67	M								
HM005	P08.0404415.M	B	P080404415	01-May-08	1	M	No				No			
HM006	P08.0405071.S	A	P080405071	19-May-08	76	F	No				No			
HM007	P80.0405729.B	B	P080405729	09-Jun-08	6	M	No				Yes	Caravan in lakes		
HM008	P08.0405942.N	A	P080405942	13-Jun-08	74	F	No				No			
HM009	P08.0406166.H	A	P080406166	20-Jun-08	62	F	No				No			
HM010	P08.0407075.N	B	P080407075	14-Jul-08	10	M	Yes	India						
HM011	P08.0408324.J	B	P,08.0408324	18-Aug-08	36	F	No				No			
HM012	P08.0408632.D	B	P,08.0408632	27-Aug-08	50	M	Yes	United arab emirates	Kuwait		Yes	Glasgow	Nuneaton	
HM013	P08.0401212.Y	B	P080401212	31-Jan-08	70	M	No							
HM015	P08.0401990.S	B	P080401990	25-Feb-08	39	F	No				No			
HM016	P08.0403118.M	B	P080403118 P080403118	27-Mar-08	64	F	No				No			
HM017	P08.0403617.W	B	P080403617	10-Apr-08	46	M	No							
HM018	P08.0403855.Q	B	P080403855	16-Apr-08	35	M	No				No			
HM019	P08.0404298.Q	B	P080404298	28-Apr-08	22	M	No				No			
HM020	P08.0404989.A	B	p080404989	16-May-08	60	F	No				No			
HM021	P08.0405504.P	B	P080405504	03-Jun-08	61	M	No				No			
HM023	P08.0405983.Y	B	P080405983	16-Jun-08	30	M	No				No			
HM024	P08.0406965.R	B	P080406965	10-Jul-08	52	M	Yes	Sharm el sheik			No			
HM025	P08.0407237.N	B	P080407237	17-Jul-08	75	M	No				No			

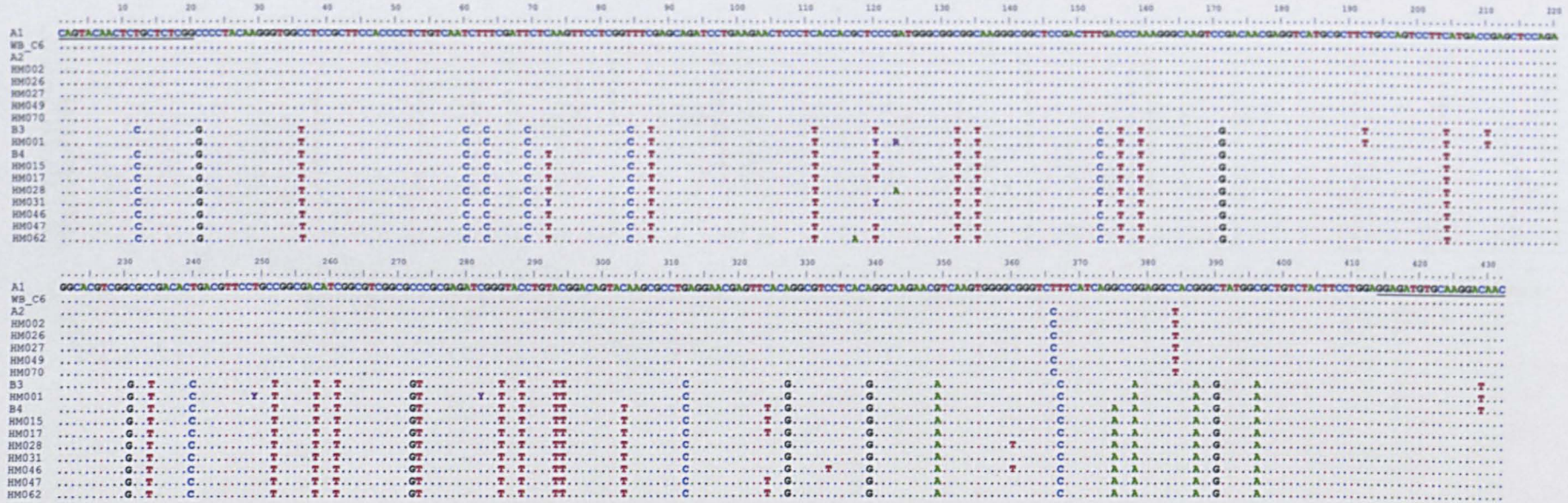
HM026	P08.0408255.P	A	P,08.0408255	16-Aug-08	25	M	Yes	Spain	Pakistan		No			
HM027	P08.0408375.P	A	P,08.0408375	18-Aug-08	2	M	No				No			
HM028	411630	B	P,08.0411630	15-Nov-08	36	F	Yes	India			No			
HM029	411016	B	P,08.0411016	30-Oct-08	7	M	Yes	Pakistan	Dubai		No			
HM030	412422	B	P,08.0412422	09-Dec-08	67	M	Yes	Egypt			No			
HM031	412185	B	P,08.0412185	01-Dec-08	50	F	Yes	India			No			
HM032	412512	B	P,08.0412512	09-Dec-08	32	F	No				No			
HM033	220733	B	P,09.0220733	21-Jan-09	46	F	No							
HM034	222390	B	P,09.0222390	04-Mar-09	32	F	No				No			
HM035	220123	A	P,09.0220123	05-Jan-09	44	M								
HM036	220750	B	P,09.0220750	21-Jan-09	29	M								
HM037	221704	B												
HM038	221752	B	P,09.0221752	17-Feb-09	61	M	No				No			
HM039	222722	B	P,09.0222722	12-Mar-09	62	F								
HM040	222658	B	P,09.0222658	11-Mar-09	35	M								
HM041	223076	A	P,09.0223076	20-Mar-09	56	M	No				No			
HM042	223112	B	P,09.0223112	23-Mar-09	27	M								
HM043	223489	B	P,09.0223489	01-Apr-09	83	M	No				No			
HM046	223872	B	P,09.0223872	09-Apr-09	2	M	No				No			
HM047	224461	B	P,09.0224461	24-Apr-09	61	F	No				No			
HM048	224342	A	P,09.0224342	22-Apr-09	78	F	No				No			
HM049	225397	A	P,09.0225397	19-May-09	62	F	Yes	Nepal			No			
HM050	225834	B	P,09.0225834	29-May-09	42	M	No				Yes	Glasgow	Manchester	Sheffield
HM051	225941	B	P,09.0225941	02-Jun-09	37	F								
HM053	225977	B												
HM054	225981	A	P,09.0225981	04-Jun-09	24	M	No				No			

			1											
HM055	226453	B	P,09.022645 3	16-Jun-09	24	M								
HM056	226478	A	P,09.022647 8	17-Jun-09	63	M	No				No			
HM059	226943	A	P,09.022694 3	29-Jun-09	43	M	No				No			
HM060	227104	B												
HM061	226962	B	P,09.022696 2	26-Jun-09	46	M								
HM062	227460	B	P,09.022746 0	10-Jul-09	35	F	Yes	France			Yes	London	Old trafford cricket ground	
HM064	228065	A												
HM065	228094	A	P,09.022809 4	26-Jul-09	68	M	No				No			
HM066	228166	B	P,09.022816 6	29-Jul-09	39	M								
HM070	228835	A	P,09.022883 5	17-Aug-09	45	F	No				No			
HM071	228930	B	P,09.022893 0	19-Aug-09	62	M	No				No			

Appendix 5 Multiple alignment of partial β-giardin nucleotide sequences of assemblage A of studied human isolates HM002, HM027, HM049 and HM070 against the reference sequences. The primers used to amplify a 511 bp fragment are underlined. The missing portion has been left dash. Dots indicate identity to A1 (isolate Portland-1) reference sequence.

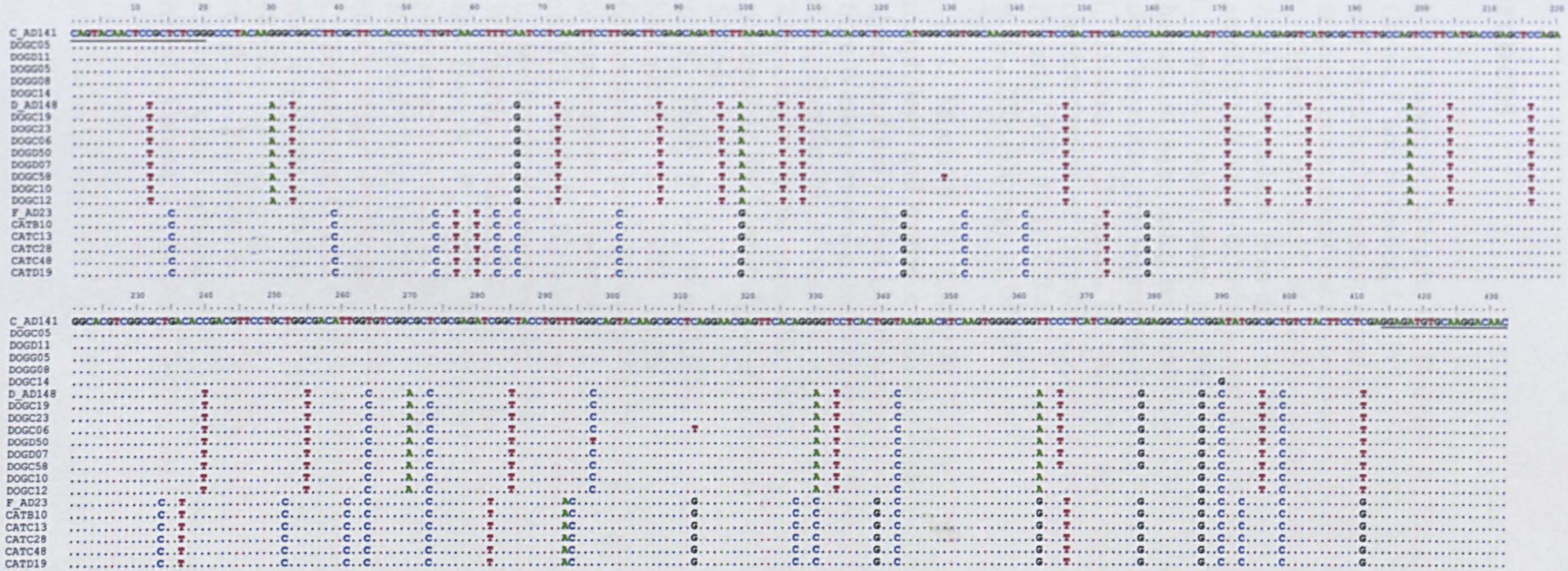
Appendix 6 Multiple alignment of partial β-giardin nucleotide sequences of assemblage B of studied human isolates HM001, HM015, HM017, HM028, HM031, HM046, HM047 and HM0042 against the reference sequences. The primers used to amplify a 511 bp fragment are underlined. Dots indicate identity to B3 (isolate BAH8) reference sequence.

Appendix 7 Multiple alignment of partial *gdh* nucleotide sequences of studied human isolates. The primers used to amplify a 432 bp fragment are underlined. Dots indicate identity to A1 (isolate AD-1) reference sequence.



Appendix 8 Multiple alignment of partial β-giardin nucleotide sequences of studied dog and cat isolates. The sequenced samples are indicated as DOG and CAT. The primers used to amplify a 511 bp fragment are underlined. The missing portion has been left dash. Dots indicate identity to C (isolate A29) reference sequence.

Appendix 9 Multiple alignment of partial *gdh* nucleotide sequences of studied dog and cat isolates. The primers used to amplify a 432 bp fragment are underlined. Dots indicate identity to the C (isolate AD141) reference sequence.



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