Molecular epidemiology of *Giardia duodenalis* in the high-income world

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Abstract

Giardia duodenalis is a gastrointestinal parasite that infects most mammals, including humans. Outcome of infection ranges from asymptomatic carriage to severe clinical disease, leading to digestive abnormalities lasting years beyond infection. In high-income countries, this parasite was once primarily thought to be contracted by individuals with a history of foreign travel. Recent evidence suggests that endemic infection is an important factor in the epidemiology of giardiasis, and consequently human patients without a history of travel should be screened for *Giardia* to aid in the understanding of endemic infection.

The first objective of the present study was to undertake a literature review of human giardiasis outbreaks to determine the primary routes of transmission in high-income countries. Outbreaks were categorised by transmission route, which included waterborne, foodborne, travel, person-to-person, zoonotic and direct faecal exposure. Waterborne transmission emerged as the route associated with the highest number of outbreak studies, followed by person-to-person transmission. This review highlighted the need for increased screening protocols in high-income countries and investigation into transmission routes other than travel.

Being able to discern between different sub-types of *Giardia*, termed assemblages, is an important aspect of transmission analysis since different assemblages show varying levels of host-specificity, with some capable of infecting both humans and animals. Current *Giardia* typing methods rely largely on PCR of marker loci and sequencing of amplicons, however these suffer from poor amplification success rates particularly when applied to non-human assemblages. In this study, recently published genomic data was used to modify and optimise one such *Giardia* marker to increase sensitivity. Using this improved marker, the success rate across multiple assemblages increased markedly and it was subsequently applied to type a large collection of UK human and companion animal field samples. This revealed an appreciable presence of zoonotic assemblages in the companion animal population and highlighted them as potential source of human infection. This study adds to the knowledge on *Giardia* epidemiology in the context of a high-income country and provides improved genotyping methodology which can be applied to future studies.

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Author's Declaration

"A scoping review of risk factors and transmission routes associated with human giardiasis outbreaks in high-income settings" was a collaborative work alongside Paul Capewell, Alison Smith-Palmer, Dominic Mellor, Willie Weir and Claire L. Alexander. It was published in Current Research in Parasitology & Vector Borne-Diseases in February 2022 (Krumrie *et al*, 2022).

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I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Sarah Krumrie

Abbreviations

µL	Microlitre
bg	*Beta-giardin*
bp	Base Pair
°C	Degrees Celsius
CA	Companion Animal
C. can	*Castor canadensis* (North American Beaver)
CI	Confidence Interval
Ct	Cycle Threshold
D	Domestic (Travel-associated)
DC	Daycare Attendance
DFC	Direct Faecal Contact
dH_2O	Distilled Water
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphates
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
FA	Farm Animal
FASTA	Text-Based Format for Nucleotide Sequence Data
FH	Food Handler
FHV	Feline Herpesvirus
g	Grams
g	Gravity
GC Content	Guanine-Cytosine Content
gdh	*Glutamate dehydrogenase*

GI	Gastrointestinal
HH	Household GI Illness
I	International (Travel-associated)
IBS	Irritable Bowel Syndrome
IgA	Immunoglobulin A
kb	Kilobases
LB	Luria-Bertani Broth
LMIC	Lower/Middle-Income Countries
Loc	Location
mL	Millilitre
mM	Millimolar
MOOSE	Meta-Analysis of Observational Studies in Epidemiology
MLST	Multilocus Sequence Typing
MSM	Men who have sex with men
ng	Nanogram
NHS	National Health Service
OECD	Organisation for Economic Co-operation and Development
OR	Odds ratio
p	P-value
PCR	Polymerase Chain Reaction
pJET	Cloning Vector
PMID	PubMed Unique Identifier
pM	Picomolar
pmol	Picomole
PSP	Pre-Analytical Sample Processing (trademark)

PrT	Pre-Treatment
PVPP	Polyvinylpolypyrrolidone
qPCR	Quantitative Polymerase Chain Reaction
Ref	Reference
RF	Reconstituted Foodstuff
RNA	Ribonucleic Acid
RW	Recreational Water
SNAP	Trademark, In-Clinic Point-of-Care Assay
SPDRL	Scottish Parasite Diagnostic and Research Laboratory
spp.	Species
SSR	Simple Sequence Repeat
ST	Sexual Transmission
taco	Dual DNA/RNA Extraction Machine (trademark)
tpi	*Triosephosphate Isomerase*
UFV	Unwashed Fruit/Vegetables
UK	United Kingdom
USA	United States of America
UT	Untreated
V	Volts
VDS	Veterinary Diagnostic Services
WA	Wild Animal
WGS	Whole Genome Sequencing
Y	Year
YC	Young Children

Chapter 1. Introduction

1.1 Public Health Significance

1.1.1 Worldwide Significance

Giardia duodenalis is a binucleate flagellated eukaryotic protozoan found worldwide that infects the gastrointestinal system of a wide variety of mammals, including humans (Adam, 2000; 2001; Caccio & Ryan, 2008). It is a ubiquitous organism that causes the disease giardiasis in both low-/middle-income and high-income countries. The World Health Organization (WHO) identified giardiasis as a "Neglected Disease" in its 2004 initiative to identify and eliminate diseases directly contributing to human sickness and death (Savioli *et al*, 2006). In low- and middle-income countries giardiasis is linked to poor sanitation, often as a result of poor quality drinking water (Aw *et al*, 2019; Daniels et al, 2018; Squire & Ryan, 2017). In high-income countries human outbreaks of varying size and sporadic cases are often caused by ingestion of contaminated water (Adam, 2001; Caccio & Ryan, 2008; Daly *et al*, 2010; Mahbubani *et al*, 1992) while citing travel as a major route of transmission, termed "travellers' diarrhoea" (ECDC, 2018; Ferguson *et al*, 2020; Morch & Hanevik, 2020). Contaminated food or food handlers are also responsible for many *Giardia* outbreaks in humans. Although it primarily causes diarrhoea, it can also result in serious long-term side effects. Giardiasis affects the growth and development of young children, evidenced by a study in India which found that children infected with *Giardia* consistently showed cognitive deficits compared to others their age (Ajjampur *et al*, 2011; Jethwa, 2015; Simsek *et al*, 2004). Additionally, a prolonged chronic colitis can result as a secondary effect of giardiasis *(Dann et al, 2018; Hanevik et al, 2009; Hanevik et al*, 2014; Wensaas *et al*, 2012) and the possibility of asymptomatic infection also exists (Caccio & Ryan, 2008; Thompson & Ash, 2016). While gastrointestinal illness on its own may not seem serious when compared to a more acutely deadly disease, the economic impact alone from an outbreak within a workforce can have a noticeable impact, both in high- and low-/middle-income countries (Mathews *et al*, 2022; Papadopoulos *et al*, 2019; Sandler *et al*, 2002; Sang *et al*, 2014). In 2016, diarrhoea was named as one of the top five causes of death worldwide (Collaborators, 2017). When explained in the wider global sense of human health

and monetary benefit, the importance of detection, treatment and baseline knowledge of disease-causing organisms such as *Giardia* begins to become clearer.

1.1.2 A Neglected Tropical Disease

In the last 30 years, infectious diseases have seen a growing amount of attention from major organisations such as the Institute of Medicine in the United States and the World Health Organisation (WHO), thanks in part to publications such as emerging infection reports and the list of Neglected Tropical Disease (Nii-Trebi, 2017; Savioli *et al*, 2006). Several factors contribute to the increase in infectious disease prevalence, many of which have also increased in recent years. Changes in environment or climate potentially alter ecosystems to allow certain organisms to flourish where they could not before, and with growing populations in a finite space, the pathogen-host interaction time and frequency has increased (Nii-Trebi, 2017). For example, malaria, a disease whose vectors are largely affected by environmental change, was the cause of over 1.1 million deaths worldwide in 2012 (Bhutta *et al*, 2014). Low-income areas are disproportionately affected by infectious disease and, according to a study by Hotez in 2005, policymakers decided neglected tropical diseases should be defined as those with a high prevalence in poverty-stricken areas (Hotez *et al*, 2020). Another reason these diseases are placed on lists of public health organisations is that there needs to be significant funding in place to research the aetiological organisms involved, which is a problem in poverty-stricken areas (Nii-Trebi, 2017). Investigation and intervention of neglected disease in low-income regions is hampered by several factors, including the lack of funding to multiple intervention areas. Pharmaceutical companies with the technology to formulate preventative medication or post-exposure treatments are disinterested in doing so where there is no opportunity for profit (Hotez *et al*, 2020). Funding for low-income countries is therefore left to academic institutions, special interest groups, or donations from foreign governments. Additionally, there is a low utilisation of health services in low-income countries, particularly by women. These services may be expensive in themselves, but they become particularly inaccessible when the price of travel and accommodation is added (Bhutta *et al*, 2014). Some other barriers to healthcare experienced in lower-/middle-income countries include long distances to travel to the nearest healthcare provider and long wait times (Harris *et al*, 2011).

In 2013, the WHO published a guide outlining five strategies to address these neglected tropical diseases, termed "Key Interventions" (WHO, 2013). They include preventive chemotherapy, innovative and intensified disease management, vector control, safe water, sanitation and hygiene, and veterinary public health services. Each of these categories requires a thorough understanding of the biology and molecular mechanisms of a disease-causing organism to succeed as an intervention. In particular, where transmission pathways are unknown, sometimes as a consequence of limited pathogen typing methods, it may be difficult for public health services to develop effective control strategies. Where the role of animals in disease transmission is unclear, assistance potentially available from veterinary professionals may be underutilised or entirely absent. Giardiasis is one such neglected disease, where isolate typing methods are currently inadequate and the role of animal hosts in the epidemiology of human disease is not sufficiently-well characterised.

1.2 Basic Biology and Life-Cycle

1.2.1 Cellular Details

Humans and animals become infected by ingesting the cystic stage of *Giardia*, which is carried *via* faeces through a variety of transmission routes. The cysts excyst in the small intestine to release the mature adult stage, which are termed trophozoites (Bernander *et al*, 2001b). The trophozoite stage is at least tetraploid (4N), having two sister nuclei each containing a full set of chromosomes (Figure 1-1: Life cycle of *Giardia duodenalis* Figure 1-1) (Adam, 2000; 2001; Bernander *et al*, 2001b; Le Blancq & Adam, 1998). Each of the two 2N nuclei in a single trophozoite replicates to become four 2N nuclei (8N), a cycle that occurs within replication during the vegetative growth stage. Another round of replication takes place between the vegetative growth stage to encysting, meaning the cyst contains four 4N nuclei (16N). Once this cyst begins the excystation process it divides into four trophozoites, each with two 2N nuclei (4N) (Bernander *et al*, 2001a; Capewell *et al*, 2021). While the *Giardia* parasite is understood to reproduce asexually on the villi of the small intestine, the involvement of non-obligate sexual or parasexual reproduction has also been suggested (Caccio & Ryan, 2008; Yu *et al*, 2002). Following encystation, the parasites exit the body into the environment *via* faeces, to be ingested and repeat the transmission cycle. These

cysts are highly environmentally resistant and can survive outside the host for days to months, depending on variables such as location, surrounding temperature and amount of organic matter present (Alum *et al*, 2014). This means removing organic matter from the environment as quickly and regularly as possible aids in disrupting the transmission cycle of *Giardia* and can help lower infection rates.

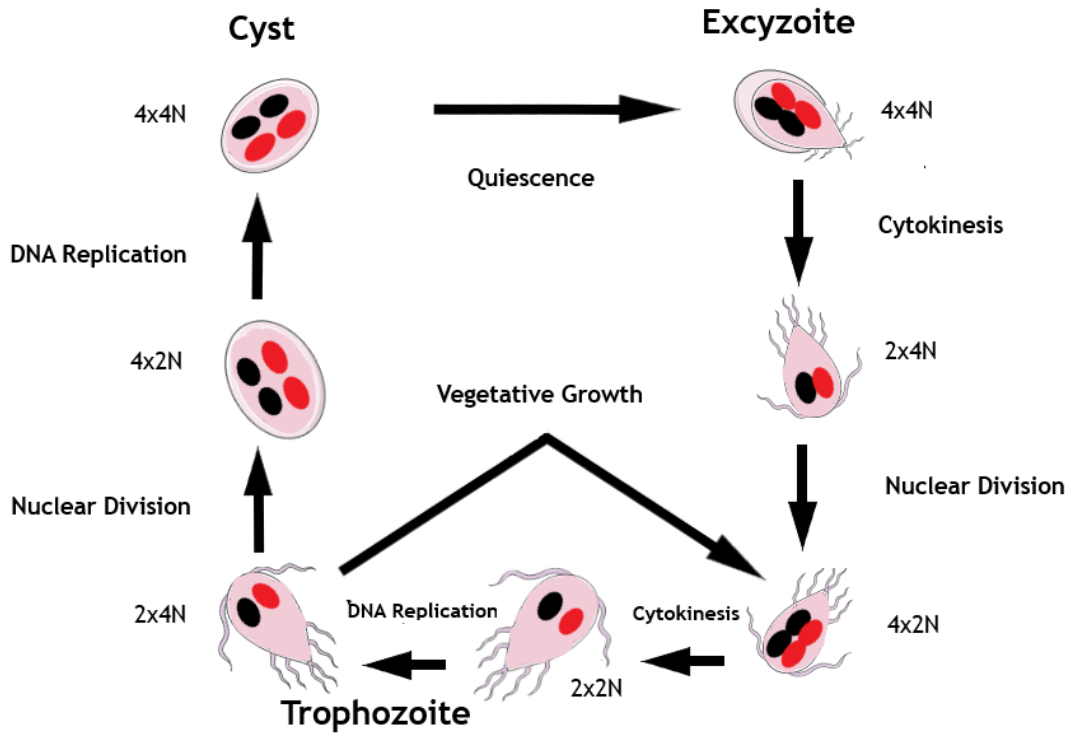

Figure 1-1: Life cycle of Giardia duodenalis

Adapted from Capewell *et al* 2020.

Although *Giardia duodenalis* is considered a single species, multiple genotypically distinct sub-types exist. These sub-types are termed “assemblages” and exhibit varying degrees of mammalian host specificity, with some assemblages found in multiple host species (Adam, 2000; 2001; Caccio & Ryan, 2008; Lebbad *et al*, 2010; Read *et al*, 2004). Eight assemblages have been defined and have been assigned the letters A through to H (Table 1-1). Assemblage A consists of two sub-assemblages, A1 and A2, which are able to infect humans along with assemblage B. Despite the ability of these assemblages to infect multiple mammals they are often identified by one host, such as assemblage F which is termed the “feline assemblage” (Caccio *et al*, 2018; Rojas-Lopez *et al*, 2022). Assemblage nomenclature is intended, in part, to signify the main host species infected. It is, however, possible for parasites to enter and attach to the small intestine of other

species although they may be unable to excyst (Caccio *et al*, 2018). The list of species able to successfully harbour each individual assemblage increases as time progresses and more studies are undertaken on a wider variety of mammals (Heyworth, 2016).

Assemblage	**Known Potential Hosts**
A	**Humans**, dog, cat, livestock (cattle, sheep, pigs, goats), horses, alpacas, deer, cetaceans, chinchillas, ferrets, beavers, jaguars, marsupials, muskox, non-human primates, seals, Australian sea lions, moose, reindeer, chickens, gulls
B	**Humans**, cats, dogs, livestock (cattle, sheep, pigs), horses, deer, chinchillas, beavers, gazelles, muskrats, ferrets, rabbits, Desmarest's hutia, marsupials, guinea pig, rock hyrax, non-human primates, chickens, seals, Australian sea lions, ostrich, dolphins, porpoises, gulls
C	**Dogs**, cattle, pigs, cetaceans, kangaroos
D	**Dogs**, cattle, foxes, cetaceans, chinchillas, kangaroos
E	**Livestock** (cattle, sheep, pigs, goats), horses, alpacas, yaks, foxes, deer, cats, humans
F	**Cats**, cetaceans, pigs
G	**Rats, mice**
H	**Seals, gulls**

Table 1-1: Summary of assemblage hosts

Primary host in bold font (Heyworth, 2016)

1.2.2 Advances in *Giardia* typing

Historically morphological differences were used to attempt to differentiate between host-specific strains of *Giardia* under microscopic examination (Feely & Erlandsen, 1985). This misguided methodology named each new morphological type as a separate species of the parasite (Adam, 2001). Microscopy was then determined to be insufficient as a method for assemblage discrimination and zymodeme analysis was adopted in addition to chromosome comparison (Adam, 2001; Adam *et al*, 1988; Meloni *et al*, 1988; 1995; Proctor *et al*, 1989). This approach, undertaken by Proctor *et al*, was an attempt to discover a correlation between clinical presentation of giardiasis with the visualised isoenzyme patterns of *Giardia* isolates from both humans and beavers using starch gel electrophoresis. It was ultimately unsuccessful, as Proctor found isolates corresponding to multiple isoenzyme patterns were able to cause clinical signs in both humans and beavers. Eventually the field settled on the use of more specific genetic techniques, such as multilocus gene sequencing using polymerase chain reaction (PCR) (Caccio *et al*, 2002; Read *et al*, 2004; Sulaiman *et al*, 2003). These methods have seen little improvement since their invention in the early 2000s, a point recognised by researchers in Brazil (Durigan *et al*, 2018). In an attempt to overcome difficulties with these markers, discussed in Section 1.2.5, they screened the limited number of *Giardia* genomic sequences publicly available on GiardiaDB for microsatellites to find new genetic targets for development (Durigan *et al*, 2018). This comprised genomes representing two assemblage A isolates (Genomes WB, DH), one assemblage B isolate (Genome GS) and one assemblage E isolate (Genome P15). The Brazilian researchers found over 1,850 simple sequence repeats (SSRs) loci in the five GiardiaDB genomes, of which 20 were polymorphic and only one was found in each of the five full genomes. This study went on to develop 60 new primers based on microsatellites, developed with ‘Primer 3 Plus’ and ‘Primer Select’ tools. Limitations of these primers include their exclusive *in silico* testing and the exclusion of isolates that did not fit exclusively into one assemblage using the three traditionally used markers. Novel techniques to discern between assemblages should ideally consider subtle differences in genotype but also be tested *in vitro* to determine if they can be used to draw epidemiological and biological conclusions. Whole genome sequencing (WGS) has also been utilised in

a few recent *Giardia* studies. One study in British Columbia, Canada, used a NexteraXT DNA Prep Kit (Illumina, San Diego, CA) to prepare a DNA library for WGS using the Illumina sequencing platform after a historic outbreak (Prystajecky *et al*, 2015). The reads were assembled to create whole genome sequences which were compared against reference genomes A, B, and E (Prystajecky *et al*, 2015). This study used stored samples from the historic human outbreak, water samples gathered periodically over four years, faecal samples from sporadic, non-travel associated human cases of giardiasis, and faecal samples from local beavers to create a pool of data. WGS was then used to demonstrate the relatedness of these samples in more depth than would have been possible with any single marker on its own, which aided the discovery that beavers in the area were harbouring a different assemblage than previously thought. These results led to heightened surveillance of the parasite in British Columbia. Following that study, another study in the same location used WGS to characterise *Giardia* in additional host species (Tsui *et al*, 2018). Whereas the previous study focussed on human and beaver samples, this study also included canine, ovine and feline samples. The researchers used MiSeq Illumina to analyse 89 isolates and the phylogenetic analysis of the results showed evidence supporting beaver involvement in two outbreaks in the area. The other animal isolates were found to be human-infective assemblages A and B, with six samples showing mixed infection. The authors note that the use of WGS overcomes one limitation of using individual loci, i.e. the inability to identify mixed infection, as there will be a preferential PCR primer binding with one of the two assemblages. They suggest important data is being lost, which could be retained using a method such as WGS.

1.2.3 Detection of unexpected assemblages

Before the genome was being explored by researchers in Brazil and Canada, important host discoveries were being made worldwide. In Spain an assemblage F isolate (putatively a feline assemblage) was found in several samples taken from cattle (Cardona *et al*, 2015). This study attempted to use two loci, *bg* and *gdh*, with which to assemblage-type 68 samples. However, *bg* was only able to amplify 4/68 (0.06%) samples, none of which were found to be assemblage F amplicons. Another study in China identified assemblage E amplicons (putatively a livestock genotype) from several rabbit hosts, which was the first reported case of its kind (Qi *et al*, 2015). In this study, all three loci in addition to the 18s subunit loci were

used on 80 microscopy-positive samples. Of these, only five samples were able to amplify at all four loci, and while these results agreed on the assigned assemblage, there was no agreement of sub-assemblages. Four of the six assemblage E amplicons isolated from these samples disagreed with at least one additional locus, while the two remaining amplicons agreed between multiple loci. Additionally, a study in Egypt discovered an assemblage E isolate in human samples using the *tpi* locus (Foronda *et al*, 2008). The authors mention the limitations of using one locus for genotyping, but the previous two studies highlight another limitation, i.e. discordance between different markers. These studies describe samples with novel host-assemblage findings, yet few samples have corroborating results from multiple markers. This is a consequence of the lack of well-characterised, high-resolution genetic markers available for *Giardia* analysis. In addition to discordance, low PCR amplification rates further hamper the reproducibility of novel results such as these. An improvement in the current panel of molecular markers is needed to increase amplification success and support novel findings.

1.2.4 Reproductive Discoveries

An issue that has plagued the *Giardia* field is that its mode of reproduction is not fully known and this has been evident since early microscopic studies. The reproductive mechanism of *Giardia* was unable to be visualised in its entirety, leading to gaps in the understanding of this process (Sagolla *et al*, 2006). One issue raised was the inability to visualise intermediate stages of mitosis, which was addressed by Sagolla *et al*. Where previously microscopy was used to visualise the intermediate stages of mitosis, Sagolla's team used three-dimensional visualisation with components tagged by immunofluorescent markers to observe mitotic events from all angles. This allowed the visualisation of unexpected events, such as the two nuclei stacking on top of each other in the middle of the trophozoite before replication occurs. *Giardia* was also observed to undergo a process called semi-open mitosis, which occurs in the prometaphase stage of replication (Lagunas-Rangel *et al*, 2021). In this phase, the central spindles and microtubules can form within the nuclear envelope, as with closed mitosis, through partial openings in strategic locations in the nuclear envelope, as with semi-open mitosis, or outwith a disassembled nuclear envelope, as with open mitosis (Asakawa *et al*, 2016; Lagunas-Rangel *et al*, 2021; Sagolla *et al*, 2006). *Giardia* was visualised as having external central spindles and microtubules

through polar openings in the nuclear envelope, with the remaining envelope intact. This process is not unique to *Giardia*; it is a phenomenon known to occur in *Drosophila melanogaster*, *Asterina miniata*, *Aspergillus nidulans*, *Ustilago maydis*, *Caenorhabditis elegans* and *Schizosaccharomyces japonicus* (Asakawa *et al*, 2016; Lagunas-Rangel *et al*, 2021). These findings are important for a number of reasons: first, a mitotic process that requires externalisation of nuclear components is an ideal target for treatment development; second, if the genetic sequence for the nuclear envelope could be identified, it could be modified for anti-protozoal effect or studied further. These discoveries served to increase the understanding of *Giardia* basic biology and have the potential to advance the work in applied areas of study.

1.2.5 Current issues with *Giardia* genotyping

The entire genome of *Giardia duodenalis* is estimated to be around $1.2x10^7$ bp with a GC content of nearly 50%, and does not contain a large amount of non-coding sequences or introns (Adam, 2000; Ankarklev *et al*, 2015; Capewell *et al*, 2021). The genomes of each nucleus within a trophozoite are not always exact copies, and there can be as many as four sites of heterozygosity per genome (Capewell *et al*, 2021). This potential for heterozygosity has led to the endeavour to discern between genetically distinct *Giardia* lineages. Genotyping technology has been utilised to gain information in multiple areas such as host-assemblage relationships, disease-assemblage relationships and epidemiological assemblage data (Monis *et al*, 2009). Multilocus sequence typing (MLST), the currently adopted genetic typing methodology used in determining these relationships, can provide useful information, but this technique is still relatively low-resolution due to the gaps in knowledge regarding genomic heterogeneity (Caccio *et al*, 2008; Capewell *et al*, 2021). A large proportion of samples cannot be typed using MLST due to PCR failure, presumably because of allelic polymorphism in the *Giardia* population. As mentioned in a review by Capewell *et al*, this may mean the PCR primers used for assemblage-typing are too specific to cope with the amount of target loci variability in field samples (Capewell *et al*, 2021). The reproducibility of results with one marker or between multiple markers is also low. Due to frequently conflicting results, it is worthwhile considering what the underlying reason may be, for example (a) poorly characterised markers, (b) the presence of mixed infection, (c) genetic exchange between assemblages or (d) a combination of the

above. These are important questions that must be addressed to facilitate effective outbreak investigation. An alternative approach, using markers specifically designed for one assemblage, may not allow for the amplification of non-target assemblages and important epidemiological information being overlooked (Ankarklev *et al*, 2015). Further assemblage classification information and more in-depth reproductive information could be gained from analysing more genetically diverse sequences with a higher degree of confidence using reliable markers (Capewell *et al*, 2021).

One study in France attempted to use two markers, *bg* and *tpi*, to assemblage-type 19 human isolates which were *Giardia* positive on microscopy (Bonhomme *et al*, 2011). All isolates were successfully amplified as assemblage A or B, and while the markers agreed on assemblage for all 19 samples, 14/19 (73.7%) disagreed on sub-assemblage. Another study in China used *gdh, bg* and *tpi* to screen 279 samples of unknown *Giardia* status from domesticated chipmunks across seven pet shops (Deng *et al*, 2018). Of 279 samples, 24 samples amplified with multiple loci: 8/279 (8.6%) were positive using *bg*, 17/279 (6.1%) were positive for *gdh* and another 17/279 (6.1%) were positive for *tpi*. The two predominant assemblages identified were A and G, and while the assemblage-assignment was agreed between at least two loci for 17 samples, 6 of those 17 were not able to amplify at a third locus and four of all 24 successfully-typed samples only be amplified at a single locus. Additionally, there was agreement on sub-assemblage for only five of the successfully amplified samples. A third study in England looked at over 400 microscopy-positive faecal samples from symptomatic patients and assemblage-typed the samples using the *gdh, bg, tp,* and 18s loci. Out of 406 samples, 218 (54%) amplified at least one marker (Minetti *et al*, 2015a). While the authors state these amplicons were unambiguously assemblage A, again sub-assemblage typing results rarely agreed among all loci. Additionally, data for only 76/218 (38.9%) is shown, suggesting the remaining samples returned ambiguous assemblage results between loci. The authors also speculate that these results indicate these human hosts are harbouring multiple sub-assemblages, without discussing the possibility of poor-quality markers. Even where assemblage data may match between loci, sub-assemblage data is often contradictory.

1.2.6 Bergen Outbreak

One of the most well-known outbreaks of *Giardia* occurred in 2004 in Bergen, Norway, which infected an estimated 5,000-6,000 people from ingesting contaminated water (Landvik, 2015). This contamination was caused by a period of increased rainfall, which, in conjunction with a compromised sewage system, pushed an increased volume of raw, contaminated water through a processing plant and to the general populous. This inspired a series of studies, not only around the time of the outbreak for the sake of outbreak investigation, but also in subsequent years to observe the cohort and resulting after effects. Before sewage and rainfall were implicated in this outbreak, dog faecal material was investigated as a potential source of infection (Landvik, 2015; Robertson *et al*, 2015). Genotypic analysis would have been useful to determine animal involvement in this case, as animal samples could have been assemblage-typed and undergone phylogenetic analysis with the outbreak samples to determine the degree to which they were related. One year after the outbreak, a study was undertaken to type *Giardia* isolates recovered from the sewage of four major processing stations servicing Bergen (Robertson *et al*, 2008). These were analysed using *gdh, bg* and *tpi*. For 3/16 (18.8%) samples no locus was amplified, 2/16 (12.5%) samples resulted in assemblage B, 1/16 (6.3%) samples resulted in assemblage A, and 9/16 (56.3%) samples amplified both A and B amplicons. Four samples failed to amplify, and the authors note these samples were the only four samples that did not undergo pre-analysis preparation such as buffer washes, centrifugation, or immunomagnetic separation. These results, corresponding to the two accepted human-infective assemblages, were not unexpected as the establishments were processing human waste from households within the city. This data is useful in that it provides an insight into the baseline epidemiological situation, regarding a potentially endemic parasite in Bergen. Other studies to stem from the Bergen outbreak focussed on those with chronic fatigue three and five years post-exposure and post-infection gastrointestinal disorders (Hanevik *et al*, 2017; Martinez *et al*, 2020). The utilisation of efficient genotypic markers in situations such as this can help identify reservoirs of infection and prevent future outbreaks.

1.3 Objectives of this work

The objectives of this work were two-fold:

1. To investigate the importance of *Giardia duodenalis* as an endemic, outbreak-associated pathogen in the context of high-income countries. This was achieved by performing a scoping review of the literature to provide a comprehensive overview of the subject and to determine the relative importance of different transmission routes.

2. To compare, genetically, companion animal and human *Giardia* isolates in a high-income country, using improved genotyping methodology. Published genomic data was utilised to increase sensitivity of a PCR-based assemblage marker and this improved assay was applied to a large collection of Scottish cat, dog and human isolates to provide new, baseline molecular epidemiological data.

Chapter 2. A scoping review of risk factors and transmission routes associated with human giardiasis outbreaks in high-income settings

2.1 Background

Giardia duodenalis (synonyms *Giardia intestinalis* and *Giardia lamblia*) is one of the leading causes of parasitic gastrointestinal disease, leading to potentially over 180 million annual cases worldwide (Torgerson *et al*, 2015). This flagellated protozoan parasite causes giardiasis, with symptoms including diarrhoea, nausea, vomiting, abdominal pain, and excessive gas production. The disease can be effectively treated with nitroheterocycles, in particular metronidazole, although there are emerging reports of metronidazole resistance (Ansell *et al*, 2015; Leitsch, 2015; Muller *et al*, 2018). Infection can result in long-term complications including irritable bowel syndrome (IBS) and chronic fatigue (Dormond *et al*, 2016; Hanevik *et al*, 2014; Litleskare *et al*, 2018). The parasite is ingested in its cystic form and remains contained until it reaches the stomach. Once exposed to stomach acid, the cyst releases vegetative trophozoites that attach to the small intestine, causing clinical signs as they replicate (Adam, 2001; Bernander *et al*, 2001a). After moving through the proximal portion of the gastrointestinal system, some trophozoites re-encyst in the jejunum before being excreted to continue the life-cycle of the parasite and infect new hosts (Adam, 2001). Although trophozoites rapidly degrade once excreted, cysts are highly robust and can last many months in the environment without a host. Transmission of infectious cysts is possible *via* a variety of different routes, including person-to-person contact, animal-to-human contact, and contaminated water and food sources. Poor quality sanitation and water filtration systems are typically thought to be responsible for transmission of cysts in lower to middle income countries (LMICs), whereas travel and food are more commonly thought to be the transmission route in higher income countries (Leung *et al*, 2019). Infection then occurs when faecal material containing infective cysts is ingested through one of these routes. The parasite has a wide host range, and a variety of subtypes exist, known as assemblages A–F. These assemblages infect many mammals and are largely host-specific, although assemblages A and B demonstrate the capacity to be zoonotic (Ryan & Caccio, 2013).

Giardiasis is supposedly less prevalent in high-income countries than LMICs, ranging from 2–7% for the former to 20–30% for the latter (Leung *et al*, 2019). The condition is diagnosed in higher income countries using a range of methods, with conventional identification involving microscopy to directly identify trophozoites and cysts excreted in faeces. However, microscopy is being replaced by more sensitive molecular methods, including polymerase chain reaction (PCR) and enzyme immunoassays (EIA) that detect parasite-specific genes or antigens. These permit the efficient and rapid screening of large numbers of samples for *Giardia* and other gastrointestinal pathogens simultaneously. In the past, diagnostic testing of patients in many higher income countries has largely been confined to testing symptomatic individuals with a history of travel to specific, perceived *Giardia*-risk countries, resulting in significant under-reporting of this pathogen (Alexander *et al*, 2017). With this increasing awareness of endemic disease in higher income countries, a greater number of samples from symptomatic cases are now being tested using more sensitive tools. As this includes patients without a history of travel, it is likely that a greater number of sporadic cases, clusters, and outbreaks will become evident. This will lead to more accurate assessment of the potential risk factors and transmission routes in higher income countries. Improving our understanding of *Giardia* transmission and raising awareness of giardiasis are essential to ensure cases receive appropriate treatment and are important for public health authorities to identify points at which interventions can be made. This is of particular concern as the parasite is easily spread between humans and has the potential to cause long-term complications (Hanevik *et al*, 2014; Litleskare *et al*, 2018). There is also evidence from LMICs that infection in young children can impact growth and development, impacting such biological processes as iron absorption, retinal morphology, and hepatic and pancreatic functionality (EPA, 1999; Lehto *et al*, 2019; Rogawski *et al*, 2017; Rogawski *et al*, 2018). With the increased recognition that there is significant under-reporting of *Giardia* in higher income countries, we hypothesise that there are underappreciated endemic sources of infection that may impact public health. Additionally, previous studies have primarily focused on one transmission route. These routes have been included and expanded upon (Baldursson & Karanis, 2011; Efstratiou *et al*, 2017; Karanis *et al*, 2007). The aim of this work was, therefore, to undertake a systematic review of the literature to identify sources and transmission routes associated with human giardiasis outbreaks in higher income

countries and establish the accuracy of the hitherto accepted assertion that giardiasis is primarily a sporadic travel-associated illness.

2.2 Search Approach

2.2.1 Literature search

The original search was performed between September 2016 up to and including November 2021, with assistance from Paul Capewell and Alison Smith-Palmer. Searches of titles and abstracts were undertaken on Medline, Embase, and PubMed databases using the following query: ((*Giardia* OR Giardiasis) AND Outbreak) OR (((*Giardia* OR Giardiasis) AND Outbreak) AND (Risk Factor OR Travel OR Pets OR Water OR Swimming Pools OR Food OR Cat OR Dog OR "companion animal")). The searches yielded a total of 254 articles.

2.2.2 Inclusion and exclusion criteria

Manuscripts were initially screened based on titles and abstracts to exclude irrelevant studies, such as those that primarily examined animal outbreaks over human outbreaks or were primarily reporting data for another pathogen but mentioned *Giardia* as a comparator. Manuscripts were also excluded if *Giardia* was not suspected as the primary pathogen of interest or if they were not originally written in English to avoid issues with translation accuracy. The full text versions of all manuscripts from the initial screen were obtained using a combination of library services and online repositories. To ensure an accurate and minimally biased systemic review, this procedure was performed independently by myself and an additional expert reviewer (Paul Capewell). The full texts of the potential manuscripts were then assessed by both reviewers in discussion using the following inclusion criteria: (i) that the manuscript reported primary outbreak data and was not a case report describing an individual patient; (ii) that the outbreak primarily focused on human cases; (iii) that the source and causative agent of an outbreak were unambiguously identified; (iv) the reported outbreak occurred in a country on the Organisation for Economic Co-Operation and Development's (OECD) list of upper-middle-income countries and territories (Figure 2-1).

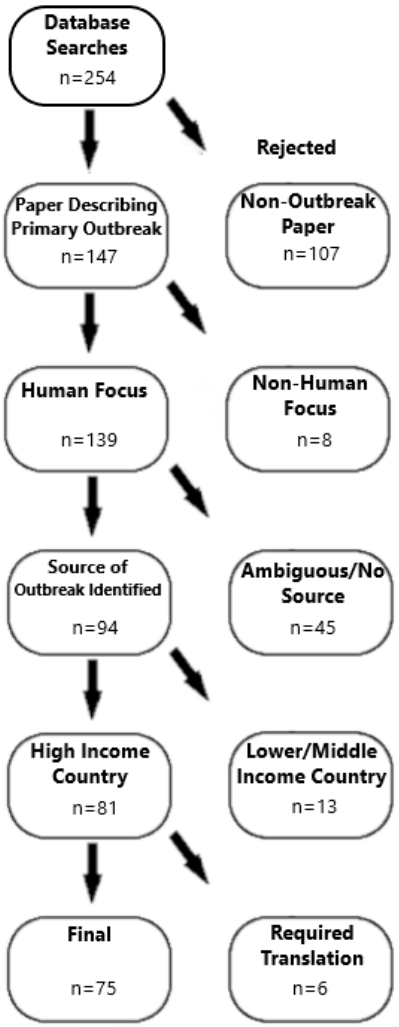

Figure 2-1: Flowchart of exclusion criteria

All study designs that met these criteria were included, including case-control and observational studies, as the primary aim of the review was to identify potential sources and transmission routes associated with *Giardia* outbreak rather than estimate the size of these risks. The identified sources and transmission routes for each outbreak were determined by each researcher independently and classed as being associated with travel, water contamination, food contamination, animal contact, person-to-person contact, or exposure to raw sewage. More refined distinctions were made within each class to provide further details (Appendix Table 1- 1). All articles could be classified into these six categories, which were generated during assessment of the literature. Due to the observational nature of the data, the MOOSE Guidelines for Meta-Analyses and Systematic Reviews of Observational Studies were applied (Stroup *et al*, 2000). Identified transmission

routes were encoded in a shared data table for ease of access, including additional metadata, such as authors, number of cases, PMID, date of study, date of publication, and country. After a final discussion between the reviewers to resolve disparities, a total of 75 papers were included in the review (Figure 2-2).

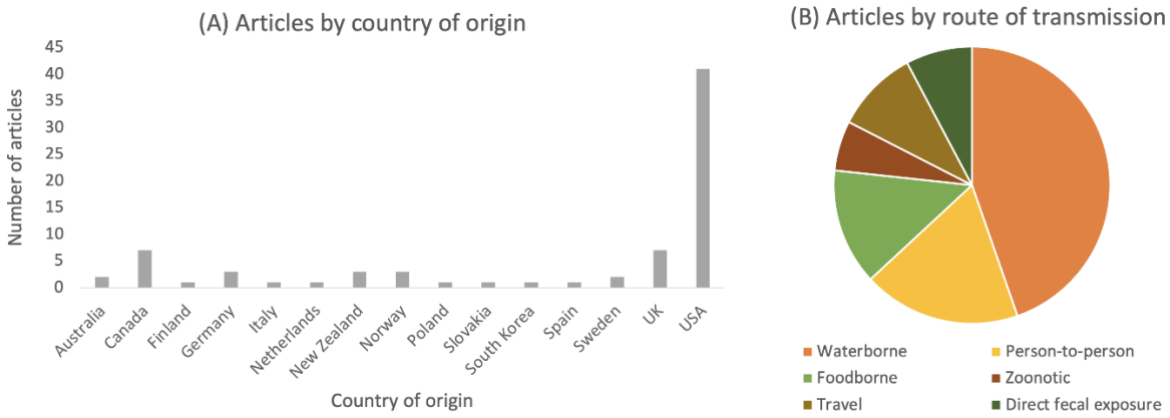

Figure 2-2: Country distribution and transmission routes cited by study (n = 75)

2.2.3 Sources of bias and heterogeneity

It was noted that very few of the manuscripts were case-control studies or presented an estimate of risk, with the majority reporting a description of a *Giardia* outbreak in a higher income country with an identified source of infection. This is likely an example of publication bias in which outbreaks without an identifiable source are not deemed sufficiently interesting to merit publication. In addition, there was significant heterogeneity in the study type, methodology, and detection tools used across the 75 papers with almost no study being directly analogous to any other. Both the apparent bias and high study heterogeneity prevent a formal meta-analysis of the data. However, as the aim of this review was to establish a list of potential outbreak sources and transmission routes in higher income countries rather than estimate risks, the systematic analysis of the data served to highlight areas in which further analysis and formal case-control experiments are required in the future.

2.3 Waterborne Transmission

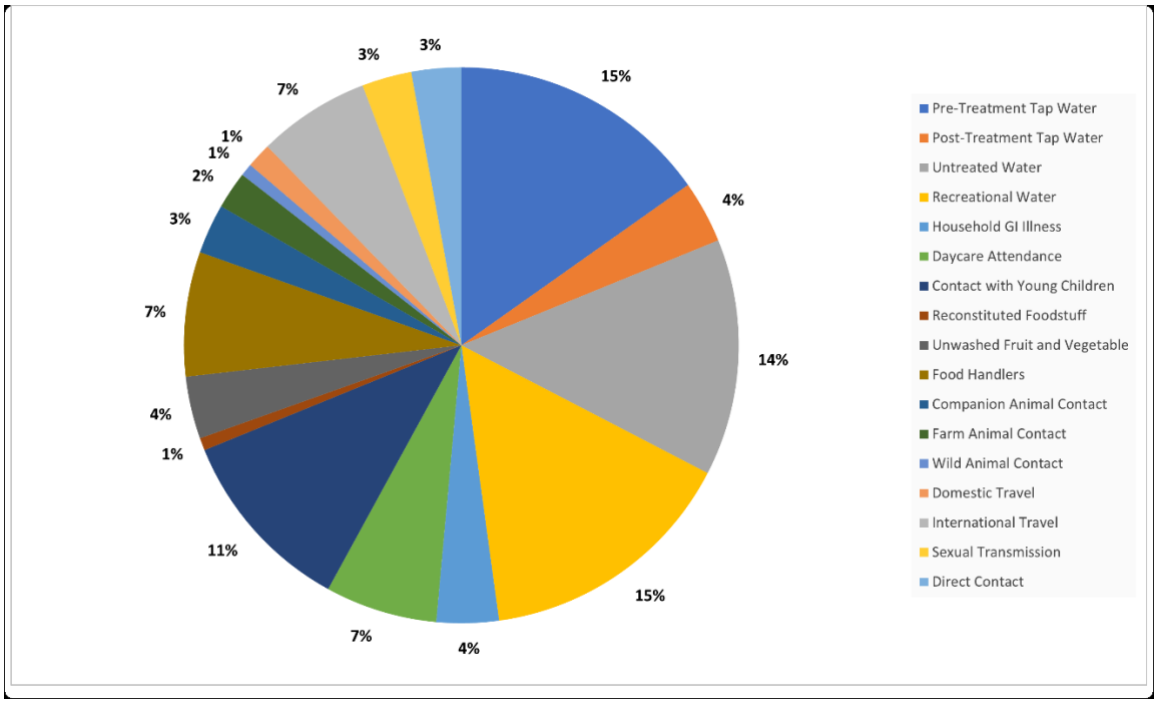

Figure 2-3: Transmission routes for giardiasis cited in studies (n = 75)

Waterborne transmission is one of the most important *Giardia* transmission routes in LMICs (Fakhri *et al*, 2021) and was found to account for the majority of outbreaks in this systematic analysis of higher income countries (Figure 2-3). This includes outbreaks in the USA, Canada, UK, Europe, the Nordics, and Southeast Asia, demonstrating that a range of water treatment approaches across a spectrum of higher income countries can be vulnerable to failure, leading to water contamination. Previously, a review of waterborne parasites in higher income countries found that *Giardia* was the second most frequently cited protozoan agent after *Cryptosporidium*, responsible for 37% of waterborne outbreaks (Efstratiou *et al*, 2017). Similarly, a review of waterborne outbreaks in Nordic countries indicated that parasites accounted for the largest outbreaks of gastrointestinal upset during the time period studied, even when compared to bacterial or viral causes (Guzman-Herrador *et al*, 2015). Of particular note with respect to waterborne outbreaks is the large number of cases per outbreak, with some having several hundred or more (Dykes *et al*, 1980; Lopez *et al*, 1980; Navin *et al*, 1985; Nygard *et al*, 2006; Weniger *et al*, 1983). Waterborne transmission of

Giardia cysts occurs *via* a variety of routes, including contaminated drinking water, swimming pools, rainwater tanks, and recreational lakes. These transmission routes increased the risk of contracting giardiasis, demonstrated both through case-control studies and outbreak investigations.

2.3.1 Drinking water

Of the 46 outbreaks described as having a water transmission route or water involvement, approximately 33 involved contaminated drinking water and led to at least 8045 laboratory confirmed cases of giardiasis in higher income countries from 1974–2016 (Table 2-1; several studies did not attribute outbreaks to case numbers and were therefore not included in the case count). Contamination of treated water by raw, untreated water or sewage was stated as a factor in at least five outbreaks. Other contributing factors cited included structural defects in water distribution systems, insufficient chlorination or poor to no filtration system, and the presence of *Giardia*-positive North American beavers (*Castor canadensis*) in the water catchment area (Dykes *et al*, 1980; Istre *et al*, 1984; Navin *et al*, 1985). This has been further explored in work by Tsui *et al*. (Tsui *et al*, 2018), who used whole genome sequencing to suggest the presence of beavers in water catchment areas and along riverbeds was a possible source of human infection *via* contaminated water but acknowledged that this was only one factor in a complex cycle of zoonotic spread. Direct contact with raw sewage following a system failure in a private residence caused at least one outbreak in Bratislava, Slovakia (Totkova *et al*, 2004). Rainwater run-off from sewer systems after severe natural events (including volcanic eruption) also led to contamination of surface water (Weniger *et al*, 1983). One German study investigated two separate sewer systems that tended to overflow when rainwater contributed to their volume, which would contaminate nearby natural bodies of water. *Giardia* was found in 12/38 (31.6%) water samples from sewer run-off, which emptied into a nearby catchment area where the local population frequently walked with their companion animals (Schreiber *et al*, 2019). This highlights how rivers and other water sources contaminated with *Giardia* from slaughterhouses and sewage run-off can pose a contamination risk for humans and animals in the area (Ma *et al*, 2019a; b).

Communal water supplies are normally treated to prevent contamination with *Cryptosporidium* and *Giardia*, but this requires carefully controlled conditions and

proper maintenance of treatment systems. Failure in any aspect of these systems can result in outbreaks due to inadequate removal of infective cysts. Outbreaks may also arise from post-treatment contamination due to pipe system damage or wastewater leakage. Welch (Welch, 2000) conducted a meta-analysis to test the hypothesis that consumption of water in rural regions of North America posed a statistically significant risk for the acquisition of giardiasis. The author notes that published reports demonstrate a higher incidence of giardiasis among people engaging in outdoor recreational activities, but there is minimal evidence for an association between this and giardiasis. The study also states that although greater emphasis is given to water purification when in the rural outdoors, the reason for increased giardiasis incidence may be due to relaxed hygiene practices on camping trips rather than raw water consumption. The use of private water supplies may also contribute to increased risk of giardiasis as they are more common in rural areas and are not subject to the same stringent water quality testing or regulations as public water supplies (Reeve *et al*, 2018; Welch, 2000). Masina *et al*. (Masina *et al*, 2019) and Ma *et al*. (Ma *et al*, 2019a; b) suggest that the use of indicator bacteria such as *E. coli* and coliforms to test the quality of tap water for ingestion may not be adequate for all pathogens present, as the absence of indicator bacteria does not necessarily indicate the absence of waterborne parasites such as *Giardia* spp. Parasites are notably more difficult to identify in water samples as they cannot be readily cultured and are found at lower concentrations in the environment.

Location	Reference	Year	No. of cases or samples (Lab confirmed)	Tap water		Untreated water	Recreational water (swimming, etc.)	Beaver involvement
				Pre-treatment	Post-treatment			
Australia	Dale *et al*. (2010)	2001-2007	12 (3)			×		
Canada	Isaac-Renton *et al*. (1999)	1996	590 (590)	×				
Canada	Isaac-Renton *et al*. (1994); Isaac-Renton *et al*. (1993)	1991-1992	124 (124)			×		×
Canada	Greensmith *et al*. (1988)	1986	59 (30)				×	
Finland	Rimhanen-Finne *et al*. (2010)	2007-2008	37 (37)	×				
Italy	Resi *et al*. (2021)	2018-2019	228 (228)		×			

Netherlands	Pijnacker *et al.* (2016)	2010–2013	219 (219)					×	
New Zealand	Wilson *et al.* (2008)	2006	1214 (1214)				×	×	
Norway	Nygard *et al.* (2006)	2004–2005	2500 (1268)	×					
South Korea	Cheun *et al.* (2013)	2010	9 (7)				×		
Sweden	Neringer *et al.* (1987)	1982	56 (56)	×					
UK	Jephcott *et al.* (1986)	1985	108 (108)		×				
UK	Gray *et al.* (1994)	1992–1993	74 (74)				×	×	
UK	Hall *et al.* (2017)	2012	4 (4)					×	
USA	Levine *et al.* (1990)	1986–1988	4 unique outbreaks[a]	×				×	
USA	Kramer *et al.* (1996)	1993–1994	9 unique outbreaks[a]	×	×		×	×	
USA	Moore *et al.* (1993)	1991–1992	8 unique outbreaks[a]	×	×		×	×	
USA	Herwaldt *et al.* (1991)	1989–1990	7 unique outbreaks[a]	×			×		
USA	Birkhead & Vogt (1989)	1983–1986	1211 (1211)	×			×		
USA	Birkhead *et al.* (1989)	1986	37 (23)	×					×
USA	Navin *et al.* (1985)	1982	324 (324)	×					×
USA	Kent *et al.* (1988)	1985–1986	703 (703)	×					× (and muskrat)
USA	Lopez *et al.* (1980)	1977	213 (213)	×					×, *C. can*
USA	Dykes *et al.* (1980)	1976	128 (128)	×					×, *C. can*
USA	Istre *et al.* (1984)	1981	20 (8)	×					
USA	Weniger *et al.* (1983)	1980	Estimated 781 (49)	×					
USA	Karon *et al.* (2011)	2007	46 (26)	×					
USA	Shaw *et al.* (1977)	1974–1975	350 (350)	×					
USA	Levy *et al.* (1998)	1995–1996	3 unique outbreaks[a]		×		×	×	
USA	Reses *et al.* (2018)	2003–2004	52 (52)				×	×	
USA	Bedard *et al.* (2016)	2009	36 (36)				×		
USA	Daly *et al.* (2010)	2007	31 (17)				×		
USA	Hopkins & Juranek (1991)	1983	31 (31)				×		
USA	Porter *et al.* (1988)	1985	9 (8)					×	
USA	Katz *et al.* (2006)	2003	149 (97)					×	
USA	Harter *et al.* (1984)	1982	70 (70)					×	
USA	Eisenstein *et al.* (2008)	2006	38 (35)					×	

Table 2-1: Giardiasis outbreaks due to waterborne transmission

[a]Number of individual cases undetermined as some studies referenced in these papers overlap with those included individually this table; cases and specific outbreak studies are not linked to one another in the original study.

2.3.2 Waterborne transmission: Recreational water

Swimming pool or recreational water was identified as the sole transmission route in seven outbreaks in higher income countries (Table 2-1), resulting in at least 463 laboratory-confirmed cases of giardiasis. Swimming in pools or natural water has previously been found to be a risk factor in several case-control studies (Dennis *et al*, 1993; Hoque *et al*, 2002; Reses *et al*, 2018; Xiao *et al*, 2017). Faecal contamination of pool water was stated as a source for four outbreaks in Canada and the USA with an increased incidence of giardiasis upon diving into the pool, due to the potential for accidental water ingestion (Greensmith *et al*, 1988; Harter *et al*, 1984; Katz *et al*, 2006; Porter *et al*, 1988). Swimming pools with additional features such as splash pads, water slides, or classes with young children in attendance accounted for several outbreaks (Eisenstein *et al*, 2008; Greensmith *et al*, 1988; Harter *et al*, 1984). While actively flowing water makes infectious agent identification difficult, *Giardia* was one of a range of pathogens identified among cases of gastrointestinal illness associated with an open swimming event in the River Thames, London (Hall *et al*, 2017).

2.4 Person-to-person Transmission

Direct or indirect person-to-person transmission was the basis for 12 outbreaks, with a laboratory confirmation of 2195 human cases (Table 2-2). Giardiasis outbreaks associated with person-to-person contact have been linked to households with young children or in childcare settings where young children are in close contact with each other, likely due to handling diapers (Hoque *et al*, 2002; Hoque *et al*, 2003; Hoque *et al*, 2001; Minetti *et al*, 2015b; Reses *et al*, 2018). Such associations highlight the importance of good personal hygiene in reducing transmission *via* regular hand washing by those caring for infants. Six outbreaks involved day-care facilities (Table 2-2), five of which involved children below five years of age. Transmission in childcare facilities was greatest when children were ambulatory but had not yet been toilet trained. Person-to-person transmission both within and out with households was identified in several studies (Table 2-2), with a consistent factor being contact with young children and/or involvement in the changing of infants' diapers. Of particular interest, one study reported a high percentage of asymptomatic cases (37 individuals out of 41 positive cases, with children aged 0–9 years most heavily affected) (Waldram *et al*, 2017) that were

only detected as a result of the household screening undertaken as part of the study. This also underlines the importance of good personal hygiene measures in the prevention of transmission.

Demonstrating the power of modern molecular genotyping approaches, Wang *et al*. (Wang *et al*, 2019) investigated the genetic diversity of *G. duodenalis* in cases in Spain between 2012 and 2018, comparing the distribution and clinical presentation of assemblages A and B between children and adults. They showed a significant difference in the distribution of assemblages between children and adults (P = 0.001) and that children under 12 years of age were more likely to have been infected by assemblage B (44/53, 83%) than assemblage A (9/53, 17%). Conversely, adults in this sample had comparable distributions of assemblages A and B (20/42, 47.6% and 22/42, 52.4% respectively). There was no significant difference in the distribution of assemblages by gender. Cases with assemblage A (4/29, 13.8%) were more likely to have asymptomatic infection than cases with assemblage B (1/66, 1.5%), with OR 10.4, 95% CI 1.108–97.625. The genotyping and subtyping results also suggest that anthroponotic transmission, such as within childcare facilities, is an important area of study for giardiasis outbreaks.

Although uncommon, sexual transmission has been identified as a potential route for some communities (Table 2-3) (Meyers *et al*, 1977; Reses *et al*, 2018). This transmission mechanism is recognised for a number of gastrointestinal pathogens including *Cryptosporidium* (Hellard *et al*, 2003), *Shigella* (Borg *et al*, 2012), and hepatitis A (Ndumbi *et al*, 2018). A study in England (Mook *et al*, 2018) used gender distributions in routine surveillance data stratified by age and region to show an excess of *Giardia* cases among males that the authors posit is linked to transmission in men who have sex with men (MSM). This has also been suggested from studies in the USA (Escobedo *et al*, 2014; Muller *et al*, 2018; Phillips *et al*, 1981; Reses *et al*, 2018). One multi-centre study testing samples from patients with acute gastroenteritis in Seattle, USA reported that enteric pathogens were detected in 56.3% of MSM cases tested. This was substantially higher than the 33.5% seen in the general population. Of these pathogens, *Giardia* was found in 20.5% of diarrheic MSM samples compared to 1.9% in the general population *(Newman et al*, 2020). Both studies used multiplex PCR panels for parasite detection.

Location	Reference	Year	No. of cases or samples (Lab confirmed)	Household GI illness	Daycare attendance	Young children
Canada	Keystone *et al.* (1978)	1976-1977	116 (116)		×	×
New Zealand	Wilson *et al.* (2008)	2006	1214 (1214)	×		
New Zealand	Hoque *et al.* (2001)	1998-1999	183 (183)			×
UK	Waldram *et al.* (2017)	2014-2015	143 (132)	×		×
UK	Ang (2000)	1999	11 (10), 3 asymptomatic		×	×
UK	Rauch *et al.* (1990)	1986-1987	27 (27), also asymptomatic outbreaks within the same population		×	
USA	Polis *et al.* (1986)	1982	39 (39)		×	×
USA	Bartlett *et al.* (1985)	1982-1983	187 (187), 105 asymptomatic		×	×
USA	Black *et al.* (1977)	1975	38 (38)		×	×
USA	Katz *et al.* (2006)	2003	149 (97), 105 *via* person-to-person			×
USA	Reses *et al.* (2018)	2003-2004	80 (80)			×
USA	White *et al.* (1989)	1986	88 (72)			×

Table 2-2: Giardiasis outbreaks due to person-to-person transmission

Location	Reference	Year	No. of cases or samples (Lab confirmed)	Sexual transmission	Direct faecal contact
Netherlands	Pijnacker *et al.* (2016)	2010-2013	219 (219)		×
New Zealand	Wilson *et al.* (2008)	2006	1214 (1214)		×
Slovakia	Totkova *et al.* (2004)	1998	7 (7)		×
USA	Newman *et al.* (2020)	2017-2018	31 (31)	×	
USA	Reses *et al.* (2018)	2003-2004	17 (17)	×	
USA	Meyers *et al.* (1977)	1975	6 (5)	×	

Table 2-3: Giardiasis outbreaks due to transmission via *direct faecal exposure*

2.5 Foodborne Transmission

Where foodborne transmission has been identified, 1401 laboratory-confirmed human cases were identified and contamination by an infected food handler was a key feature in these outbreaks. Few reports suggest the possibility of food items being intrinsically infected (Dawson, 2005; Dixon *et al*, 2013; Rose & Slifko, 1999; Slifko *et al*, 2000). Ten foodborne outbreaks of giardiasis were identified in this analysis across higher income countries (Table 2-4), with asymptomatic food handlers or people asymptomatic at the time of food preparation who later developed giardiasis contributing to eight of these. This included an outbreak at a private party (Porter *et al*, 1988); those who consumed fruit salad were seven times more likely to have been ill than those who did not. It was noted that the household had a child in diapers and a pet rabbit present in the kitchen where the fruit salad was prepared, both of whom were positive for *Giardia*. Therefore, it was likely that the food preparer became infected by the child and/or rabbit and then contaminated food due to poor hand hygiene. The contaminated food became the primary transmission route for the outbreak. In another outbreak, no individual food item was identified; the assemblage and subtype from one of the asymptomatic food handlers matched the two outbreak cases for which genotyping was available (Figgatt *et al*, 2017). An outbreak among UK tourists residing at a hotel in Greece was linked to several risk factors, including the consumption of raw vegetables (Hardie *et al*, 1999). Salads were identified as a risk factor in two case-control studies of sporadic giardiasis (Espelage *et al*, 2010; Stuart *et al*, 2003), while another study identified inadequate washing of raw fruits and vegetables as a risk factor (de Lucio *et al*, 2017). This route of transmission is further supported by the identification of *Giardia* in 10 of 19 salad products tested in a study from Spain (Amoros *et al*, 2010); however, rates of positivity were lower (10 of 475 samples) in a Norwegian study (Robertson & Gjerde, 2001). Conversely, two studies of sporadic cases in the USA (Reses *et al*, 2018) and UK (Minetti *et al*, 2015b) found eating raw fruit and vegetables was inversely associated with giardiasis. Reses *et al*. (Reses *et al*, 2018) suggested repeated exposure *via* contaminated raw produce could provide protective immunity and that this inverse association could reflect increased healthy behaviours among controls compared to cases. Individuals who frequently consume fruit and vegetables might

possess better general health habits than those who do not and could be less likely to contract giardiasis or develop a systemic infection.

Location	Reference	Year	No. of cases or samples (Lab confirmed)	Unwashed fruit and vegetables	Food handlers
Germany	Espelage *et al.* (2010)	2007-2008	24 (24)	×	
New Zealand	Wilson *et al.* (2008)	2006	1214 (1214)		×
Spain	de Lucio *et al.* (2017)	2014	6 (6), also 16 (16) dogs 2 (2) cats	×	
USA	Porter *et al.* (1990)	1986	10 (8)		×
USA	Figgatt *et al.* (2017)	2015	20 (20)		×
USA	Quick *et al.* (1992)	1990	27 (11)		×
USA	White *et al.* (1989)	1986	88 (72)		×
USA	Petersen *et al.* (1988)	1985	13 (11)		×
USA	Mintz *et al.* (1993)	1990	27 (18)		×
USA	Osterholm *et al.* (1981)	1979	31 (17)		×

Table 2-4: Giardiasis outbreaks due to foodborne transmission

2.6 Zoonotic Transmission

The search results yielded two studies each for farm animal and companion animal contact transmission routes for giardiasis, affecting 408 people and the elderly at a rate of 5193/10000 people (Table 2-5) (Brunn *et al*, 2019; Jagai *et al*, 2010; Rehbein *et al*, 2019; Wojcik-Fatla *et al*, 2018). Until relatively recently, the lack of robust molecular genotyping for *Giardia* has hampered work to fully understand zoonotic transmission. One study conducted in the USA (Jagai *et al*, 2010) on the impact of cattle density on rates of *Cryptosporidium* and *Giardia* concluded that higher annual rates of giardiasis were recorded in rural areas with low population density, and these populations were likely to be at greater risk of protozoan infections regardless of cattle density (Jagai *et al*, 2010). It did, however, find strong seasonal patterns, with areas with a large cattle-to-human population ratio showing a peak in *Cryptosporidium* and *Giardia* infections during late October. Conversely, a lack of association with cattle was reported from a study of children

and cattle in Spain (Cardona *et al*, 2011) despite another study in the same area detecting *Giardia* in 18.8% of cattle fecal samples (Cardona *et al*, 2015). Brunn *et al*. (Brunn *et al*, 2019) investigated the associations between livestock reservoirs and sporadic cases of giardiasis in Ontario, Canada. Livestock reservoirs were investigated by testing either dairy, beef, or swine farms every month. Case crossover analysis found that livestock reservoirs were associated with an increased risk of human giardiasis with a one-week lag period (OR: 1.65, 95% CI: 1.23–2.22, P = 0.001). Assemblage typing data confirmed that zoonotic assemblages A and B were present in the livestock reservoir, which further supports the likelihood of zoonotic transmission (Brunn *et al*, 2019). This study is supported by another project undertaken in Scotland that demonstrated the presence of human assemblages in both beef and dairy cattle (Bartley *et al*, 2019). A separate study among veterinarians in Poland suggested the risk of transmission between animals and humans was low (Wojcik-Fatla *et al*, 2018), but an Australian study (Zajaczkowski *et al*, 2018) found contact with domestic, farm animals, or wildlife to be a risk factor. One study investigated shedding of *Giardia* cysts from pet owners (3/69; 4%) who had either cats or dogs; one household pair of human and dog samples had similar, although not identical, assemblage B genetic sequences, suggesting possible transmission. In this study, more dog than cat fecal samples were found to be *Giardia* positive (39% vs 14% respectively) *(Rehbein et al*, 2019) (Table 2-5). Likewise, a study conducted in northern Spain comprising 63 households with domestic cats and dogs found no evidence that they were a significant reservoir for human infection (de Lucio *et al*, 2017), nor were domestic or farm animals in a study in Germany (Espelage *et al*, 2010). A review of *Giardia* in eastern Europe suggested assemblages A and B were common among domestic animals (Plutzer *et al*, 2018). Assemblage A is thought to be more likely zoonotically transmitted to humans (Horton *et al*, 2019) than assemblage B. This concept is supported by a multivariate analysis from England (Minetti *et al*, 2015b) that found dog ownership was a significant risk factor for developing giardiasis, although this effect was limited to contracting assemblage A infections. In summary, it appears that the involvement of animals in the transmission of *Giardia* is variable and depends on local factors that require further investigation with accurate genotyping tools.

It is also important to note that of the previously described outbreaks in which drinking water was involved, six involved *C. canadensis* beavers that were positive for *Giardia*, which may have contributed to multiple cases of human giardiasis (Birkhead *et al*, 1989; Dykes *et al*, 1980; Isaac-Renton *et al*, 1993; Isaac-Renton *et al*, 1994; Kent *et al*, 1988; Lopez *et al*, 1980; Navin *et al*, 1985) (Table 2-1). Contaminated water in these cases was found to be insufficiently filtered and/or treated, suggesting the impact of wild animals in the transmission can be alleviated with proper system maintenance. In outbreaks in which beavers were involved, assemblage typing was not always available but, notably, when beavers were removed from the vicinity of the water supply, there were no further cases of giardiasis (Isaac-Renton *et al*, 1993; Isaac-Renton *et al*, 1994; Navin *et al*, 1985). While the evidence does not support zoonotic transmission as a major risk for human infections when compared with other transmission routes, it is a route that should be considered, especially when positioning reservoirs and designing water distribution networks.

Location	Reference	Year	No. of cases or samples (Lab confirmed)	Animal contact	
				Companion	Farm
Canada	Brunn *et al.* (2019)	2006-2013	403		×
Germany	Rehbein *et al.* (2019)	2019	3 (3)	×	
Poland	Wojcik-Fatla *et al.* (2018)	2018	2 (2)	Occupational exposure in veterinarians	
USA	Jagai *et al.* (2010)	1991-2004	5193 (5193) per 10,000 elderly		×

Table 2-5: Giardiasis outbreaks due to zoonotic transmission

2.7 Travel-association

International travel was associated with outbreaks in a small number of studies, which affected 1288 people as a primary transmission route (Table 2-6) (Gray *et al*, 1994; Wilson *et al*, 2008) and was suggested as a risk factor in some analyses, although this was not universal. In one of two Australian studies of giardiasis risk factors, international travel was only significant in univariate analysis and not in multivariable analysis (Zajaczkowski *et al*, 2018). International travel was not

considered a risk factor in a study from Spain (de Lucio *et al*, 2017). The risk identified with both international and domestic travel may be related to activities undertaken in the destination and the resulting water or environmental exposures. Some of the studies included in this review were case-control studies or outbreak investigations that excluded any cases of giardiasis with a travel history from the study cohorts, so this was unable to be explored as a risk factor.

A study in England showed assemblage B to be the type most frequently isolated from human samples where companion animals were not involved, accounting for 64% of cases compared to 33% for assemblage A. Cases of mixed assemblages were rare (Minetti *et al*, 2015a), which is consistent with studies in several other countries. A study in Spain also found assemblage B was more common than assemblage A (66/95, 69.5% and 29/95, 30.5% respectively) (Wang *et al*, 2019). The opposite was found to be true in a Scottish study of 30 *Giardia*-positive cases, where assemblage A was isolated most frequently (21/30, 72%). This was followed by assemblage B and mixed infections of assemblages A and B (4/30, 14% and 3/30, 10% respectively) (Alexander *et al*, 2014). This difference in predominant assemblage by country may also contribute to travel-associated giardiasis, due to traveller exposure to novel assemblages as they move to different regions. Another factor which is not mentioned is a potential selection bias that affects who receives *Giardia* screening tests, which until recently was predominantly those with a history of travel.

Location	Reference	Year	No. of cases or samples (Lab confirmed)	International travel
New Zealand	Wilson *et al*. (2008)	2006	1214 (1214)	×
UK	Gray *et al*. (1994)	1992-1993	74 (74)	×

Table 2-6: Giardiasis outbreaks due to travel-associated transmission

2.8 Multiple Transmission Routes

Multiple transmission routes for *Giardia* were described in 11 studies, which included 1308 laboratory confirmed human cases (Table 2-7). Among these, international travel was the single most important factor identified in seven

studies. However, it should be noted that many countries in which these studies were based require a history of foreign travel before testing for *Giardia*, adding an element of bias into these multivariate analyses. Other risk factors reflect those described above for person-to-person transmission, contaminated water, and animal and environmental exposures. One study also identified taking antibiotics and having a chronic gastrointestinal condition (Reses *et al*, 2018) while another showed primary immunodeficiencies such as that of immunoglobulin A (IgA) (Agarwal & Mayer, 2013) as risk factors for giardiasis acquisition.

Loc	Ref	Y	No. of cases or samples (Lab confirmed)	Travel-associated		Waterborne			Foodborne			Person-to-person			Faecal exposure		Animal contact/other			
				D	I	Tap Water PrT	UT	RW	RF	UFV	FH	HH	DC	YC	ST	DFC	CA	FA	WA	Other
Australia	Zajaczkowski *et al.* (2018)	2016	68 (68)		×							×		×			×	×	×	
UK	Hardie *et al.* (1999)	1997	58 (58)		×	×			×	×										
UK	Stuart *et al.* (2003)	1998-1999	192 (192)			×		×		×										
UK	Minetti *et al.* (2015)[a]	2012-2013	236 (150)		×		×	×						×			×			Reporting IBS symptoms, taking indigestion medication
USA	Reses *et al.* (2018)	2003-2004	213 (213)		×		×	×				×	×	×	×					Taking antibiotics/having a chronic GI condition
New Zealand	Hoque *et al.* (2002)	1998-1999	183 (183)	×	×		×	×						×		×				
New Zealand	Hoque *et al.* (2003)	1999-2000	69 (69) children under 5				×	×						×						
Sweden	Andersson *et al.* (1972)	1971	30 (30)		×						×									
USA	Lopez *et al.* (1978)	1976	27 (27)		×	×				×	×									
USA	Dennis *et al.* (1993)	1984-1985	273 (273)				×	×				×	×							
USA	Novotny *et al.* (1990)	1983	45 (45)	×									×[b]							

Table 2-7: Giardiasis outbreaks due to multiple modes of transmission

[a]Study included two separate multivariate analyses, both with and without international travel as a risk factor

[b]Found to be a factor with a family size ⩾ 4 people

Abbreviations: Loc, location; Ref, Reference; Y, Year; Travel-associated: I, international; D, domestic; Waterborne: PrT, pre-treatment; UT, untreated; RW, recreational water; Foodborne: RF, reconstituted foodstuff; UFV, unwashed fruit/vegetables; FH, food handler; Person-to-person: HH, household GI illness; DC, day-care attendance; YC, young children; Faecal exposure: ST, sexual transmission; DFC, direct faecal contact; Animal contact/other: CA, companion animal; FA, farm animal; WA, wild animal; IBS, irritable bowel syndrome; GI, gastrointestinal

2.9 Conclusion

This review challenges the hypothesis that *Giardia* outbreaks in higher income countries are primarily associated with foreign travel and shows that transmission can occur through a wide range of local routes. This likely reflects endemic populations of *Giardia* that have been overlooked due to an insistence of a history of foreign travel before testing in several higher income countries. Of these routes, contaminated water was the most frequently identified route of *Giardia* transmission in the literature, primarily due to insufficient treatment or post-treatment contamination due to poor maintenance or practices. Water-linked outbreaks are also common to LMICs, but the situations are not directly comparable as poverty and a lack of proper sanitation are the major causes of high giardiasis prevalence in LMICs rather than a temporary disruption in water quality. This suggests that continued investment in water distribution networks in higher income countries is essential to control the disease and there is a need to avoid complacency. This systematic study also highlights a lack of robust case-control studies for assessing the risk of *Giardia* in higher income countries. Without such analyses, it was not possible to perform a detailed meta-analysis in this review as has been done for LMICs (Fakhri *et al*, 2021). This was exacerbated by extremely high heterogeneity in study methods and design. It was also noted very few studies examined a range of possible transmission routes for an outbreak, with most focusing on water supply or travel. It is likely that many researchers similarly limit themselves and if an origin is not one of these two common routes, an outbreak is unlikely to be reported, further adding to the publication bias. This may explain the large number of outbreaks linked to water in the literature. This review therefore highlights the need for more in-depth studies with consistent methodology to improve our understanding of this pathogen in higher income

countries. It also underscores the need for full and publicly available epidemiological examinations of *Giardia* outbreaks to avoid such publication bias. Our understanding of the various transmission pathways is further hampered by the lack of studies that include in-depth molecular data, such as assemblage typing, that could be used to understand zoonotic transmission. Indeed, it was noted that only three studies reported molecular genotyping in their results. Wider employment of molecular genotyping and improved tools to determine specific variants will improve surveillance of sporadic cases and help identify outbreaks and associated risk factors. Despite these caveats, just focusing on reported sources and transmission routes rather than estimating risks, our systematic analysis suggests that there are numerous sources and routes for *Giardia* outbreaks in higher income countries, particularly due to failures in water treatment and infrastructure.

Chapter 3. Bridging section

As *Giardia duodenalis* is increasingly identified in developed countries due to the increased sensitivity of detection techniques and a greater awareness that transmission is not necessarily linked to a history of foreign travel (Minetti *et al*, 2016), further infection analysis will inevitably follow. In addition to standard epidemiological analysis, the increased application of molecular methods to genetically characterise parasite isolates will facilitate the investigation of sporadic and outbreak-associated cases. This may help in the identification of an infectious source, a route of transmission, and perhaps even a timeline of disease progression through a population. For this type of genotypic analysis to be efficacious, the genotyping assay deployed needs to be reliable and to discriminate between assemblages. This is particularly important where zoonotic transmission or outbreaks may occur, as biosecurity measures can be put into place to prevent spread from domestic or wild animals. In instances of human outbreaks, connections between individual cases can be established and potentially traced to a source, if an appropriate assay is utilised. Similar methodology can be applied to the investigation of giardiasis outbreaks in animals.

One major issue with *Giardia* is its poorly understood reproductive biology, especially the role of recombination in the generation of parasite diversity. The historic approach of using a singular PCR assay to discern between all assemblages is associated with a poor success rate, which compromises not only ongoing attempts to understand the reproductive biology of this parasite, but efforts to document its transmission and prevalence. The following chapter details efforts to improve a commonly used molecular marker, namely *tpi*, for assemblage-typing of *Giardia* isolates. A particular limitation of current protocols based on this marker is their failure to PCR amplify a substantial proportion of field samples. Using published genomic sequence data for this locus, the goal of this work was to improve the sensitivity of this marker in terms of increasing the genetic diversity it encompasses, thereby improving the PCR success rate. Broad application of an improved marker such as this has the potential to increase the base knowledge of *Giardia duodenalis* not only in higher income countries, but also in LMICs.

Chapter 4. Molecular characterisation of *Giardia duodenalis* from human and companion animal sources in the United Kingdom using modified molecular markers

4.1 Introduction

4.1.1 General Background

Giardia duodenalis is a protozoal parasite that has been under increasing scrutiny since its inclusion in the WHO's neglected disease initiative in 2004 (Savioli *et al*, 2006). The route of infection is faecal-oral and there is growing evidence of zoonotic transmission, increasing the importance of biosecurity in its control (Adam, 2000; 2001; Heyworth, 2016). It can cause gastrointestinal upset as well as several other long-term gastrointestinal and cognitive sequelae, placing it in a category of importance for children in particular (Ajjampur *et al*, 2011; Dann *et al*, 2018; Hanevik *et al*, 2009; Hanevik *et al*, 2014; Jethwa, 2015; Simsek *et al*, 2004; Wensaas *et al*, 2012). Asymptomatic infections also commonly occur (Caccio & Ryan, 2008; Thompson & Ash, 2016). Detection of *Giardia* is mainly performed by qPCR, microscopy or ELISA. While quantitative information can be gained from qPCR assays, qualitative PCR when combined with Sanger sequencing can provide useful genetic information on isolate genotype, or 'assemblage' (Caccio *et al*, 2002; Read *et al*, 2004; Savioli *et al*, 2006; Verweij *et al*, 2003).

4.1.2 Genetic Background

Giardia is recognised as being ubiquitous and detrimental to health and there has been an increasing call for an improvement in methods for detection and genetic characterisation (Caccio & Ryan, 2008; Caccio *et al*, 2005; Durigan *et al*, 2018; Savioli *et al*, 2006). Correct identification of assemblage types can aid in highlighting outbreak sources, determining or predicting directionality of infection and general epidemiological monitoring of the parasite. Assemblage A is a potentially zoonotic human type which may be sub-divided into sub-assemblages A1 and A2. A1 has the potential to infect humans as well as a range of other mammals, whereas A2 is more generally found in human hosts. Assemblage B also infects human and other mammals, assemblages C and D are considered the canine assemblages, assemblage E is primarily found in livestock, assemblage F in felines,

assemblage G in murines and assemblage H in seals and gulls (Table 1.1) (Heyworth, 2016). The genetic loci used for typing should be present in all genotypes, exhibit an appreciable level of polymorphism among isolates and, ideally, be single copy in the parasite genome to help ensure detection methods are consistent, reproducible and informative.

4.1.3 Limitations in genotyping

Throughout the 1990s, the primary method for assigning assemblages to *Giardia* isolates was isoenzyme or allozyme electrophoresis, which required a large amount of parasite to be cultured *in vitro* (Monis *et al*, 1999; Thompson, 2004). However, attempts to culture *Giardia* from field samples are often unsuccessful due to excystation failure and microbial contamination (Cruz *et al*, 2003; Nash, 2019). The entire small ribosomal subunit locus was initially evaluated as a genetic marker by restriction enzyme mapping and by PCR in the 1990s (van Keulen *et al*, 1991; van Keulen *et al*, 1998; van Keulen *et al*, 1993). Subsequently, conventional PCR of this locus was investigated as a method of detection followed by amplicon sequencing and phylogenetic comparison to classify isolates. This protocol was developed to determine to what extent different assemblages are restricted to a particular host species, which assemblages have zoonotic potential and to broadly gauge genetic variability between assemblages. While this PCR was initially used to target the 18S subunit locus, additional loci were later assessed to increase the genotyping success rate and to provide additional genetic discrimination.

The genes *beta giardin* (*bg*), *triosephosphate isomerase* (*tpi*) and *glutamate dehydrogenase* (*gdh*) were evaluated by Caccio, Sulaiman and Read respectively (Caccio *et al*, 2002; Read *et al*, 2004; Sulaiman *et al*, 2003) for their degree of polymorphism and found to be sufficient to draw phylogenetic inferences to assemblage and sub-assemblage level. However, genetic markers based on these genes have proven to be inconsistent in terms of PCR success rate and genetic classification and only limited further development of markers based on *bg* and *tpi* has been undertaken. Thus, many studies have been reliant on the original published markers (Abd El-Latif *et al*, 2020; Abe *et al*, 2010; Caccio & Ryan, 2008; Colli *et al*, 2015; Daly *et al*, 2010; ElBakri *et al*, 2021; Lebbad *et al*, 2010; Nolan *et al*, 2017; Robertson *et al*, 2007; Volotao *et al*, 2007; Yang *et al*, 2010). Many of the current PCR primers contain several degenerate bases, which increases the

possibility of off-target primer binding. While this can accommodate a degree of genetic variation within and between assemblages, it decreases the ability of all primers in a reaction to bind to template, as only a proportion of the primer mix will match the template at degenerate positions. Additionally, when phylogenetic comparisons are undertaken using each of these genes, conflicting results often arise (Caccio *et al*, 2008). For example, *tpi* and *gdh* showed assemblage ambiguity when compared with one another and when compared with the 18S genotyping results (Read *et al*, 2004; Traub *et al*, 2004). Due to its conflict with other markers and low amplification success, *tpi* is a good candidate for further development (Zajaczkowski *et al*, 2021). If isolates cannot be amplified or definitively placed within a specific assemblage, it limits the capacity of the marker to be applied to outbreak analysis and elucidate transmission pathways (Thompson & Ash, 2016).

4.1.4 Developing Primers

To develop new primers, a representative collection of *Giardia*-positive samples is needed from the field, as opposed to a limited number of laboratory-cultivated isolates. Additionally, a consistent and reproducible method of DNA extraction is required to maximise the amount of *Giardia* DNA recovered from samples (Thompson & Ash, 2016). Improved genotyping methods should encompass as much *Giardia* genetic diversity as possible and should be tested on a range of field samples to determine if they have utility in drawing epidemiological and biological conclusions.

4.1.5 Study Objectives

The first objective of the present study was to refine an existing genetic marker to improve sensitivity, in terms of ability to detect a range of genotypes, when applied to a panel of *Giardia* qPCR positive field samples. The second aim was to utilise the improved markers to characterise a national collection of companion animal and human samples to evaluate host specificity of *Giardia duodenalis* in a high-income country, i.e. the United Kingdom.

Firstly, an optimal method for DNA extraction from *Giardia* cysts in faecal material was identified, as *Giardia* cysts are relatively robust (Adam, 2000; 2001; Caccio *et al*, 2005) and may be present in low numbers. Although the literature describes many techniques for extracting genetic material from the cysts, an optimal

method has not yet been agreed upon. Secondly, published sequences of *Giardia* genes were assessed to identify allelic polymorphisms between and within assemblages, which may have been contributing to a lack of PCR amplification for some isolates. Once this was completed, the primer sequences were redesigned based on the genetic diversity found at the binding sites of the target loci. This allowed the development of adjusted primers, for which the amplification parameters were optimised. The new primers were applied to a wide panel of field isolates which, in addition to experimentally validating the markers, provided new insights into host-specificity of assemblages in the context of a high-income country.

4.2 Materials and Methods

4.2.1 Parasite Material

Faecal samples from companion animals were obtained from the University of Glasgow's Veterinary Diagnostic Services (VDS). This comprised samples sent to the laboratory to investigate infectious causes of diarrhoea in animals attending a variety of veterinary clinics primarily in the United Kingdom between the 9th January 2018 and the 7th June 2021. DNA extracts of faecal samples which were found to be *Giardia* positive by a diagnostic qPCR (Verweij *et al*, 2003) were retained for the present study and were stored at 4 °C. 125 feline and canine samples with Ct values ranging from 17 to 39 were collected.

Human faecal samples containing *Giardia* were obtained from the Scottish Parasite Diagnostic and Research Laboratory (SPDRL), which forms part of the National Health Service (NHS) in Scotland. Historically, only foreign travel-associated cases of diarrhoea were tested for *Giardia*, however following a recent change in policy, *Giardia* antigen ELISA testing is now routinely undertaken on all samples submitted to NHS laboratories for diarrhoea investigation. A total of 79 samples from the Glasgow and Clyde areas were collected between September 2019 and March 2020, and a sub-sample of each was stored in Faeces Stabilization Buffer (Stratec) at 4 °C before DNA extraction for the current study.

4.2.2 DNA Extraction

A preliminary comparison of extraction methods was performed using 0.2 g sub-samples of companion animal faecal material. Using the standard manufacturer-

recommended protocols, results were compared for (a) the taco Nucleic Acid Automatic Extraction System (Genereach), (b) repeated freeze-thaw using liquid nitrogen followed by a PSP stool kit and (c) bead beating with a Tissuelyser followed by a PSP stool kit. A comparison of Ct values from a *Giardia*-specific qPCR on the various extracts indicated that the taco method generated the highest concentration of recovered *Giardia* DNA and it was therefore selected for use in this study. Thus, for each companion animal sample, approximately 0.2 g of faecal material was placed into an Eppendorf tube containing 1 mL of lysis buffer containing 3% Polyvinylpolypyrrolidone (PVPP). The buffer consisted of 0.24 M Triton X-100, 0.36 M diaminoethanetetra-acetic acid disodium salt dihydrate and 10 M guanidine thiocyanate in 0.1 M Tris (pH 6.4). 100 µL of a solution containing a known quantity of feline herpesvirus (FHV) in extraction buffer was added, to act as a control for DNA extraction. The tube was vortexed to mix and left to sit for ten minutes, with another brief vortex five minutes later. After this, the sample was centrifuged for one minute at 16.2 *g* (Eppendorf Centrifuge 5415D). The taco Nucleic Acid Automatic Extraction System was then employed, which utilised magnetic bead separation technology. 200 µL of supernatant was then loaded into the left-most well of the pre-loaded taco plates, which contained lysis buffer and magnetic beads, according to manufacturer's instructions. Each plate contains 48 pre-loaded wells and is used for the simultaneous extraction of eight samples. The wells contain a series of washing buffers, with the final well containing a proprietary elution buffer. This plate is loaded into a taco machine which performs a dual DNA/RNA extraction cycle over the course of 30 minutes. If a sample was to be utilised for Sanger sequencing following PCR analysis, the buffer in the final cell of the plate was replaced with 200 µL of dH_2O. DNA extracts were then removed from the right-most well and placed into new Eppendorf tubes. Human samples were extracted using a Stratec PSP Spin Stool DNA Plus Kit according to manufacturer's instructions. A different extraction method was used for these samples as there was no access to the taco machine in the human ethics-approved laboratory where they were extracted.

4.2.3 Designing and Optimising Primers

The five whole-genome sequences available in GiardiaDB (https://giardiadb.org) were queried and five *tpi* gene sequences were downloaded as FASTA files together with 1 kb of upstream and downstream sequence data. The sequences

were then aligned and trimmed to the published *tpi* primer sites (Sulaiman *et al*, 2003) using Geneious Prime (Dotmatics). The published primers for *tpi* were directly compared with the corresponding target loci in *Giardia duodenalis* genomic sequences present in GiardiaDB using ClustalX2 (Larkin *et al*, 2007). New primers were designed by modifying the existing primers to match bases in the full genomic alignment (Figure 4-1). New *tpi* primers were ordered from Eurofins Genomics (Ebersberg, Germany) and temperature gradients and concentration grids performed on four samples of canine origin and three of feline origin with Ct values ranging from 17 to 33 to determine the optimal PCR conditions. A *Giardia*-rich positive control DNA sample representing assemblage A1 (genome WB clone 6) derived from sterile, lab-cultivated trophozoites at a diluted concentration of 1:200 was used as template in the first round of a nested PCR. To determine the optimal annealing temperature for both rounds, an annealing range of 53.2-66.7 °C was tested first with the internal primers then with the external primers, with a constant 1 pmol concentration of forward and reverse primers (Figure 4-2). The concentration grid tested all combinations of forward/reverse primers at concentrations of 4 pmol, 2 pmol, 1 pmol and 0.5 pmol in a 20 µL total reaction volume again with the internal primers first then with the external primers (Figure 4-3). Optimal annealing temperature and primer concentrations were determined before the PCR assay was applied to field sample material.

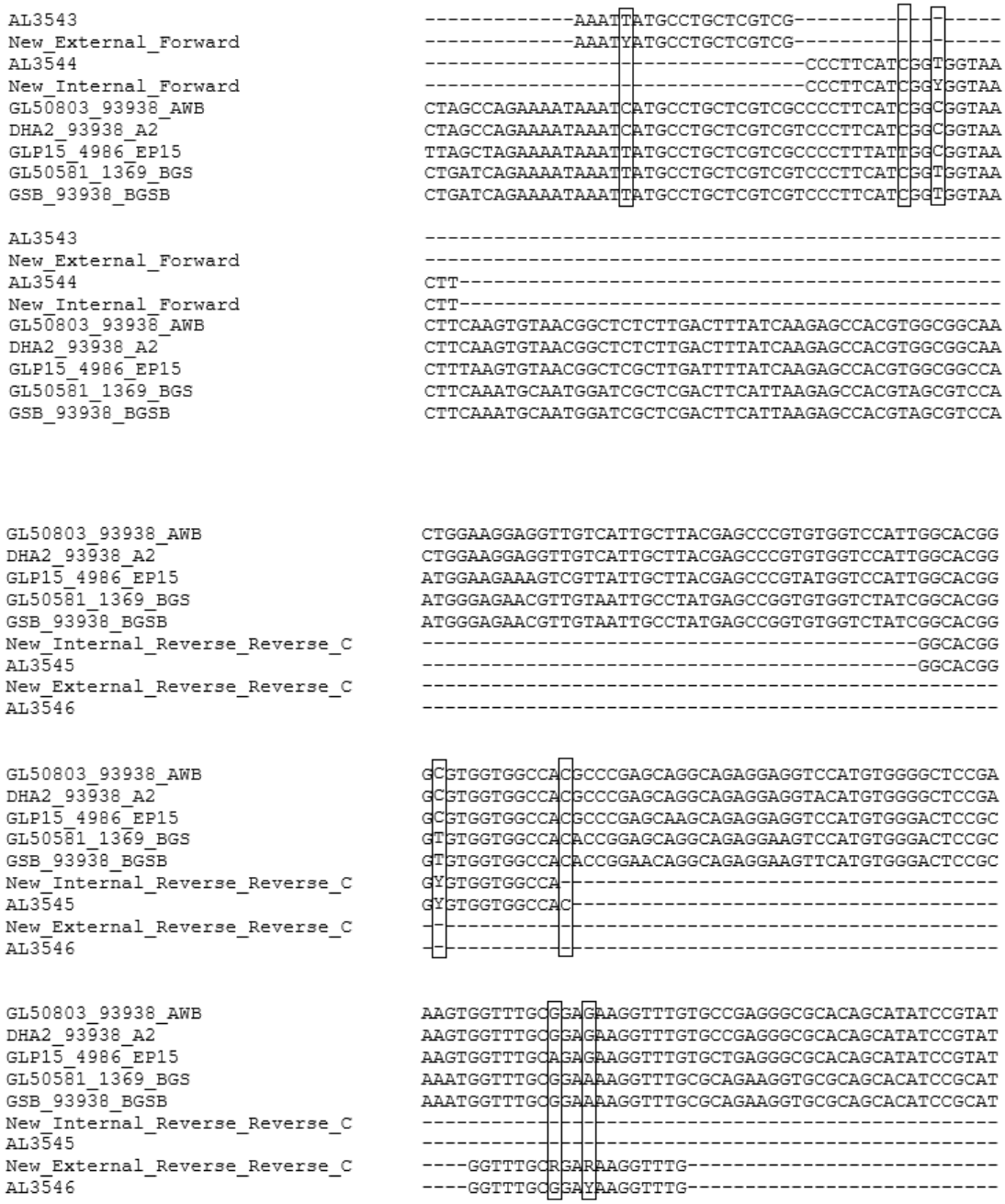

Figure 4-1: Published and modified primers aligned to full Giardia *sequences*

Alignment of five full *Giardia* genomes along with published and modified primers with differences outlined by a box. GL50803_93938_AWB: Assemblage A1; DHA2_93938_A2: Assemblage A2; GLP15_4986_EP15: Assemblage E; GL50591_1369_BGS: Assemblage B; GSB_93938_BGSB: Assemblage B.

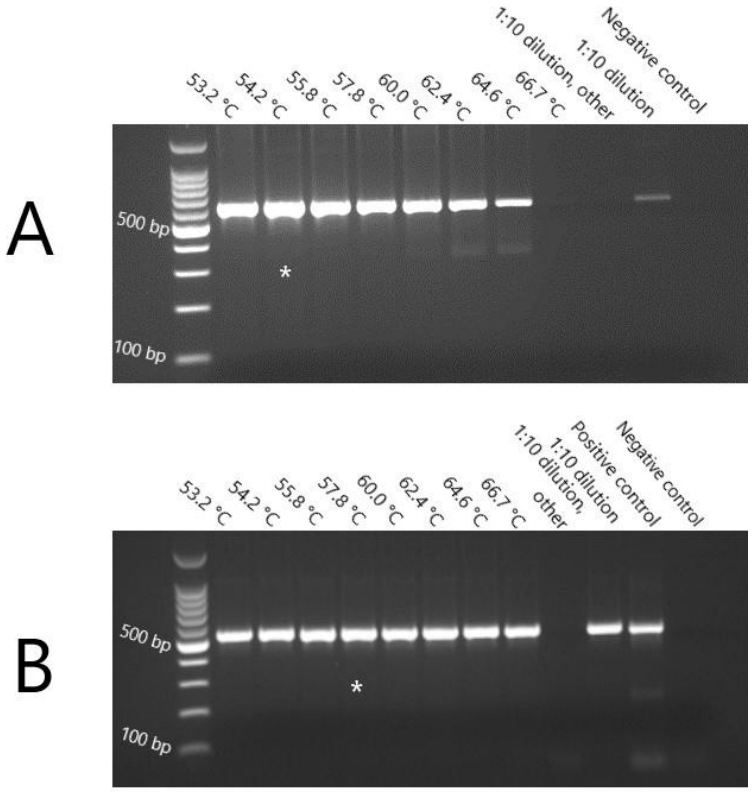

Figure 4-2: Temperature gradient using modified tpi *primers*

Optimisation of annealing temperature using purified DNA extract. A: First round of the nested PCR using the modified tpi primers. B: Second round of the nested PCR using the modified tpi primers. *: Brightest band indicating the selected conditions. "Other" refers to a different DNA sample than that used in the remaining columns for simple comparison. The positive control was DNA extract of a WB line of assemblage A sterile trophozoites (ATCC 50803) diluted to 1:20.

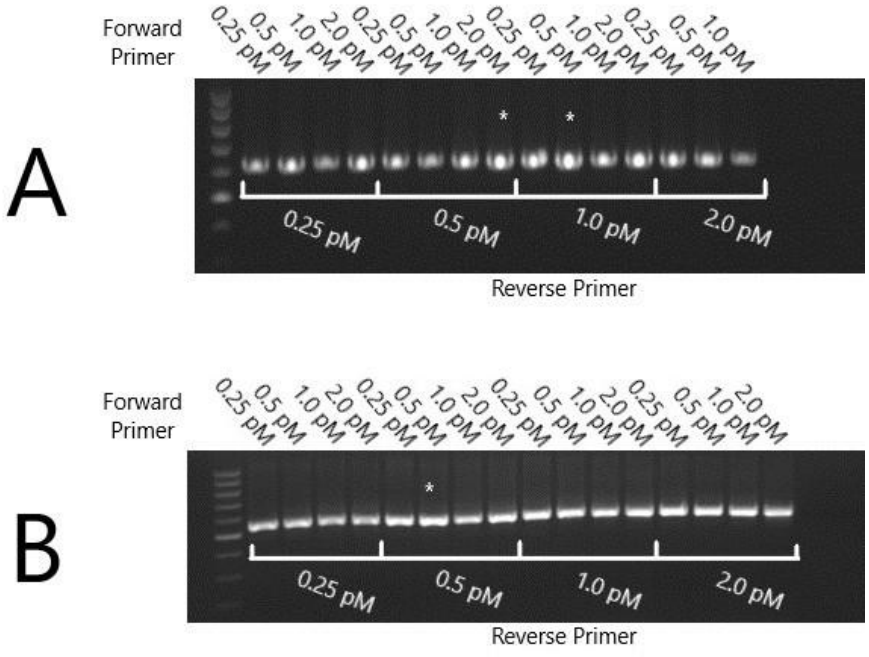

Figure 4-3: Primer concentration grid using modified tpi *primers*

Optimisation of primer concentration using purified DNA extract. A: First round of the nested PCR using the modified tpi primers. B: Second round of the nested PCR using the modified tpi primers. *: Brightest band indicating the selected conditions

4.2.4 Novel *TPI* Assay

As the original *tpi* PCR assay was developed as a nested protocol, the same approach was applied in the current work. Both rounds comprised a total 20 µL reaction volume including 3 µL of template DNA, 0.5 U of HotStart *Taq* (Qiagen) and 25mM dNTPs. While the first round used 0.5 pM and 2 pM of forward and reverse primers respectively, the second round used 0.5 pM of both forward and reverse primers. The first round involved an initial denaturation step of 5 minutes at 95 ˚C followed by 40 cycles of 94 ˚C, 54.2 ˚C and 72 ˚C for 1 minute each, ending with 10 minutes at 72 ˚C. The product was diluted 1:1000 before being added to the reaction mix of the second round, which comprised 5 minutes at 95 ˚C followed by 40 cycles of 94 ˚C, 57.8 ˚C and 72 ˚C for 1 minute each, ending with 10 minutes at 72 ˚C.

Once the second round of the PCR was completed, 15 µL of the product and 3 µL of loading dye was subjected to electrophoresis on a 1 % TBE agarose gel at 100 V for 45 minutes using a 100 bp ladder. The target sequence was 531 bp, and any band appearing around this location was excised using a Qiagen gel purification kit and sent to Eurofins for sequencing.

4.2.5 Amplicon Cloning and Sequencing

A PCR Thermofisher pJET cloning kit was used per the manufacturer's instructions to clone and amplify low concentration amplicons. The kit was used with 1 µL sample for the ligation reaction transformed into *Escherichia coli*. Three plates per sample of LB-agar spiked with ampicillin at a final concentration of 100 µL/mL were set up, and half the plate was seeded with one drop of transformed *E. coli* while the other half was seeded with three drops. The plates were incubated overnight at 37.5 ˚C. Three large colonies from each plate were picked for each sample and incubated overnight in 5 mL of LB. The broth was centrifuged to pellet the *E. coli* and the supernatant discarded. The pellet was resuspended into 50 µL water and plasmids were purified using a Qiagen miniprep kit. The samples were analysed on a Qubit 4 (ThermoFisher Scientific) to confirm there was at least 25 ng

of plasmid DNA for a 500 bp target product, which was then diluted to 5 ng/µL for Sanger sequencing using a Mix2seq kit (Eurofins). Two tubes of 15 µL extract were sent to Eurofins for sequencing, containing either 2 µL of the forward or reverse primer at 10 pmol/µL.

4.2.6 Phylogenetic and Statistical Analysis

Amplicon sequences along with reference genomes WB-A1, DH-A2, GS-B, KT728520_C, P15-E and KP866788_F were aligned using CLUSTAL Omega (Sievers *et al*, 2011), trimmed to the same length and sample phylogeny estimated using RAxML (Stamatakis, 2014) and maximum likelihood with 100 bootstraps. Outputted Newick trees were visualised using FigTree (Rambaut, 2014). Diagnostic assay Ct values of amplifying and non-amplifying samples were compared using a Wilcoxon Rank Sum method.

4.3 Results

4.3.1 Primer Analysis and Redesign

The published primer sequences were compared with available complete *Giardia* genomic sequences at the *tpi* locus to detect mismatches in the primer sequences that may explain PCR failure (Figure 4-1). Whenever a mismatch between a primer base position and any of the genomes of assemblages A, B or E was detected, a suitable alternative to encompass all genomic sequences was inserted in its place. In total, six mismatching positions were identified between the published primers and the full genomes. These bases were either replaced with appropriate degenerate bases or the primer was shifted slightly. Care was taken to avoid hairpin formation, provide optimal GC content and match annealing temperatures (Northwestern University's OligoCalc: Oligonucleotide Properties Calculator). For one primer, one base was not amended as the replacement base provided an unsuitable melting temperature (under 50 °C) and the primer could not be shifted without significantly altering melting temperature. A single base was also removed from the end of the internal reverse primer to bring the melting temperature from 68-70 °C to 66-68 °C. The newly designed primers for first round (external) amplification were 5′-AAATYATGCCTGCTCGTCG-3′ (Forward) and 5′-CAAACCTTYTCYGCAAACC-3′ (Reverse). The primers used for the internal second

round were 5′-CCCTTCATCGGYGGTAACTT-3′ (Forward) and 5′-TGGCCACCACRCCCGTGCC-3′ (Reverse) (Y and R notations, Cornish-Bowden, 1985).

4.3.2 Optimisation of New Primers

The newly designed primers were tested to find the optimal annealing temperature and oligo concentration for each round of the nested PCR assay. For the first-round external primers, the optimal annealing temperature was found to be 54.2 °C, with concentrations of 0.5 pM and 2 pM for forward and reverse primers, respectively. The second-round internal primers were found to have an optimum annealing temperature of 57.8 °C with concentrations of 0.5 pM each for forward and reverse primers. Following a series of test dilutions of 1:100, 1:500 and 1:1000, the optimal concentration of the primary product for the second-round reaction was found to be 1:1000. Following optimisation, both sets of markers were tested on the seven field samples picked to represent a range of parasite loads, as inferred from diagnostic PCR Ct values, and host species together with the DNA extract of the sterile trophozoites as a positive control. These seven samples were used with the initial PCR primers at the beginning of the project and throughout the troubleshooting process, then retested with the modified primers at the end of designing and optimising (Figure 4-). The published assay was able to generate clear, convincing bands from three samples together with weak amplicons from a further two, which were insufficient for Sanger sequencing. In contrast, the novel assay was able to generate strong bands from each of the seven DNA samples. These samples were sequenced and found to represent assemblages A, C and F. Sequence data is available on the GenBank database under accession numbers 2645542-OP860417 through 2645542-OP860514.

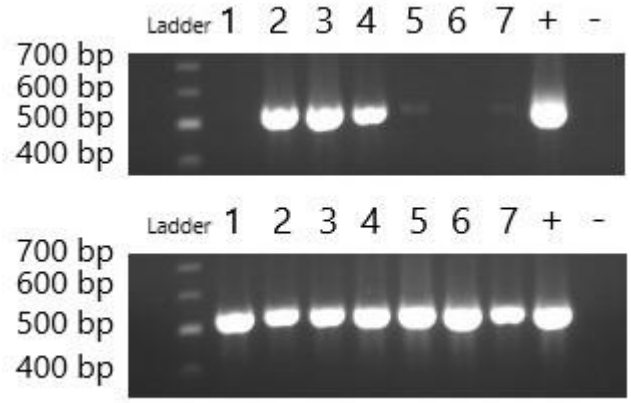

Figure 4-4: Comparison of published and modified tpi *primers*

Target product at 531 bp. Top photo: published primers using published conditions on seven field samples from Veterinary Diagnostic Services, Bottom photo: modified primers using optimised conditions on the same seven field samples

4.3.3 Genotyping of Scottish human and animal-derived *Giardia*

The redesigned and optimised *tpi* PCR assay was applied to 174 companion animal and 79 human faecal samples positive for *Giardia* by qPCR and ELISA respectively. Of these samples, 73 companion animal samples and 37 human samples generated bands in the expected location and these were sent for Sanger sequencing. This corresponded to genotyping success rates of 41.95% and 45.6% respectively. Using 99 samples for which full-length good-quality sequence was obtained, a cladogram was constructed incorporating published sequences representing assemblages A1, A2, B, C, and F trimmed to the *tpi* locus as reference and *Giardia muris* as an outgroup (Figure 4-4).

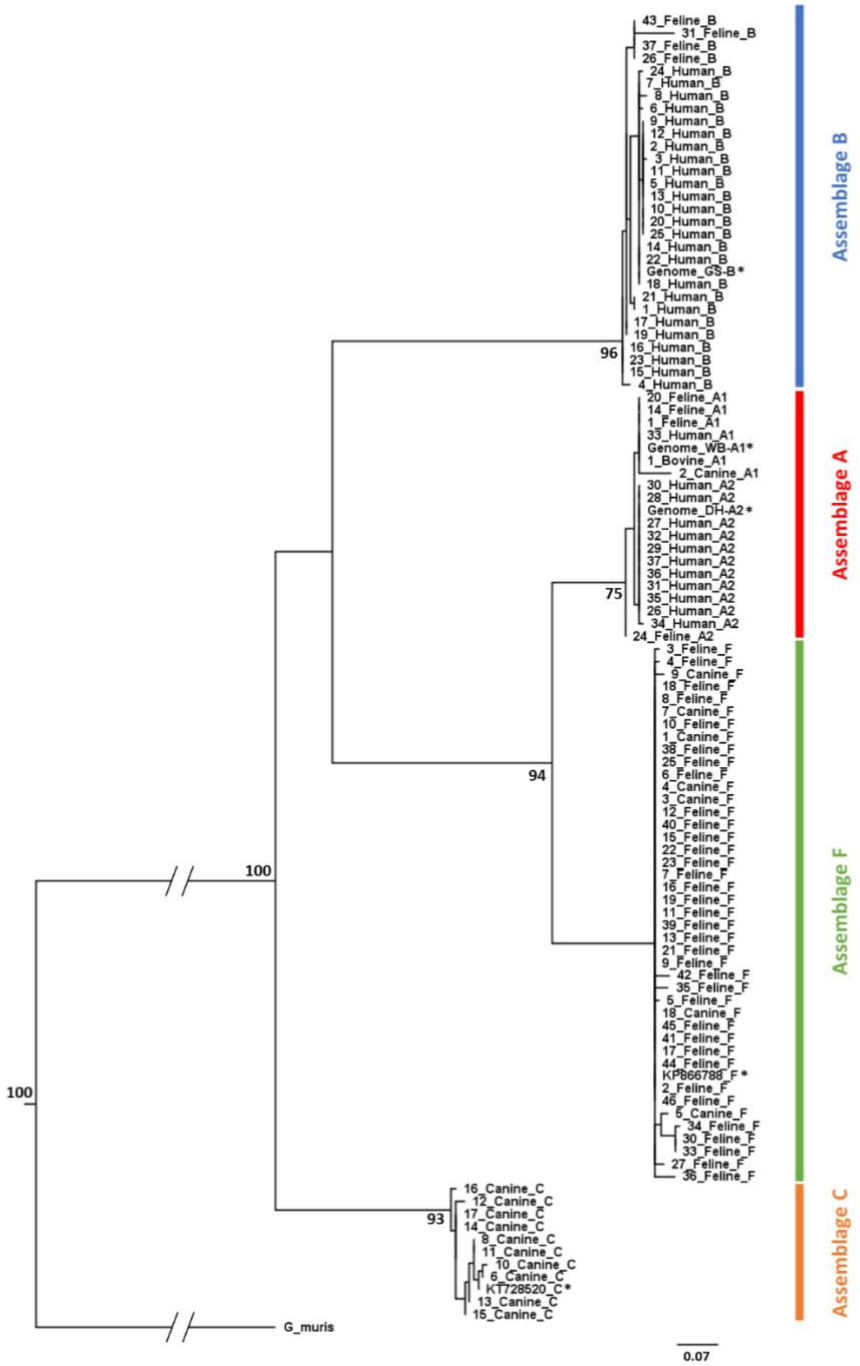

Figure 4-4: Phylogenetic tree of field sample results

Phylogenetic tree representing the assemblage distribution of the field sample amplicons generated. Bootstrap values on major branches are included. The scale of the genetic distance is indicated. * indicates reference genome. Genome_GS-B: Assemblage B. Genome_WB-A1: Assemblage A1. Genome_DH-A2: Assemblage A2. KP866788_F: Assemblage F. KT728520_C: Assemblage C. G_Muris: *Giardia muris* genome.

Two feline assemblage F sequences were excluded due to insufficient sequence length. The field samples formed into four discrete clusters corresponding to the

assemblage A, B, C and F reference sequences, supporting the anticipated phylogeny of *Giardia* and demonstrating the utility of the modified primers for assemblage-typing. Isolates representing different assemblages cluster into monophyletic groups and while there is strong bootstrap support for most major branches of the tree, there is some ambiguity as to the relative position of B, C and A/F clades. The tree supports the current delineation of *G. duodenalis* into assemblages *via* the representative sequences of assemblages A, B, C, and F. The phylogenetic tree revealed that the amplicons generated in this study represented a mixture of assemblages A, B, C and F. Human samples generated 12 assemblage A (one A1 and 11 A2) and 25 assemblage B amplicons, these two human-associated assemblages being anticipated in the human samples. Five companion animal samples generated assemblage A amplicons, comprising four feline and one canine samples, and four amplicons from feline samples were placed in assemblage B. Three of four feline samples and the canine sample categorised as A were sub-typed as A1 and the remaining feline sample was sub-typed as A2. The panel of canine samples, anticipated to correspond to either of the putatively canine-specific assemblage C or D, resulted in a mixture of assemblages C, A and F. Amplicons derived from feline samples, expected to correspond with putatively feline-specific assemblage F, were found to be primarily of assemblage F, although four corresponded to assemblage B (Table 4-1: Summary of field sample results). Eight companion animal samples produced faint bands which were too weak to sequence.

Assemblage	Total Amplified per Species
A	Human n=12 (A1 n=1, A2 n=11) Canine n=1 (A1 n=1, A2 n=0) Feline n= 4 (A1 n=3, A2 n=1)
B	Human n=25 Canine n=0 Feline n= 4
C	Human n=0 Canine n=10 Feline n=0
F	Human n=0 Canine n=8 Feline n=38

Table 4-1: Summary of field sample results

Table demonstrates the number of samples successfully sequenced (n) out of total number of samples tested (N). Humans N = 79. Canine N = 52. Feline = 122.

The Ct values of the companion animal samples which could be typed ranged from 17 to 37, while those that could not ranged from 23 to 36. Overall, the Ct values of the non-*tpi* amplifying samples was found to be significantly higher (P = <0.001, Wilcoxon Test), indicating that parasite DNA concentration within samples is a contributing factor explaining the success or failure of PCR and consequently the ability to genotype samples.

4.4 Discussion

The novel *tpi*-based genotyping assay described here may be used to classify human and animal derived isolates into the currently accepted assemblages with improved sensitivity in terms of the proportion of samples that can be genotyped when compared with the lower rates 23.2% (19/82) and 20% (9/45) in the Rafiei and Wang studies (Rafiei *et al*, 2020; Wang *et al*, 2017; Zajaczkowski *et al*, 2021). However, the failure to amplify from a marked proportion of field samples reflects the ongoing challenge of genotyping *Giardia* isolates. This is an issue shared with markers based on *bg* and *gdh* loci unless the assemblages of interest are limited to A and B, which appear to be associated with a higher success rate (Calegar *et al*, 2022; Correa *et al*, 2020; Rafiei *et al*, 2020; Zajaczkowski *et al*, 2021). The tendency of *tpi* to be used in studies where animal samples are involved helped the decision to begin modifying the existing primers for this locus (Caccio *et al*, 2008; Lebbad *et al*, 2010; Zou *et al*, 2021). We demonstrate that this new assay can amplify a wider range of field samples than the published protocol, as illustrated in Figure 4-2. The higher level of success of the new primers illustrates that amplification failure associated with the existing primers may be explained in some cases by hitherto unappreciated polymorphism at the primer binding site, which would cause a mismatch in bases and prevent PCR amplification.

The current trend in publishing *Giardia* PCR amplification success rates allows the comparison of these modified primers to the original published primers. The amplification rate of companion animal and human samples was 73/174 (41.95%) and 37/79 (46.8%) respectively. When compared with the success rates of *tpi* quoted in similar studies, the companion animal success rate of *tpi* in this study was consistently much higher and the human success rate was either higher or

lower, depending on the study (Correa *et al*, 2020; Rehbein *et al*, 2019; Sommer *et al*, 2018; Wu *et al*, 2022; Zajaczkowski *et al*, 2021). In the present study, samples with a higher parasite load were associated with a slightly higher likelihood of genotyping success. This is logical and an even stronger correlation may have been observed if the primers had been able to capture more of the allelic polymorphism suspected to exist in the *Giardia* population. It is possible the greater amount of parasite genetic material in lower Ct samples increases the likelihood of partial or imperfect binding, sufficient to initiate a PCR reaction. The increased parasite load may also simply ensure there is enough genetic material to which the primers can bind.

While many of the *Giardia* assemblages detected in this study were of the anticipated type given their host, some unexpected results were generated. Several canine samples contained assemblage F genotypes; this has not been documented previously, although assemblage F has been found in cetaceans and pigs (Heyworth, 2016). While this may represent true cross-species infection, one may speculate that it could be explained by dogs ingesting *Giardia*-positive feline faecal material and experiencing a transient infection. Judging by the Ct range of these samples, which ranged from 24 to 37, both scenarios may be possible. This result does, however, call into question how strictly host-specific this and potentially other assemblages truly are. In 2016 Heyworth published a paper detailing the various mammalian hosts found with each assemblage. The only assemblage that remained strictly within its host niche was assemblage G, which until that point had still only been found in rodents (Heyworth, 2016). The findings of the present study and others (Caccio *et al*, 2018; Cardona *et al*, 2011; Deng *et al*, 2018; Foronda *et al*, 2008; Qi *et al*, 2015) suggest that the idea of host-specificity should perhaps be better considered as host-propensity. As more samples from different hosts are typed, the likelihood of finding different assemblages in different hosts can be better quantified in different geographical areas with varied epidemiological situations. In line with these ideals, four feline-derived samples were found to contain classic human assemblages; these comprised three assemblage A and one assemblage B samples and this echoes the findings of previous studies utilising samples from a combination of low- and high-income countries (Adam, 2000; 2001; Caccio & Ryan, 2008; Lebbad *et al*, 2010; Read *et al*, 2004). Assemblage C was detected in a range of canine samples in the

present study. In terms of the efficacy of the presented *tpi* assay, this is an encouraging finding as no assemblage C genome was available for the genomic analysis.

While the putative dog and cat-specific genotypes were not identified in humans, the finding of human-infective assemblages A and B in companion animals, specifically cats, raises the possibility of zoonotic disease transmission in the domestic setting. These findings highlight the importance of applying suitable hygiene measures when handling diarrhoeic companion animals suspected of or diagnosed with *Giardia* infection even in high-income countries. Heyworth's study cites that assemblages A and B have been found in companion animals in several countries, which further supports the need for appropriate biosecurity measures around diarrhoeic animals or where companion animals may be around food preparation or eating surfaces such as in cat cafes (Covacin *et al*, 2011; Lebbad *et al*, 2010; Li *et al*, 2013; Suzuki *et al*, 2011; Volotao *et al*, 2011).

The field trial of an oral canine *Giardia* vaccine in 2016 provided new and important insights into the potential of companion animals to pose a risk of zoonotic transmission in a developing country. This study showed a dramatic reduction in infection rate and protective effect in companion animals while simultaneously reducing the rate of infection in children (Serradell *et al*, 2016). The reduction in childhood infection occurred as a secondary effect due to the treatment of the human-infective strain harboured by the animals in the area. This vaccine has yet to enter mass-production. In another study, dogs in urban settings in the USA without any clinical signs were also found to harbour assemblage A and B strains, meaning they have the potential to shed strains which may be human-infective. This highlights the notion that biosecurity should not be limited to diarrhoeic animals (Covacin *et al*, 2011). Our study found that 8/46 (17.4%) of successfully sequenced feline samples contained potentially human-infective strains, based on assemblage typing. In high-income countries where felines are often kept indoors as pets, this underscores the need to wash hands after cleaning litter boxes, disinfect litter boxes regularly and dispose of waste safely. Common sense hand washing before eating and waste disposal measures should be applied with all companion animals to prevent the spread of any infectious material.

This project has highlighted the need to appreciate assemblage types in terms of differing host propensity rather than absolute species-specificity and it would be advantageous if epidemiological models were to incorporate this concept. Additionally, increasing the resolution of markers in terms of sub-assemblage typing would help appreciate and quantify the zoonotic risk that particular strains pose. With more refined genetic markers, medical and public health officials may discover the actual risk of zoonotic infection may be higher than estimated and more detailed, evidence-based public health advice may be developed to limit parasite spread.

4.5 Conclusion

This study details a novel *tpi*-based genotyping assay for *Giardia*, accounting, so far as possible, for genetic variation in the parasite genome, based on published sequence data. Genetic polymorphism in the parasite population appears to be a major factor in determining the success or failure of PCR-based genotyping methods and further investigation into other genetically informative loci is warranted to underpin development of an effective multi-locus genotyping approach. However, as evidenced in this study, even a single-marker based approach can provide new insights into the molecular epidemiology of giardiasis. Several human-infectious assemblages were found in companion animals using this single-marker, which provides further evidence for zoonotic infection and a basis for further research into potential non-human reservoirs.

By integrating new information from the ever-expanding number of genomic sequences into the improvement of *Giardia* genetic markers, it may be predicted that the rate of genotyping success will increase together with confidence in assigning assemblages. It is possible that amount of diversity in the field population of *Giardia* is sufficiently great that, even at single genetic loci, several assemblage-specific PCRs may be required, which could be developed as a multiplex PCR. The development of higher resolution markers will allow directionality and origin of infection to be inferred more easily which would allow public health bodies to develop biosecurity measures specific to the major routes of transmission in their region. In high-income countries, this may mean paying particular attention to companion animals and water purification (Krumrie *et al*, 2022). While attention is classically paid to water as a transmission route in low-

income countries (Aw *et al*, 2019; Saaed & Ongerth, 2019; Squire & Ryan, 2017), the availability of knowledge of companion animal infection may also influence biosecurity advice surrounding free-roaming animals to include washing hands whenever contact is made with the animal or with soil in which an animal is known to defaecate.

As more comprehensive genotyping methodology is developed, the veracity of the current assemblage paradigm can be reviewed and revised if necessary. With more complete genomic information and refined detection techniques, the molecular epidemiology of *Giardia* can be explored in different areas of the world, addressing its neglected status with the WHO.

Chapter 5. General Discussion

5.1 Challenging Historic Notions

In the first part of this thesis, outbreak studies in high-income countries with confirmed *Giardia* aetiologies were analysed for transmission routes. While previously travel-associated transmission was thought to contribute to a high number of *Giardia* outbreaks in these countries, the literature review indicated that waterborne and person-to-person transmission involving young, ambulatory children was the key underlying factor. This shows how *Giardia duodenalis* is still a poorly understood parasite, especially in high-income countries where the extent of its endemic nature is still being explored. Part of the reason for this poor understanding is the perpetuation and historically held ideas about transmission that continue unchallenged, coupled with low resolution markers that are not improved or rejected completely in the pursuit of better methods. This thesis has not only challenged the idea that historically held notions should be consistently questioned with ever-evolving molecular typing methods, but highlighted the apathy of the *Giardia* community to press forward with the development of novel techniques that would provide more complete and useful information about the organism, such as WGS. In this thesis, we have demonstrated *Giardia* to be an endemic parasite transmitted by means other than travel. Currently it is rarely included in basic diarrhoeal screening protocols from those without a history of travel because the cause is believed unlikely to be giardiasis. Medical and public health professionals will eventually need a rapid, easily accessible method with which to genetically characterise samples to gain important epidemiological and biological insights and to generate more definitive information on *Giardia*'s endemic nature and host specificity. It is unknown whether in the future isolate phenotype, in terms of pathogenicity or drug resistance, can be inferred from genomic data. If pathogenicity is linked to genotype, the creation of suitable and easy-to-access knowledge bases will be essential in exploiting this type of information.

While only one marker was optimised in this project due to time constraints, ideally markers based on *beta-giardin* and *glutamate dehydrogenase* would also have been redesigned and optimised. One locus provides sufficient information for assemblage typing, but three loci which are applied using the same extraction

techniques could potentially provide additional supportive information and increased genetic resolution. While the reliance on a single marker is a limitation of the present study, the distinct clustering observed in the cladogram provides reassurance that assemblage assignment is valid. If all markers could be improved and applied regularly, one may hypothesise that new routes of transmission and pockets of infection may be discovered.

5.2 The Limitations of Published Studies

A considerable limitation at the beginning of the project was the lack of published success rates of current genetic markers when applied to non-human samples. The practice of not sharing *Giardia* genotyping success rates has decreased in recent years. However, the lack of published results in this area meant that a significant proportion of project time was devoted to repeating the work of others. More generally, across the scientific discipline, a substantial amount of time is spent repeating techniques that have been previously attempted but not published, as competition for research funding and publication pressures create a bias toward obtaining and publishing positive, potentially non-reproducible results (Baker, 2016; Jarvis & Williams, 2016). Yet it may be argued that by publishing negative results, time and effort would be spared, projects would progress more rapidly, the amount of animals involved in science could be minimised and trends could be discovered in negative data (Boorman *et al*, 2015; Gundogan & Agha, 2016; Matosin *et al*, 2014). This project discovered some small, novel fragments of information in the process of troubleshooting, such as the most effective of three extraction techniques, but time could have been better spent testing more samples, modifying the two other loci or looking for new genotyping techniques. Perhaps high-impact scientific journals should accept more negative result studies, making better use of research funds thereby allowing science to progress even more rapidly.

5.3 Future Genotyping Technology

Despite the improvements documented in this study, the feasibility of using a single nested PCR for each assemblage for even one locus, let alone three, in a public health surveillance setting is low due to time and resources required, balanced against the likelihood of generating actionable information. This is particularly true in LMICs, where funding may not be as readily available to public

health services. Designing assemblage-specific primers may be challenging due to the large amount of polymorphism that has gone undetected thus far. One may hypothesise that without the future publication of additional wide-scale *Giardia* WGS datasets, any improvements in marker design will be incremental in nature. Even if samples can be amplified, the three loci commonly used to assign amplicons to their respective assemblages often disagree (Heyworth, 2016), yet the *Giardia* community still uses them to draw important epidemiological information. An immediate question would be whether each locus is identifying different components of a mixed infection or if the assemblages are not as genetically isolated as currently assumed. An alternative method that would provide useful fundamental and practical information is whole genome sequencing, a technique that has been used by *Salmonella* reference laboratories to detect and sequence various serotypes after other molecular techniques failed to capture the genetic variability of the bacteria (Ibrahim & Morin, 2018). This is a technique widely available to government and public health laboratories and, with recent advances in technology, is much less time consuming and labour intensive than a series of nested PCRs. This would provide a large amount of baseline sequence data from which we could learn more about *Giardia*. Once this data is gathered, a comprehensive database of complete genomes would be helpful for epidemiological analysis and other research purposes. To date, WGS has only been applied to cultured *Giardia* isolates and with only a proportion of isolates capable of adapting to culture, an inevitable bias exists in the generation of sequencing libraries. This may be overcome in the future with the development of DNA capture methods for *Giardia*, which offer the potential of enabling WGS from limited levels of template DNA. If this system could be developed, this would still necessitate *Giardia* WGS being a two-step endeavour, which is considerably more involved than, for example, sequencing bacteria.

5.4 Inadequacy of Current Genotyping Markers

The inability to type a large proportion of field isolates of *Giardia* means that the zoonotic potential of this parasite and relationship of genotype to virulence phenotype cannot be fully explored. This raises the question of what other pathogens are being overlooked or underestimated due to poor detection techniques and pinned on low- or middle-income countries as regions of origin,

such as *Schistosomes* or *Leishmania*, which are both considered neglected tropical pathogens associated with poverty (Engels & Zhou, 2020). Many outbreak studies in the chapter 2 review were excluded from formal analysis because there was not definitive evidence to state a clear aetiology, which is an important public health aspect. If no aetiology is identified in an outbreak, preventative measures cannot be as accurately applied and trends for a particular organism cannot be extrapolated from data. While disease-causing organisms are difficult to isolate from some locations, such as where rapidly moving water is involved, the situation is not helped by the baseline inability to detect the pathogen consistently and accurately in a laboratory setting. If these PCR markers were improved or another more reliable method, such as WGS, was developed, the quality of evidence provided in outbreak studies would undoubtedly improve.

5.5 Protecting Children

One of the important transmission routes that emerged from the review was from children to other children or from children to adults. This is aided, in part, by the small cyst diameter of 5 by 7 to 10 µm and the cyst's immediately infective nature upon excretion, coupled with the potential for infection with ingestion of as few as ten cysts (Adam, 2001). This highlights the potential for faecal pathogens to be transmitted in households with children and also suggests more appropriate biosecurity measures should be applied in institutions where children are commonly present. These discoveries mirror those of *Salmonella*, for which children are an important reservoir and where strict biosecurity measures are able to restrict spread (Bula-Rudas *et al*, 2015). These measures are especially important given the developmental deficits that can arise from infant giardiasis and the ease with which spread can be halted. Ensuring day care facilities use the correct disinfectant, regularly decontaminating splash pads where children are known to play, and heightened biosecurity around swimming pools at times when children are present could potentially disrupt the transmission of infective cysts by a significant amount. While it is difficult to monitor the frequency and thorough nature with which washing occurs at an individual level, more general, population-based cleanliness measures can be applied although this can only happen if the biology and transmission of pathogens such as *Giardia* are more completely understood. Countries should be working to learn everything they can about pathogens that threaten the health and future of their children.

5.6 Issues with Treatment

Treatment of *Giardia* is rapidly becoming an issue, due to both failure of treatment and treatment resistance (Carter *et al*, 2018; Lalle & Hanevik, 2018; Leitsch, 2015; Morch & Hanevik, 2020). Treatment failure is defined as the failure of an intervention to produce eradication of a parasite or organism. This is often the result of human error, such as inappropriate medication dosage, failure to complete a course of treatment, or lack of adherence to dosage advice (Lalle & Hanevik, 2018). In contrast, treatment resistance is defined as the ability of an organism to survive and/or multiply despite the administration of an appropriate dosage of an agent meant to counteract this activity (Carter *et al*, 2018; Lalle & Hanevik, 2018). Treatment resistance is becoming an increasing concern within the medical community and is being monitored more frequently by public health officials (Lalle & Hanevik, 2018; Leitsch, 2015). The first line treatment for humans has been metronidazole with albendazole as a second line or concurrent treatment (Carter *et al*, 2018; Lalle & Hanevik, 2018; Leitsch, 2015), and metronidazole or fenbendazole in companion animals (Thompson *et al*, 2008). Metronidazole has been used since the 1960s as a defence against *Giardia*, which is yet another demonstration of how this parasite has been left behind in scientific progress (Lalle & Hanevik, 2018). One area of discussion in the veterinary community is whether to treat apparently asymptomatic cases of *Giardia*, given that inappropriate treatment may contribute to resistance (Tysnes *et al*, 2014). Appropriate and effective assemblage-typing markers may help determine whether host apathogenic host species/assemblage combinations can be defined and this may in turn reduce unnecessary treatment and the development of resistance. If a host is not truly harbouring an assemblage commonly found in its species and is showing no clinical signs, treatment can be foregone. It may be hoped that once higher-resolution markers are available, a database relating treatment success with *Giardia* genetic determinants can be developed, a tactic used to combat multi-drug resistant *Staphylococcus aureus* (Lowy, 2003).

5.7 Concluding Remarks

The WHO's top Neglected Tropical Diseases list is important in drawing attention to pathogens which are not receiving sufficient research interest and financial support, and these particularly affect developing countries. These pathogens,

such as *Giardia*, do not recognise or respect international borders and can also cause underappreciated endemic disease in higher income countries. With limited resources being directed towards these diseases, it is particularly important that both positive and negative study results find their way into the public domain to avoid unnecessary duplication of effort. This would accelerate research activities, in particular the development of higher-quality molecular typing methods, which in turn will allow better-informed intervention by human and veterinary public health bodies.

Appendix

PMID	Title	Authors	Citation	Journal/Book	Publication Year	DOI	Location	Study Year
28402185	Giardiasis Outbreak Associated with Asymptomatic Food Handlers in New York State, 2015	Figgatt M, Mergen K, Kimelstein D, Mahoney DM, Newman A, Nicholas D, Ricupero K, Cafiero T, Corry D, Ade J, Kurpiel P, Madison-Antenucci S, Anand M.	J Food Prot. 2017 Apr 12:837-841. doi: 10.4315/0362-028X.JFP-16-415. Online ahead of print.	J Food Prot	2017	10.4315/0362-028X.JFP-16-415	NY, USA	2015
18990929	Outbreak of giardiasis and cryptosporidiosis associated with a neighborhood interactive water fountain--Florida, 2006	Eisenstein L, Bodager D, Ginzl D.	J Environ Health. 2008 Oct;71(3):18-22; quiz 49-50.	J Environ Health	2008		FL, USA	2006
24159537	The first outbreak of giardiasis with drinking water in Korea	Cheun HI, Kim CH, Cho SH, Ma DW, Goo BL, Na MS, Youn SK, Lee WJ.	Osong Public Health Res Perspect. 2013 Apr;4(2):89-92. doi: 10.1016/j.phrp.2013.03.003.	Osong Public Health Res Perspect	2013	10.1016/j.phrp.2013.03.003	South Korea	2010
19751538	Outbreak of giardiasis associated with a community drinking-water source	Daly ER, Roy SJ, Blaney DD, Manning JS, Hill VR, Xiao L, Stull JW.	Epidemiol Infect. 2010 Apr;138(4):491-500. doi: 10.1017/S0950268809990744. Epub 2009 Sep 15.	Epidemiol Infect	2010	10.1017/S0950268809990744	NH, USA	2007
29739483	Risk factors for sporadic *Giardia* infection in the USA: a case-control	Reses HE, Gargano JW, Liang JL, Cronquist A, Smith K, Collier SA, Roy SL,	Epidemiol Infect. 2018 Jul;146(9):1071-1078. doi: 10.1017/S09	Epidemiol Infect	2018	10.1017/S0950268818001073	CO & MN, USA	2003-2004

	study in Colorado and Minnesota	Vanden Eng J, Bogard A, Lee B, Hlavsa MC, Rosenberg ES, Fullerton KE, Beach MJ, Yoder JS.	5026881800 1073. Epub 2018 May 9.					
20587 126	Giardiasis outbreak at a camp after installation of a slow-sand filtration water-treatment system	Karon AE, Hanni KD, Mohle-Boetani JC, Beretti RA, Hill VR, Arrowood M, Johnston SP, Xiao L, Vugia DJ.	Epidemiol Infect. 2011 May;139(5): 713-7. doi: 10.1017/S09 5026881000 1573. Epub 2010 Jun 29.	Epidemiol Infect	2011	10.101 7/S09 50268 81000 1573	CA, USA	2007
18957 775	A water contamination incident in Oslo, Norway during October 2007; a basis for discussion of boil-water notices and the potential for post-treatment contamination of drinking water supplies	Robertson L, Gjerde B, Hansen EF, Stachurska-Hagen T.	J Water Health. 2009 Mar;7(1):55-66. doi: 10.2166/wh. 2009.014.	J Water Health	2009	10.216 6/wh. 2009.0 14	Norway	2007
20429 718	Contaminated water caused the first outbreak of giardiasis in Finland, 2007: a descriptive study	Rimhanen-Finne R, Hänninen ML, Vuento R, Laine J, Jokiranta TS, Snellman M, Pitkänen T, Miettinen I, Kuusi M.	Scand J Infect Dis. 2010 Aug;42(8):61 3-9. doi: 10.3109/003 6554100377 4608.	Scand J Infect Dis	2010	10.310 9/003 65541 00377 4608	Finland	2007-2008
90724 1	A communitywid e outbreak of giardiasis with evidence of transmission by a municipal water supply	Shaw PK, Brodsky RE, Lyman DO, Wood BT, Hibler CP, Healy GR, Macleod KI, Stahl W, Schultz MG.	Ann Intern Med. 1977 Oct;87(4):42 6-32. doi: 10.7326/000 3-4819-87-4-426.	Ann Intern Med	1977	10.732 6/000 3-4819-87-4-426	NY, USA	1974-1975
16569 269	Prolonged outbreak of giardiasis with	Katz DE, Heisey-Grove D, Beach M, Dicker RC, Matyas BT.	Epidemiol Infect. 2006 Oct;134(5):9 35-41. doi:	Epidemiol Infect	2006	10.101 7/S09 50268	MA, USA	2003

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Appendix Table 1- 1: MOOSE Sources

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