Molecular epidemiology and geospatial analyses of *Giardia intestinalis* in humans in NSW, Australia.

By

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Certificate of original authorship

I, **Patricia Zajaczkowski** declare that this thesis is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Life Sciences at the University of Technology Sydney.

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Publications arising from thesis

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Mazumdar, S., Fletcher-Lartey, S.M., Zajaczkowski, P. and Jalaludin, B. (2020). Giardiasis notifications are associated with socioeconomic status in Sydney, Australia: a spatial analysis. *Australian and New Zealand Journal of Public Health* **44**, 508-513.

This published paper was written by Dr Soumya Mazumdar and Dr Stephanie Fletcher-Lartey, and as such is not included as part of this PhD thesis. This work was prompted as part of an exploratory analysis of the data used in the Zajaczkowski *et al*., 2018 study (Chapter 2).

Zajaczkowski, P., Mazumdar, S., Conaty, S., Ellis, J.T. and Fletcher-Lartey, S.M. (2018). Epidemiology and associated risk factors of giardiasis in a peri-urban setting in New South Wales Australia. *Epidemiology and Infection* **147**, e15.

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Zajaczkowski, P., Mazumdar, S., Mahimbo, A., Alexander, K., Lee, R., Stark, D., Wehrhahn, M., Fletcher-Lartey, S.M. and Ellis, J.T. (2022) Geospatial distribution and molecular epidemiology of *Giardia intestinalis* assemblages in humans in NSW, Australia. *Australian Society for Parasitology Annual Conference*; Cairns, Australia.

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Supplementary Information

The thesis chapters 2 - 5 refer to supplementary material that helps to support and guide the reader when understanding the methodologies used as well as the results obtained for each chapter.
For Chapter 2, the supplementary information is accessible via the following link (https://doi.org/10.1017/S0950268818002637) or is also accessible at the URL specified at the end of the chapter. For chapters 3 to 5, the supplementary data is located at the end of each respective chapter.

Thesis Abstract

Giardia intestinalis (also known as *Giardia lamblia* or *Giardia duodenalis*) is a protozoan parasite that causes communicable gastrointestinal disease and has received increasing attention due to its rising prevalence worldwide. The majority of current molecular and epidemiological research on giardiasis is concentrated on *G. intestinalis* infection in developing countries, where prevalence rates remain the highest. Infection of *G. intestinalis* can occur with only a few cysts, meaning that overcrowded conditions, poor access to water, sanitation and hygiene facilities, and contaminated water and food sources result in increased infection risk. The extent to which these risk factors are relevant in Australia is relatively unknown, however ongoing surveillance has seen *G. intestinalis* cases in humans more than double in the last 20 years. The re-emergence of this parasite is concerning, as it suggests there are unique risk factors and disease reservoirs present in the Australian setting that are contributing to this rise in case numbers.

To further complicate matters, *G. intestinalis* has been molecularly categorised into eight genetically diverse assemblages, all of which have varying host specificities. Of particular importance are *G. intestinalis* assemblage A and assemblage B, which have been isolated from human and animal hosts worldwide. Current molecular assays used in typing *G. intestinalis* are often unreliable and have variable amplification success. It remains difficult to genetically characterise *G. intestinalis* in clinical samples, and harder still to understand the transmission cycles and virulence of these assemblages.

When read collectively, the chapters in this body of work address the main research objective, which is to comprehensively investigate the molecular epidemiology of *G. intestinalis* cases in NSW, Australia. The initial chapter presents a comprehensive review outlining our current knowledge of the two human-specific *G. intestinalis* assemblages A and B. It highlights the vast genetic differences seen between the assemblages and summarises the advantages and shortcomings of commonly used PCR-assays for genotyping *G. intestinalis*. An in-depth epidemiological analysis of *G. intestinalis* cases in South-Western Sydney (SWS) was undertaken in Chapter 2, aiming to describe the epidemiology and identify sources of exposure in an urban setting in Australia. Building upon the previous work, Chapters 3 and 4 combined epidemiological and molecular data to identify the prominent assemblages circulating in Australia. Powerful geospatial tools were utilised to visualise the spatial and temporal distributions of *G. intestinalis* cases and assemblages and to further identify hotspots of increased infection risk. Chapter 5 concludes this body of work with an overall review of the molecular epidemiology of human *G. intestinalis* infection in

Australia, with an emphasis on recent epidemiological findings. It demonstrates that the identified risk factors of giardiasis are fundamentally unique to Australia. In the public health sphere this would allow for reliable outbreak detection, help identify drug-resistant isolates and answer long-standing questions concerning the zoonotic potential of *G. intestinalis* assemblages A and B.

Exegesis

The primary objective of this body of research was to combine molecular epidemiological tools and geospatial analyses to describe and explore the distribution of *G. intestinalis* cases and assemblages A and B in NSW, Australia. This project consists of five chapters, each of which represents an independent study with relevant background, methodologies, results, discussions and concluding remarks included. When read in succession, these papers outline the backbone of the research, and logically lead into the next chapter to address each research aim. Chapters 1 and 2 have been published in separate, international journals, hence do differ regarding the formatting and referencing style. The remaining 3 chapters have been formatted for journal submission and are intended to be admitted for review.

The following paragraphs provide a brief overview for each chapter, addressing the main objectives of each paper, the methodologies used and how each work is interconnected as part of the main project.

Chapter 1 presents an up-to-date literature review detailing the current molecular assays used to genotype *G. intestinalis* and provides background information on two genetically unique assemblages that are commonly seen in human infections, assemblage A and B. While comparative genomics have allowed researchers to successfully genotype *G. intestinalis* at the assemblage, sub-assemblage and even sub-type level, there remains complications regarding the reliability of common genetic markers such as the beta-giardin (*bg*), glutamate dehydrogenase (*gdh*) and triosephosphate isomerase (*tpi*). As these markers differ vastly in their genetic variability, there are increasing reports of variable PCR-amplification success, preferential amplification of one assemblage over another and inconsistent typing results depending on whichever genetic marker and primer-set is used. For this reason, this review recommends that a minimum of two loci should be implemented in future molecular studies to allow for reliable results.

Chapter 2 is a matched case-control study that involved the recruitment of *Giardia*-positive participants from South-Western Sydney (SWS), one of the largest health districts in New South Wales (NSW). As very few Australian studies have documented the prevalence of giardiasis, or identified the risk factors that drive local transmission, this research is a first of its kind. The SWS area is often overlooked in epidemiological studies despite being the largest growing health district in Sydney. It contains both rural and urban areas and is one of the most demographically diverse districts. In this chapter, univariate and multivariable analyses are run to compare *G. intestinalis*

cases with controls, and to detect significant risk factors that are specific to SWS. The main findings from this chapter indicate that common risk factors of giardiasis, such as drinking non-municipal water, have no significance in an Australian setting. For this reason, the study recommends further investigation into the *G. intestinalis* assemblages in circulation in NSW, and the potential sources of infection.

Chapter 3 builds upon the previous chapter by incorporating data from *G. intestinalis* positive cases within the entire state of NSW rather than just the district of SWS. The study uses two molecular typing methods targeting the small subunit ribosomal RNA (SSU-rRNA) and triosephosphate isomerase (*tpi*) loci. *Giardia intestinalis* assemblages that are successfully amplified are coded into data on SPSS software and incorporated into comprehensive epidemiological analyses. This combination of molecular epidemiology aimed to determine the distribution, sources of transmission and patterns of infection of *G. intestinalis* assemblages in the context of NSW, Australia. Interestingly, groupings of cases were observed in urban communities, and seasonal trends suggested that outdoor water activities play a larger role in transmitting *G. intestinalis* than previously suggested.

Chapter 4 reads as a continuation of the preceding chapter. Chapter 3 had previously identified geographical trends of *G. intestinalis* infection across NSW, observing 'hotspots' of cases in metropolitan Sydney. To substantiate these potential spatial clusters, a combination of geospatial tools was applied to the data. Tools such as hotspot analyses, spatial autocorrelation and purely spatial cluster detection are only recently gaining popularity in epidemiological and public health studies. However, use of these spatial tools regarding protozoan parasites such as *G. intestinalis* are incredibly rare. Chapter 4 details how geographical and temporal differences across NSW ultimately influence *G. intestinalis* incidence and distribution. Using previous assemblage data from Chapter 3, this paper geographically maps the various genetic types of *G. intestinalis* within NSW. Geospatial analyses such as these are essential for sporadic case and outbreak surveillance, and when used in conjunction with molecular data can become a powerful tool for disease prevention and control.

Chapter 5 provides an extensive look into the molecular epidemiology of *G. intestinalis*, specifically in the context of developed countries such as Australia. As infrastructure in Australia's cities and regional centres continue to rapidly urbanise, it is more important than ever to continue surveillance on enteric protozoa such as *G. intestinalis*. Issues of overcrowding, waterborne-outbreaks and cases imported from overseas play a large role in the re-emergence and increasing

trends of *G. intestinalis* infection in Australia. The chapter also delves into current issues regarding molecular typing and highlights the necessity for routine genotyping of *G. intestinalis* infections in diagnostic laboratories.

Chapter 1

The controversies surrounding *Giardia intestinalis* assemblages A and B

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Certificate:

I certify that the following chapter is largely my own work, although the contributions of other authors are duly recognised. The contribution of other authors are detailed as follows:

- By providing suggestions on topics to be reviewed
- By proof reading draft manuscripts
- By providing suggestions to improve layout, structure and writing style
- By correcting spelling and grammatical errors in drafts

Otherwise, the main composition of this work is credited to me.

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The controversies surrounding *Giardia intestinalis* assemblages A and B

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ABSTRACT

Giardia intestinalis continues to be one of the most encountered parasitic diseases around the world. Although more frequently detected in developing countries, *Giardia* infections nonetheless pose significant public health problems in developed countries as well. Molecular characterisation of *Giardia* isolates from humans and animals reveals that there are two genetically different assemblages (known as assemblage A and B) that cause human infections. However, the current molecular assays used to genotype *G. intestinalis* isolates are quite controversial. This is in part due to a complex phenomenon where assemblages are incorrectly typed and underreported depending on which targeted locus is sequenced. In this review, we outline current knowledge based on molecular epidemiological studies and raise questions as to the reliability of current genotyping assays and a lack of a globally accepted method. Additionally, we discuss the clinical symptoms caused by *G. intestinalis* infection and how these symptoms vary depending on the assemblage infecting an individual. We also introduce the host-parasite factors that play a role in the subsequent clinical presentation of an infected person, and explore which assemblages are most seen globally.

1. Introduction

Giardia intestinalis (also known as *Giardia lamblia* or *Giardia duodenalis*) is a protozoan parasite, commonly reported throughout the world as the most important non-viral cause of human diarrhea. Annually, it affects an estimated 280 million people worldwide; however, incidence of the disease is highest in developing countries (Esch & Petersen, 2013). Some studies theorise that the true number of *G. intestinalis* infections globally is much higher as cases can go unreported due to high rates of asymptomatic infection with the parasite (Fletcher et al., 2012). Interestingly, locally acquired cases and outbreaks of *Giardia* are continually reported in developed countries. In the USA and Canada, giardiasis continues to be one of the most reported causes of intestinal parasite infection despite there being an overall decline in infection rates (Pardhan-Ali et al., 2012; Coffey et al., 2020). Meanwhile in New South Wales (NSW), Australia, ongoing disease surveillance has seen *Giardia* cases more than double in the last 20 years (NSW NCIMS, 2021). In urbanised communities, *G. intestinalis* infection is generally seen in children, particularly those attending day-care centres. Giardiasis has also been linked to waterborne outbreaks involving non-potable drinking sources and swimming pools.

Giardia intestinalis is split into eight unique, genetic assemblages (assemblages A to H) which can only be separated by molecular genotyping. Assemblages A and B have been described as having broad host distribution, being isolated from not only humans, but other mammals making them potential zoonotic genotypes (Adam et al., 2013). The remaining assemblages C-H are specific to animal hosts. Comparative genomic analyses based on the sequenced genomes of assemblage A (isolate WB) and assemblage B (isolate GS) have shown that the two assemblages share only 77% nucleotide identity in protein-coding regions (Jerlström-Hultqvist et al., 2010). Interestingly, there is a stronger similarity observed between assemblage A and assemblage E isolates, and assemblage B shows greater phylogenetic distance from both genomes (Xu et al., 2012; Adam et al., 2013). The differences between the two human isolates implies that assemblages A and B should be regarded as two separate *Giardia* species (Franzén et al., 2009).

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Despite the advances made in genetically characterising *G. intestinalis*, there is continuing ambiguity surrounding the associations with specific symptoms and prevalence rates for assemblages A and B. This is in part due to a lack of standard molecular methodology used by researchers when genotyping *G. intestinalis* samples. Additionally, it is not common practice for clinical laboratories to genotype patient samples, and so the brunt of *G. intestinalis* cases remained uncategorised. The most used markers, including the small subunit ribosomal RNA (SSU-rRNA), glutamate dehydrogenase (*gdh*), triosephosphate isomerase (*tpi*) and *β*-giardin (*bg*) are known to show conflicting genotyping results when used independently, and even more so in samples involving mixed assemblages. In this paper, we review the correlation between *G. intestinalis* assemblages and disease pattern, explore the potential of zoonotic transmission and summarise the advantages and shortcomings of commonly used biomarkers for discriminating *Giardia* assemblages.

2. Perceptions of zoonotic potential

For the most part, it is acknowledged that there is limited epidemiological evidence and too much ambiguity surrounding host specificity to confidently support zoonotic transmission of *G. intestinalis* assemblages A and B (Bowman & Lucio-Forster, 2010; Plutzer et al., 2010; Helmy et al., 2014). While these assemblages have been found in both humans and animals, the question of the zoonotic potential of pets, livestock and wildlife remains unclear.

2.1. Giardiasis in wildlife

Multiple epidemiological investigations focused on waterborne outbreaks have implicated wild animals as having transmitted giardiasis to humans (Sulaiman et al., 2003; Sroka et al., 2015). An Australian investigation employed PCR coupled single-strand conformation polymorphism (SSCP) and phylogenetic analyses of loci in the triosephosphate isomerase (*tpi*) gene to characterise *G. intestinalis* found in wildlife living near major drinking-water catchments (Nolan et al., 2013). The study successfully defined 28 distinct sequence types all of which represented assemblage A. One of these assemblage A genetic variants had been previously reported from humans (Lasek-Nesselquist, 2009; Wielinga et al., 2011), cattle (Feng et al., 2008), cats (Suzuki et al., 2011), dogs (Lasek-Nesselquist, 2009), white-tailed deer (Trout et al., 2003) and gulls (Lasek-Nesselquist et al., 2008) worldwide, suggesting that there is a potential for zoonotic infections to occur, or at the very least that wildlife are possible reservoirs for zoonotic *Giardia*. Interestingly, this Australian study also identified marsupials infected with novel assemblage A variants which contained one to two polymorphic nucleotide positions (Nolan et al., 2013). Not only is it difficult to assign these genetic variants to a specific genotype but it raises questions as to whether these novel assemblage A subtypes are transmissible to humans. It is suggested that this genetic variability exists among *G. intestinalis* genotypes due to mixed infections and allelic divergence (Cacciò & Ryan, 2008). There is an obvious need to develop a more robust classification system for these new subtypes.

Earlier investigations have also successfully isolated the human-specific assemblages A and B from the faeces of beavers and muskrats found in water samples around the animal habitats (Weniger et al., 1983; Crabtree et al., 1996; Appelbee et al., 2005). However, it must be noted that these previous studies inferred that waterborne infections by *Giardia* species were from marsupials or aquatic mammal sources, but evidence of causation was lacking. It is just as likely that the wildlife acquired the zoonotic genotypes from drinking water sources contaminated with human faeces, or even perhaps agricultural runoff suggesting humans may be considered a major reservoir of giardiasis for wildlife. Reverse zoonotic transmission is also plausible and must be considered to fully understand giardiasis epidemiology (Palmer et al., 2008).

2.2. Giardiasis in livestock

Hoofed animals such as cattle, sheep and pigs are infected with assemblage E; however, various worldwide studies show an increasing trend of isolating the zoonotic assemblage A from infected livestock suggesting that zoonotic transmission from animals to humans can occur (Minetti et al., 2014; Adam et al., 2016; Zhang et al., 2016). Yet most of these studies were focused mainly on young calves despite previous literature reporting that the distribution of assemblages A and E are associated with cattle age (Trout et al., 2007; Mark-Carew et al., 2012; Bartley et al., 2019). Assemblage A is predominantly detected in young cattle while assemblage E is more often found in older livestock. Interestingly, an Indian study was able to identify the zoonotic sub-assemblage AI in both calves and dairy farm workers (Khan et al., 2011), despite humans being predominantly infected with sub-assemblage AII (Faria et al., 2017; Lecová et al., 2018; Hernández et al., 2019). Moreover, there was a significant correlation (P-value < 0.0001) between sub-assemblage AI and individuals who bred livestock observed in a Syrian study (Skhal et al., 2017). While sub-assemblage AI is regarded as having a broad host range and has most commonly been reported in cats, pigs, sheep, and cattle (Peng et al., 2016; Li et al., 2017; Wang et al., 2017; Lecová et al., 2020), the occurrence of this zoonotic sub-assemblage in humans suggests that cattle can contribute to contamination of the environment and thus to human infections indirectly.

Yet authors of other studies remain unconvinced that livestock is the source of transmission for *G. intestinalis* in humans, particularly in communities living in developed countries (Thompson & Monis, 2011; Ryan & Cacciò, 2013). In countries like Australia, the USA and Germany, there is limited exposure between farmers and their livestock, and stronger control measures are implemented to prevent contaminated agricultural runoff leading into water systems. This is reflected in the molecular studies from these countries which observe a higher prevalence of the non-zoonotic, host-specific assemblage E in livestock (Trout et al., 2004; Ng et al., 2011; Gillhuber et al., 2013). Further molecular studies are needed to understand the risk that cattle pose, as well as to properly define the transmission dynamics between livestock and humans.

2.3. Giardiasis in dogs

The possible role of companion animals as a source of *G. intestinalis* infection in humans is still unclear as studies have yet to provide any causal evidence of zoonoses. In Australia, *G. intestinalis* was observed in 9.4% of domestic dogs and only assemblages C and D were detected (Palmer et al., 2008). On the other hand, a recent Australian study found that individuals who handle domestic animals have a significant risk of infection (OR = 2.04) (Zajaczkowski et al., 2018) and a report from Germany observed 60% of dogs with *G. intestinalis* were predominantly infected with the human-defined assemblage A (Leonhard et al., 2007). Likewise, studies in England, Malaysia and Thailand support the zoonotic potential of assemblage A and implicate domestic animals as reservoirs of zoonotic *Giardia* (Traub et al., 2009; Anuar et al., 2014; Minetti et al., 2015a). It has been theorised that transmission of potentially zoonotic assemblages between humans and their pets is favoured in domestic households as there is a higher chance of contact between humans and canines (Thompson & Monis, 2004; Feng & Xiao, 2011). Likewise, dogs that are kennelled or housed independently have been reported to have a higher prevalence of assemblages A and B (Dado et al., 2012). Although it appears that domestic canines have a high potential zoonotic risk, without further molecular investigations that can confirm identical genotypes between owner and pet, as well as case-control studies that can establish the initial transmission source, the zoonotic potential of *G. intestinalis* assemblages will remain unclear.

In a recent multi-locus genotyping study, humans living in rural Cambodian communities were found to be predominantly infected with

BIII (72.5%) followed by AII (27.5%) (Inpankaew et al., 2014). Molecular characterisation of the canine population reported only 2% of dogs harbouring sub-assemblage BIII suggesting that the animals have a minimal risk for transmitting to humans (Inpankaew et al., 2014). Interestingly, the dogs in the study were semi-domesticated and shared contact with humans; however, it is possible that the canine-specific assemblages outcompeted assemblages A and B (Inpankaew et al., 2014; Schär et al., 2014).

3. International prevalence of *Giardia* assemblages

The prevalence of giardiasis varies from 2% in developed countries to 70% in developing countries (Geurden et al., 2009; Júlio et al., 2012; Fletcher et al., 2013; Choy et al., 2014). Higher rates of disease in developing nations are attributed to a combination of local risk factors including lack of basic sanitation and hygiene facilities, inadequate access to potable drinking water and poor housing (Slack, 2012; Fletcher et al., 2013). As developed countries have better access to good quality sanitation and hygiene facilities, there is a misconception that *G. intestinalis* infection in industrialised countries is mainly associated with international travel to developing nations (Schlagenhauf et al., 2015). However, it is likely that endemic giardiasis cases are being underestimated, particularly because returning travellers are more likely to be tested for *G. intestinalis* infection, compared with those without a travel history (Zajaczkowski et al., 2018). Indeed, a Scottish study found that a total of 93% (26/28) *Giardia*-positive cases would have been omitted from routine screening as they did not have a recent travel history or travelled to a 'low-risk' region (Currie et al., 2017). Emerging studies have also observed the possibility that majority of giardiasis cases in industrialised countries are in fact a result of endemic transmission and local risk factors (Espelage et al., 2010; Plutzer et al., 2010; Minetti et al., 2015b; Woschke et al., 2021). A German case-control study observed more than half of *G. intestinalis* infections were acquired by individuals who did not report travelling overseas prior to illness onset (Espelage et al., 2010).

There is still a lot of uncertainty surrounding the distribution of *G. intestinalis* assemblages around the world mainly because there are limited molecular epidemiological studies of giardiasis in humans and the results are often difficult to compare. While *G. intestinalis* assemblages A and B are globally distributed, most studies based in both developed and developing regions agree that assemblage B is the most commonly found in human infections (Kohli et al., 2008; Breathnach et al., 2010; Tungtrongchitr et al., 2010). Higher prevalence of assemblage B has been reported in Canada (Iqbal et al., 2015), China (Yu et al., 2019), England (Minetti et al., 2015a), Kenya (Mbae et al., 2016), Spain (Wang et al., 2019). Assemblage B infections have a higher parasitic load and increased rate of cyst shedding which would explain the higher detection rates in comparison to assemblage A infections (Kohli et al., 2008). Yet higher rates of assemblage A infections have been observed in countries such as Brazil (Souza et al., 2007), Ethiopia (Damitie et al., 2018), Iran (Kasaei et al., 2018; Mahmoudi et al., 2020), New Zealand (Winkworth et al., 2008), Syria (Skhal et al., 2017) and Romania (Costache et al., 2020). Due to limited molecular epidemiological studies of giardiasis in humans, an accurate geographical pattern for individual *G. intestinalis* assemblages cannot be concluded. However, a recent study has noted that the occurrence of mixed-assemblage infections appears to be higher in developing countries as opposed to developed regions of the world (Samie et al., 2020). This can be attributed to overcrowded living conditions and higher contact among infected individuals, allowing assemblages A and B to persist and to re-circulate in these communities (Asher et al., 2014).

There are very few studies that can explain why the prevalence of *G. intestinalis* assemblages seen in communities vary so widely. This may be due to a lack of sensitive surveillance systems that monitor giardiasis prevalence. Most likely, there are several factors at play that influence genotype dominance. These factors could be linked to differences in wildlife populations and the potential for zoonotic disease transmission, as well as cultural, human behavioural and climatic variances across regions in the world. It is proposed that the host's age or gender can also alter the transmission dynamics and the distribution of assemblages A and B. A study in England observed that assemblage A was more common in adults aged greater than or equal to 65 years-old, while assemblage B was more prevalent in children (Minetti et al., 2015a). Likewise, studies in Spain and Egypt observed children were more commonly infected by assemblage B than adults (El Basha et al., 2016; Wang et al., 2019). It is theorised that older individuals develop a potential immunity to *G. intestinalis* assemblage B, which would explain why assemblage A infections tend to be recorded in adults rather than children. It is also possible that these age-related differences are the result of other factors, such as different typing techniques, sample sizes and target populations used during the studies.

4. Molecular tools for genetic characterisation of *G. intestinalis*

There is an increasing use of molecular PCR being applied to study *Giardia* from a variety of mammalian species. This method provides a highly sensitive and specific approach to diagnostics and unlike conventional methods, can accurately characterise *Giardia* at the species- and assemblage-level. PCR-based restriction fragment length polymorphism (PCR-RFLP) targeting the *bg*, *gdh* or *tpi* genetic markers is one of the earliest molecular tools used by investigators to genotype *G. intestinalis* (Aydin et al., 2004; Almeida et al., 2006; Gelanew et al., 2007; Pelayo et al., 2008; Lebbad et al., 2011; Sarkari et al., 2012; Rafiei et al., 2020). Studies that have utilised a PCR-RFLP method commonly detect a higher prevalence of inter-assemblage mixed infections that are not always caught by standard PCR (Amar et al., 2002; Read et al., 2004; Lalle et al., 2005; Van der Giessen et al., 2006). Yet advancements in DNA sequencing technologies means the PCR-RFLP method is rapidly becoming obsolete. It is a far more time-consuming procedure and is often subject to contamination.

PCR amplification of *G. intestinalis* involves using assemblage-specific primers and partially sequencing one or more of the following loci; small subunit ribosomal RNA (SSU rRNA), glutamate dehydrogenase (*gdh*), triosephosphate isomerase (*tpi*) or *β*-giardin (*bg*) (see Table 1). Other targeted loci that are used albeit less commonly are the elongation factor 1-alpha (*ef-1*) (Traub et al., 2004), the GLORF-C4 (C4) (Yong et al., 2002) and the intergenomic rDNA spacer region (IGS) (Lee et al., 2006). Although most genotyping studies use a single gene to characterise their isolates, there remains a crucial need for the development of a novel genotyping method that targets more than one locus for identifying *G. intestinalis* assemblages. Most studies that have characterised *G. intestinalis* by testing at single genetic loci have shown inconsistent subtyping results (Read et al., 2004; Cooper et al., 2007; Gelanew et al., 2007). One study observed that three of the loci (SSU rRNA, *tpi* and *gdh*) classified human isolates as assemblage A. However, genotyping of those same isolates at the *bg* gene as a single gene target showed a higher similarity to assemblage B (Cacciò et al., 2008).

4.1. The occurrence of mixed-assemblage infections

'Assemblage swapping' has been identified in clinical, animal, and environmental samples and is thought to be a result of mixed-assemblage infections (Almeida et al., 2010). Mixed-assemblage infections containing both assemblage A and B are rarely identified accurately, and most studies report only a 3–10% prevalence (Kohli et al., 2008). There is speculation that mixed assemblage infections are being underreported due to the preferential amplification of one assemblage over the other depending on the primer set being used (Wielinga & Thompson, 2007). Moreover, primers used for typing *G. intestinalis* not only differ in their specificities (see Table 1) but have been reported to have variable amplification success rates (Costache et al., 2020; Chourabi et al., 2021; Iwashita et al., 2021).

Table 1
Common molecular methods used for genotyping *Giardia intestinalis* assemblages A and B

Loci targeted for genotyping[a]	Genotyping method[b]	Name of primer/probe	Primer or probe sequence (5′-3′)	Amplicon size (bp)	Specificity	Reference
SSU rRNA	Multiplex real-time (Scorpion) PCR	ScA	Probe (HEX-CCCGGCGCATGGCTTC GTCCTTGCCGGG-BHQ1-HEG-ATCC TGCCGGAGCGCGACG)		A & B	Haque et al. (2005); Ng et al. (2005); Kohli et al. (2008); Alam et al. (2011)
		ScB	Probe (FAM-CGGGCATGCATGGCCCG-BHQ1-HEG-CGGTCGATCCTGCCGGAATC)			
SSU rRNA	Nested PCR and sequencing	Primary: r37fw	CAAGGACRMAAGCCATGC	847	A & B	Breathnach et al. (2010)
		Primary: r883rev	CCTTCAAGTTYCAGCCTTGC			
		Secondary: r275fw	GGAGRATCAGGGTTYGACTC	506		
		Secondary: r780rev	GTTTACGSYCGGGAATACG			
SSU rRNA	Nested PCR and sequencing	Primary: RH11	CATCCGGTCGATCCTGCC	292	A & B	Hopkins et al. (1997); Read et al. (2002); Mohammed Mahdy et al. (2009); Jerez Puebla et al. (2017)
		Primary: RH4	AGTCGAACCCTGATTCTCCGCCAGG			
		Secondary: GiarF	GACGCTCTCCCCAAGGAC	130		
		Secondary: GiarR	CTGCGTCACGCTGCTCG			
bg	Single and/or nested-PCR-RFLP	Primary: G7	AAGCCCGACGACCTCACCCGCAGTGC	753	A & B	Cacciò et al. (2002); Lalle et al. (2005); Almeida et al. (2006); El Basha et al. (2016)
		Primary: G759	GAGGCCGCCCTGGATCTTCGAGACGAC			
		Secondary: BGiF/G99	GAACGAGATCGAGGTCCG	511		
		Secondary: BGiR/G609	CTC GAC GAG CTT CGT GTT			
		RE: *Hae*III		50, 113, 150, 200 (A); 24 26, 84, 113 117, 150 (B)		
gdh	Semi-nested PCR-RFLP	Primary: GDHeF	TCAACGTYAAYCGYGGYTTCCGT		A (AI, AII) & B (BIII, BIV)	Read et al. (2004); Sarkari et al. (2012); Kashinahanji et al. (2019); Tembo et al. (2020)
		Primary: GDHiR	GTTRTCCTTGCACATCTCC			
		Secondary: GDHiF	CAGTACAACTCYGCTCTCGG	432		
		Secondary: GDHiR	GTTRTCCTTGCACATCTCC			
		RE: *Bsp*LI (*Nla*IV) and *Rsa*I				
gdh	Nested PCR and sequencing	Primary: Gdh1	TTCCGTRTYCAGTACAACTC	754	A (AI, AII, AIII) & B (BIII, BIV)	Cacciò et al. (2008); Wang et al. (2019)
		Primary: Gdh2	ACCTCGTTCTGRGTGGCGCA			
		Secondary: Gdh3	ATGACYGAGCTYCAGAGGCACGT	530		
		Secondary: Gdh4	GTGGCGCARGGCATGATGCA			
tpi	Nested-PCR and/or assemblage-specific PCR	Primary: AL3543	AAATIATGCCTGCTCGTCG	605	A & B	Sulaiman et al. (2003); Geurden et al. (2008); Levecke et al. (2009); Bahrami et al. (2017); Jerez Puebla et al. (2017); Kashinahanji et al. (2019)
		Primary: AL3546	CAAACCTTITCCGCAAACC			
		Secondary: AL3544	CCCTTCATCGGIGGTAACTT	532		
		Secondary: AL3545	GTGGCCACCACICCCGTGCC			
		Assemblage A-specific primers				
		Af	CGCCGTACACCTGTCA	332 (A)		
		Ar	AGCAATGACAACCTCCTTCC			
		Assemblage B-specific primers				
		Bf	GTTGTTGTTGCTCCCTCCTTT	400 (B)		
		Br	CCGGCTCATAGGCAATTACA			
tpi	Semi-nested PCR and sequencing	Primary: tpi1f	CCCTTCATCGGYGGTAAC		A (AI, AII) & B (BIII, BIV)	Breathnach et al. (2010)
		Primary: tpi479r	CCCGTGCCRATRGACCACAC			
		Secondary: tpi300r	AGRGCACGCTTMGCCTTC	339		
tpi	Semi-nested, duplex PCR	Assemblage A-specific primers			A (AI, AII) & B	Amar et al. (2002); Sahagún et al. (2008); Ajjampur et al. (2009); Breathnach et al. (2010)
		Primary: TPIA-F	CGAGACAAGTGTTGAGATGC	576 (A)		
		Primary: TPIA-R	CGTGTTGTAAGCTCTTGACC			
		Secondary: TPIA-IF	CCAAGAAGGCTAAGCGTGC	476 (A)		
		Secondary: TPIA-R	CGTGTTGTAAGCTCTTGACC			
		Assemblage B-specific primers				
		Primary: TPIB-F	GTTGCTCCCTCCTTTGTGCA	208 (B)		
		Primary: TPIB-R	GCGAGACCAATGAGCAGAGT			
		Secondary: TPIB-IF	GCACAGAACGTGTATCTGG	140 (B)		
		Secondary: TPIB-R	GCGAGACCAATGAGCAGAGT			
		RE: *Rsa*I		437, 39 (AI); 235, 202, 39 (AII)		

Table 1 (*continued*)

Loci targeted for genotyping[a]	Genotyping method[b]	Name of primer/probe	Primer or probe sequence (5′-3′)	Amplicon size (bp)	Specificity	Reference
tpi	PCR-RFLP	Forward primer	TGGACTGGCGAGACAAG	540	A & B	Aydin et al. (2004)
		Reverse primer	TCCGGCTTGAGGGAAGC			
		RE: *Xho*I		540 (A); 442, 241 (B)		
tpi	Real-time (TaqMan-MGB) PCR	GDAT	Probe (VIC-CCATTGCGGCA AACA-MGB-NFQ)		A & B	Elwin et al. (2014)
		GDBT	Probe (FAM-AATATTGCTCAG CTCGAG-MGB-NFQ)			
tpi	Assemblage-specific PCR	Assemblage A-specific primers			A & B	Vanni et al. (2012); Belkessa et al. (2021)
		4E1-HP-AF	AAAGAGATAGTTCGCGATGTC	165 (A)		
		4E1-HP-AR	ATTAACAAACAGGGAGACGTATG			
		Assemblage B-specific primers				
		4E1-HP-BF	GAAGTCATCTCTGGGGCAAG	272 (B)		
		4E1-HP-BR	GAAGTCTAGATAAACGTGTCGG			
Tif and *Cath*	Real-time PCR-HMC	TIF-assemblage AF	AGAAGTGTCCTGGACTGGGTCT	168 (A)	A & B	Van Lith et al. (2015); Woschke et al. (2021)
		TIF-assemblage AR	CGTGGAATTGTCAATCGTTAAAC			
		CATH-assemblage BF	GCGATTTTCCGCGGAAGGTTGT	99 (B)		
		CATH-assemblage BR	AGAGGGCATCATAAACATAAACC			
IGS region of rDNA	Nested PCR -gel electrophoresis	Primary: GLF	GACCGTCGTGAGACAGGTTAG		A (AI, AII) & B	Lee et al. (2006); Hussein et al. (2017)
		Primary: GSR	CCTGCTGCCGTCCTTGGATG			
		Assemblage AI-specific primers				
		GA1F	GGTTTGCCGTGATATGCA	176 (AI)		
		GABR	AGCACCTTGTCCTATAYAGT			
		Assemblage AII-specific primers				
		GA2F	CCCTCCAGAGCAGMRTGAGA	261 (AII)		
		GABR	AGCACCTTGTCCTATAYAGT			
		Assemblage B-specific primers				
		GBF	GRCAGGTGTGCTCAGGTG	319 (B)		
		GABR	AGCACCTTGTCCTATAYAGT			
IGS region of rDNA	Real-time PCR-HMC	Primers as listed above (GLF, GSR, GA1F, GA2F, GBF and GABR)			A (AI, AII) & B	Al-Mohammed (2011)
orfC4, tpi and *gdh*	Real-time PCR	Assemblage A-specific primers			A & B	Almeida et al. (2010)
		ORFC4-AF	CTGTAGACAGGGCCCAGGCC	103 (A)		
		ORFC4-AR	ATGATGTCCCCTGCCCTTAAT			
		TPI-AF	TCGTCATTGCCCCTTCCGCC	77 (A)		
		TPI-AR	CGCTGCTATCCTCAACTG			
		GDH-AF	CCGGCAACGTTGCCCAGTTT	180 (A)		
		GDH-AR	ACTTGTCCTTGAACTCGGA			
		Assemblage B-specific primers				
		ORFC4-BF	ACTGTCCATTTCTATCTGAG	171 (B)		
		ORFC4-BR	GGATTCCATTGGCCTCCACCT			
		TPI-BF	GATGAACGCAAGGCCAATAA	77 (B)		
		TPI-BR	GATTCTCCAATCTCCTTCTT			
		GDH-BF	CGTATTGGCGTCGGCGGT	133 (B)		
		GDH-BR	TGTGGCCTCTGGTCTGATAG			
bg, tpi, gdh and SSU rRNA	MLST-PCR -sequencing[c]	*bg* primers: G7, G759, BGiF/G99, BGiR/G609 *tpi* primers: AL3543, AL3546, AL3544, AL3545 *gdh* primers: GDHeF, GDHiR, GDHiF, GDHiR SSU rRNA primers: RH11, RH4, GiarF, GiarR	As listed above	As listed above	A (AI, AII, AIII) & B (BIII, BIV)	Cacciò et al. (2008); Lebbad et al. (2008); Pelayo et al. (2008); Tungtrongchitr et al. (2010); Lebbad et al. (2011); Gillhuber et al. (2013); Minetti et al. (2015b); Faria et al. (2017); Skhal et al. (2017); Wang et al. (2019); Costache et al. (2020); Rafiei et al. (2020); Chourabi et al. (2021)

[a] *tpi*, triosephosphate isomerase; *gdh*, glutamate dehydrogenase; *bg*, *β*-giardin; SSU rRNA, small subunit ribosomal ribonucleic acid; IGS, intergenic spacer; *Tif*, translation initiation factor gene; *Cath*, cathepsin L precursor gene.

[b] PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; MLST, multilocus sequence typing; HMC, high resolution melting curve analysis.

[c] In this paper, MLST refers to genotyping that has amplified sequences at three or more loci: *tpi*, *gdh*, *bg* and SSU rRNA.

Interestingly, PCR-based studies that use assemblage-specific primers targeting the *tpi* locus are more likely to observe mixed-assemblage infections in comparison to other PCR methodologies (Huey et al., 2013; Elhadad et al., 2021) (see Table 2). Huey et al. (2013) originally noted a mixed-infection rate of 64%; however, the rate was underestimated when using a multi-locus genotyping approach on the same isolates. Multi-locus PCR remains the foremost tool for efficient genotyping of *G. intestinalis*; but it is clearly not without its biases, particularly when dealing with mixed assemblage infections.

Mixed infections can be a result of genetic exchanges occurring between assemblages in a single cyst, forming hybrids known as recombinants (Lasek-Nesselquist, 2009; Almeida et al., 2010). While originally it was presumed that the reproduction of *Giardia* was exclusively asexual, whole-genome sequencing (WGS) studies have identified homologs of genes involved in meiosis (Ramesh et al., 2005; Poxleitner et al., 2008). This suggests that the fusion of two nuclei, or karyogamy, and ultimately somatic recombination is possible in the cyst stage. Additionally, population genetic data (Cooper et al., 2007; Kosuwin et al., 2010) and epidemiological studies (Teodorovic et al., 2007; Lasek-Nesselquist et al., 2009) have indicated strong evidence for recombination within and between *G. intestinalis* assemblage isolates. While sexual recombination in *G. intestinalis* is theoretically possible, there has yet to be confirmation of meiosis through direct observation. It is likely that the mechanism of sexual reproduction is an infrequent and/or rare phenomenon that can happen only under specific host conditions.

Another strong point in favour of *Giardia* being a sexually reproducing organism is the overall low levels of allelic sequence divergence seen in both assemblages (Morrison et al., 2007; Teodorovic et al., 2007; Lasek-Nesselquist et al., 2009; Jerlström-Hultqvist et al., 2010). Allelic sequence heterogeneity (ASH) levels are reported as low as 0.01–0.03% in assemblage A and 0.4–0.5% in assemblage B (Cooper et al., 2007; Morrison et al., 2007; Teodorovic et al., 2007; Adam et al., 2013; Xu et al., 2020). It is assumed that sexually reproducing organisms maintain a lower level of allelic heterozygosity by the need for chromosome pairing during meiosis. In contrast, asexual organisms with a ploidy of two or more have highly divergent allelic sequences because of independently evolving nuclei (Birky Jr., 2010).

It is also important to note that *G. intestinalis* assemblage B possesses higher ASH in comparison to assemblage A (Cacciò et al., 2008; Lalle et al., 2009; Ankarklev et al., 2012; Huey et al., 2013). This high polymorphism in assemblage B is reflected in the high frequency of double peaks seen in sequence chromatograms. Oftentimes, this makes assigning a subtype to assemblage B isolates difficult. Some studies have also indicated that the *bg*, *gdh* and *tpi* markers are more likely to show heterogeneous templates due to their highly polymorphic nature (Wielinga & Thompson, 2007). A genotyping study did not detect mixed-infection profiles when using the conserved SSU rRNA target, and only identified two intra-assemblage mixed infections when running a *gdh* assay (Hussein et al., 2009). A novel multi-locus genotyping method is needed; one that utilises polymorphic genetic markers to reliably identify assemblages but without masking true mixed infections. Ideally assemblage-specific primer sets should be incorporated into a multi-locus genotyping method.

4.2. Comparing common molecular methods used for genotyping *G. intestinalis*

Markers used in genotyping *G. intestinalis* isolates differ vastly in terms of their genetic variability, as well as test sensitivity and specificity (Wielinga & Thompson, 2007). The SSU rRNA gene remains one of the most used markers for genotyping *G. intestinalis* assemblages due to its highly conserved sequence, multi-copy nature and subsequent high amplification rate in PCR. Most genotyping studies that target the SSU rRNA utilise a nested-PCR methodology using primers RH11/RH4 and GiarF/GiarR developed by Hopkins et al. (1997) and Read et al. (2002). Due to high copy numbers in the SSU rRNA gene, these nested primers are found to be incredibly sensitive. This is particularly advantageous when dealing with low quantities of parasite load or high amounts of PCR inhibitors. Evidence of this was seen in a study by Minetti et al. (2015a) where *Giardia*-positive specimens were successfully typed at the SSU rRNA gene, despite having previously failed to amplify when using other loci (*bg*, *gdh*, *tpi*). Although SSU rRNA gene remains a commonly used option for typing, it is recommended that it be used strictly for confirming the presence or absence of *Giardia* DNA in specimens rather than for sub-typing purposes. This is because a major limitation of using any SSU rRNA primer is the low genetic variation between assemblages making it impossible to differentiate between sub-assemblages (see Table 1).

Sub-typing (meaning typing at the level of sub-assemblages) *G. intestinalis* isolates is only possible when using the single-copy genes *tpi*, *gdh* or *bg*. In comparison to the SSU rRNA gene, the *tpi* and *gdh* markers have the highest discriminatory power, followed by the *bg* gene meaning that they support major assemblage and sub-assemblage typing. There are several primer sets used for *tpi* assays (see Table 1); however, the most used are the nested-PCR primers (AL3543/AL3546 and AL3544/AL3545) described by Sulaiman et al. (2003). Later studies then incorporated assemblage-specific primers (Af/Ar and Bf/Br) to be used alongside the primer sets of Sulaiman et al. (2003), and this allowed for the detection of assemblages A and B (Geurden et al., 2008; Levecke et al., 2009). Assemblage-specific primers such as these are advantageous for multiple reasons. First, the assemblages are identified by differing PCR product lengths and this allows researchers to genotype *G. intestinalis* isolates without the need for sequence analyses. This makes it a time and cost-efficient method. Secondly, these primers can detect mixed assemblages more effectively than standard PCR primers (Sahagún et al., 2008; Ajjampur et al., 2009; Breathnach et al., 2010; Huey et al., 2013). Using general primers can often overlook mixed assemblage cases because of the variable proportions of assemblages A and B DNA. Despite the popularity of the *tpi* marker for genotyping purposes, there is evidence that *tpi* primers have variable amplification success. MLST studies that have utilised the AL3543/AL3546 primer set have observed low amplification rates for *tpi* genes when comparing to *bg* and *gdh* (Chourabi et al., 2021; Iwashita et al., 2021). Yet another MLST study using the same primers found that typing at the *tpi* gene had the highest amplification success compared to the *gdh* and ITS regions (Costache et al., 2020). Several factors may be causing this lack of reliability, including the DNA yield and method of DNA extraction, as well as the possibility of DNA contaminants and inhibitors (Faria et al., 2017). It may also be a result of nucleotide mismatches that are affecting PCR primer-binding sites and leading to the non-amplification of some isolates (Capewell et al., 2021). This only accentuates the idea that current single locus-based typing of *G. intestinalis* is limited and may in fact be missing essential genetic data. Several studies have recommended utilising a multi-locus sequence typing (MLST) methodology involving targeting a combination of commonly used markers (*bg*, *gdh*, *tpi* and SSU rRNA) to increase successful PCR chances. This process allows subtypes of each locus to be combined into a multi-locus genotype (MLG) (Cacciò et al., 2008).

Typing *G. intestinalis* at the *gdh* marker involves using the semi-nested primers *GDH*eF/*GDH*iF and *GDH*iR, which can discriminate between sub-assemblages AI, AII, BIII and BIV once digested with enzymes (see Table 1) (Read et al., 2004). This PCR-RFLP method is widely used as it offers a cheap, but effective alternative to subtyping by sequence analysis. However, similarly with other PCR-RFLP, this assay has a limited ability to detect genetic variations and can often miss novel sub-types, some that often differ by one nucleotide (Cacciò et al., 2008). As such, it is recommended that the nested primers *Gdh*1/*Gdh*2 and *Gdh*3/*Gdh*4 are used as an alternative to the PCR-RFLP assay (Cacciò et al., 2008). These primers offer greater discrimination through sequencing and can discriminate between all major assemblages and sub-assemblages (see Table 1). However, amplifying single-copy genes such as *gdh*, *bg* and *tpi* is not without disadvantages. The high polymorphism seen in these genetic markers can make it difficult to discriminate between assemblage B

Table 2
Associations seen between *Giardia* assemblages and clinical symptoms

Country	Study outline and sample size	Locus targeted	Genotypes detected (%)	Symptoms observed and assemblage associations	Reference
Australia	Children aged under 5 years and attending day-care centres. *Giardia* genotyping data were obtained from 23 children ($n = 23$)	SSU-rRNA	Assemblage A (30%) Assemblage B (70%)	‣ Children infected with assemblage A were 26 times more likely to have diarrhoea ($P < 0.005$). ‣ An association was also found between assemblage B and asymptomatic infection in children less than 5 years of age.	Read et al. (2002)
Bangladesh	Patients with diarrhoea admitted to a research institution ($n = 211$)	SSU-rRNA	‣ Symptomatic cases ($n = 40$) Assemblage A (20%) Assemblage B (80%) ‣ Asymptomatic cases ($n = 171$) Assemblage A (4%) Assemblage B (96%)	‣ 7 times more likely to have diarrhoea with an assemblage A infection (OR = 6.88; $P = 0.001$)[a]. ‣ Assemblage B infection was statistically associated with asymptomatic infection and occurred at a significant rate in the population (18.0%; $P < 0.0001$).	Haque et al. (2005)
	Stool samples collected from diarrhoeal and non-diarrhoeal patients and genotyped by qPCR ($n = 117$)	SSU-rRNA	‣ Diarrhoeal cases ($n = 57$) Assemblage A (25%) Assemblage B (68%) Mixed A+B (7%) ‣ Non-diarrhoeal cases ($n = 60$) Assemblage A (2%) Assemblage B (95%) Mixed A+B (3%)	‣ Strong association between diarrhoea and assemblage A infection ($P = 0.01$). ‣ Assemblage B infection was associated with non-diarrhoeal, asymptomatic infections ($P = 0.01$).	Alam et al. (2011)
Brazil	Stool specimens collected from 47 children at 3-month intervals and during diarrhoeal episodes ($n = 58$)	SSU-rRNA	Assemblage A (16%) Assemblage B (74%) Mixed A+B (10%)	‣ No significant difference in diarrhoeal symptoms experienced.	Kohli et al. (2008)
Cuba	*G. intestinalis*-positive stool samples collected from primary-school children and genotyped successfully 20 ($n = 20$)	*bg*, *gdh*	Assemblage A (45%) Assemblage B (55%)	‣ Significant association between *Giardia* symptoms (diarrhoea and/or at least two of the following: nausea, vomiting, loss of appetite, weight loss, abdominal pain) and assemblage B infection ($P = 0.017$).	Pelayo et al. (2008)
	Giardia-positive stool samples collected from children remitted to hospital with gastrointestinal symptoms. Fifty-two ($n = 52$) of the samples were genotyped successfully	*tpi*, SSU-rRNA	Assemblage A (25%) Assemblage B (54%)	‣ Significant association between diarrhoea and assemblage B infection ($P = 0.02$)[b].	Jerez Puebla et al. (2017)
Egypt	Samples acquired from school children ranging in age from 5 to 15 years. *Giardia*-positive faecal samples were submitted for genotyping ($n = 65$)	IGS region rDNA	‣ Symptomatic cases ($n = 30$) Assemblage A (53%) Assemblage B (40%) Mixed A+B (7%) ‣ Asymptomatic cases ($n = 35$) Assemblage A (80%) Assemblage B (20%)	‣ Sub-assemblage AI was less common in symptomatic children as opposed to asymptomatic children (53.3 *vs* 66.6%; $P = 0.013$). ‣ Sub-assemblage AII was only reported among asymptomatic children. ‣ There was a significant relationship between symptomatic children and assemblage B ($P = 0.013$).	Hussein et al. (2017)
	Samples collected from patients (aged 2–55 years-old) complaining of diarrhoea ($n = 60$)	*bg*	Assemblage A (37%) Assemblage B (63%)	‣ Cases infected with assemblage A were significantly more likely to report severe and recurring diarrhoea ($P < 0.001$) and dehydration ($P < 0.001$).	El Basha et al. (2016)
England	Patients with gastroenteritis and a *Giardia* infection ($n = 150$)	*bg*, *gdh*, *tpi* and SSU-rRNA	Assemblage A (31%) Assemblage B (67%) Mixed A+B (2%)	‣ Cases infected with assemblage B were significantly more likely to report vomiting ($P = 0.030$), abdominal pain ($P = 0.041$), bloating ($P = 0.012$) and loss of appetite ($P = 0.020$).	Minetti et al. (2015b)
Ethiopia	*G. intestinalis*-positive stool samples collected from hospital patients and children attending day-care centres/primary schools ($n = 59$)	*bg*, *gdh*, *tpi*	Assemblage A (52%) Assemblage B (22%) Mixed A+B (14%) Mixed A+F (12%)	‣ Significant association between nausea, abdominal pain, diarrhoea, and assemblage B infection (OR = 12; $P = 0.007$).	Gelanew et al. (2007)
India	Children living in a community birth cohort with giardial diarrhoea ($n = 101$)	*tpi*	‣ Symptomatic cases ($n = 50$)	‣ Association between diarrhoea and assemblage A infection ($P = 0.074$).	Ajjampur et al. (2009)

(*continued on next page*)

Table 2 (*continued*)

Country	Study outline and sample size	Locus targeted	Genotypes detected (%)	Symptoms observed and assemblage associations	Reference
			Assemblage A (10%) Assemblage B (80%) Mixed A+B (10%) ‣ Asymptomatic cases ($n = 51$) Assemblage A (4%) Assemblage B (94%) Mixed A+B (2%)		
Iran	Faecal samples collected from individuals referred to medical laboratories. Genotyping was performed on ($n = 23$) PCR products	*tpi*	Assemblage A (52%) Assemblage B (48%)	‣ No significant association was observed between assemblages and clinical manifestations.	Bahrami et al. (2017)
	Giardia-positive stool samples collected from clinical laboratories ($n = 172$)	*gdh*	Assemblage A (74%) Assemblage B (21%) Mixed A+B (5%)	‣ Significant association between assemblage AII and abdominal pain, nausea, and vomiting.	Sarkari et al. (2012)
	Voluntary participants from rural and urban communities. A total of 24 *Giardia*-positive cases were genotyped at least at a single locus ($n = 24$)	*bg*, *gdh*, *tpi*	Assemblage A (50%) Assemblage B (50%)	‣ No significant association was observed between assemblages and clinical manifestations.	Rafiei et al. (2020)
	Human stool samples gathered from individuals admitted to medical and health care facilities. Twenty-three samples were successfully genotyped ($n = 23$)	*gdh*, *tpi*	Assemblage A (78%) Assemblage B (22%)	‣ No significant association was observed between assemblages and clinical manifestations.	Kashinahanji et al. (2019)
Malaysia	Indigenous individuals living in village communities ($n = 42$)	SSU-rRNA	Assemblage A (2%) Assemblage B (98%)	‣ Two times more likely to have diarrhoea, abdominal pain, vomiting and nausea with an assemblage B infection (OR = 2.4; $P = 0.019$).	Mohammed Mahdy et al. (2009)
Nicaragua	Human stool samples from diarrhoeal patients and healthy individuals ($n = 112$)	*bg*, *gdh*	Assemblage A (21%) Assemblage B (79%)	‣ No association was observed between symptoms experienced and assemblage type.	Lebbad et al. (2008)
Portugal	Asymptomatic individuals including adults and children under 12 years of age ($n = 7$)	*bg*	Assemblage A (29%) Assemblage B (71%)	‣ Asymptomatic infections seen in individuals infected with assemblages A and B.	Almeida et al. (2006)
Saudi Arabia	Primary-school children aged 6 12 years ($n = 40$)	IGS region rDNA	‣ Symptomatic cases ($n = 24$) Assemblage A (29%) Assemblage B (63%) Mixed A+B (8%) ‣ Asymptomatic cases ($n = 16$) Assemblage A (100%) Assemblage B (0%)	‣ Symptomatic giardiasis was significantly associated with assemblage B (OR = 25.874; $P < 0.001$).	Al-Mohammed (2011)
Spain	Patients with symptomatic or asymptomatic giardiasis ($n = 108$)	*tpi*	Assemblage A (40%) Assemblage B (56%) Mixed A+B (4%)	‣ Significant association between symptomatic infections and assemblage A ($P < 0.05$). ‣ A significant association was also found between asymptomatic infections and assemblage B ($P < 0.05$). ‣ These correlations were only significant in children 5 years-old and younger.	Sahagún et al. (2008)
	Samples collected from out-patients (aged 1 75 years) from multiple hospitals ($n = 97$)	*bg*, *gdh*, *tpi*	Assemblage A (30%) Assemblage B (68%) Mixed A+B (2%)	‣ Significant association between asymptomatic infection and assemblage A (OR = 10.4; $P = 0.029$). ‣ Frequency of abdominal pain occurrence was higher in assemblage B patients (98.5 *vs* 86.2%; $P = 0.029$).	Wang et al. (2019)
Sweden	Patients with intestinal symptoms who consulted a physician and individuals who attended health check-ups ($n = 207$)	*bg*, *gdh*, *tpi*	Assemblage A (35%) Assemblage B (62%) Mixed A+B (3%)	‣ Correlation between flatulence and assemblage B infections in children aged 6 years and younger ($P = 0.006$).	Lebbad et al. (2011)
Syria	Samples collected from three main hospitals. Forty patients with symptomatic giardiasis participated in the study ($n = 40$)	*bg*, *gdh*	Assemblage A (67.5%) Assemblage B (10%) Mixed A+B (22.5%)	‣ Significant association between weight loss and sub-assemblage AII ($P = 0.05$).	Skhal et al. (2017)
Thailand	*Giardia*-positive stool samples from individuals with and without gastrointestinal symptoms ($n = 61$)	*bg*, *gdh*, *tpi*	Assemblage A (8%) Assemblage B (51%) Mixed A+B (41%)	‣ All assemblage A infections were symptomatic; however, there was no significant association between assemblages and symptomatic giardiasis.	Tungtrongchitr et al. (2010)

Table 2 (*continued*)

Country	Study outline and sample size	Locus targeted	Genotypes detected (%)	Symptoms observed and assemblage associations	Reference
Turkey	Samples from patients with symptomatic/asymptomatic giardiasis were included in the study ($n = 56$). Forty-four samples were stool specimens: symptomatic patients ($n = 20$); asymptomatic patients ($n = 24$). Twelve samples were duodenal aspirates: symptomatic patients ($n = 6$); asymptomatic patients ($n = 6$)	*tpi*	‣ Stool specimens ($n = 44$) Assemblage A (43%) Assemblage B (57%) ‣ Duodenal aspirates ($n = 12$) Assemblage A (50%) Assemblage B (50%)	‣ Strong association between diarrhoeal symptoms and assemblage A infection (17 out of 20 isolates; $P < 0.001$). ‣ Majority of asymptomatic infections are associated with assemblage B (22 out of 24 isolates).	Aydin et al. (2004)
United Arab Emirates	*Giardia*-positive stool samples from healthy individuals residing in Sharjah, UAE ($n = 67$)	*tpi*	Assemblage A (45.7%) Assemblage B (41.3%) Mixed A+B (13.0%)	‣ Strong association between the presence of diarrhoea and assemblage B infection (OR = 10.533; $P = 0.001$). ‣ An association was also found between mixed infections (A+B) and diarrhoea (OR = 8.899; $P = 0.003$).	ElBakri et al. (2014)
United Kingdom	*Giardia*-positive stool samples collected from hospital diagnostic laboratories ($n = 199$)	*tpi*	Assemblage A (24%) Assemblage B (73%) Mixed A+B (3%)	‣ Fever was significantly more common in assemblage A infections (OR = 5.091; $P = 0.018$). ‣ Longer illness or asymptomatic infection was linked with assemblage B infection; however, this was not statistically significant.	Breathnach et al. (2010)

[a] This result was more significant when mixed assemblage cases (A and B) and mixed intestinal infections were excluded from the analysis.
[b] This result was only significant when mixed intestinal infections were excluded from the analysis.

subtypes (Cacciò et al., 2008; Lebbad et al., 2008; Lalle et al., 2009; Ankarklev et al., 2012; Huey et al., 2013). Additionally, amplifying single-copy genes often leads to discordant data, as it has been reported that certain isolates are amplified at one locus but not at another (Traub et al., 2004; Cooper et al., 2007; Gelanew et al., 2007).

Studies have also characterised *G. intestinalis* isolates by targeting the IGS region of rDNA in single-locus PCR assays (Lee et al., 2006; Al-Mohammed, 2011), as well as by incorporating this marker in MLST (Hussein et al., 2017; Costache et al., 2020). The IGS is a multi-copy and highly variable region that spans the 5.8S rDNA and two internal transcribed spacers (ITS1-5.8S-ITS2), making it suitable for classifying *G. intestinalis* into the major assemblages. In fact, a recent report has observed a strong agreement between *tpi* and IGS loci when classifying *G. intestinalis* into assemblages; both markers revealed 100% concordant results (Jerez Puebla et al., 2020). Assemblage-specific primers targeting the IGS region of rDNA have been successfully used to identify *G. intestinalis* assemblages without the need for sequence analyses (Lee et al., 2006; Hussein et al., 2017). These primers (GLF/GLR) and the pairs (GA1F/GABR, GA2F/GABR and GBF/GABR) are specific for assemblages AI, AII and B (see Table 1). The GLF/GLR primers are not only highly specific but sensitive (92.3%) and have shown greater polymorphism than the *tpi* gene (Hussein et al., 2017). Between assemblages A and B, the difference in their nucleotide sequences was found to be 25%, approximately 200 bp (Lee et al., 2006). More recently, the primers GLF/GLR were incorporated into a real-time PCR reaction using high-resolution melting curve analyses (HRM) (see Table 1) (Al-Mohammed, 2011). This method allows investigators to genotype *G. intestinalis* using a one-step, closed-tube method without needing sequencing or electrophoresis, all within 80 min. It must be noted that caution should be taken when sequencing the IGS region of rDNA as there are currently insufficient sequence data of reference isolates available for comparison within GenBank. For this reason, it is recommended that future studies avoid single loci genotyping methods, and at the very least, target a combination of *tpi* and IGS loci. Both loci are highly polymorphic and provide discriminating sub-genotype and phylogenetic data. It is also suggested to take advantage of the multi-copy nature of SSU rRNA gene and target this locus alongside the *tpi* and IGS region. This will ensure a high success of PCR amplification in specimens that might otherwise fail to amplify.

4.3. Novel molecular methods used for genotyping G. intestinalis

In addition to standard PCR, there is an urgent need for the development of novel molecular methods that can discriminately and genetically classify *G. intestinalis* into assemblage types. It is essential that a molecular-based classification system, such as a PCR-based barcoding approach, is introduced as the standard. DNA barcoding is a highly discriminatory tool used for genomic, epidemiological and transcriptomic research as it allows researchers to make direct genetic comparisons within and among *Giardia* sequences (Almeida et al., 2010). Such comparative genomic studies can also predict links between phenotypic traits seen in *G. intestinalis* assemblages and parasite-host interplay, virulence, and pathogenicity.

The strength of this PCR-based barcoding system increases with the number of genes targeted, so it is imperative that *G. intestinalis* sequence data are extended to help identify further genetic markers. Currently, identifying novel genetic markers is difficult due to the limited published sequence information for alternative loci. High throughput next-generation sequencing (NGS) comparing the genetically diverse profiles of each *G. intestinalis* assemblage will help to identify new genetic markers showing sufficient assemblage and/or intra-assemblage differences. Once these novel markers are found, they can be used as additional 'barcodes' for future genotyping (Minetti et al., 2015a).

While other studies have developed barcoding techniques used for identifying and subtyping protozoan parasites (Scicluna et al., 2006;

Nzelu et al., 2015); to date, only one study has genetically characterised *Giardia* isolates using systematic DNA barcoding (Nolan et al., 2011). This study successfully employed SSCP based methods and restriction endonuclease fingerprinting (REF) to analyse sequence variation within and among *Giardia* amplicons (Nolan et al., 2011). Isolates were characterised by targeting common genetic markers (*tpi*, *gdh* and *bg*). Interestingly, there were no disparities found when assigning *G. intestinalis* assemblages unlike previous multi-locus genotyping studies.

5. Clinical differences associated with *G. intestinalis* assemblages A and B

The clinical appearance of giardiasis is quite variable, and while some patients will develop clinical symptoms, others will remain asymptomatic. Symptomatic cases mainly suffer from acute and/or chronic diarrhoea, stomach cramps, nausea, vomiting, flatulence, dehydration and weight loss (Muhsen & Levine, 2012). Although symptoms are mainly non-life threatening, individuals that are immunocompromised, infants and young children can suffer from malabsorption, malnutrition and debilitating fatigue often leading to subsequent growth retardation, stunting and impaired cognitive development (Adam et al., 2013). It is still unclear why *G. intestinalis* infections manifest such variable clinical symptoms, although most studies hypothesise that host-parasite factors and the genetic differences within a parasite play a major role in subsequent clinical presentation (Tungtrongchitr et al., 2010).

Complex interactions between co-infecting enteropathogens and host molecular responses have also been suggested to influence *Giardia* disease manifestations. While co-infections with *Vibrio cholerae* (Mukherjee et al., 2014) and norovirus (Becker-Dreps et al., 2014) are particularly common, some investigations have observed a synergistic relationship between *G. intestinalis* and rotavirus (Bhavnani et al., 2012; Vasco et al., 2014). An Ecuadorian study found that individuals living in rural settings and co-infected with *Giardia* + rotavirus were associated with acute diarrhoeal illness, as opposed to being infected with either pathogen alone (OR = 24; 95% CI: 1.9–302) (Vasco et al., 2014). Interestingly, it has been suggested that *G. intestinalis* may protect against diarrhoea by competing with other enteric pathogens (Muhsen et al., 2014). However, little is still known about the biological interactions between *G. intestinalis* and co-infecting pathogens, and how these might influence outward symptoms.

While there is evidence that *G. intestinalis* can disrupt and alter intestinal microbiota resulting in symptoms similar to irritable bowel disease (IBS) and increased pathogenicity, there is still limited information regarding how *G. intestinalis* assemblages directly or indirectly influence the gut microbiome (Barash et al., 2017; Beatty et al., 2017). Comparative whole-genome sequence (WGS) analyses have identified significant genetic diversity between the two assemblages (Franzén et al., 2009), and these differences may be associated with symptomology. In particular, the variant-specific surface proteins (VSP) genes which are associated with antigenic variation and immune evasion, were found to differ between the two isolates (Ankarklev et al., 2010). There is speculation that persistent infection and chronic disease is directly related to antigenic variation in VSP (Prucca et al., 2008). Additionally, assemblage A was found to grow faster, encyst/excyst more efficiently *in vitro* and was found to cause more tissue lesions and intestinal microbiota changes in mice than assemblage B isolates (Bernander et al., 2001; Reiner et al., 2008; Pavanelli et al., 2018). Whether assemblage A is truly associated with severe clinical symptoms is yet undecided and while these studies have increased our understanding of the parasitic mechanisms involved in *G. intestinalis*, it remains difficult to determine whether these differences between assemblages is true for all *G. intestinalis* isolates. Interestingly, there appears to be a correlation between symptomatic giardiasis and the age of the host. Children aged less than 5 years and the elderly appear to suffer from more severe symptoms, which is likely to be the result of a weaker immune system (Sahagún et al., 2008; Tungtrongchitr et al., 2010). The virulence of assemblage A and B in humans may also be related to parasite factors including growth rates, metabolic products or toxins produced and drug resistance.

Currently there is no clear correlation between assemblages and symptoms with only limited studies on this topic. Assemblage A infection has reportedly been affiliated with more serious clinical symptoms in Australia, Bangladesh, Egypt, India, Iran, Turkey, Syria, and Great Britain (Read et al., 2002; Aydin et al., 2004; Haque et al., 2005; Ajjampur et al., 2009; Breathnach et al., 2010; Alam et al., 2011; Sarkari et al., 2012; El Basha et al., 2016; Skhal et al., 2017). However, the complete opposite has been suggested in other studies (Homan & Mank, 2001; Gelanew et al., 2007; Pelayo et al., 2008; Mohammed Mahdy et al., 2009; Al-Mohammed, 2011; ElBakri et al., 2014; Hussein et al., 2017; Wang et al., 2019). Furthermore, there were no associations with either assemblage in Brazil, Nicaragua, Iran and Thailand (Almeida et al., 2006; Kohli et al., 2008; Lebbad et al., 2008; Tungtrongchitr et al., 2010; Rafiei et al., 2020) (see Table 2). Whether these conflicting results can be made clear by differences in study methodology or due to the frequent occurrence of mixed-assemblage infections is an issue that needs further investigation.

6. Emerging interest in *G. intestinalis* sub-types

Allozyme analyses and recent genetic analyses at the *gdh* locus have revealed the existence of sub-genetic structures located within *G. intestinalis* assemblages A (AI, AII and AIII) and B (BIII and BIV) (Feng & Xiao, 2011; Ryan & Cacciò, 2013). It is well documented that sub-assemblage AI is mainly zoonotic, AII has anthroponotic transmission (Faria et al., 2017; Hernández et al., 2019) and AIII is mainly restricted to wild hoofed animals (Cacciò et al., 2008; Feng & Xiao, 2011; Iwashita et al., 2021). While sub-assemblage AII is predominant in humans, it has also been reported in animals suggesting zoonotic transmission is possible (Ryan & Cacciò, 2013). Sub-assemblages BIII and BIV are commonly found in humans.

With the introduction of MLST analyses targeting the *bg*, *gdh* and *tpi* loci, there has been a rapid discovery of several subtypes (Feng & Xiao, 2011; Xiao & Feng, 2017). Within assemblage A there are a total of six subtypes (A1-A6), and these are further organised within sub-assemblage AI (A1 and A5), sub-assemblage AII (A2-A4) and sub-assemblage AIII (A6) (Feng & Xiao, 2011; Xiao & Feng, 2017). These subtypes often differ by a single point mutation, which makes subtyping assemblage B almost impossible due to the presence of extensive genetic variability and ASH.

Currently, it has become increasingly important to standardise a classification system to provide a better divison of assemblage A subtypes. It has been suggested to use a multi-locus genotype (MLG) profile as the naming scheme, which is a combination of three subtypes characterised at each of the genetic loci targeted in MLG analysis (these being the *bg*, *gdh* and *tpi* loci). Based on this system, Cacciò & Ryan (2008) suggested 10 different MLGs for assemblage A (AI-1, AI-2, AII-1, AII-2, AII-3, AII-4, AII-5, AII-6, AII-7 and AIII-1). More recent investigations have identified novel MLGs—AII-8 and AII-9 (Minetti et al., 2015a; Faria et al., 2017), and Chourabi et al. (2021) discovered a novel MLG AII (profile: A2/A2/novel A2) in two isolates. Humans can be infected with an array of very diverse assemblage A subtypes, and the MLST approach should be consistently used in all future molecular epidemiological studies in other geographical regions. Additionally, the development of new target regions of the genome with lower substitution rates is necessary to successfully subtype assemblage B.

7. Conclusions and perspectives

Giardia intestinalis is one of the most common protozoan parasites causing disease in developed countries. To properly manage, treat and prevent cases of human giardiasis, it is essential that we fully understand the molecular profile of this parasite. Studies have now confirmed that *G. intestinalis* is categorised into eight assemblages, two of which are established as human-infecting (A and B). There are vast genetic and phenotypic differences between assemblages A and B which is reflected

in the differences seen in assemblage prevalence and zoonotic potential. Although various molecular studies have isolated these assemblages from other mammals including wildlife, domestic pets, and livestock, the zoonotic potential of *G. intestinalis* is still poorly understood (Leonhard et al., 2007; Sroka et al., 2015; Adam et al., 2016). This is in part due to a lack of molecular epidemiological and comparative studies that have been able to identify a direct transmission of giardiasis between animals and humans. It remains important to correctly identify *G. intestinalis* assemblages, particularly in a zoonotic context as it allows for better disease regulation and helps to identify sources of exposure in giardiasis outbreaks, especially in areas where wildlife-human interactions are common. There is a vital need for more multi-locus genotyping and sub-genotyping studies to be done on human and animal *G. intestinalis* infections. Although most literature agrees that assemblage B is more virulent and therefore more likely to manifest severely (Mohammed Mahdy et al., 2009; Al-Mohammed, 2011; Jerez Puebla et al., 2017), other studies have observed associations between assemblage A and gastrointestinal symptoms (Breathnach et al., 2010; Shahriari et al., 2012). These contrasting results can be a result of different typing techniques, sample sizes and target populations used during the studies. Likewise, results may be dependent on the differences in parasite populations and assemblage predominance across varying geographical regions. Certainly, recent studies have noted that using current molecular tools for genotyping *G. intestinalis* assemblages are unrealistic, as they depend entirely on which loci are targeted and how many markers are used in the analysis (Traub et al., 2004; Cooper et al., 2007; Gelanew et al., 2007). Using only single locus typing can often draw inconsistent conclusions depending on the interpretation of the genotyping data. As such, studies aiming to genotype *G. intestinalis* isolates must remain cautious when interpreting results obtained from single locus typing. Moving forward, whole-genome analyses will play a significant role in identifying appropriate, novel genetic markers to be used for *G. intestinalis* typing. In the meantime, it is recommended that genotyping analyses are undertaken based on a minimum of two loci to allow for consistent results. Furthermore, loci that are not only polymorphic but maintain a high level of agreement should be used as opposed to conservative markers. Ideally, the use of a MLST system that includes the *tpi* and IGS loci is encouraged at least until a worthwhile barcoding system can be developed.

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CRediT author statement

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Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter 2

Epidemiology and associated risk factors of giardiasis in a peri-urban setting in New South Wales Australia

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Certificate:

I certify that the following chapter is largely my own work, although the contributions of other authors are duly recognised. The contribution of other authors are detailed as follows:

- Suggestions on study design
- By providing suggestions on topics to be reviewed
- By proof reading draft manuscripts
- By providing suggestions to improve layout, structure and writing style
- By correcting spelling and grammatical errors in drafts

Otherwise, the main composition of this work is credited to me.

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Epidemiology and associated risk factors of giardiasis in a peri-urban setting in New South Wales Australia

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Abstract

Giardiasis is one of the most important non-viral causes of human diarrhoea. Yet, little is known about the epidemiology of giardiasis in the context of developed countries such as Australia and there is a limited information about local sources of exposure to inform prevention strategies in New South Wales. This study aimed to (1) describe the epidemiology of giardiasis and (2) identify potential modifiable risk factors associated with giardiasis that are unique to south-western Sydney, Australia. A 1:2 matched case-control study of 190 confirmed giardiasis cases notified to the South-Western Local Health District Public Health Unit from January to December 2016 was employed to investigate the risk factors for giardiasis. Two groups of controls were selected to increase response rate; Pertussis cases and neighbourhood (NBH) controls. A matched analysis was carried out for both control groups separately. Variables with a significant odds ratio (OR) in the univariate analysis were placed into a multivariable regression for each matched group, respectively. In the regression model with the NBH controls, age and sex were controlled as potential confounders. Identified risk factors included being under 5 years of age (aOR = 7.08; 95% confidence intervals (CI) 1.02–49.36), having a household member diagnosed with a gastrointestinal illness (aOR = 15.89; 95% CI 1.53–164.60) and having contact with farm animals, domestic animals or wildlife (aOR = 3.03; 95% CI 1.08–8.54). Cases that travelled overseas were at increased risk of infection (aOR = 19.89; 95% CI 2.00–197.37) when compared with Pertussis cases. This study provides an update on the epidemiology and associated risk factors of a neglected tropical disease, which can inform enhanced surveillance and prevention strategies in the developed metropolitan areas.

Introduction

Giardia duodenalis (also known as *Giardia lamblia* or *Giardia intestinalis*) is one of the most common enteroparasites affecting humans with an estimated 280 million people being infected each year, around the world [1]. It is a protozoan parasite that causes infection in the bowel and clinically manifests as a diarrhoeal illness. Additionally, giardiasis has been associated with the development of chronic diarrhoea or irritable bowel syndrome, debilitating fatigue and reactive arthritis [2]. Giardiasis is not a life-threatening disease, however, infections may often go unnoticed due to many cases having a lack of symptoms. If left without treatment, the infection can become serious; impairing the development of children and resulting in a failure to thrive [3]. Certainly, giardiasis contributes negatively to public health development of endemic countries and causes devastating socio-economic loss. In 2004, *G. duodenalis* was officially included in the WHO Neglected Diseases Initiative [4]. Meanwhile, in Australia, giardiasis is a notifiable disease in several states and territories including New South Wales (NSW) [5].

Giardiasis is the most common notifiable parasitic infection in NSW. While the burden of disease is greater in developing settings with poor access to water, sanitation and hygiene (WASH) facilities, sporadic cases occur in developed countries including Australia and outbreaks are not uncommon [6]. In 2014, nearly 3000 cases were notified by laboratories in NSW [7] and 3434 cases reported in 2015 [7]. South Western Sydney (SWS) accounts for approximately 6% of cases state-wide. Amongst hospitalised patients, giardiasis was the second most commonly identified enteric protozoa, affecting mainly school age and young children [8]. In Australia, giardiasis is frequently associated with waterborne infections, day care centre disease outbreaks and travel-associated diarrhoea.

Few Australian studies have documented the prevalence of giardiasis; however, there are no recent studies that have examined the risk factors that drive local transmission of giardiasis [9, 10]. The aim of this study was to describe the epidemiology of giardiasis and to identify the risk factors and sources of exposure associated with the disease in the SWS region of

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NSW. The study provides information on the impact of giardiasis on human health in SWS and a better understanding of the epidemiology and associated risk factors that can inform public health control strategies.

Materials and methods

Study site

The South-Western Sydney Local Health District (SWSLHD) was the research site. The SWSLHD includes seven Local Government Areas (LGA): Bankstown, Camden, Campbelltown, Fairfield, Liverpool, Wingecarribee and Wollondilly (see Supplementary Fig. S1).

The SWSLHD is the largest and fastest growing District in metropolitan Sydney. It has a large population of approximately 900 000, has a diverse geography, including significant populations in both rural and urban areas and approximately 46% of the population speak a language other than English at home. Public Health surveillance data can provide an example of what could be occurring across the NSW state.

Study design and data collection

Case-control survey

A 1:2 case-control study of risk factors was designed with the prospective recruitment of cases and controls. Cases were all confirmed cases of giardiasis notified to the SWSLHD Public Health Unit (PHU) from 1 January 2016 to 31 December 2016. A study questionnaire was developed based on a comprehensive review of the literature and was used to collect data from all cases and controls who agreed to participate in the study. Both case and control questionnaires are accessible online as Supplementary Material on the Cambridge Core website. The questionnaire asked about various socio-demographic features, self-reported clinical symptoms, information about care seeking behaviour and treatment received, the number of household members or other close contacts with similar symptoms and a range of exposures experienced 3 weeks before illness onset (for cases) or a similar time frame for controls. Enhanced data collection for this study also included additional details on potential confounders including country of birth, language spoken at home, highest educational attainment and occupation of the parents (for cases residing with their parents).

Recruitment and selection of participants

Laboratories are required under the *NSW Public Health Act 2010* to notify PHUs of cases of giardiasis. As per the NSW Control Guideline protocols for investigation, once a giardiasis case was notified to the SWSLHD PHU, staff contacted the diagnosing doctor of the giardiasis case to request permission to contact the case or the parent or guardian (for persons under 16-years-old), to interview the case.

Cases

A 'case' was a person who had laboratory definitive evidence for the detection of *G. duodenalis* cysts or trophozoites in stool samples or samples of duodenal contents. Informed consent was provided by the case or their parent (for persons under 16 years); with parents/guardians asked to complete the responses on behalf of children 12-years-old or younger and to provide consent for children 13–15 years to answer their own questions.

Controls

A 'control' was defined as a person resident in SWSLHD and who did not have a history of a positive *Giardia* test in the previous 3 months (due to the possibility of chronic infection with *Giardia*). In order to improve the response rate and reduce selection bias, three different sets of controls were identified for the study.

(1) Control group 1: Neighbourhood controls (NBH):
Confirmed giardiasis cases were grouped into (i) urban and (ii) regional areas based on Australian Bureau of Standards regional classification. The aim was to identify 10 controls for each case to increase the likelihood of at least one household responding to the questionnaire. The following sampling strategy was employed.
   (i) Urban: A list of all addresses in SWSLHD geocoded to latitude-longitude coordinates was obtained from the Geocoded National Address File. This dataset is available for free from 'Public Sector Mapping Agencies' Australia. A 500 m radius buffer (due to the dense population in urban areas) was drawn around each case's address using Geographic Information System tools (e.g. see Supplementary Fig. S2). Ten houses were then randomly selected from the list of addresses for each buffer.
   (ii) Rural: The procedure followed was the same as for urban areas, except that 5 km buffers were used to account for population sparseness.
   A letter with the Patient Information Statement and control questionnaire were sent to the selected household, with a request that the person with the next birthday in the household complete the questionnaire. The completed questionnaire was to be returned by post in the self-addressed envelope provided.

(2) Control group 2: Pertussis case:
Confirmed Pertussis cases notified in the same year, within the same age range (±5 years), residing within the same LGA but not on the same street as the corresponding giardiasis case were identified. If there were two or more persons meeting the criteria, one would be selected by simple random sampling using a random sampling function in Excel. Where no age match was available for the same LGA, one was selected from the closest LGA. Each control was contacted by telephone and once consent was obtained, the individual was interviewed with the standardised control questionnaire. If the person refused to participate in the study or was uncontactable after three phone calls, then the person was listed as a non-response.

(3) Control group 3: Friend Control:
This recruitment method yielded no controls and was not considered further.

Sample size

Based on surveillance data, it was estimated that the SWSLHD PHU received an average of 147 giardiasis notifications annually between the years 2012 and 2015. In a 1:3 unmatched design with a two-sided confidence level of 95% ($z_{\alpha/2} = 1.96$) with power ($z_{\beta} = 0.80$) of 80% and an estimated prevalence of a risk factor of 17% in controls and 40% in cases, at least 35 cases and 105 controls were needed to detect a significant risk of exposure (odds ratio (OR) >3.25) [11]. Oversampling of cases and controls was performed to accommodate for any non-responses or incompleteness in the data. As such, a total of 50 cases and 150 controls were needed.

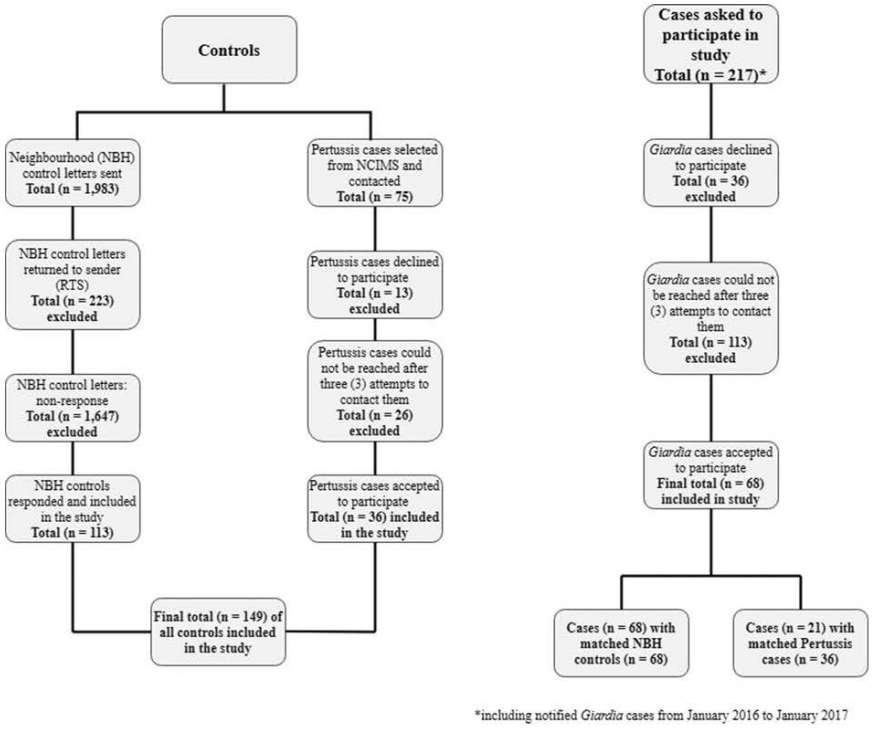

Fig. 1. A flowchart summary of the two different control types (neighbourhood control and Pertussis case) and the number of cases used in the study.

Matched case-control analysis

Survey data were entered into an outbreak questionnaire developed using the Notifiable Conditions Information Management System (NCIMS) and analysed using IBM SPSS Statistics version 23.0 [12]. Pertussis cases were matched to cases by age (±5 years) and location; NBH controls were matched to cases by location (urban or rural). Univariate analysis was carried out to compare cases with each control group separately and an adjusted estimate of the OR and their 95% confidence intervals (CI) were calculated from matched pairs of cases and controls for various risk factors.

For each case-control group, variables with a significant OR in the univariate analysis were placed into a multivariable regression for each matched group respectively. No potential confounders were identified in the regression model with the Pertussis cases. In the regression model with the NBH controls, age and sex were controlled as potential confounders. A backward stepwise elimination process was employed, using a likelihood ratio test to produce the most parsimonious model [13]. All variables with a Wald χ^2 statistically significant at the P-value of <0.05 were considered significant. OR and 95% CI for the association were reported. Cases for whom we could not identify suitable matching control subjects were excluded from the matched case-control analysis.

Results

Of the 217 giardiasis cases invited to participate in the study, 68 (31.3%) consented to be interviewed for the study (see Fig. 1). Letters were mailed to 1983 randomly selected households residing in the same neighbourhood as cases (Fig. 1). Of these, 113 controls (5.7%) returned a completed questionnaire and were included in the study. A total of 75 Pertussis cases were selected from NCIMS and contacted via telephone. Of these, 36 (48.0%) agreed to be interviewed for the study and, 26 (34.7%) could not be contacted after three telephone call attempts. To reduce the risk of selection bias, two separate matched analyses were done: one which combined 21 cases and 36 Pertussis cases and the other matched 68 cases and 68 NBH controls.

Demographic characteristics

The distribution of the cases and controls by age and gender is presented in Table 1. Cases and controls were similar with regard to language spoken at home, highest level of education and indigenous status. Cases and controls mainly originated from urban areas in SWS as opposed to rural. More than half of case-patients (40 or 58.8%), compared with 27 (40.3%) NBH controls and 15

Table 1. Demographics of cases and controls

Demographics	Cases % (*N* = 68)	Neighbourhood controls % (*N* = 113)	*P*-value	Cases % (*N* = 21)	Pertussis cases (*N* = 36)	*P*-value
Median age in years (range)	8.0 (Range 0–83; Mean 18.3 (13.6–23.0) ± 19.36)	57.5 (Range 0–84; Mean 51.0 (46.0–56.1) ± 20.8)	NR[a]	7.0 (Range 1–70; Mean 19.4 (10.0–28.9) ± 20.8)	8.0 (Range 1–65; Mean 16.3 (10.2–22.3) ± 17.9)	NR
0–4 years	36.8% (25)	2.9% (2)	0.001*	28.6% (6)	41.7% (15)	0.242
5 years or older	63.2% (43)	97.1% (66)		71.4% (15)	58.3% (21)	
Gender						
Male	58.8% (40)	39.7% (27)	0.027*	47.6% (10)	41.7% (15)	0.662
Female	41.2% (28)	60.3% (41)		52.4% (11)	58.3% (21)	
Residence						
Urban	86.8% (59)	86.8% (59)	1.000	76.2% (16)	75.0% (27)	0.920
Rural	13.2% (9)	13.2% (9)		23.8% (5)	25.0% (9)	
Language spoken at home						
English	86.6% (58)	100.0% (10)	NR	76.2% (16)	100.0% (4)	NR
Arabic	3.0% (2)	0.0% (0)		0.0% (0)	0.0% (0)	
Hindi	3.0% (2)	0.0% (0)		9.5% (2)	0.0% (0)	
Other languages[b]	7.4% (5)	0.0% (0)		14.3% (3)	0.0% (0)	
Aboriginality						
Aboriginal but not Torres Strait Islander	0.0% (0)	1.5% (1)	NR	0.0% (0)	2.8% (1)	NR
Not Aboriginal and Torres Strait Islander	98.5% (67)	83.8% (57)		95.2% (20)	97.2% (35)	
Both Aboriginal and Torres Strait Islander	1.5% (1)	0.0% (0)		4.8% (1)	0.0% (0)	
Highest Level of Education[c]						
No formal education	4.4% (3)	2.9% (2)	NR	4.8% (1)	0.0% (0)	NR
Primary or elementary school (Year K-6 or equivalent)	1.5% (1)	4.4% (3)		4.8% (1)	2.8% (1)	
Secondary school (Year 7–12 or equivalent)	22.1% (15)	22.1% (15)		9.5% (2)	41.7% (15)	
Vocational (e.g. TAFE or skills training)	27.9% (19)	33.8% (23)		28.6% (6)	25.0% (9)	
University	36.8% (25)	35.3% (24)		42.9% (9)	30.6% (11)	
Other form of education	7.3% (5)	1.5% (1)		9.5% (2)	0.0% (0)	

[a]NR not reported and/or calculated.
[b]Other languages spoken by one person each: Bengali, Cantonese, Macedonian, Mandarin and Spanish.
[c]If the case was under 12 years of age, the educational level was provided by the parent/head of household answering the survey.
*Statistically significant ($P < 0.05$).

(41.7%) Pertussis cases were males. The age distributions varied between cases and controls with the median age being 8 (±19.4) years for giardiasis cases, 58 (±20.8) years for NBH controls and for Pertussis cases, 8 (±17.9) years (see Table 1).

In comparison with the cases, there were significantly fewer NBH controls aged 0–4 years (36.8% *vs* 2.9%). Conversely, significantly more Pertussis cases were aged 0–4 years (28.6% *vs* 41.7%). There were also significantly more older females as NBH controls in comparison with the Pertussis cases which had significantly more children aged <5 years.

Univariate analyses revealed that males were significantly more likely to be cases when compared with NBH controls, hence sex was controlled as a potential confounder in the multivariable analysis. When controlling for sex in the multivariable analysis, cases aged under 5 years had a seven times greater risk of *Giardia* infection (aOR = 7.08; 95% CI 1.02–49.36) when compared with NBH controls. There was no difference between the ages and genders of giardiasis cases and Pertussis cases.

Risk factors for giardiasis

Univariate analysis of the comparison between NBH controls and cases revealed that cases who, (a) were males aged under 5 years, (b) visited their/parent's country of birth, (c) had a child that attends childcare, (d) had a household member diagnosed with a gastrointestinal illness, (e) were individuals who swim in pools, (f) had contact with domestic animals, wildlife or livestock and (g) were individuals who visited a farm, zoo or wildlife park, were at increased risk for giardiasis ($P < 0.05$) (Table 2). Those who temporarily stored their water in jars, bottles or cisterns at home and for those who consumed green salad or lettuce on a daily basis were at a decreased risk ($P < 0.05$) (Table 2). When age and location were controlled in the multivariable analysis, all variables lost their significance except for having a member of household diagnosed with a gastrointestinal illness and having contact with the farm, domestic or wild animals. Those who reported swimming in pools had an elevated risk, but this was not significant ($P = 0.06$) (Table 2).

The univariate analysis matching cases with the second group of controls (i.e. Pertussis cases) found that giardiasis cases were more likely to have travelled overseas and had a household member diagnosed with a gastrointestinal illness. Notably, there was a negative association found between giardiasis cases and living in close proximity to wildlife. All three variables except travelling overseas and outside Australia lost their significance in the multivariable analysis (Table 2).

Discussion

This matched case-control study represents the value of continuing to monitor giardiasis in south-western Sydney and other parts of NSW and recommends further studies to examine the genotypes in circulation and their potential for zoonotic transmission. The results from this study indicate that some common risk factors of *Giardia* infection seen in other developed countries were not found to be significant risk factors in south-western Sydney.

Notably, the multivariable analyses among cases and NBH controls and cases and Pertussis cases found no significant association between giardiasis and those using water sourced from alternative supplies such as roof-harvested rainwater (RHRW), tank water or bore wells. An overall low number of individuals reporting drinking non-municipal water long-term may lead to this lack of association [14]. However, the result is in keeping with other Australian studies that could not identify untreated RHRW tanks as sources of infection for giardiasis, which is likely due to the fact that RHRW tanks are likely to be mainly used for potable replacement for flushing toilets, washing clothes, or watering gardens [14, 15].

Furthermore, while initial univariate analyses between cases and NBH controls found a significant association between giardiasis and those who reported swimming in pools (chlorinated, salt-water or non-chlorinated) 3 months prior to illness onset, this significance was lost in the multivariable model that controlled for age and sex. This suggests there may be a relationship between age, sex and swimming that is confounding their association with giardiasis infection in this setting. On the other hand, there are multiple studies that have established the association between swimming in pools and giardiasis infection [16–18].

Giardiasis cases were also more likely to have a household member diagnosed with a gastrointestinal illness when compared with NBH controls. A similar risk found in the univariate analysis with Pertussis cases, may be due to a low response rate. Notwithstanding, studies in Turkey and other countries have reported an increased risk of infection amongst household members infected with giardiasis [19, 20]. This indicates a potential for person-to-person transmission of infection occurring within households in SWS with infected family members (or household members) serving as sources of infection. There is also the prospect of transmission through food or water prepared by the infected individual. This study emphasises the importance of screening all household members for giardiasis once a case has been diagnosed.

In this study, the multivariable analysis found a seven times greater risk of infection for those aged under 5 years. However, when compared with Pertussis cases, the risk was insignificant. While other case-control studies have observed no significant risk associated with age, it is more likely that this result is due to the small participant numbers in the Pertussis cases group. Individuals of all age groups can be infected by *G. duodenalis* although the majority of literature maintains that giardiasis is most prevalent in school-age and younger children [21, 22]. Children tend to have a higher exposure to contaminated faeces particularly in close-contact facilities such as childcare centres putting them at greater risk of infection [16, 23, 24].

While univariate analyses among cases and NBH controls observed that males were at an increased risk of giardiasis, this association lost its significance in the multivariable analysis after being controlled for sex and age and was likely due to the fact that there were overwhelmingly more females among NBH controls [25, 26].

Cases coming in contact with domestic animals, farm animals and even wildlife were at increased risk of infection when compared with NBH controls, but not when compared with Pertussis cases. The lack of significance, when compared with the Pertussis cases, may be due to a lack of difference in exposure between the two groups, hence diluting the risk. The possible role of animals as a source of *G. duodenalis* infection to humans is still unclear, although most studies agree that animals can play an indirect role in transmission [6, 27]. Molecular investigations on *G. duodenalis* and the potential for zoonotic transmission observed that humans can only be infected with human-specific assemblages (A or B) and not from animal-adapted genotypes (C–H) [28]. A possible explanation for the present results is that animals are carriers of assemblages A or B and act as vehicles

Table 2. Univariate and multivariable analysis of risk factors for *G. duodenalis* infection

Risk Factors	Cases % (*N* = 68)	Neighbourhood Controls % (*N* = 68)	Unadjusted OR[a] (95% CI)	Adjusted OR[b] (95% CI)	Cases % (*N* = 21)	Pertussis cases % (*N* = 35)	Unadjusted OR (95% CI)	Adjusted OR[c] (95% CI)
Gender								
Male	58.8% (40)	40.3% (27)	2.17 (1.09–4.30)*	1.31 (0.47–3.67)	47.6% (10)	41.7% (15)	1.27 (0.43–3.76)	NR[d]
Female	41.2% (28)	60.3% (41)			52.4% (11)	58.3% (21)		
Age Category								
0–4 years	36.8% (25)	2.9% (2)	19.19 (4.32–85.18)*	7.06 (1.02–49.36)*	28.6% (6)	41.7% (15)	0.56 (0.18–1.78)	NR
5 years or older	63.2% (43)	97.1% (66)			71.4% (15)	58.3% (21)		
Travel within Australia								
Yes	8.8% (6)	10.8% (7)	0.80 (0.25–2.53)	NR	9.5% (2)	8.3% (3)	1.16 (0.18–7.56)	NR
Travel overseas								
Yes	19.1% (13)	11.9% (8)	1.74 (0.67–4.53)	NR	23.8% (5)	2.8% (1)	10.94 (1.18–101.41)*	19.89 (2.00–197.37)*
Visit country of birth or parent's country of birth								
Yes	76.9% (10)	18.8% (3)	14.44 (2.39–87.40)*	NR	80.0% (4)	0.0% (0)	NR	NR
Countries visited overseas								
South & Southeast Asia	38.5% (5)	37.5% (3)	NR	NR	20.0% (1)	100.0% (1)	NR	NR
West Central Asia/North Africa	7.7% (1)	12.5% (1)			20.0% (1)	0.0% (0)		
Oceania	30.8% (4)	25.0% (2)			40.0% (2)	0.0% (0)		
Europe	0.0% (0)	12.5% (1)			0.0% (0)	0.0% (0)		
Latin America & Caribbean	7.7% (1)	12.5% (1)			0.0% (0)	0.0% (0)		
Multiple Regions	15.4% (2)	0.0% (0)			20.0% (1)	0.0% (0)		
Camp or caravan								
Yes	9.0% (6)	10.4% (7)	0.84 (0.27–2.66)	NR	9.5% (2)	0.0% (0)	NR	NR
Children at home attending childcare								
Yes	50.0% (34)	7.7% (5)	12.00 (4.29–33.57)*	2.46 (0.63–9.70)	42.9% (9)	30.6% (11)	1.71 (0.56–5.21)	NR
Member of household diagnosed with a gastrointestinal illness								
Yes	24.2% (16)	1.5% (1)	21.44 (2.75–167.08)*	13.89 (1.53–164.60)*	21.1% (4)	5.6% (2)	4.53 (0.73–27.50)*	NR
Unfiltered or non-boiled tap water								
Yes	65.2% (43)	73.5% (50)	NR	NR	65.0% (13)	58.3% (21)	1.33 (0.43–4.12)	NR
Filtered tap water								
Yes	45.5% (30)	33.8% (22)	NR	NR	40.0% (8)	38.9% (14)	1.05 (0.34–3.20)	NR
Sydney water connected to home								
Yes	91.7% (55)	86.8% (59)	1.68 (0.53–5.32)	NR	82.4% (14)	91.2% (31)	0.45 (0.08–2.52)	NR
Roof-harvested rain water to home								

Yes	5.9% (4)	13.2% (9)	0.41 (0.12–1.40)	NR	9.5% (2)	2.8% (1)	3.68 (0.31–43.32)	NR
Bore water or shallow well water used in home								
Yes	0.0% (0)	1.5% (1)	NR	NR	0.0% (0)	0.0% (0)	NR	NR
Tank water used in home								
Yes	22.1% (15)	10.3% (7)	2.47 (0.94–6.50)	NR	19.0% (4)	27.8% (10)	0.61 (0.17–2.27)	NR
Temporary storage of water in jars, bottles, cisterns at home								
Yes	1.6% (1)	32.4% (22)	0.03 (0.00–0.26)*	NR	0.0% (0)	2.8% (1)	NR	NR
Swimming in pool								
Yes	57.6% (38)	28.4% (19)	3.43 (1.67–7.06)*	2.63 (0.95–7.27)	52.4% (11)	52.8% (19)	0.98 (0.34–2.89)	NR
Swimming in river, lake, lagoon, pond or similar setting								
Yes	13.2% (9)	4.4% (3)	3.31 (0.85–12.79)	NR	9.5% (2)	13.9% (5)	0.65 (0.12–3.71)	NR
Swimming in the ocean								
Yes	10.3% (7)	16.2% (11)	0.60 (0.22–1.64)	NR	14.3% (3)	16.7% (6)	0.83 (0.19–3.75)	NR
Always wash hands before eating								
Yes	60.3% (41)	67.2% (45)	0.74 (0.37–1.50)	NR	61.9% (13)	69.4% (25)	0.72 (0.23–2.22)	NR
Always wash hands after playing with animals								
Yes	80.3% (49)	76.5% (52)	1.26 (0.54–2.92)	NR	76.5% (13)	74.3% (26)	1.13 (0.29–4.38)	NR
Changing nappies of child/children								
Yes	13.8% (9)	20.9% (14)	0.61 (0.24–1.52)	NR	14.3% (3)	8.3% (3)	1.83 (0.34–10.04)	NR
Engaging in sexual activity with contact with faeces								
Yes	1.8% (1)	1.5% (1)	1.20 (0.07–19.37)	NR	0.0% (0)	0.0% (0)	NR	NR
Onsite septic system at home								
Yes	12.1% (8)	14.9% (10)	0.79 (0.29–2.14)	NR	15.8% (3)	22.9% (8)	0.63 (0.15–2.74)	NR
Contact with farm/domestic animal/wildlife								
Yes	61.8% (42)	32.4% (22)	3.38 (1.67–6.84)*	3.03 (1.08–8.54)*	71.4% (15)	72.2% (26)	0.96 (0.29–3.18)	NR
Visited a farm, zoo, wildlife park								
Yes	28.4% (19)	9.1% (6)	3.96 (1.47–10.69)*	NR	19.0% (4)	38.9% (14)	0.37 (0.10–1.33)	NR
Wildlife in close proximity to house								
Yes	26.5% (18)	17.9% (12)	1.65 (0.72–3.76)	NR	14.3% (3)	41.7% (15)	0.23 (0.06–0.94)*	0.54 (0.11–2.65)
Consumes green salad/lettuce daily								
Yes	17.9% (12)	38.8% (26)	0.34 (0.16–0.76)*	0.48 (0.15–1.52)	14.3% (3)	22.2% (8)	0.58 (0.14–2.49)	NR

*Statistically significant ($P < 0.05$).
[a]Unadjusted odds ratio.
[b]Odds ratio from multivariable model adjusted for sex and age and all exposures that have been previously reported to be associated with giardiasis and showed a significant association ($P < 0.05$) in the univariate model.
[c]Odds ratio from multivariable model adjusted for sex and all exposures that have been previously reported to be associated with giardiasis and showed a significant association ($P < 0.05$) in the univariate model.
[d]NR not reported and/or calculated.

for mechanical transmission to humans who come in contact with animal's faeces at parks or wildlife settings where hand-washing facilities may not be available [29], or other environmental exposures to cysts attached to the fur of domestic animals [30].

Interestingly, the vast majority (80.9%) of *G. duodenalis* cases did not report travelling overseas within the 3 months prior to illness onset suggesting that most of the giardiasis cases were locally acquired. This is the first case-control study to examine travel history amongst giardiasis cases in this setting and is consistent with other case-control studies conducted in other developed countries [16, 23, 31]. However, multivariable analyses found that when compared with Pertussis cases, giardiasis cases were 20 times more likely to have been travelling overseas. The most popular countries visited were in South & South-East Asia, West Central Asia/North Africa and Oceania. Overseas travel to endemic regions is widely believed to be the principal risk factor for giardiasis in developed countries. However, due to detection bias associated with physicians testing for giardiasis more commonly among returning travellers, overseas acquired infection rate is likely to be overestimated; and consequently underestimating locally acquired giardiasis [32].

There are some limitations to this study. Although care was taken to recruit controls representative of the source population of cases, some selection bias may exist among controls. There was a larger response rate among older females residents in urban areas in SWS, indicating that women were more likely to respond to the NBH control questionnaire. There was also an underrepresentation of children seen in the NBH controls when compared with Pertussis cases. This selection bias emphasised the sex and age differences between cases and NBH controls and could explain why some exposures were also present among the control group, thus diluting the exposure rates amongst cases. A matched analysis was done to reduce selection bias and improve internal validity, by controlling for the sex, age and region of residence differences between cases and NBH controls. The matched design reduced the risk of error from the confounding effect of age, sex and location but due to the resulting close matching on these variables, their effects on giardiasis risk could not be assessed. However, controlling for these well-known confounders was valuable as it allowed the assessment of other risk factors without their confounding influences. Admission risk bias is a potential problem with Pertussis cases, which were selected based on being a group of patients available through NCIMS, did not have gastrointestinal symptoms or diagnosed with giardiasis and hence they may have a different exposure profile to the general population. Since giardiasis cases matched to pertussis cases were quite similar in sex distribution, there was no association and hence no further need for controlling this variable. Like most studies that utilises surveillance data as a sampling frame, only symptomatic *G. duodenalis* cases that sought medical attention and had a positive laboratory test were included in the study. This means that this study represents only a proportion of the overall burden of the disease in the community. Cases with undiagnosed and asymptomatic giardiasis would not have been considered. Therefore, this study cannot be generalised to all of Australia and must be interpreted in the context of these limitations.

Conclusion

The study showed an increased risk of giardiasis in children aged under 5 years, amongst individuals who have a household member diagnosed with a gastrointestinal illness and have contact with domestic animals, wildlife or livestock. The study also found that cases who travelled overseas were at a greater risk of infection. There is a need to educate residents living in urban areas in SWS on the potential of person-to-person transmission of giardiasis; particularly if a household member is ill with gastroenteritis. Targeted intervention and health messages are needed for the parents/carers of younger children especially during high-risk seasons such as warmer months, with emphasis on potential risks and appropriate hygiene practices when visiting farms and wildlife parks or where contact with animals is to be expected. Likewise, people travelling overseas to endemic countries should be appropriately informed of the risks and possible control strategies that can be implemented. This study illustrates the value of continuing to monitor giardiasis in south-western Sydney and other parts of NSW and recommends further studies to examine the genotypes in circulation and their potential for zoonotic transmission.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0950268818002637.

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Conflict of interest. None.

Ethical standards. Ethical approval for this study was received from the South-Western Sydney Local Health District Human Research Ethics Committee which is accredited by the NSW Ministry of Health (HREC approval number: HE16/079 LNR).

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Chapter 3

Genotyping and epidemiological distribution of *Giardia intestinalis* assemblages in NSW, Australia

Certificate:

I certify that the following chapter is largely my own work, although the contributions of other authors are duly recognised. The contribution of other authors are detailed as follows:

- Suggestions on study design
- By providing suggestions on topics to be reviewed
- By proof reading draft manuscripts
- By providing suggestions to improve layout, structure and writing style
- By correcting spelling and grammatical errors in drafts

Otherwise, the main composition of this work is credited to me.

I hereby certify that the above statements are true and correct:

Patricia Zajaczkowski, PhD Candidate	Production Note: Signature removed prior to publication.	17/11/2022
Dr. Rogan Lee, Co-Author	Production Note: Signature removed prior to publication.	28/11/22
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Genotyping and epidemiological distribution of *Giardia intestinalis* in NSW, Australia

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3.1. Abstract

Giardiasis is the most common enteric protozoan infection notifiable in New South Wales (NSW), Australia and surveillance by NSW Health had shown a steady increase (prior to the COVID-19 pandemic) in the number of cases reported since 2012. The reasons for this currently remain unknown, and the epidemiological significance of the various *Giardia* assemblages is still unclear in Sydney and more broadly NSW. Contradictory results have been published worldwide regarding the geographical distribution and clinical manifestation of the assemblages. This study aimed to evaluate the predominance of *Giardia intestinalis* assemblages causing human infection in NSW. Individual faecal specimens (*n* = 169) were collected from participating hospitals and private laboratories, and the presence of *Giardia* cysts and co-infections were confirmed by real-time multiplex-PCR. Samples were genotyped by sequence analysis of the triosephosphate isomerase (*tpi*) gene and the small subunit rRNA DNA (SSU-rRNA). Genotyping results showed that most samples belong to only assemblage B (46.9%, *n* = 76), and a small percentage of cases were infected with only assemblage A (9.3%, *n* = 15). Surprisingly, mixtures of assemblages A and B in individuals were relatively common (43.8%, *n* = 71). Co-infections were observed in 49.1% (*n* = 83) of all *G. intestinalis*-positive faecal samples with the most common co-infection being *Blastocystis hominis* (31.3%, *n* = 25), followed by *Dientamoeba fragilis* (15.0%, *n* = 12). Although giardiasis was more prevalent in males (55.2%, *n* = 85), the assemblage distribution between the sexes appeared uniform. The age distribution was bimodal, with peaks in 0–15year-olds and in adults in their 30s. The overall largest number of cases were collected from patients aged 30-49 years (33.8%, *n* = 49). Interestingly, females aged 5 years-old and under had a greater risk of assemblage B infection than their male counterparts (OR = 2.61; 95% CI 1.12-6.07; *p* = 0.001), and females aged greater than 5 years-old were significantly more likely to have a mixed assemblage A+B infection (OR = 2.16; 95% CI 0.90-5.19; *p* = 0.012). No significant correlation was demonstrated between a given assemblage and the occurrence of clinical symptoms. This study provides new insights into the molecular diversity of giardiasis in NSW, Australia, and can help to inform enhanced surveillance and prevention strategies in developed metropolitan areas.

3.2. Introduction

Giardia intestinalis continues to be the most encountered parasitic disease around the world. Although more frequently detected in developing countries with limited access to clean water, sanitation, and hygiene facilities, ongoing disease surveillance in developed nations has observed a sharp increase in the number of *Giardia* sp. associated-waterborne outbreaks of gastrointestinal illness [1,2]. Sporadic cases are also on the rise in countries like Australia. In New South Wales (NSW), Australia, giardiasis remains the most common notifiable parasitic infection in NSW with an average (before the COVID-19 pandemic) of around 3000 cases notified by laboratories each year. It may be that these case numbers are reflective of better diagnostic methodologies used in pathology laboratories and the implementation of sensitive assays such as multiplex-PCR. Although it should be noted that culture-independent DNA-based testing methods were only implemented in Australian diagnostic laboratories in late 2013 and onwards, the complete impact of these testing methods on *Giardia* case notifications remains unquantified. The increase of children attending child-care centres across NSW may also play a part in larger case numbers. By state, NSW has the largest share of children attending child-care centres, and use of these centres has increased by 27.8% just within the past ten years [3]. However, only a few Australian studies have examined the risk factors that drive local transmission of giardiasis [4–6].

Annual notifications of *G. intestinalis* peak between January and April each year and are highest among children aged 0 to 4 years, and adults aged 30 to 39 years [7]. Incidence rates (per 100,000 population) also vary across communities, ranging from 11.2 to 63.2; and high rates (~39.0) are reported from both rural and urban health districts [8]. In NSW, it remains unclear if associations exist between high-risk age groups. A recent epidemiological study found that among hospitalised patients in NSW, giardiasis was the second most identified enteric protozoa (after *Blastocystis* spp.) affecting mainly children at school age and younger [9]. Additionally, a more recent study based in south-western Sydney (SWS) confirmed that children aged five-years of age and under were seven times at greater risk of contracting giardiasis [6]. Despite this, it remains unclear whether there are other factors at play, or even whether fluctuations across NSW communities are reflecting different disease dynamics. Recent molecular studies have determined that *G. intestinalis* can be split into eight morphologically identical genetic assemblages (assemblages A to H) which can only be distinguished by molecular typing methods [10,11]. Both assemblages A and B are potentially zoonotic; they are responsible for most human infections however they have also been successfully isolated from other mammals. The remaining assemblages (C to H) are host-specific although in some rare cases, have been detected in humans. Previous work has shown conflicting results

regarding the relationship between *G. intestinalis* assemblages A and B and their clinical presentation. Some studies have suggested that assemblage A is associated with more severe clinical symptoms in Peru, Bangladesh, and Spain [12–14], however the opposite has also been suggested by others [15]. Currently studies from Australia have yet to find a clear correlation between assemblages and symptoms in humans despite several genotyping studies being reported [16–20]. Two studies, however, did investigate a link between clinical symptoms and assemblage type [21,22]. Both studies were based in Western Australia. Read *et al*. (2002) [22] observed a strong association between assemblage A infection and diarrhoea, while Yang *et al*. (2010) did not find similar correlations [21]. It is difficult to ascertain whether these conflicting results were the result of differences in study methodology, however it is an issue that needs further investigation. Clinical symptoms of *G. intestinalis* infection can differ according to each individual, and some cases can even remain asymptomatic. Symptoms can include acute and/or chronic diarrhoea, stomach cramps, nausea, vomiting, dehydration, and weight loss [23]. Although it is still unclear why certain cases remain asymptomatic, it has been suggested that host-parasite factors and the genotypic differences within a parasite can influence the subsequent clinical presentation of an infected individual [24,25].

The aim of this study was to identify *G. intestinalis* assemblages contributing to human infections in NSW, Australia, and to detect any significant associations between assemblages and the demographic, clinical and geographical factors. This study provides information on the impact of giardiasis on human health in NSW, and a better understanding of the continuing rise in cases. This will increase the capacity of NSW to apply advanced analyses to disease surveillance and will inform the application of similar methodologies to other intestinal protozoan diseases in NSW.

3.3. Materials and methods

3.3.1. Faecal specimen collection

Faecal specimens were collected between June 2016 and December 2019 from individuals who had tested positive for *Giardia* species. Samples were collected from two hospitals, the Centre for Infectious Diseases and Microbiology (CIDM) at Westmead Hospital, NSW and SydPath at St. Vincent's Hospital, NSW. To mitigate potential geographical bias, samples were also collected from two private pathology laboratories, namely Laverty Pathology and Douglass Hanly Moir Pathology (DHM), both situated in NSW, Australia. These private laboratories cover a broader geographical scope within NSW when compared to the hospital laboratories.

Diagnosis of *Giardia* in the hospital pathology laboratories involved a combination of multiplex-PCR detection and immunoassays [26–28]. In both private pathology clinics, *Giardia* diagnosis was made by visualising *Giardia* cysts and/ or trophozoites in faecal smears of prepared concentrates using microscopy. Stool samples from DHM were initially prepared using the Mini-Parasep® solvent-free (SF) (Apacor, England, UK) faecal parasite concentrator with a formalin and Triton X/ethyl acetate solution. Both private laboratories also utilised commercial antigen tests such as the Remel ProSpecT™ *Giardia*/*Cryptosporidium* microplate immunoassay (Thermo Fisher Scientific) to detect positive antigens.

For each positive stool sample collected, the corresponding patient's gender, age and post-code region of residence was obtained from the electronic Medical Records (eMR). To protect the sensitive personal information of the patients, no identifiers were collected from the eMR, and ages of the patients were replaced by age groups to further reduce the possibility of re-identification. Ethics approval for the conduct of this study was received from the South-Western Sydney Local Health District Human Research Ethics Committee (HREC) for each of the two hospitals and two private pathology laboratories and ratified by HREC of the University of Technology Sydney (UTS).

All patient ages were categorised into one of the six age groups: ≤5, 6-15, 16-29, 30-49, 50-69 and 70+ years. The history of the patient's symptoms and potential risk factors were collected from the clinical notes recorded on laboratory requests or medical records. All faecal samples were transported to UTS, provided a unique identification number, and stored unpreserved at 4°C before DNA extraction. Note that for individuals with multiple faecal samples collected at the same time, only one sample was included in the analyses.

3.3.2. Multiplex RT-PCR

To confirm the presence of *G. intestinalis* and to detect any co-infections within the collected samples, the specimens were analysed by a multiplexed real-time PCR (RT-PCR) EasyScreen™ assay (Genetic Signatures, Newtown, Australia) at Sydpath at St. Vincent's Hospital, Sydney, Australia [26]. The EasyScreen™ kit tests for a variety of enteric pathogens including common enteric protozoan parasites: (i) *Dientamoeba fragilis*, (ii) *Cryptosporidium* spp., (iii) *Blastocystis hominis*, (iv) *Entamoeba* complex, (v) *Giardia intestinalis*; bacterial pathogens: (i) *Salmonella* spp., (ii) *Campylobacter* spp., (iii) *Shigella* spp., (iv) *Yersinia enterocolitica*, (v) toxigenic *Clostridium difficile* and (vi) *Listeria monocytogenes*; and viruses: (i) Norovirus group I, (ii) Norovirus group II, (iii) Adenovirus hexon, (iv) Adenovirus 40/41, (v) Rotavirus A and B, (vi) Astrovirus (group 1-7) and (vii) Sapovirus.

3.3.3. Genomic DNA extraction

DNA was extracted directly from 150mg of the faecal sample using an ISOLATE II Fecal DNA Kit (Bioline, Sydney, Australia) following the manufacturer's instructions with only minor modifications: the DNA was washed three times with Fecal DNA Wash Buffer (rather than once as per the manufacturer's instructions). Elution was accomplished by adding 100µl elution buffer. The eluted DNA was stored at -20°C until PCR amplification. Samples with sterile water were used as a negative control to monitor contamination during nucleic acid extraction. Samples spiked with *Cryptosporidium* spp. DNA templates were used as a positive control during the extraction process. DNA concentration and purity was determined via 260/280 and 260/230 ratios measured on the NanoDrop™ One microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, United States).

3.3.4. Nested PCR amplification of the G. intestinalis SSU-rRNA

Primers originally designed by Hopkins *et al*. (1997) amplify the SSU-rRNA gene of *G. intestinalis* producing a 292 bp product from the primary PCR reaction, and a 130 bp product from the secondary PCR reaction [29]. Due to the small product size and innate low genetic variation within the SSU-rRNA gene, distinguishing between assemblage A and B sequences is entirely dependent on identifying four single nucleotide polymorphisms (SNPs). To increase discriminatory power, new primers were designed to capture an area with a greater distribution of polymorphisms between assemblages A and B. The Clustal W multiple sequence alignment program was used to align SSU-rDNA sequence reads of assemblages A and B, and a total of seven SNPs were identified between the two assemblages [30].

A 447 bp fragment of the SSU-rRNA gene was first amplified using the previously described forward primer RH11 [29] and the newly designed reverse primer RH4.1: TGGCACCAGACCTTGCCCT. This reaction was followed by a secondary amplification step, which used the internal primer GiarF [22] and the newly designed reverse primer GiarR.1: ACTCCCCGTCGCTGCCT. Both PCR amplifications were prepared in a final volume of 50µl and carried out using conditions previously described [29]. Negative controls (no template added) and positive controls (containing DNA from previously sequenced and confirmed *G. intestinalis* samples) were included in each assay reaction. Reactions were performed on an Eppendorf Mastercycler® Nexus (Sigma-Aldrich).

Specificity of the novel primers was tested using a panel of three protozoan parasite-positive and two bacteria-positive clinical samples previously submitted to St Vincent's Hospital (including *Cryptosporidium parvum*, *Dientamoeba fragilis*, *Blastocystis hominis*, *Campylobacter* spp. and *Clostridium* spp.). Sensitivity was estimated using a series of 10-fold dilutions of DNA from extracted *G. intestinalis* DNA samples to assess the lowest detection threshold of each PCR assay. Reaction templates corresponded to decreasing concentrations from 10^{2} to 10^{-3} ng/µL DNA per PCR tube.

To confirm successful amplification, 4 µL of the PCR product was subjected to electrophoresis on a 2.0% agarose gel containing GelRed® Nucleic Acid Gel Stain (Sigma-Aldrich). PCR products of the correct band length (363 bp) were purified by using a PCR purification kit (Qiagen, GmbH. Germany) and sequenced (Macrogen, Seoul, Korea) on both strands using the PCR primers. Sequence data was trimmed and analysed using SeqTrace and for each PCR product, a consensus contig was generated from the sequence data [31]. The final sequences were then compared to sequences (>99% similarity) contained in GenBank using the nucleotide-BLAST tool (nBLAST). The identification of homologous sequences allowed determination of the *G. intestinalis* assemblage.

3.3.5. Assemblage-specific nested PCR amplification of the tpi gene

Two *G. intestinalis* assemblage-specific nested PCR assays were used to amplify the triose phosphate isomerase (TPI) gene. A 605 bp fragment of the gene was first amplified using previously described primers AL3543 and AL3546 [32]. The secondary PCR reaction involved two separate assays using assemblage-specific primers: the assemblage A-specific primers Af and Ar amplifying a 332 bp PCR product [33] and the assemblage B-specific primers Bf and Br amplifying a 400 bp product [34]. Both PCR amplifications were prepared in a final volume of 50µl and carried

out using conditions previously described [34]. Negative controls (no template added) and positive controls (containing DNA from previously sequenced and confirmed *G. intestinalis* samples) were included in each assay reaction. Reactions were performed on an Eppendorf Mastercycler® Nexus (Sigma-Aldrich) and PCR products were separated by electrophoresis in a 2.0% agarose gel containing GelRed® Nucleic Acid Gel Stain (Sigma-Aldrich).

3.3.6. Statistical analyses and mapping of spatial data

Statistical analysis was performed using SPSS® Statistics 27 (IBM, USA). Categorical variables are reported in terms of percentages, with corresponding Confidence Intervals (CI) at 95%. The existence of association between categorical variables was evaluated using Pearson's Chi-Square test (or Fisher's Exact test for sparse data). Statistical significance was set as a p-value <0.05. Positive human cases of giardiasis in NSW were also geographically mapped using ArcGIS. Case postcode data was initially geocoded using ArcGIS, then spatially joined to two polygon layers: NSW Local Government Area boundaries (2019) [33] and Local Health District boundaries (2014) [34]. New South Wales is divided into eight metropolitan Local Health Districts (LHDs) and seven rural/regional LHDs [35]. The LHDs are further split into 128 Local Government Areas (LGAs). Cases were then aggregated according to the postcode-matched LGA and LHD, to calculate the total number of *G. intestinalis* cases and *G. intestinalis* assemblages for each region in NSW.

3.4. Results

3.4.1. Detection and identification of G. intestinalis assemblages

During the study period, a total of 410 *G. intestinalis*-positive faecal samples were collected, although 107 had missing data and were excluded from the study. From the total 303, a little over half of the *G. intestinalis*-positive samples, were chosen to be genotyped in the current study (55.8%; *n* = 169). To prevent selection bias, this subset of samples (*n* = 169) was deliberately selected to accurately reflect the demographic characteristics (i.e. age, sex, and location) of the broader population diagnosed with *G. intestinalis* during the study period. The subset of samples primarily originated from private pathology laboratories (87.0%) as opposed to hospital laboratories (13.0%). The *tpi* assemblage A/B-specific PCR amplified in 147/169 (87.0%) of these samples; 18.4% (*n* = 27) were only assemblage A, 54.4% (*n* = 80) were only assemblage B and 27.2% (*n* = 40) were classified as mixed assemblages. The SSU-rRNA PCR amplified 136/169 (80.0%) of the specimens; of which 18.4% (*n* = 25) were only assemblage A, 73.5% (*n* = 100) were only assemblage B and 8.1% (*n* = 11) were mixed assemblages A+B.

Further genetic characterisation of *G. intestinalis* involved combining the results of both the assemblage-specific PCR (*tpi*) and the nested PCR (SSU-rRNA). Characterising a sample as a single assemblage (either assemblage A or B) would require both PCR assays to have an identical result. A mixed assemblage was defined by either an identical A+B result for both PCR assays or discordant results from each assay. Among the subset of *G. intestinalis*-positive samples that were collected, 162 (95.9%) were successfully amplified at one or more loci; of which 9.3% (*n* = 15) were only assemblage A, 46.9% (*n* = 76) were only assemblage B and 43.8% (*n* = 71) were mixed infections of assemblage A+B.

Co-infecting pathogens were detected in 49.1% (*n* = 83) of all *G. intestinalis*-positive faecal samples. Of these co-infections, 56.6% (*n* = 47) were parasitic, 12.0% (*n* = 10) were bacterial, and 8.4% (*n* = 7) were viral. Joint parasitic/viral coinfections were also identified in 14.5% (*n* = 12), followed by parasitic/bacterial coinfections (4.8%, *n* = 4), bacterial/viral coinfections (2.4%, *n* = 2) and parasitic/bacterial/viral coinfections (1.2%, *n* = 1). Overall, the most common pathogens detected were the enteric protozoa *Blastocystis hominis* (31.3%, *n* = 25) and *Dientamoeba fragilis* (15.0%, *n* = 12), followed by the pathogens *Campylobacter* spp. (5.0%, *n* = 4) and *Enterovirus* (5.0%, *n* = 4). Other coinfections are reported in Supplementary Table S3. 1.

3.4.2. Socio-demographics of G. intestinalis assemblage cases

Age and gender information were obtained for 145 of the *G. intestinalis* positive cases and are presented in Figure 3. 1. Those infected with *G. intestinalis* were aged between 0 years to over 70 years. A bimodal distribution is seen in Figure 3. 1, and the peaks coincided with children aged ≤5 years, and adults in their 30s. Cases aged 30-49 (33.8%, *n* = 49) and 50-69 years (20.7%, *n* = 30) made up the largest age groups, followed closely by those ≤5 years (20.0%, n = 29).

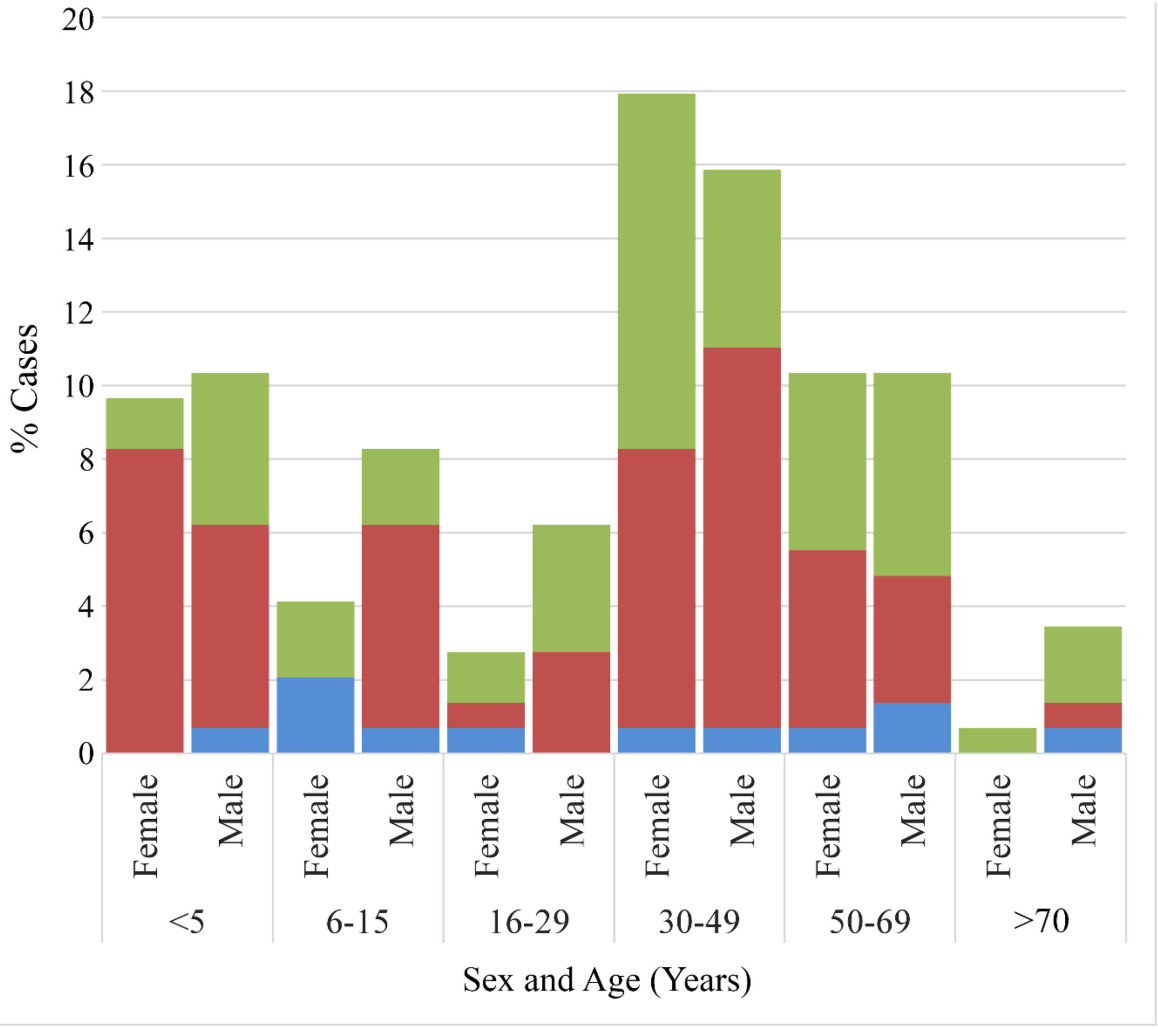

Figure 3. 1. Distribution of *G. intestinalis* assemblages A, B and A+B (*n* = 145) by age and sex (%). Assemblage A, Blue; assemblage B, Red; mixed-assemblage A+B, Green.

Overall, Giardiasis was observed in more males (55.2%, *n* = 85) than females (44.8%, *n* = 69), although there was no significant difference between these two groups (Table 3. 1). Within male and female groups, the assemblage distribution appeared uniform. For males, single infections with assemblages A or B were identified in 54% and 58% cases respectively. Among females, 46% cases were assemblage A only infections, whilst 43% cases were only B. Mixed assemblage A+B cases were identified in 53% males and 47% females. Assemblages were distributed across all age groups; however, single assemblage A infections were mostly seen in children aged 6-15 years, and adults aged 50 years and greater. In comparison, single assemblage B infections were more common among middle-aged individuals aged 30-49 years old, and children aged 5 years and under. When categorising the cases into two age categories (≤5 and >5 years), it was found that children ≤5 years-old were more commonly infected by assemblage B only (OR = 2.74; 95% CI 1.15-6.51; $p = 0.020$) than assemblage A only (Table 3. 1). Additionally, females aged ≤5 years-old had a greater risk of assemblage B-only infection than their male counterparts (OR = 2.61; 95% CI 1.12-6.07; $p = 0.001$).

Table 3. 1. Distribution of *G. intestinalis* assemblages based on age and sex.

Demographics	Assemblage A				Assemblage B				Assemblage A+B				Total, % (*n*)
	% (*n*)	*p*-value	OR	95% CI	% (*n*)	*p*-value	OR	95% CI	% (*n*)	*p*-value	OR	95% CI	
Sex													
Male	53.8% (7)	0.919	0.942	0.30-2.95	57.5% (42)	0.579	1.197	0.63-2.26	52.9% (36)	0.617	0.85	0.45-1.61	55.2% (85)
Female	46.2% (6)	-	-	-	42.5% (31)	-	-	-	47.1% (32)	-	-	-	44.8% (69)
Age (years)													
≤5	8.3% (1)	0.275	-	-	27.8% (20)	0.104	-	-	13.1% (8)	0.299	-	-	20.0% (29)
6-15	33.3% (4)	-	-	-	11.1% (8)	-	-	-	9.8% (6)	-	-	-	12.4% (18)
16-29	8.3% (1)	-	-	-	6.9% (5)	-	-	-	11.5% (7)	-	-	-	9.0% (13)
30-49	17.0% (2)	-	-	-	36.1% (26)	-	-	-	34.4% (21)	-	-	-	33.8% (49)

Table 3. 1. Continued.

Demographics	Assemblage A				Assemblage B				Assemblage A+B				Total, % (*n*)
	% (*n*)	*p*-value	OR	95% CI	% (*n*)	*p*-value	OR	95% CI	% (*n*)	*p*-value	OR	95% CI	
Age (years)													
50-69	25.0% (3)	-	-	-	16.7% (12)	-	-	-	24.6% (15)	-	-	-	20.7% (30)
70+	8.3% (1)	-	-	-	1.4% (1)	-	-	-	6.6% (4)	-	-	-	4.1% (6)
Age (years – 2 categories)													
≤5	8.3% (1)	0.263	0.341	0.04-2.75	27.8% (20)	0.020*	2.74	1.15-6.51	13.1% (8)	0.077	0.45	0.19-1.11	20.0% (29)
>5	91.7% (11)	-	-	-	72.2% (52)	-	-	-	86.9% (53)	-	-	-	80.0% (116)

**p*-value <0.05 is significant

3.4.3. Clinical and travel history of G. intestinalis cases

Common clinical symptoms identified from the *G. intestinalis* cases included diarrhoea (79.0%, *n* = 49), abdominal pain (21.0%, *n* = 13) and bloating (19.4%, *n* = 12) (Table 3. 2). Vomiting and/or nausea, weight loss and fatigue were also reported, albeit rarely (8.1%, 1.6% and 1.6% respectively). A few individuals positive for *G. intestinalis* also reported being asymptomatic (14.5% (*n* = 9)) at the time of sampling. These included cases who had recently travelled overseas and/or arrived in the country as a refugee (*n* = 3), as well as family members, friends, and household members of confirmed giardiasis cases (*n* = 5). The remaining asymptomatic case (1.6%) had reported having a lowered immunity. Sixty-two cases whose isolates were successfully genotyped (and who did not have coinfections with other entero-pathogens) had clinical data available (Table 3. 2). More cases from whom assemblage B and mixed assemblages A+B were identified reported symptoms (*n* = 32 and *n* = 27, respectively), however comparisons could not be made with assemblage A as clinical data was only available for three individuals. Of the nine asymptomatic cases, five had mixed assemblages A+B, three had assemblage B, and one had assemblage A. Overall, no significant association was found between symptoms and infection with specific *G. intestinalis* assemblages.

Regarding the travel history of cases, 7.8% (*n* = 10/129) of cases reported travelling overseas prior to illness onset. Out of the 10 cases, none were infected with assemblage A only, 20.0% had assemblage B and the remaining 80.0% were found to have mixed A+B infection. Additionally, those who had travelled overseas were six times more likely to be infected with mixed assemblages A+B (OR = 5.917; 95% CI 1.20-29.08, *p* = 0.02) as opposed to single infections.

Table 3. 2. *G. intestinalis* assemblages and recorded clinical symptoms.

Clinical data[a]	Assemblage A (*n* = 3)				Assemblage B (*n* = 32)				Assemblage A+B (*n* = 27)				Total, % (*n*)
	% (*n*)	*p*-value	OR	95% CI	% (*n*)	*p*-value	OR	95% CI	% (*n*)	*p*-value	OR	95% CI	
Asymptomatic	33.3% (1)	0.381	3.188	0.258-39.360	9.4% (3)	0.205	0.414	0.093-1.832	18.5% (5)	0.334	1.761	0.424-7.315	14.5% (9)
Diarrhoea	33.3% (1)	0.109	0.115	0.010-1.380	84.4% (27)	0.286	1. 964	0.562-6.862	77.8% (21)	0.831	0.875	0.256-2.989	79.0% (49)
Bloating	0.0% (0)	0.518	-	-	12.5% (4)	0.158	0.393	0.105-1.476	29.6% (8)	0.072	3.263	0.864-12.328	19.4% (12)
Abdominal pain	33.3% (1)	0.513	1.958	0.164-23.449	15.6% (5)	0.286	0.509	0.146-1.780	25.9% (7)	0.400	1.692	0.494-5.789	21.0% (13)
Vomiting and/or nausea	0.0% (0)	0.774	-	-	6.3% (2)	0.469	0.600	0.093-3.867	11.1% (3)	0.376	2.063	0.320-13.313	8.1% (5)
Weight loss	0.0% (0)	0.952	-	-	0.0% (0)	0.484	-	-	3.7% (1)	0.435	-	-	1.6% (1)

[a]Excluding clinical data for samples with coinfections with other entero-pathogens

Table 3. 2. Continued.

Clinical data[a]	Assemblage A (*n* = 3)				Assemblage B (*n* = 32)				Assemblage A+B (*n* = 27)				Total, % (*n*)
	% (*n*)	*p*-value	OR	95% CI	% (*n*)	*p*-value	OR	95% CI	% (*n*)	*p*-value	OR	95% CI	
Fatigue	0.0% (0)	0.952	-	-	3.1% (1)	0.516	-	-	0.0% (0)	0.565	-	-	1.6% (1)
Immunocompromised	0.0% (0)	0.952	-	-	0.0% (0)	0.484	-	-	3.7% (1)	0.435	-	-	1.6% (1)
Other symptoms	0.0% (0)	0.733	-	-	12.5% (4)	0.367	2.000	0.339-11.817	7.4% (2)	0.468	0.620	0.105-3.666	9.7% (6)

[a]Excluding clinical data for samples with coinfections with other entero-pathogens

3.4.4. Spatial and seasonal distribution of G. intestinalis assemblages across NSW LHDs

Giardiasis cases (*n* = 141) collected between 2016 and 2019 were aggregated into assemblage groups and geographically mapped across NSW (Figure 3. 2). Cases appeared in metropolitan areas of Sydney, the Blue Mountains (west of Sydney), and regional inland and coastal centres of NSW. Most cases, however, were from metropolitan areas, in particular the Northern Sydney district. The surrounding areas of inner-west Sydney also showed a high frequency of giardiasis infection (Figure 3. 2). In the regional/rural areas, cases were often seen in the Newcastle and lower Hunter region as well as mid-western regional locations including Dubbo, Orange, and Bathurst. Allocation of postcode data to Local Health Districts supported this: a total of 66.0% (*n* = 95) samples were from metropolitan LHDs (see Table 3. 3). The Northern Sydney LHD accounted for over a quarter (29.5%, *n* = 28) of all metropolitan samples, and of these 35.7% (*n* = 10) were from the Northern Beaches LGA. A total of 34.0% (*n* = 49) of cases were from rural/regional LHDs, including Western NSW (38.8%, *n* = 19), Hunter New England (24.5%, *n* = 12) and the Far West (16.3%, *n* = 8). A smaller number of regional cases were from Southern NSW (*n* = 4), Murrumbidgee (*n* = 3), and Mid North Coast (*n* = 3).

Using ArcGIS, the *G. intestinalis* assemblages (*n* = 141) were mapped to Local Government Area (LGAs) boundaries. The maps (Figure 3. 2) showed that the assemblages were distributed across the entirety of NSW and did not show obvious geographic clustering. There were no correlations found between region of residence and specific assemblage type, and most infections were commonly reported in metropolitan regions of Sydney and the eastern coast of NSW. There were, however, significant associations found between seasons and assemblage B only infections ($p = 0.004$) as well as mixed assemblage infections ($p = 0.005$). In metropolitan NSW, single assemblage A infections were not observed in Autumn, and were only detected in Summer, Spring and Winter ($p = 0.048$) (Figure 3. 3). Additionally, mixed assemblage infections were most often found in Spring ($p = 0.004$), whilst single assemblage B infections made up most cases in Summer, Autumn, and Winter. In rural/regional areas of NSW (Figure 3. 3), single assemblage B infections made up 31.0% of all cases, and peaked in Autumn and Winter.

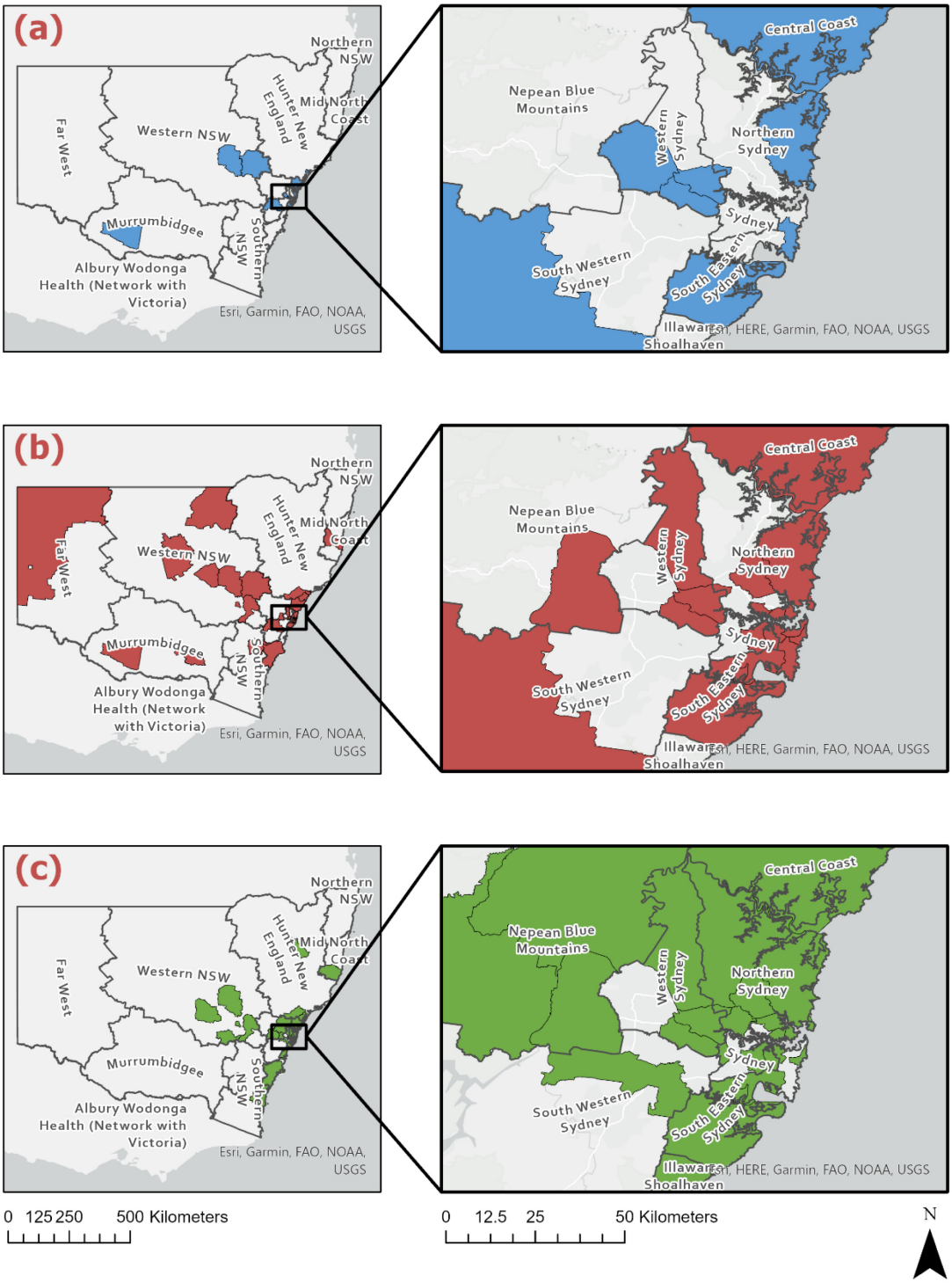

Figure 3. 2. Geospatial distribution of *G. intestinalis* assemblages A, B and A+B across NSW LHDs. This figure shows the geospatial distribution of (a) *G. intestinalis* assemblage A (Blue), (b) *G. intestinalis* assemblage B (Red) and (c) *G. intestinalis* mixed-assemblage A+B (Green) across NSW LHDs.

Table 3. 3. Distribution of *G. intestinalis* assemblages based on region of residence in NSW.

Local Health District (LHD) of residence	**Assemblage A**				**Assemblage B**				**Assemblage A+B**				**Total, % (*n*)**
	% (*n*)	***p*-value**	**OR**	**95% CI**	**% (*n*)**	***p*-value**	**OR**	**95% CI**	**% (*n*)**	***p*-value**	**OR**	**95% CI**	
Central Coast	7.7% (1)	0.568	-	-	12.7% (9)	0.833	-	-	5.0% (3)	0.350	-	-	9.0% (13)
Hunter New England	7.7% (1)	-	-	-	7.0% (5)	-	-	-	10.0% (6)	-	-	-	8.3% (12)
Nepean Blue Mountains	0.0% (0)	-	-	-	4.2% (3)	-	-	-	5.0% (3)	-	-	-	4.2% (6)
Northern Sydney	7.7% (1)	-	-	-	16.9% (12)	-	-	-	25.0% (15)	-	-	-	19.4% (28)
South-Eastern Sydney	15.4% (2)	-	-	-	11.3% (8)	-	-	-	10.0% (6)	-	-	-	11.1% (16)
South-Western Sydney	7.7% (1)	-	-	-	2.8% (2)	-	-	-	1.7% (1)	-	-	-	2.8% (4)
Sydney	0.0% (0)	-	-	-	9.9% (7)	-	-	-	5.0% (3)	-	-	-	6.9% (10)

Table 3. 3. Continued.

Local Health District (LHD) of residence	**Assemblage A**				**Assemblage B**				**Assemblage A+B**				**Total, % (*n*)**
	% (*n*)	***p*-value**	**OR**	**95% CI**	**% (*n*)**	***p*-value**	**OR**	**95% CI**	**% (*n*)**	***p*-value**	**OR**	**95% CI**	
Western Sydney	23.1% (3)	-	-	-	8.5% (6)	-	-	-	6.7% (4)	-	-	-	9.0% (13)
Far West	7.7% (1)	-	-	-	7.0% (5)	-	-	-	3.3% (2)	-	-	-	5.6% (8)
Illawarra Shoalhaven	0.0% (0)	-	-	-	1.4% (1)	-	-	-	6.7% (4)	-	-	-	3.5% (5)
Mid-North Coast	0.0% (0)	-	-	-	1.4% (1)	-	-	-	3.3% (2)	-	-	-	2.1% (3)
Murrumbidgee	7.7% (1)	-	-	-	2.8% (2)	-	-	-	0.0% (0)	-	-	-	2.1% (3)
Southern NSW	0.0% (0)	-	-	-	2.8% (2)	-	-	-	3.3% (2)	-	-	-	2.8% (4)
Western NSW	15.4% (2)	-	-	-	11.3% (8)	-	-	-	15.0% (9)	-	-	-	13.2% (19)

Table 3. 3. Continued.

Local Health District (LHD) of residence	**Assemblage A**				**Assemblage B**				**Assemblage A+B**				**Total, % (*n*)**
	% (*n*)	***p*-value**	**OR**	**95% CI**	**% (*n*)**	***p*-value**	**OR**	**95% CI**	**% (*n*)**	***p*-value**	**OR**	**95% CI**	
Residence (2 categories)													
Metropolitan	61.5% (8)	0.597	1.06	0.31-3.65	67.6% (48)	0.808	1.091	0.54-2.20	65.0% (39)	0.763	0.897	0.44-1.82	66.0% (95)
Regional/Rural	38.5% (5)	-	-	-	32.4% (23)	-	-	-	35.0% (21)	-	-	-	34.0% (49)

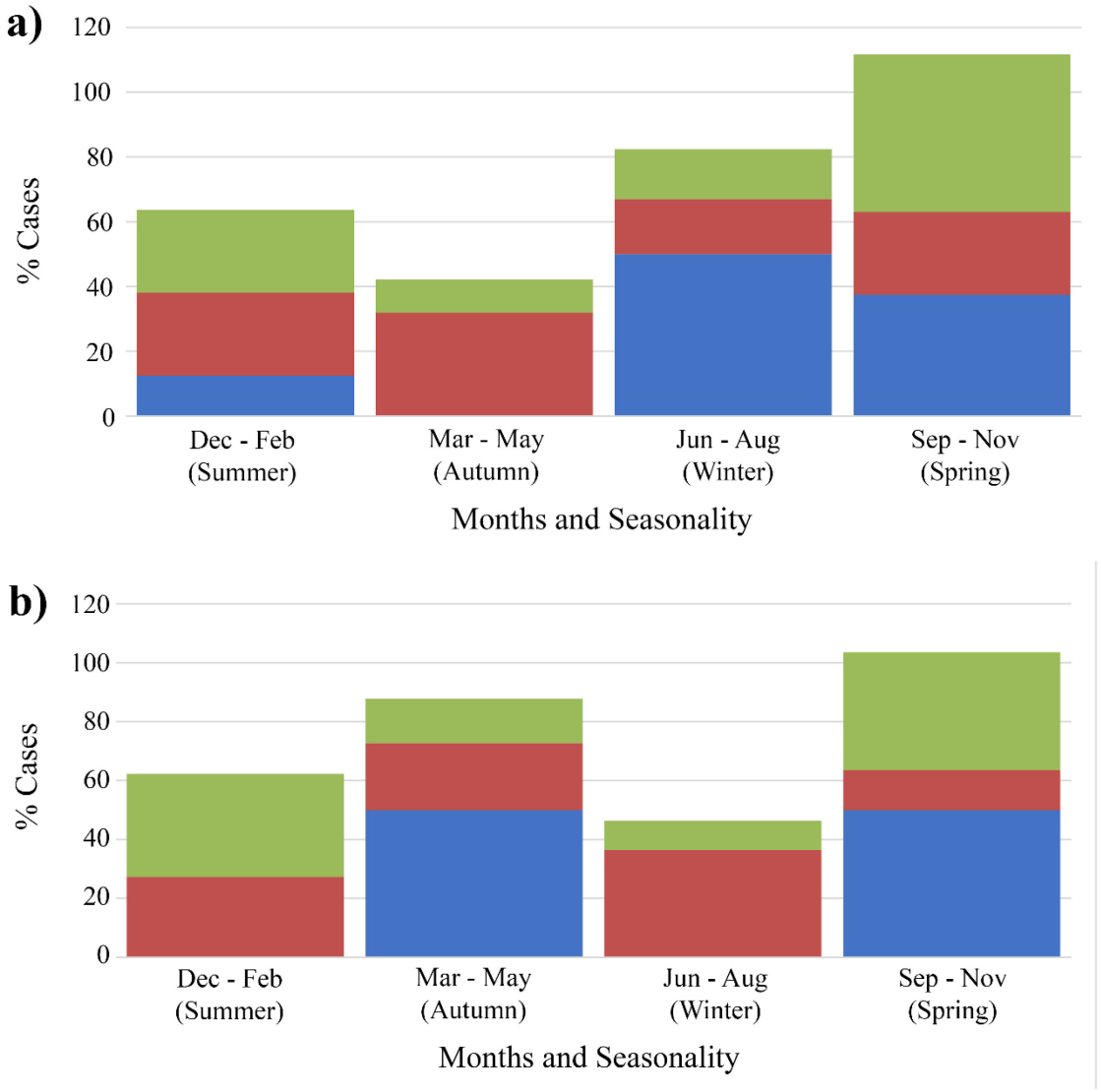

Figure 3. 3. Distributions of *G. intestinalis* assemblages and seasonal dispersal across metropolitan and rural/ regional LHDs. This figure shows the seasonal distribution of genotyped cases occurring in (a) metropolitan LHDs (n = 94) and (b) regional/ rural LHDs (n = 46) in NSW between 2016 and 2019. Assemblage A, Blue; assemblage B, Red; mixed-assemblage A+B, Green.

3.5. Discussion

The present study aimed to identify the genetic diversity of *G. intestinalis* in humans across metropolitan and rural/regional areas of NSW, Australia during 2016-2019. Faecal samples that were positive for *G. intestinalis* were collected (n = 410) from participating hospital and private pathology laboratories participating in the study. From this pool, a representative subset of samples was chosen for genotyping (n = 169) and 95.9% (n = 162) were successfully amplified using PCR. The genotyping results indicate the presence of both assemblages A and B in NSW. Assemblage B infections were predominant, accounting for 46.9%, followed by mixed infections of assemblages A and B at 43.8%, and single assemblage A infections at 9.3%. There is still a lot of controversy surrounding the distribution of assemblages around the world mainly because the results are difficult to compare. Studies in Australia, Austria and Sweden determined that assemblage B was the most dominant [22,35,36]. Assemblage B infections are not only more virulent than assemblage A but have been reported more commonly in giardiasis outbreaks [37]. The higher virulence of assemblage B infections suggests that those infected are more likely to seek medical assistance, which in turn could account for the higher detection rates. High re-infection rates have also been attributed to assemblage B infections, wherein poor hygiene and environmental contamination allows this assemblage to recirculate within a community [38].

Of note, is the high number of mixed-assemblage infections identified (43.8%). In other studies, mixed-assemblage infections are rarely identified accurately, and most studies report only a 3–10% prevalence [39]. PCR-based studies that use assemblage-specific primers targeting the *tpi* locus are often more likely to observe mixed-assemblage infections in comparison to other PCR methodologies [40,41]. In part this is due to the polymorphic nature of the *tpi* markers that allow them to reliably distinguish between assemblage A and B isolates. While standard primer sets may overlook mixed assemblage cases due to the variable proportions of assemblage A and B DNA, assemblage-specific primers excel in this regard [42]. This was confirmed in the present study, where the *tpi*-PCR successfully genotyped 27.2% mixed assemblage A+B infections, in comparison to the 8.1% obtained by the SSU-rRNA-PCR assay.

In the present study, cases of *G. intestinalis* infection were found across all age groups ranging from 0 years to over 70 years. Data shows that the number of cases peaked at ages ≤5 years and 30-49 years regardless of gender (Figure 3. 1). Similar bimodal age distributions have been observed in *G. intestinalis* surveillance reports from the United States [43,44], and England [45]. Although the distribution of *G. intestinalis* assemblages was found across all age groups in NSW; adults aged in

their 30s and 40s, and children under five years of age maintained a higher prevalence of assemblage B. In fact, children under five years-old were more commonly infected by assemblage B (OR = 2.74; 95% CI 1.15-6.51; $p = 0.020$) than assemblage A. This strong association between assemblage B infection and children under 5 years might be indicative of age-specific risk factors and transmission routes. The exposure of assemblage B infections to children can occur either through high-risk activities such as day-care attendance or schooling, as well as poor hygiene behaviours [46,47]. Children with assemblage B infections have also demonstrated a higher level of cyst shedding, which would facilitate a faster spread within institutional settings and areas where children frequent [39]. In Spain, *G. intestinalis*-positive children were 10 times more likely to be infected with assemblage B in comparison to adults [48]. Additionally, a Brazilian study observed a predominance of assemblage B infection in middle-aged adults aged 30 to 39 years old [49], and this was echoed in a similar study in England [14]. Children are likely to be playing a critical role in an ongoing transmission cycle of assemblage B infection, aiding the spread of infection by contaminating family members, day-care centre staff and other attendees.

Co-infecting pathogens were detected in nearly half (49.1%, $n = 83$) of all-*G. intestinalis*-positive faecal samples. Infection of *G. intestinalis* with concomitant infections with a variety of gut bacteria, viruses and parasites are incredibly common in most countries. Most co-infections were parasitic (56.6%, $n = 47$), and bacterial (12.0%, $n = 10$). Overall, the most common pathogens detected were the enteric protozoa *Blastocystis hominis* (31.3%, $n = 25$) and *Dientamoeba fragilis* (15.0%, $n = 12$), followed by *Campylobacter* spp. (5.0%, $n = 4$) and *Enterovirus* (5.0%, $n = 4$). Infection of *G. intestinalis* with concomitant infections with a variety of gut bacteria, viruses and parasites are incredibly common in most countries. In India, *Giardia* infections with *Vibrio cholerae* and rotavirus were commonly identified in children aged under ten years old [50] while in Nicaragua, the majority of *G. intestinalis* cases (70.4%) were co-infected with either *Norovirus*, *Sapovirus* or enteropathogenic *Escherichia coli* (EPEC) [51]. In the United States, multiple parasites including *G. intestinalis*, *Cryptosporidium* spp., and *Entamoeba* spp. were responsible for drinking water outbreaks [52]. Interestingly, a study in Uganda observed a link between *Giardia* assemblage B and *Helicobacter pylori* infection [53]. There are limited studies on the associations between *G. intestinalis* assemblages and coinfecting pathogens, so comparisons remain difficult to make. In the present study, no associations were found between assemblage type and co-infecting pathogen. The high level of co-infections can be explained as most enteric pathogens are transmitted via the same route of infection: the faecal-oral route. This underscores the value of continued examination of faecal specimen from symptomatic persons for multiple pathogens in

developed settings such as Australia, where the practice appears to be diminishing in clinical settings.

In the present study, it was found that clinical symptoms were not associated with assemblage type. This was consistent with previous studies in Brazil [39], Iran [54–56], Thailand [57] and China [58]. Despite this, there have been other studies that have reported a close association between assemblages and clinical symptoms [59–61]. Assemblage B has been associated with severe diarrhoea, vomiting, abdominal pain, and bloating [48,60,62,63], although it is equally plausible that since younger children and their middle-aged parents appear to be predominantly affected with this assemblage and are more likely to seek care, these assemblages become overrepresented among notified cases. In other studies, assemblage A has been affiliated with more serious clinical symptoms [12,22,45,64,65]. It remains difficult to determine a true correlation between assemblages and symptoms. It may be that the virulence of assemblages A and B in humans relies on a variety of factors, including human host age and gender, parasite growth rates, metabolic products, or toxins and even drug resistance.

Another interesting finding was that only 8.0% of *G. intestinalis* positive cases reported travelling overseas prior to illness onset, suggesting that most giardiasis cases in NSW are a result of endemic transmission. There is a misconception that *G. intestinalis* infection in industrialised countries is mainly associated with international travel to developing nations. Several studies have observed that most giardiasis cases in industrialised countries are in fact a result of endemic transmission and local risk factors [66–68]. In the present study, individuals with a history of overseas travel were six times more likely to be infected with mixed assemblages A and B (OR = 5.917; 95% CI 1.20-29.08, $p = 0.02$) as opposed to being infected with a single assemblage infection. This is a novel finding which has not been observed elsewhere, and it remains important to investigate this further. However, a recent study [69] noted that the occurrence of mixed-assemblage infections is higher in developing countries as opposed to developed regions of the world. Exposure to conditions where environments are contaminated with human faeces, have poorer access to or less well-maintained hygiene facilities may be more common in developing settings, which increases the risk of both assemblages co-circulating in communities and being picked up by travellers.

Analyses of 141 cases of sporadic human giardiasis showed that infections were widely dispersed across eastern regions of Sydney and NSW, where the majority of the NSW population resides [70] (Figure 3. 2). Among the NSW LHDs, most (66.0%) sporadic cases occurred in metropolitan LHDs. These findings are consistent with historical state-wide surveillance trends, that identified

significant positive associations between area-level advantage and an increased likelihood of giardiasis notifications [71]. However, it cannot be ignored that the high incidence rates of giardiasis detected in urban Sydney may also be artifactual, particularly as these locations often have highly transient populations. It must also be considered that individuals residing in metropolitan areas have better access to primary health care facilities and greater access or inclination to submitting stool samples for testing when compared with those living in rural areas. In addition to this, densely populated cities such as Sydney have a higher risk of exposure to an infected individual, whether that be through contaminated environment, wastewater, sewage or recreational waters or transmission through day-care centres, schools, and other institutional settings. The plausibility of this was confirmed by a study in the US that found a positive correlation between giardiasis prevalence and population density and population size [72].

Seasonal trends in the dispersal patterns of *G. intestinalis* assemblages were also observed (Figure 3.3.a and Figure 3.3.b). Single assemblage A cases were not detected in metropolitan LHDs during autumn ($p = 0.048$). Alternatively, single assemblage A cases were also missing in regional areas across summer and winter. This finding may be artefactual and is likely due to the lower numbers of assemblage A cases identified throughout the study. Overall, the giardiasis infection rates peaked in spring and dropped in early autumn and winter. This is consistent with other reports of seasonality [73,74]. A peak incidence of giardiasis in NSW during October through to December coincides with high prevalence of outdoor and higher risk activities in these warmer months.

3.6. Conclusions

This study provides new insights into the molecular diversity of *G. intestinalis* in NSW, Australia, and helps to inform enhanced surveillance and prevention strategies in developed metropolitan areas. During the study period, a higher prevalence of assemblage B was observed among human cases in NSW. Factors which possibly influence this higher incidence in NSW may be behavioral, climatic, environmental, or related to the virulence or assemblage of the parasite. Higher numbers of mixed assemblage infections were also identified, which is a novel finding for a developed country like Australia. The distribution of assemblages A and B remained relatively uniform across genders and no clear differences were observed in clinical presentation between assemblages; however, assemblage B was more commonly observed among children. Further high-powered studies are needed to investigate the prevalence and clinical manifestations of assemblage B in children. While most giardiasis cases were transmitted locally, those individuals that had reported travelling overseas prior to illness onset were six times more likely to be infected with mixed assemblages A and B as opposed to single assemblages. This novel discovery underscores the importance of additional investigation into 'travel' as a risk factor for Australians, particularly delving into the differences observed in giardiasis transmission dynamics between endemic and international cases. Among metropolitan LHDs, *G. intestinalis* cases were consistently identified in the Nepean Blue Mountains, Northern Sydney, Western Sydney, South-eastern Sydney, Sydney CBD, and Central Coast regions, which persisted throughout all seasons, and has highlighted these locations as potential disease hotspots in NSW. It remains essential to improve our knowledge of giardiasis and its molecular epidemiology among host populations; to help better inform surveillance strategies and response actions aimed at preventing further spread of infection. Further studies involving the geospatial and spatiotemporal distribution of *G. intestinalis* assemblages is recommended, and in particular targeting the metropolitan and urban areas of NSW.

3.7. Supplementary data

Supplementary Table S3. 1. Co-infecting pathogens identified in *G. intestinalis* positive faecal samples.

	Assemblage type, % (*n*)			
Co-infecting pathogen(s)[a]	**A**	**B**	**A+B**	**Total, % (*n*)**
Adenovirus* & *Dientamoeba fragilis	0.0% (0)	5.0% (2)	0.0% (0)	**2.5% (2)**
Adenovirus* & *Enterovirus	0.0% (0)	2.5% (1)	0.0% (0)	**1.3% (1)**
Adenovirus*, *Blastocystis hominis* & *Dientamoeba fragilis	0.0% (0)	2.5% (1)	0.0% (0)	**1.3% (1)**
Astrovirus* & *Blastocystis hominis	0.0% (0)	0.0% (0)	3.2% (1)	**1.3% (1)**
Astrovirus* & *Sapovirus	0.0% (0)	2.5% (1)	0.0% (0)	**1.3% (1)**
***Astrovirus*, *Blastocystis hominis*, *Sapovirus* & *Shigella* spp.**	0.0% (0)	2.5% (1)	0.0% (0)	**1.3% (1)**
Blastocystis hominis	22.2% (2)	32.5% (13)	32.3% (10)	**31.3% (25)**
Blastocystis hominis* & *Bocavirus	0.0% (0)	2.5% (1)	0.0% (0)	**1.3% (1)**
***Blastocystis hominis* & *Campylobacter* spp.**	0.0% (0)	0.0% (0)	3.2% (1)	**1.3% (1)**
Blastocystis hominis* & *Dientamoeba fragilis	11.1% (1)	10.0% (4)	9.7% (3)	**10.0% (8)**

[a]As reported by the real-time PCR (RT-PCR) EasyScreen™ assay

Supplementary Table S3. 1. Continued.

	Assemblage type, % (*n*)			
Co-infecting pathogen(s)[a]	**A**	**B**	**A+B**	**Total, % (*n*)**
Blastocystis hominis* & *Enterovirus	0.0% (0)	2.5% (1)	0.0% (0)	**1.3% (1)**
***Blastocystis hominis* & *Shigella* spp.**	0.0% (0)	0.0% (0)	3.2% (1)	**1.3% (1)**
Blastocystis hominis*, *Dientamoeba fragilis*, *Enterovirus* & *Sapovirus	0.0% (0)	2.5% (1)	0.0% (0)	**1.3% (1)**
***Campylobacter* spp.**	11.1% (1)	0.0% (0)	9.7% (3)	**5.0% (4)**
***Campylobacter* spp., *Enterovirus* & *Shigella* spp.**	0.0% (0)	2.5% (1)	3.2% (1)	**2.5% (2)**
Clostridium difficile	0.0% (0)	7.5% (3)	0.0% (0)	**3.8% (3)**
***Cryptosporidium* spp.**	11.1% (*n* = 1)	0.0% (*n* = 0)	3.2% (*n* = 1)	**2.5% (*n* = 2)**
Dientamoeba fragilis	22.2% (*n* = 2)	12.5% (*n* = 5)	16.1% (*n* = 5)	**15.0% (*n* = 12)**
Dientamoeba fragilis* & *Enterovirus	11.1% (*n* = 1)	0.0% (*n* = 0)	0.0% (*n* = 0)	**1.3% (*n* = 1)**
***Dientamoeba fragilis* & *Norovirus* GII**	0.0% (*n* = 0)	2.5% (*n* = 1)	0.0% (*n* = 0)	**1.3% (*n* = 1)**
Enterovirus	11.1% (*n* = 1)	5.0% (*n* = 2)	3.2% (*n* = 1)	**5.0% (*n* = 4)**

[a]As reported by the real-time PCR (RT-PCR) EasyScreen™ assay

Supplementary Table S3. 1. Continued.

	Assemblage type, % (*n*)			
Co-infecting pathogen(s)[a]	**A**	**B**	**A+B**	**Total, % (*n*)**
***Norovirus* GII**	0.0% (*n* = 0)	2.5% (*n* = 1)	0.0% (*n* = 0)	**1.3% (*n* = 1)**
***Salmonella* spp.**	0.0% (*n* = 0)	0.0% (*n* = 0)	3.2% (*n* = 1)	**1.3% (*n* = 1)**
Sapovirus	0.0% (*n* = 0)	0.0% (*n* = 0)	6.5% (*n* = 2)	**2.5% (*n* = 2)**
Yersinia enterocolitica	0.0% (*n* = 0)	2.5% (*n* = 1)	3.2% (*n* = 1)	**2.5% (*n* = 2)**
Total	**100.0% (*n* = 9)**	**100.0% (*n* = 40)**	**100.0% (*n* = 31)**	**100.0% (*n* = 80)**

[a]As reported by the real-time PCR (RT-PCR) EasyScreen™ assay

3.8. Acknowledgements

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3.10. Ethical approval

Ethics approval for the conduct of this study was received from the South-Western Sydney Local Health District Human Research Ethics Committee (HREC approval number: HE18/059 LNR) for each of the two hospitals and two private pathology laboratories and ratified by HREC of the University of Technology Sydney (UTS approval number: ETH21-5951).

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Chapter 4

Towards an understanding of the epidemiology of giardiasis through geospatial analyses of human *Giardia intestinalis* infections in NSW, Australia

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Certificate:

I certify that the following chapter is largely my own work, although the contributions of other authors are duly recognised. The contribution of other authors are detailed as follows:

- Suggestions on study design
- By providing suggestions on topics to be reviewed
- By providing advice on geospatial analyses and parameters
- By proof reading draft manuscripts
- By providing suggestions to improve layout, structure and writing style
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Towards an understanding of the epidemiology of giardiasis through geospatial analyses of human *Giardia intestinalis* infections in NSW, Australia

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4.1. Abstract

Giardia intestinalis is a leading cause of diarrhoeal disease in Australia and remains a substantial health burden to human populations in New South Wales (NSW). For appropriate disease control interventions to be developed for diseases like giardiasis, it becomes necessary to not only understand the spatial distribution of this parasite but to identify those areas at a higher (or lower) risk of infection. The aim of this study was to identify and investigate high-risk spatial clustering of giardiasis across Local Government Areas (LGAs) in NSW, Australia. Separate spatial models were also designed for age categories (0-4yrs, 5-14yrs, 15-64yrs and 65yrs and older), sex (male, female) and *G. intestinalis* molecular assemblage type (A and B) to identify any clusters specific to those variables. Cases of giardiasis were analysed at the post-code level. Global and local clustering of infections were evaluated using the Moran's *I* autocorrelation method in ArcGIS Pro 2.9.0. The Hot Spot analysis (Getis-Ord G_i* statistic) tool was also employed to locally identify statistically significant hotspots of giardiasis. Purely spatial and space-time clusters were investigated using Poisson scan statistic models on SaTScan™. Space-time scan statistics were applied to identify seasonal, yearly, and monthly patterns in respect to space and time. Geographical clusters of high *G. intestinalis* incidence were identified in Northern Sydney and South-Eastern Sydney (RR = 1.83; $p \leq 0.01$) as well as Western NSW and Hunter New England (RR = 2.55; $p \leq 0.05$). The space-time analyses also detected three significant clusters of *G. intestinalis* during spring, summer, and early autumn. These clusters aggregated in metropolitan Sydney and regional NSW. Additionally, a large cluster (130.18 km in radius) of primarily male *Giardia*-positive cases in regional Western NSW (RR = 13.28; $p \leq 0.01$) and a smaller cluster (7.15 km in radius) of female *Giardia*-positive cases along the north-eastern Sydney coastline (RR = 5.23; $p \leq 0.05$) were identified in the separate spatial model for sex categories. Clustering in the Central Coast and Northern Beaches LGAs was also seen for those aged 15-64 years old (RR = 4.39; $p \leq 0.05$). No significant clusters were identified for *G. intestinalis* assemblages A or B. Based on the findings of this study, there is a clear rationale for where future giardiasis prevention efforts in NSW should be prioritised. In addition, factors such as biological sex appear to correlate strongly with one's geospatial environment, and this in turn helps public health systems to target vulnerable communities at risk.

4.2. Introduction

Giardia intestinalis is a protozoan parasite recognised as a major contributor to sporadic and epidemic diarrhoeal disease in humans. There are an estimated annual total of 280 million clinical cases of giardiasis worldwide, and consequently this disease was included in the WHO Neglected Diseases Initiative since 2004 [1]. The *G. intestinalis* parasite is primarily spread by the faecal oral route from person-to-person or via ingestion of faecal-contaminated untreated water. As such, giardiasis is often seen in countries with poor sanitation, hygiene practices and a lack of access to adequate drinking water and water-treatment facilities. Symptoms of giardiasis might include short-term diarrhoea, abdominal pain, fatigue, nausea, vomiting, dehydration, and weight loss [2]. Long term consequences of *G. intestinalis* infection have also been reported including extra-intestinal manifestations (such as reactive arthritis [3–5]), a failure to thrive and growth stunting due to nutritional deficiencies [6,7], as well as post-infectious irritable bowel syndrome (IBS) [8].

Giardia intestinalis is considered a species complex and can be classified into eight molecularly distinguishable assemblages A through to H. Only assemblages A and B have been previously identified in both human and animal hosts suggesting potential zoonotic transmission. The other assemblages (C to H) have been found purely in animal hosts. *G. intestinalis* assemblages A and B have been identified worldwide, although most studies generally agree that assemblage B is the most prevalent in human infections [9–11]. Consistent with this, a recent genotyping study conducted in New South Wales (NSW), Australia, revealed that the majority of *G. intestinalis* infections were attributed to assemblage B (46.9%), with mixed A+B assemblages following closely at 43.8%. Additionally, the study highlighted the widespread distribution of sporadic human giardiasis cases across metropolitan NSW, particularly in the greater Sydney region (Zajaczkowski *et al*., see Chapter 3). However, there are no studies that have applied Geographic Information Systems (GIS) epidemiological methods to identify geospatial and temporal clusters of *G. intestinalis* assemblages in Australia, including NSW.

In NSW, Australia the Public Health Act 2010 [12] requires laboratories, hospitals, doctors, and institutional facilities at risk to routinely report cases and outbreaks of *G. intestinalis* infection to public health units (PHUs). Most giardiasis outbreaks in NSW are related to institutional outbreaks and are common in aged-care facilities, early education, and child-care facilities. Giardiasis is also often associated with drinking water outbreaks linked to small tank or bore water supplies rather than municipal drinking water [13]. Overall, in NSW, giardiasis is responsible for an annual estimate of 37.8 cases per 100,000 [14] and over the last twenty years, giardiasis cases have more

than doubled, peaking at 3,455 cases in 2016 [15]. Notification data has found that the highest rates of giardiasis are consistently located in the urbanised Northern Sydney and South-Eastern Sydney regions. Despite this, there is epidemiological evidence suggesting that wildlife, cattle, and livestock may play a role in transmitting giardiasis in regional areas of Australia and NSW [16–18]. The state of NSW is incredibly vast and varied in socioeconomic grounds, demographics, climate, and environmental risk factors. It consists of urban coastal strips, the metropolitan Sydney region, and regional and rural agricultural plains. Identifying hotspots where there is a higher density of giardiasis occurrences in comparison to the surrounding areas is necessary to understand the spatial distribution of this disease and to help identify disease sources, as well as to design prevention strategies.

The aim of this study was to provide a better picture of the distribution of giardiasis in NSW, Australia through the years from mid-2016 to 2019. This was done by utilising spatial autocorrelation tools, hotspot analyses, purely spatial and temporal, as well as space-time analyses to identify clusters and trends in the collected data. Additionally, purely spatial Poisson analyses for clustering using *G. intestinalis* case data adjusted for age, sex and assemblage type was developed to evaluate the distribution of these variables across NSW, Australia. GIS are powerful tools that not only assist in spatially modelling health surveillance data but provide researchers with a clearer picture of the trends of disease transmission. This in turn can inform decision-makers and public health systems to implement management policies and targeted disease control programs.

4.3. Materials and methods

4.3.1. Study area

This study was carried out in NSW, which is situated on the south-eastern coast of Australia. NSW has an area of 801,150 km^2, with a total approximate population of 8,186,800 people in 2021 [19] making it Australia's most populous state. NSW is divided into 15 local health districts (LHDs); eight cover the Sydney metropolitan region and seven cover rural and regional NSW (Supplementary Figure S4. 1). Within the LHDs are a further 128 local government areas (LGAs), each of which encompass multiple suburbs or localities. Climate varies substantially across NSW. The eastern-most coastal regions of the state are temperate, the subtropical north-eastern regions are humid, and the inland regions are semi-arid often with minimal rainfall during the hotter months. Seasons are as follows; summer (December – February), autumn (March – May), winter (June – August) and spring (September – November).

4.3.2. Data collection

In NSW, Giardiasis has been listed as a notifiable disease since August 1998 [20] and all laboratory-confirmed *Giardia* cases are legally required to be notified to public health authorities in NSW [12]. For this study, *Giardia*-positive specimens were acquired during June 2016 to December 2019 from two public hospitals: the Centre for Infectious Diseases and Microbiology (CIDM) at Westmead Hospital, NSW and SydPath at St. Vincent's Hospital, NSW. To mitigate potential geographical bias, specimens were also provided by two pathology clinics: Laverty Pathology and Douglass Hanly Moir Pathology (DHM). These pathology providers are two of the largest in NSW, having established a widespread network of laboratories across regional and metropolitan areas of the state. For each *Giardia* sample collected, the corresponding patient's gender, age, disease onset date and post-code of residence was obtained from the electronic Medical Records (eMR). In addition to this, a previous genotyping study (Zajaczkowski *et al*., see Chapter 3) provided the molecular assemblage type (assemblage A or B, or a mixed A+B) for a portion (n = 129) of the collected *G. intestinalis* samples. In this study, the mixed assemblages A+B were counted as one assemblage A and one assemblage B. All data were fully anonymised, and no direct identifiers were collected from the eMR. Cases were coded with unique case IDs; the case ages were aggregated into four age categories (0-4yrs, 5-14yrs, 15-64yrs and 65yrs and older). This age-categorisation has been used in a previous spatial analysis study [21]. Dates of disease onset were classified as months and years to further reduce the possibility of re-identification.

All *G. intestinalis* positive cases that had postcode data and were collected between June 2016 to December 2019 were geocoded using ArcGIS Pro 2.9.0 [22]. This involved matching case postcode data to its central latitude and longitude coordinates. Each *G. intestinalis* case was then spatially attributed to specific LGAs in which they were located. To reduce spatial distortion, the 'GDA2020 New South Wales Lambert' projected coordinate system was selected when mapping in ArcGIS Pro 2.9.0 [23].

A subset of *Giardia*-positive specimens acquired during June 2016 to December 2019 were genotyped in an earlier study (Zajaczkowski *et al*., see Chapter 3), and a molecular assemblage type (assemblage A or B, or a mixed A+B) was assigned to each case (n = 129). In the current study, the mixed assemblages A+B were counted as one assemblage A and one assemblage B.

Ethical approval for this study was received from the South-Western Sydney Local Health District Human Research Ethics Committee (HREC) which is accredited by the NSW Ministry of Health (HREC approval number: HE18/059 LNR), and the University of Technology Sydney (UTS approval number: ETH21-5951).

4.3.3. Data analysis

4.3.3.1. Spatial Autocorrelation (Global and local Moran's *I*)

Global clustering of *G. intestinalis* cases was evaluated using spatial autocorrelation (Global Moran's *I*) statistical toolsets in ArcGIS Pro 2.9.0 [22,24]. The Global Moran's *I* statistic compares the values of neighbouring locations and assesses whether the values stored on the geographic features are clustered, random, or dispersed. The Moran's *I* Index value, which ranges from -1 to 1 measures the overall spatial autocorrelation of the dataset [25]. The null hypothesis is that the pattern is random (Moran's $I = 0$), or that no spatial autocorrelation is present. A positive Moran's *I* (+1) signifies clustering, which suggests that neighbouring features tend to have similar values. Alternatively, a negative Moran's *I* (-1) signifies a dispersal pattern, which suggests that neighbouring features tend to have different values. The Global Moran's *I* tool also calculates a z-score and *p*-value which are used to assess the significance of the Index.

Local Moran's *I* statistics were also employed to detect local spatial clusters and outliers of giardiasis between LGAs and their neighbours. This Local Moran's *I* calculates a z-score and a *p*-value which are then used to determine the statistical significance of the computed local Moran's *I* Index values. A statistically significant positive local Moran's *I* value suggests there is spatial clustering classified as either a hotspot (high-high) or a coldspot (low-low). Alternatively, a

statistically significant negative local Moran's *I* value implies there is a spatial outlier (high-low and low-high) [26].

The Incremental Spatial Autocorrelation (ISA) tool was implemented to identify the scale or geographic distance at which *G. intestinalis* cases are most clustered across NSW. The ISA analysis runs the Global Moran's *I* tool at multiple increasing distances and measures the strength of spatial clustering between each distance. Output data displays the z-score and *p*-value associated with each distance. The largest statistically significant z-score was selected as the optimal cut-off distance band for further Global and local Moran's *I* analyses, and for the detection of hotspots using the Getis-Ord* tool.

As the LGAs in NSW are not uniform in area size, the 'zone of indifference' was selected as the conceptualisation of spatial relationships parameter in both spatial autocorrelation analyses. This parameter avoids forming strict neighbourhood boundaries and forms a more accurate representation of the data [27,28]. Euclidean distance method was used for all three analyses.

4.3.3.2. Detection of hotspot areas of *G. intestinalis* infection in NSW

The Hot Spot Analysis (Getis-Ord G_i* statistic) tool was employed in ArcGIS Pro 2.9.0 [22] to locally identify statistically significant hotspots (clusters of *G. intestinalis* cases with high values) and coldspots (clusters of *G. intestinalis* cases with low values). Statistical significance is indicated by the test statistic (z-score) and *p*-value. A positive z-score implies there is clustering of high values (hotspot) and the larger the z-score, the greater the clustering is. The opposite is true for negative z-scores. The Hot Spot Analysis also provides a confidence level bin value (G_i Bin) ranging from -3 to 3. Values of -3 to -1 indicate a coldspot with 99% confidence while 1 to 3 indicates a hotspot with 99% confidence. Any values of -1 to 1 are non-significant [29]. Both hotspots and coldspots are further classified as 99%, 95% and 90% confidence levels which indicates the strength of the clustering.

The Getis-Ord G_i* was adjusted similarly to the parameters used in the spatial autocorrelation analyses. As before, the 'zone of indifference' was selected as the conceptualisation of spatial relationships. The Euclidean distance method was chosen to calculate the straight-line distances between the geographic points of *G. intestinalis* cases. As previously mentioned, the distance with the largest statistically significant z-score from the ISA analysis was selected as the optimal distance band when detecting hotspots in NSW.

4.3.3.3. Purely spatial clusters

Purely spatial clusters were analysed using the SaTScan™ spatial statistic developed by Kulldorff [30]. This scan-statistic analysis required three sets of data (1) the case file which reports the numbers of *G. intestinalis* cases for each postcode area (2) the population file which includes the populations estimates for each postcode area obtained from the 2016 General Community Profile (GCP) of Postal Areas (POA) in NSW [31] (3) and the coordinates file which provides the latitude and longitude-coordinates for each postcode centroid as previously calculated by ArcGIS Pro 2.9.0. Spatial scan statistics identify clusters by scanning the study area with an overlapping circular window. The size of the window is initially set in the parameters. In this case, the maximum spatial cluster size was set to 20% of the population at risk [32] and a circle radius of 200km or less.

Separate spatial models were also designed for all variables to identify any significant clustering. In total, there were four spatial models for each age category (1) 0-4yrs, (2) 5-14yrs, (3) 15-64yrs and (4) 65yrs and older; two spatial models for sex (1) male, (2) female; and two for *G. intestinalis* molecular assemblage type (1) A and (2) B. The estimated population counts for every postcode in NSW were modified for each of the four age groups, as well as males and females [31]. The population counts for *G. intestinalis* assemblage types were obtained for each LHD from the Communicable Diseases Branch of NSW Health [33]. These models were run using a maximum spatial cluster size set as 3% of the population at risk and a circle radius of 200km or less.

4.3.3.4. Space-time and purely temporal clusters

When using the SaTScan™ spatial statistic, the space-time scan statistic and purely temporal cluster scans required a case file, population file and coordinates file. In the case of temporal and space-time analyses, the case file also required that the *G. intestinalis* cases be stratified by the time of diagnosis by month and year.

The space-time scan statistic was employed to identify the most likely clusters of both high and low-rate areas, as well as detect these clusters through space and time. This space-time permutation model uses a cylindrical window wherein the circular base of the cylinder represents the space or geography of the cases, and the height of the cylinder represents the time period of possible clusters. In this way, when the cylindrical window scans and overlaps across the study region (NSW), it is moving in both space and time. The maximum spatial cluster size was 20% of the population at risk with a circle radius of 200km. The maximum temporal window was set at 20% of the study period.

The purely temporal scan statistic was used to observe any high or low rates of clustering in the data regarding time. As this tool does not identify spatial clusters, it does not use a circular window, but rather uses a cylindrical window of differing heights (time) that scans across the study area. In this study, two temporal scans were run; (1) one had time aggregation set to month (2) and the other had time aggregation set to year. Regarding the temporal scanning window, the parameters were set to include 20% or less of the study period or one year of the study period respectively.

SaTScan™ can identify secondary clusters in addition to the initial cluster in both the purely spatial and space-time scan statistic analyses. In this study, the cluster with the maximum likelihood ratio test statistic was the most likely cluster (or the primary cluster), and the clusters with lower likelihood ratios were regarded as secondary clusters. Additionally, the criteria for reporting hierarchical clusters were set to the restrictive option of no geographical overlap. This would disregard any secondary clusters if they intersected with a previous cluster. Note that no secondary clusters are reported for purely temporal analyses.

Each of the SaTScan™ cluster analyses were tested through the assumption of a discrete Poisson model [30]. Additionally, the *p*-value was obtained by Monte Carlo hypothesis testing and the number of replications was limited to 999. Statistically significant results were considered as $p \leq 0.05$. Results from the cluster analyses were imported and visualised in ArcGIS Pro 2.9.0.

4.4. Results

Of the total of 128 local government areas (LGAs) in the study area, data were obtained from 64 LGAs; 56% (36/64) of which covered metropolitan regions of NSW, and 44% (28/64) of which spanned across rural and regional areas of NSW. Overall, a total of 410 laboratory-confirmed *G. intestinalis* cases were reported by the participating laboratories in this study across NSW during the 2016 - 2019 study period. Most of the cases were provided by the community pathology laboratories (92.0%, 377/410), as opposed to the hospitals (8.0%, 33/410). A total of 99 cases were excluded from the study due to data incompleteness (e.g., missing post-code data) and an additional 8 cases were excluded as the postcodes originated from outside NSW, leaving 303 *G. intestinalis* cases in total. Both males (54.5%, 165/303) and females (45.5%, 138/303) were represented in the total cases. The 15–64-year-old age category made up most *G. intestinalis* cases (55.1%, 167/303), followed by children aged 0-4 years (20.1%, 61/303) and 5-14 years (17.2%, 52/303). The smallest age group were individuals aged 65 years and older (7.6%, 23/303). Out of the 303 *Giardia*-positive cases included in the study, a total of 129 were previously genotyped by Zajaczkowski *et al*., (Zajaczkowski *et al*., see Chapter 3). These cases were made up of assemblages A (n = 12), B (n = 62) and a combination of A+B (n = 55). To increase the number of individual assemblages in each analysis group, the combined A+B assemblages were counted twice, as one assemblage A and one assemblage B. This led to a new total of 184 individual assemblages made up of assemblages A (n = 67) and assemblages B (n = 117).

4.4.1. Global and Local Moran's I autocorrelation

Spatial autocorrelation was tested at 20 km distance intervals and beginning at 50 km, using the ISA tool. This tool identified 21 statistically significant z-scores (ranging from 1.93 to 3.43) between 150 km and 550 km. The z-scores peaked at a distance band of 290 km (Z = 3.43, global Moran's I = 0.08, $p \leq 0.01$). The peak distance value (290 km) was used as the cut-off distance band for Global and local Moran's I analyses and the Getis-Ord* tool. The Global Moran's I analysis was used to identify significant spatial autocorrelation in the total number of giardiasis cases collected during the study period (n = 303). This analysis found that *G. intestinalis* infections were clustered in NSW (Z = 3.55, global Moran's I = 0.09, $p \leq 0.01$) (see Supplementary Figure S4. 2).

As there was a significant spatial pattern identified in the data, the Local Moran's *I* analysis was employed to identify clusters. Overall, there were 33 clusters identified: 22 High-High clusters and 11 Low-Low clusters. High-High clusters were predominantly located in metropolitan LGAs, as opposed to Low-Low clusters which were exclusively found in regional NSW. Additionally, 26 Low-High outliers were identified across both metropolitan and regional LGAs (Figure 4. 1). The High-High and Low-Low clusters are indicative of neighbouring features in the study area with equally high or low attribute values, respectively. The outliers (Low-High and High-Low) are defined by neighbouring features with dissimilar values. For example, the Low-High outlier is categorised by high-risk neighbours surrounding a lower risk area [22,25].

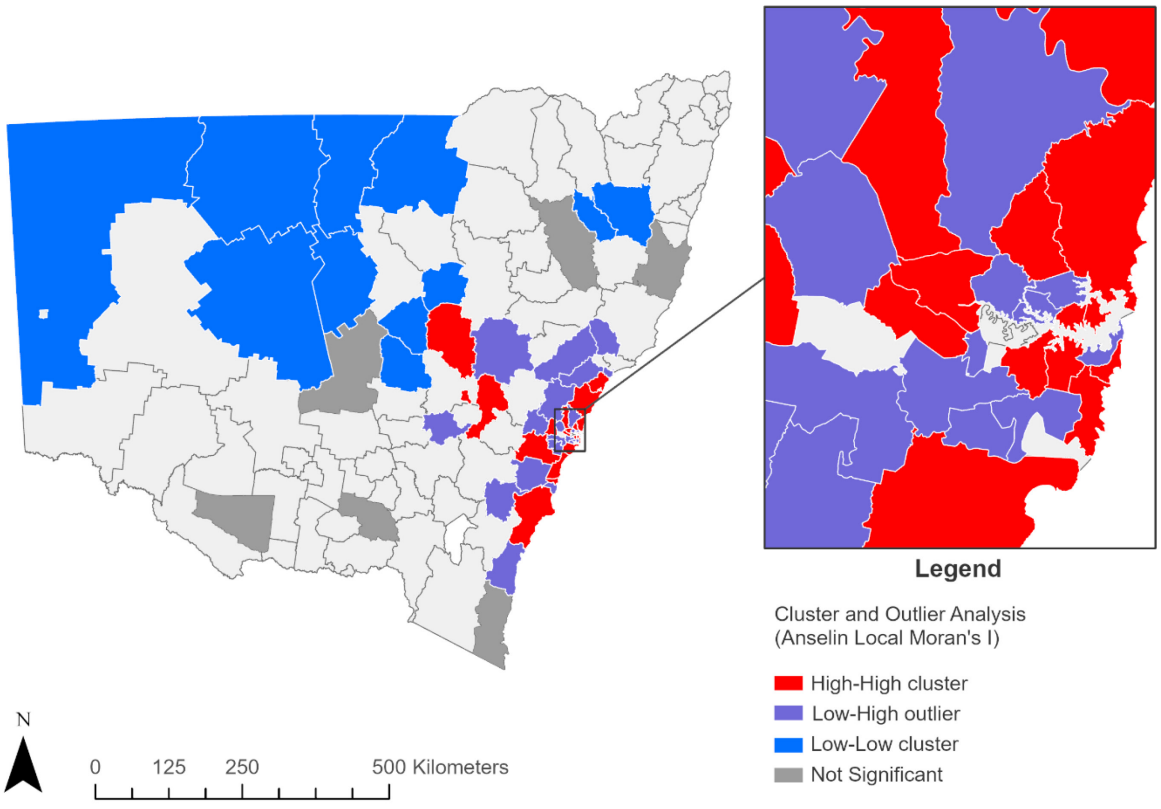


Figure 4. 1. Mapping of giardiasis local spatial clusters (Local Moran's *I*) in NSW between June 2016 and December 2019. A Euclidean distance band of 290 km was used, and the type of cluster identified is colour coded according to the scale provided. This analysis shows that *G. intestinalis* infections are significantly clustered in NSW.

4.4.2. Hotspot Analysis (Getis-Ord)*

Hotspot identification of giardiasis cases using the Getis-Ord G_i* statistic is shown in Figure 4. 2. Hotspots for *G. intestinalis* cases were seen across both metropolitan and regional areas of NSW although hotspots with 99% confidence levels were located almost exclusively in urban localities. The regional areas included Hunter New England (LGAs: Cessnock, Lake Macquarie and Newcastle), Southern NSW (LGA: Goulburn Mulwaree) and Western NSW (LGA: Cowra). In comparison, hotspots with 95% confidence levels were concentrated in the western-most regional parts of the state including Bathurst, Dubbo, Mid-Western Regional, and Orange. These hotspots were also identified in Hunter New England, Dungog, and Singleton in particular, and Hawkesbury in the Nepean Blue Mountains. Hotspots with 90% confidence levels were not identified by the Getis-Ord* analysis. Coldspots with 90% confidence were also noted in six LGAs in Western NSW, as well as Armidale Regional in the far north-east of the state. One coldspot with 95% confidence levels was detected in Gilgandra, a regional LGA found in the central west of NSW.

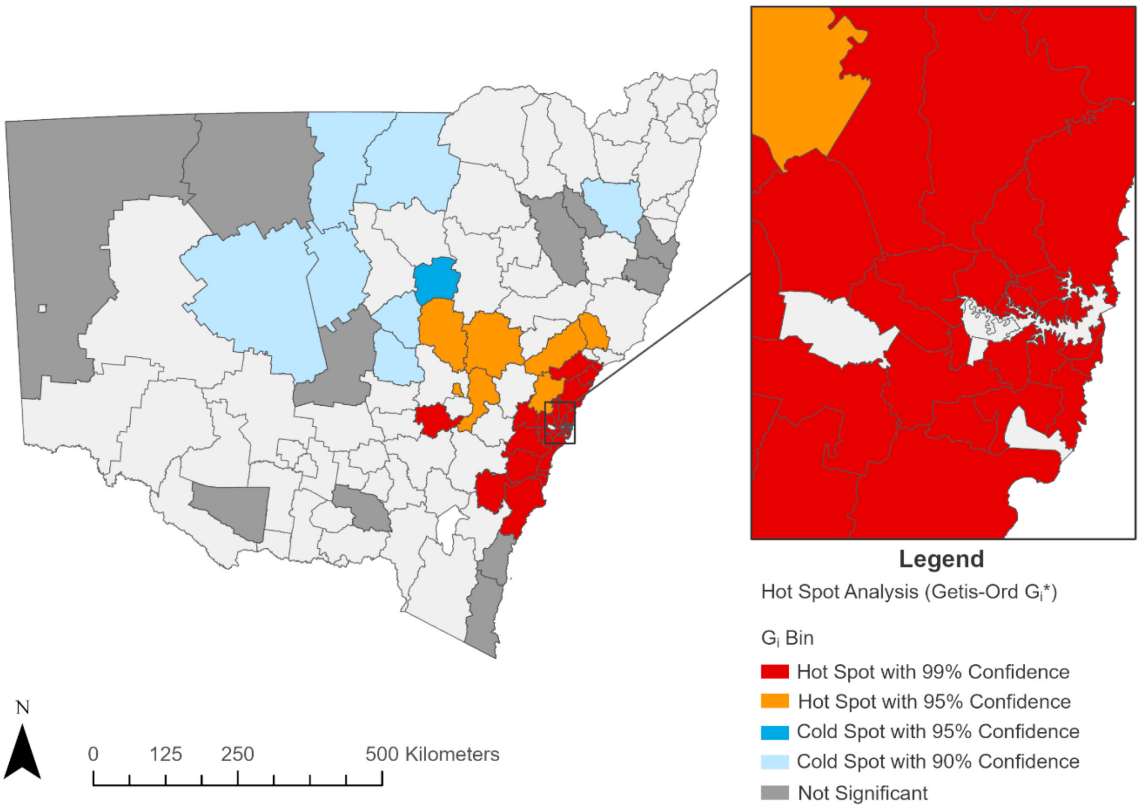


Figure 4. 2. Mapping of giardiasis hotspots in NSW between June 2016 and December 2019 using the Hotspot Analysis (Getis-Ord*) statistical tool. Hotspots and coldspots are colour coded according to the scale provided.

4.4.3. Purely spatial clusters analysis

The purely spatial clusters were analysed by spatial scan statistics using a discrete Poisson model. There were three significant clusters found in the study period, all with a *p*-value equalling or less than 0.05 (Table 4. 1, Figure 4. 3). The first cluster was the smallest (14.04 km radius) and was observed in Western Sydney, South-Western Sydney and on the western outskirts of the Sydney district. This cluster included 11 LGAs (RR = 0.41) with an approximate total population of 2 million individuals. The second cluster was larger (16.25 km in radius) and covered 10 LGAs (RR = 1.83). These LGAs included, Hunters Hill, Ku-ring-gai, Lane Cove, Mosman, North Sydney, the Northern Beaches, the city of Sydney, Waverley, Willoughby, and Woollahra. The final cluster was the largest (192.00 km in radius), and unlike the previous two clusters, covered only regional/ rural LGAs. There were 16 LGAs included in cluster 3 (RR = 2.55), which were predominantly located in Western NSW, and partially crossed into Hunter New England.

To observe any clustering consistent with sex (male, female), age category (0-4yrs, 5-14yrs, 15-64yrs and 65≥yrs) and/or molecular *G. intestinalis* assemblage type (A, B), separate purely spatial analyses were run for each categorical variable. As seen in Table 4. 1 and Figure 4. 4, three significant clusters were identified. A large cluster (130.18 km in radius) of male *Giardia*-positive cases was found in regional Western NSW (RR = 13.28; $p \leq 0.01$). The spatial analyses were also run for the female category and identified a primary cluster (7.15 km in radius) of female *Giardia*-positive cases along the state coastline (Central Coast and Northern Sydney) (RR = 5.23; $p \leq 0.05$). Finally, a separate spatial scan was run for all four age categories and detected one primary cluster (12.78 km) of individuals aged 15-64 years old spanning from the Central Coast to the Northern Beaches in Sydney (RR = 4.39; $p \leq 0.05$). No significant clusters were identified for the *G. intestinalis* assemblage types A or B.

Table 4. 1. Significant clusters of *G. intestinalis* incidence in NSW at the LGA level as identified by the purely spatial scan statistic.

Variable	SaTScan cluster	Radius (km)/ LGA (*n*)	Observed cases	Expected cases	RR	LLR	*p*-value
-	1	14.04 (10)	28.0	59.73	0.41	12.50	≤0.01
	2	16.25 (11)	76.0	46.76	1.83	9.41	≤0.01
	3	192.00 (9)	27.0	11.19	2.55	8.41	≤0.05
Gender (Female)	1	7.15 (1)	8.0	1.61	5.23	6.61	≤0.05
Gender (Male)	1	130.18 (5)	6.0	0.47	13.28	9.87	≤0.01
Age (15-64yrs)	1	12.78 (1)	10.0	2.39	4.39	6.89	≤0.05

RR: Relative risk; LLR: Log-Likelihood ratio

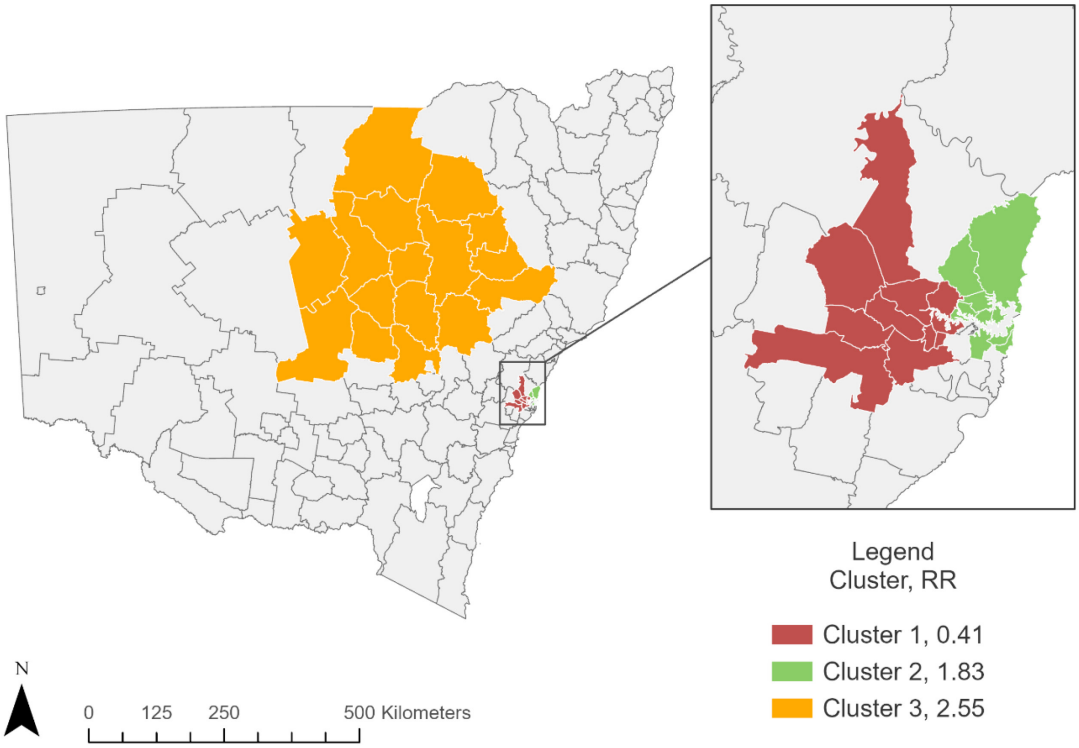


Figure 4. 3. Mapping of purely spatial clusters of giardiasis in NSW between June 2016 and December 2019. Three statistically significant clusters were identified. These included Cluster 1 (Western Sydney and South-Western Sydney), Cluster 2 (Northern Sydney and South-Eastern Sydney) and Cluster 3 (Western NSW and Hunter New England).

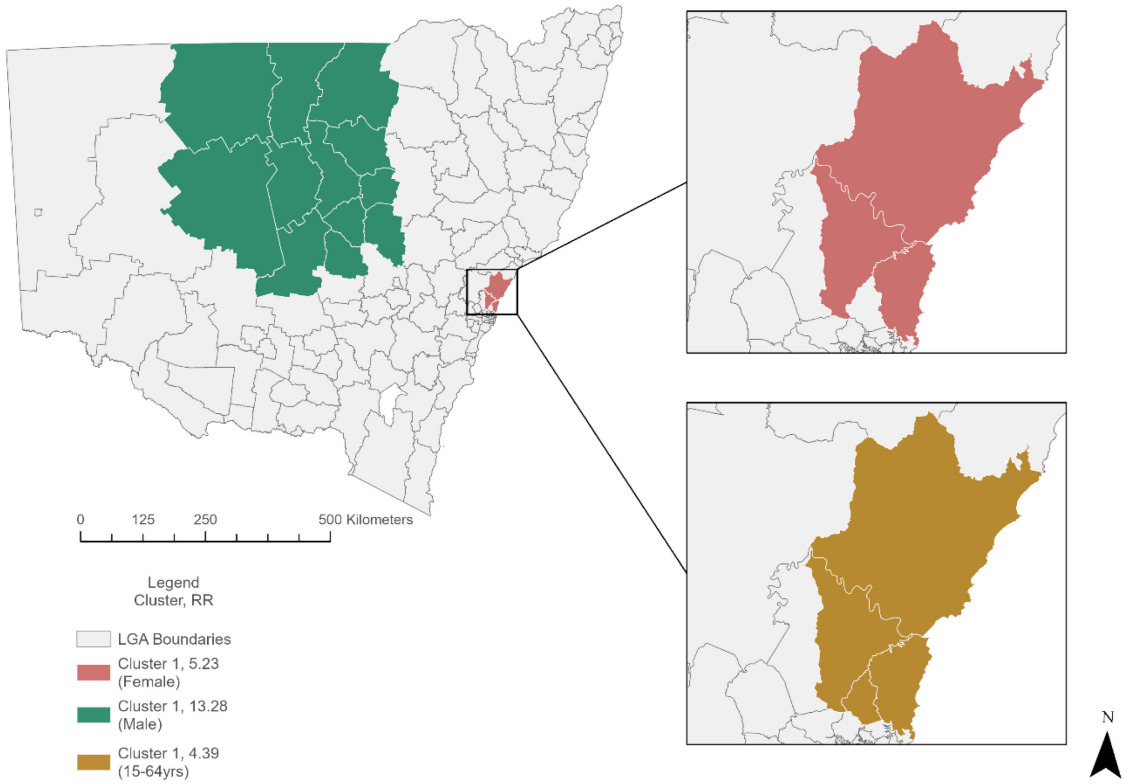


Figure 4. 4. Mapping of purely spatial clusters of giardiasis (sex and age category) in NSW between June 2016 and December 2019. Separate analyses were run for each sex and age category, and three main clusters were identified. These included (1) Cluster 1 identified relating to males in regional NSW (Western NSW), (2) Cluster 1 identified to females (Northern Sydney and the Central Coast) and (3) Cluster 1 identified relating to 15-64 age group (Northern Sydney and the Central Coast).

4.4.4. Temporal and Space-time clusters analysis

Space-time analysis detected three significant clusters (Table 4. 2). The first cluster included Northern and South-Eastern Sydney districts. The time frame for this cluster encompassed the warmer months in Australia (October 2017 – February 2018) and continued into the cooler autumn months (March 2018 – May 2018) (RR = 5.89; $p \leq 0.01$). A second, larger cluster was detected spanning across regional Western NSW and Hunter New England, as well as the semi-urban district of Nepean Blue Mountains. The time frame for this cluster was also from October 2017 to May 2018, with a RR = 8.57; $p \leq 0.01$. The third cluster was recorded only in the summer month of December 2017 and was in regional Hunter New England and the Mid-North Coast areas (RR = 5.50; $p \leq 0.01$).

Additional scan-statistic analyses found purely temporal clustering in the year 2019 (RR = 0.09; $p \leq 0.01$), and from October 2017 to May 2018 (RR = 5.62; $p \leq 0.01$) (Table 4. 2). Additionally, the seasonal analysis found that the risk of giardiasis was significantly reduced between the winter months of June and July across all years (RR = 0.48; $p \leq 0.01$).

Table 4. 2. Significant temporal and spatiotemporal clusters of giardiasis in NSW between 2016 and 2019.

Method	SaTScan cluster	Timeframe	Cluster location (LGA)	LHD	Observed cases	Expected cases	RR	*p*-value
Purely temporal (year)	1	2019/01 to 2019/12	All	All	10.0	83.93	0.09	≤0.01
Purely temporal (month)	1	2017/10 to 2018/05	All	All	169.0	55.88	5.62	≤0.01
Seasonal	1	Jun - Jul	All	All	30.0	56.11	0.48	≤0.01
Space-time	1	2017/10 to 2018/05	Hunters Hill, Inner West, Ku-ring-gai, Lane Cove, Mosman, North Sydney, Northern Beaches, Randwick, Ryde, Sydney, Waverley, Willoughby, Woollahra	South Eastern Sydney, Northern Sydney, Sydney	53.0	10.53	5.89	≤0.01

RR: Relative risk

Table 4. 2. Continued.

Method	SaTScan cluster	Timeframe	Cluster location (LGA)	LHD	Observed cases	Expected cases	RR	p-value
Space-time	2	2017/10 to 2018/05	Bathurst Regional, Blue Mountains, Cowra, Dubbo Regional, Gilgandra, Lachlan, Mid-Western Regional, Narromine, Orange, Parkes, Singleton	Hunter New England, Nepean Blue Mountains, Western NSW	25.0	3.15	8.57	≤0.01
Space-time	3	2017/12	Armidale Regional, Dungog, Kempsey, Lake Macquarie, Maitland, Newcastle, Port Macquarie-Hastings, Tamworth Regional, Uralla	Hunter New England, Mid North Coast	9.0	0.66	14.10	≤0.01

RR: Relative risk

4.5. Discussion

This study sought to utilise GIS tools including ArcGIS Pro 2.9.0 [22] and the SaTScan™ spatial statistic [30] to explore the spatial and temporal epidemiology of *G. intestinalis* infection in humans across metropolitan and rural/ regional NSW, in Australia. Geospatial analyses were also utilised to distinguish potential clusters of *G. intestinalis* assemblages A and B. Differences between these molecular assemblages have been described before regarding host demographics and clinical symptoms (Zajaczkowski *et al*., see Chapter 3). However, it has yet to be seen if these genetic assemblages are specific to certain regions in NSW, and ultimately are influenced by environmental risk factors. The results from this study not only identified priority regions in NSW but identified where preventative interventions can be targeted to have the greatest public health impact.

Spatial clustering analyses using the SaTScan™ spatial statistic [30] found no significant aggregation of *G. intestinalis* assemblages A and B in NSW. While this might suggest that the geospatial distribution of both assemblages in NSW is random, it must be considered that other risk factors and population demographics can directly influence disease incidence. It is difficult to compare these results with previous studies, as there continues to be little research done that has aimed to search for spatial trends in *G. intestinalis* assemblage distribution. The potentially zoonotic assemblage A has been identified in schoolchildren, their family members and household pets (dogs) residing in urban Mexico [34]. Similarly, dogs living in urban environments of Australia have been found with assemblage A infections [35]. The studies do suggest that contact with domestic dogs living in urban households is a risk factor for giardiasis. Whether the transmission of this assemblage is directly zoonotic, or an indirect form of transmission is yet to be identified. In contrast, an Argentinian study observed that children from rural households were more likely to be identified with *G. intestinalis* assemblage B [36]. The study also noted that the children who used wells as their source of drinking water were also more likely to be infected with assemblage B than those who drank from piped water. This predominance of one assemblage over another in a specific region reflects the complex circulation of *G. intestinalis* in the environment.

In this study, the Moran's *I* and Getis-Ord* statistical tools revealed that the spatial structure of *G. intestinalis* case distribution was statistically significant and highly clustered in NSW, Australia. Hotspots with 99% confidence levels were identified in all eight metropolitan LHDs and almost all the metropolitan LGAs excluding the City of Lithgow and Hawkesbury (Figure 4. 2). Further analyses using the SaTScan™ spatial statistic [30] confirmed purely spatial clusters of *G.*

intestinalis cases (Table 4. 1, Figure 4. 3). The primary cluster was classified as having a low trend, in which the rate of giardiasis was decreasing inside the cluster as opposed to the increasing rates outside the cluster. This low-rate cluster was observed in the suburban regions of Western Sydney and South-Western Sydney (RR = 0.41; $p \leq 0.01$). While this low risk for *G. intestinalis* infection may be the effect of a small population size, it is still important to note that a previous geospatial study found that those residing in South-Western Sydney had significantly lower giardiasis notification rates in comparison to other LHDs [21]. This might be due to potential issues of affordability and a lack of access to local general practitioners (GPs) for disadvantaged patients [37,38]. Indeed, the Australian Bureau of Statistics (ABS) Socio-economic Indexes for Areas (SEIFA) has identified communities of socioeconomic disadvantage in parts of South-Western Sydney [39]. It is also worth mentioning that South-Western Sydney and Western Sydney are two of the most culturally and linguistically diverse LHDs in NSW. Almost half of the population in Western Sydney (49.8%) and South-Western Sydney (45.2%) were born overseas, and over 33,000 migrants settled in these areas in 2017 alone [40,41]. Ultimately it can be difficult for migrants from non-English speaking backgrounds to navigate Australia's public health care system and receive the proper treatment. Moreover, South-Western Sydney and Western Sydney are geographically located inland (see Supplementary Figure S4. 1), and as such have fewer opportunities for outdoor recreational water exposure in comparison to the coastal cities on the eastern border of NSW. It is well documented that *Giardia intestinalis* cysts spread easily to humans through ingesting contaminated water sources and recreational water-borne outbreaks are particularly common in Australia [13,42,43].

The SaTScan™ spatial statistic further identified two significant clusters of positive correlation to giardiasis in NSW; (1) one cluster was in metropolitan Northern Sydney and South-Eastern Sydney and saw individuals with an almost two times greater risk of *G. intestinalis* infection (RR = 1.83; $p \leq 0.01$) and (2) the other cluster was identified in regional Western NSW and Hunter New England (RR = 2.55; $p \leq 0.05$) (Table 4. 1). The cluster identified in metropolitan Sydney was not surprising, as annual surveillance data collated by the Communicable Diseases Branch of NSW reports both Northern Sydney and South Eastern Sydney as the LHDs with the highest incidence rates (IR) of giardiasis (~78.9 and ~64.8 IR per 100,000 respectively) [14,44]. A similar trend was seen in New Zealand, which reported the highest rates of giardiasis from the populous urban area of Auckland [45]. A more recent case-study based in the USA also indicated a significant relationship between urbanity, population density and incidence of *G. intestinalis* infection [46]. These increased notification rates in urban locations might be a result of better local GP access as opposed to those living in rural areas. In addition to this, urban areas are more likely to be serviced by GPs with

advanced training, greater diagnostic proficiency, and spatial access to surgeries [38]. Individuals living in coastal urban towns and cities also have greater access to outdoor recreation, especially with potentially contaminated water sources such as beaches, ocean pools, water parks/ playgrounds and estuaries where fishing remains a popular activity. Northern Sydney and South-Eastern Sydney are also popular locations for tourism (both domestic and international), so it is possible that the high number of visitors to these LHDs could be responsible for importing giardiasis. In fact, a previous epidemiological case-control study in Sydney found that giardiasis cases were 20 times more likely to have been travelling overseas in comparison to the control cases [42].

Overall, there is strong evidence that human activity is the primary source of *G. intestinalis* infection in urban areas. Swimming at beaches and public recreational water sources that are contaminated with *G. intestinalis* cysts may in fact be contributing to high infection rates. This is particularly true as *G. intestinalis* cysts are highly resilient in water and have shown resistance to chlorine treatments [47]. Attending day-care centres and schools in densely populated communities can also lead to a higher chance of exposure to an infected individual [48–50]. Likewise, parents or caregivers of toddlers are often at risk of infection due to nappy handling [51,52]. Women of reproductive age (i.e., 15-49 years) [53] in particular have been found to be at risk of giardiasis as they tend to play a greater active role in nursing younger children [54–56] than males. The Australian workforce of early childhood teachers and educators is also largely dominated by women aged in their 30's [57]. This is reflected in the purely spatial Poisson analyses which found significant clustering of female giardiasis cases in the Central Coast and Northern Sydney regions, and an overlapping cluster of cases aged 15-64 years (Figure 4. 4).

In comparison, a significant cluster of male cases was identified in regional western NSW near the Bogan, Cobar, and Gilgandra government areas (Figure 4. 4). As the cases of *G. intestinalis* tended to be spread out in these regional LGAs due to large surface areas and low population counts, this cluster had a larger radius of 130.18 km. A radius restriction of 200 km was implemented to avoid any overly large clusters. The cluster of male cases appeared a logical outcome of this study; regional NSW is made up of livestock and crop farmland, and males are more likely to work in the Australian agricultural workforce in comparison to females [58]. While this could suggest potential occupational exposure of males to giardiasis in NSW, it is difficult to determine precisely whether the exposure occurred through contact with livestock or wildlife, via the application of agricultural manure or even by drinking contaminated water sourced from local rainwater tanks or bore wells. Data on these variables were not available for analysis during the study. No further clusters were identified in Western NSW for any age category. This might suggest that not only working-aged

men are at a higher risk of giardiasis in regional NSW, but potentially male children and the elderly. If true, there are other risk factors that must be considered including drinking contaminated bore water or rainwater from tanks, or even having contact with domestic pets. In the United States, county-level giardiasis incidence rates were directly related to the use of private wells [59] and in rural Western Australia, the majority of *G. intestinalis* human cases shared the same assemblage type as that identified previously in marsupials and wild foxes, indicating a potential for zoonotic transmission [60]. Although in the present study significant clustering was identified in both urban and rural LGAs, it is likely that risk factors associated with rural NSW are not the same as those in city environments. Utilising GIS as a tool to distinguish and identify common risk factors specific to rural and metropolitan localities can be beneficial to public health units in preventing and minimising health risks to the relevant communities.

Purely temporal results saw *G. intestinalis* infection outbreaks between October 2017 to May 2018 (Table 4. 2). Climate data from the Bureau of Meteorology recorded that the year 2017 was one of the warmest and driest years on record for NSW, although the months of October and December saw above average rainfall across the state [61]. These heavy rains continued into 2018 until the end of March. Overall, the year of 2018 was substantially warmer for both mean and daytime temperatures [62]. Previous studies have reported positive correlations between heavy rainfall conditions, high humidity, and an increase of *G. intestinalis* infections [55,63–65]. More specifically, anthroponotic transmission of *G. intestinalis* cysts is possible when high levels of rainfall overflow onto manure contaminated soil and causes runoff into surface water [66]. Likewise, heavy rainfall can often lead to sewage overflow and contamination of drinking and recreational waters. It is suggested that the constant cycle of wet weather conditions and hot/ dry temperatures in 2017 and 2018 will have facilitated an increased use of these contaminated recreational water sources by people, leading to the higher rates of giardiasis. Interestingly, there was a negative yearly seasonal occurrence of sporadic *G. intestinalis* infection in the year 2019 (RR = 0.09; $p \leq 0.01$), although this may have been a result from the lower number of cases collected from participating hospitals and laboratories (Table 4. 2). The negative association may also be due to 2019 being the driest and warmest year on record for NSW, with state-wide rainfall being 55% below the average [67]. The year of 2019 was also heavily affected by heatwaves and bushfires in the northeast, which may have prevented individuals from travelling to high-risk areas.

Space-time analyses identified clusters of giardiasis during the warmer seasons (spring and summer) and continuing into the autumn months. These clusters were identified across NSW, in South-Eastern Sydney, Northern Sydney, Central Sydney, Hunter New England and Western NSW (Table 4. 2). A similar seasonal trend with *G. intestinalis* incidence peaking in the summer and early autumn months has been observed in Canada [68], the USA [69,70] and in previous research from NSW, Australia (Zajaczkowski *et al*., see Chapter 3). Additionally, a meta-analysis of surveillance studies has concluded that rising temperatures are significantly linked with increases in bacterial and protozoal infections [71]. As both urban and rural LHDs showed this summer/autumn seasonal trend, it can be assumed that risk factors for *G. intestinalis* transmission in NSW are associated with seasonal patterns. An additional significant space-time cluster was identified in the summer month of December (RR = 14.10; $p \leq 0.01$) spanning across two regional LHDs; Hunter New England and the Mid North Coast (Table 4. 2). These regions are popular destinations for recreational water-based activities for interstate tourists. Increased human exposure to this pathogen through contaminated recreational waters, and increased travelling/ tourism activities to rural recreational areas during the summer holiday period is feasible.

With the use of geospatial analyses, epidemiologists and policy makers can effectively target high-risk areas with prevention/ intervention programs and make the relevant changes to public health policy. In the case of this study, local interventions are recommended for males in Western NSW; particularly those who own agricultural farmland and live or work near water and/or wildlife. Similarly, targeted prevention programs should be aimed towards working-age females residing in the Central Coast and Northern Sydney. To raise awareness about the disease, local health authorities must disseminate information packages about *G. intestinalis* infection to these high-risk areas including day-care centres, schools, and public swimming pools. Additional factsheets aimed towards households with children should promote correct hand-washing techniques for children. While NSW has an incident response protocol regarding public swimming pools and spa pools [72], it remains difficult to police every indoor and outdoor pool establishment. Ultimately, swimming pool water-quality and control measures are left to the discretion of the pool operator. It is recommended that additional infection control practices are provided to individuals wanting to swim in public/ private pool venues, rivers, lakes, estuaries, beaches, and ocean pools. These individuals should be encouraged to avoid swimming while ill, including the two weeks after illness onset. Geospatial and GIS analysis techniques are powerful exploratory tools that can identify and help visualise high (or low) occurrences of disease infection amongst selected study areas. When used alongside geocoded health surveillance data these geospatial tools not only have the potential

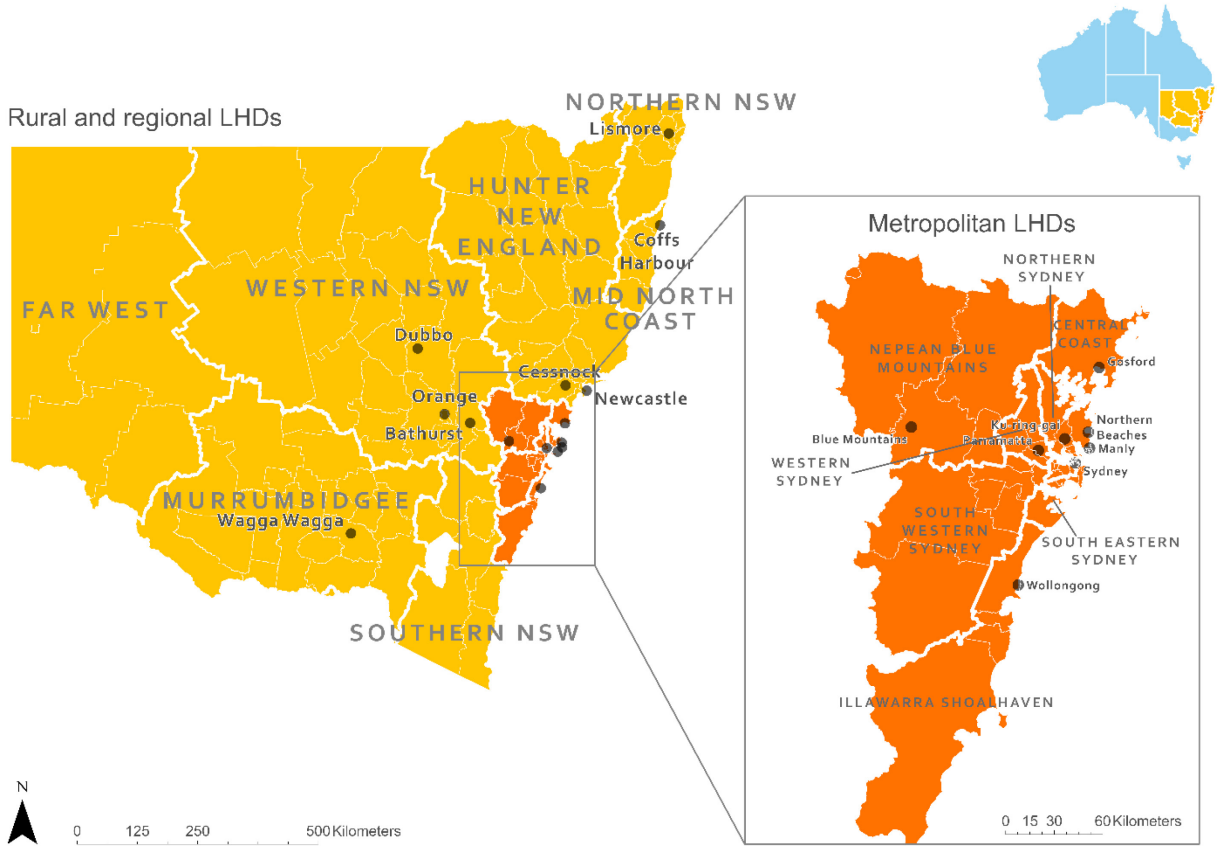
to identify the locations most at risk, but also inform public health services of the socio-demographic characteristics and associated risk factors that influence the disease cluster.

However, this study does have some limitations. Other than the age, gender, post-code data and assemblage type provided for this study, other additional information on each *G. intestinalis* case was not available and therefore were not included in the analysis. Risk factors such as whether the case travelled overseas prior to illness onset, had contact with non-potable water sources and/ or potential contact with livestock, wildlife, or domestic animals would have provided greater insight into whether these risks are associated with certain areas of NSW and whether they influence the intensity of the clustering in SaTScan. Additionally, no outbreak data of *G. intestinalis* infection was initially recorded, so it was not feasible to remove this data from the spatial analyses. Including data of potential giardiasis outbreaks in the SaTScan statistical analyses could affect the overall results. A group of giardiasis cases limited in the same area and timeframe could lead to false clusters being reported. In this study, the quality of the data was limited as hospital and laboratory notified cases of *G. intestinalis* will under-represent the actual numbers of cases in NSW. Though it is worth mentioning that the two community pathology laboratories (Laverty and DHM) operate across NSW, and as a result collected the majority of data in this study. It is assumed that greater notifications of *G. intestinalis* infection would stem from advantaged LGAs with easier access to local GPs. Additionally, individuals with higher socioeconomic status typically benefit from better healthcare services than their counterparts [37,38]. While a circular scan statistic was utilised in the purely spatial and space-time analyses in SaTScan, it does have limitations in detecting irregularly shaped clusters. An alternative would be to use the elliptic scan statistic which has a long and narrow shape to identify non-circular clusters [73]. Although due to the large surface area of NSW, it was more logical to use a circular scan statistic to cover more of the study region.

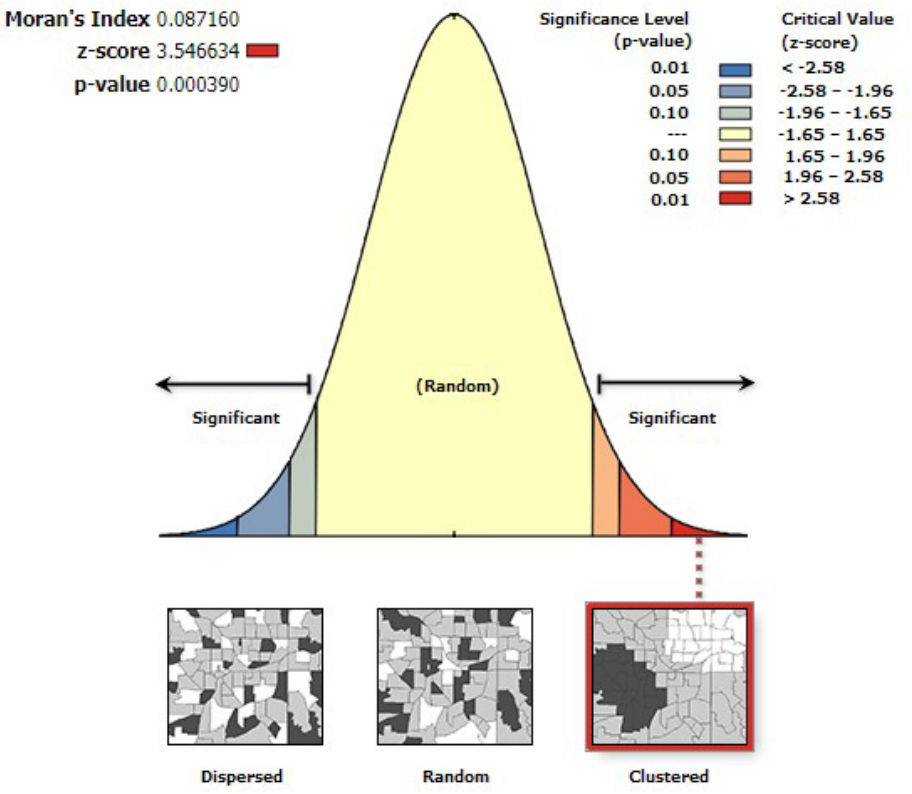
4.6. Conclusion

This study provides a first attempt to visually identify and describe the geospatial and temporal characteristics of *G. intestinalis* cases as well as the molecular assemblages A and B. In utilising SaTScan to analyse spatial, temporal and space-time data, and ArcGIS to help build maps and manipulate data with several available GIS tools, we succeeded in identifying geographical clusters and trends representing high rates of giardiasis in Northern Sydney, South-Eastern Sydney, Western NSW, and Hunter New England. Spatiotemporal analyses also detected four significant clusters during the warmer seasons and leading into early autumn months. These clusters were observed in metropolitan Sydney and rural Western and Southern NSW, indicating that there are common seasonal risk factors between these regions that are of public health importance. Males residing in regional NSW and adult females in urban Sydney also appeared to be at higher risk of infection. This implies that there are gender specific risk factors that need to be addressed for public health intervention. Further investigations need to be performed regarding occupation risks for day-care and childcare workers as well as agricultural farmers. Interestingly, *G. intestinalis* assemblage distribution was found to be randomly dispersed in NSW. This study will assist policy makers in implementing targeted interventions in specific geographic areas of NSW, thereby ensuring that limited resources are used to the utmost efficiency.

4.7. Supplementary figures



Supplementary Figure S4. 1 NSW local health districts (LHDs). A map overview of NSW, showing the 15 local health districts (LHDs). The Sydney metropolitan region is made up of eight LHDs: Central Coast, Illawarra Shoalhaven, Nepean Blue Mountains, Northern Sydney, South-Eastern Sydney, South-Western Sydney, Sydney, and Western Sydney. The rural and regional NSW LHDs include the Far West, Hunter New England, Mid North Coast, Murrumbidgee, Northern NSW, and Western NSW.



Supplementary Figure S4. 2 Spatial Autocorrelation Moran's *I* (Z = 3.55, global Moran's *I* = 0.09, $p \leq 0.01$) [22].

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4.10. Ethical approval

Ethics approval for the conduct of this study was received from the South-Western Sydney Local Health District Human Research Ethics Committee (HREC) which is accredited by the NSW Ministry of Health (HREC approval number: HE18/059 LNR), and the University of Technology Sydney (UTS approval number: ETH21-5951).

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Chapter 5

The re-emergence of human *Giardia intestinalis* infection in Australia, with an emphasis on recent molecular epidemiological findings

Certificate:

I certify that the following chapter is largely my own work, although the contributions of other authors are duly recognised. The contribution of other authors are detailed as follows:

- By providing suggestions on topics to be reviewed
- By providing advice on geospatial analyses and parameters
- By proof reading draft manuscripts
- By providing suggestions to improve layout, structure and writing style
- By correcting spelling and grammatical errors in drafts

Otherwise, the main composition of this work is credited to me.

I hereby certify that the above statements are true and correct:

Patricia Zajaczkowski, PhD Candidate

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17/11/2022

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The re-emergence of human *Giardia intestinalis* infection in Australia, with an emphasis on recent molecular epidemiological findings

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5.1. Abstract

Giardia intestinalis is a protozoan parasite that causes mild to severe diarrhoeal illness in humans. Although more frequently detected in developing countries, giardiasis has received considerable attention across the developed world due to a steady rise in prevalence, even as improved health care and disease surveillance systems have been implemented. The re-emergence of *G. intestinalis* is concerning, as it suggests there are unique risk factors and disease reservoirs present that are contributing to this rise in case numbers. It must also be considered that recent global changes including rapid urbanisation and climate change are major determinants for the increased risk of giardiasis in developed countries. However, reliably identifying, and characterising sporadic cases and outbreaks of giardiasis remains difficult. This is partly because current molecular diagnostic tools for typing *G. intestinalis* require at least two genetic markers for reliable PCR amplification. This review investigates the current body of knowledge on genetic sub-structuring within this 'species complex'. It further explores the important developments made in understanding of the molecular epidemiology of giardiasis in recent years and addresses the long-standing question of why giardiasis cases are continuously rising in number across human populations within Australia.

5.2. Introduction

Diarrhoeal diseases are a leading cause of child mortality and morbidity worldwide. Annually, there are an estimated 1.7 billion cases of diarrhoeal disease in children under five, of which 525,000 lead to death [1]. While there are several viral and bacterial pathogens that cause severe diarrhoeal illness in humans, a substantial number of cases are caused by protozoan parasites such as *Giardia intestinalis* (syn. *Giardia lamblia* and *Giardia duodenalis*). Owing to an estimated 280 million infections annually [2], this parasite is often associated with poverty, poor sanitation, and hygiene, as well as a lack of access to safe drinking water [3–5]. As low-income countries are most at risk, *G. intestinalis* has been included in the WHO Neglected Diseases Initiative to control the transmission of this parasite and improve global awareness of this disease [6].

In Australia, the infection rate of human giardiasis cases is estimated between 2 – 8% [7–9], and approximately 600,000 sporadic cases are reported each year [10]. While it is not regarded as a nationally notifiable disease, most government states and territories of Australia require that cases of *G. intestinalis* infection be recorded to each relevant public health unit (PHU). Australia's most populous state, New South Wales (NSW), reports an average of 3000 cases of giardiasis each year [11], making it one of the most common notifiable gastrointestinal diseases of humans (Supplementary Figure S5. 1). In comparison, surveillance reporting in Western Australia (WA) often detects more than 700 cases of giardiasis annually [12], suggesting that communities within Australia may also differ in agent, host and environmental determinants. In the context of developed countries, giardiasis is increasingly considered a re-emerging disease. Prior to the COVID-19 pandemic, rates of this disease have been increasing across northern Europe, the British Isles [13] as well as New Zealand [14] and Australia [11]. The success of this parasite can be attributed to its low infectious dose and the hardiness of its inactive cyst form – this allows for the easy spread of cysts between person to person, as well as contaminated food and water sources. Therefore, it is not surprising that giardiasis is closely associated with clustered outbreaks of diarrhoea within child-care settings and similar institutional facilities (Zajaczkowski *et al*., Chapter 3). Waterborne outbreaks of *G. intestinalis* infection are also consistently reported in developed countries suggesting that contaminated water sources, whether that be for drinking or recreational purposes, are a major factor in the re-emergence of giardiasis [15]. Recent global changes must also be considered as a reason for the increased risk of giardiasis. Rapid urbanisation, mass migrations and climate change may affect the dynamics and spread of diseases within endemic and global populations.

Molecular advances in DNA amplification, sequence-based technologies and genotyping capabilities have the potential to revolutionise our understanding of the molecular profile of *G. intestinalis*. When used in combination with epidemiological data, it becomes possible to not only describe the distribution of genetically characterised cases within human populations, but to explore the potential of zoonotic transmission and point-source outbreaks endemic to Australia. Currently, it is accepted that *G. intestinalis* is a species complex; an assemblage of species that are genetically related albeit morphologically identical. There are a total of eight assemblages documented, and these are referred to as assemblages A to H. Further genetic typing of these assemblages has revealed the presence of sub-assemblages [16,17] and sub-types [18], all which exhibit adaptation to different hosts. Here, we review the current diagnostic and molecular epidemiologic tools used for characterising *G. intestinalis* assemblages, explore the molecular epidemiology of human giardiasis with a strong focus on current and future global challenges, and address the barriers preventing the proper diagnosis and treatment of giardiasis in individuals residing in disadvantaged communities within Australia.

5.3. Diagnosis of *Giardia intestinalis* in clinical samples

In Australia, routine diagnosis of *G. intestinalis* infections relies on a multitude of assays, tools, and techniques. Traditional light microscopy to detect *G. intestinalis* cysts or trophozoites in duodenal or faecal samples remains the standard in most clinical laboratories [19,20]. The stool specimens may be examined as fresh smears to detect living, motile trophozoites or as concentrated samples using formalin-ethyl acetate, formalin, or polyvinyl alcohol [21]. Samples may also be stained with iodine, trichrome, iron-haematoxylin or Giemsa to enhance detection of *G. intestinalis* cysts and trophozoites, and to help differentiate from other microorganisms, protists, or debris [19,21]. As microscopic diagnosis often lacks sensitivity and/ or specificity, commercial immunological methods are additionally employed for the detection of *G. intestinalis* antigens in biological samples [22]. The two most common techniques include the enzyme-linked immunosorbent assay (ELISA) and direct fluorescent-antibody (DFA) tests [21,23,24]. These methods are found to be advantageous over light microscopy, having the ability to perform mass screening of multiple stool samples in a rapid, inexpensive, and labour-effective manner [23,25].

In recent years, several molecular methods based on polymerase chain reaction (PCR) for protozoan parasites have been developed. In Australia, a number of diagnostic and hospital pathology laboratories have transitioned to using real-time PCR (RT-PCR) and multiplex real-time PCR (MT-PCR) in combination with commercially available assays to diagnose a variety of enteric pathogens

[26,27]. These tools are the gold-standards for protozoan parasite detection and in comparison to conventional methods, allow for rapid identification from clinical samples and can achieve sensitivity and specificity levels of 100% [26,28–30]. The high sensitivity of PCR allows detection of *G. intestinalis* in mixed infections with other enteric protozoa, bacteria, and viruses. Since most enteric pathogens are transmitted via the faecal-oral route, and thus often share exposure sources, concomitant infections with *G. intestinalis* infection are well documented globally [31–33]. A recent study in Australia found that nearly half (49.1%) of all *G. intestinalis* infections in humans had co-infecting pathogens, of which majority were other protozoan parasites such as *Blastocystis hominis*, *Dientamoeba fragilis* and *Campylobacter* spp. (Zajaczkowski *et al*., Chapter 3). This was echoed in an earlier investigation into waterborne outbreaks in the United States, which documented multiple co-infecting parasites including *G. intestinalis*, *Cryptosporidium* spp., and *Entamoeba* spp. [34].

5.4. *Giardia intestinalis*: a "species complex"

Previous allozyme and DNA sequence analyses have classified *G. intestinalis* as a species complex that comprises of eight, genetically unique lineages referred to as assemblage A to H. While humans are primarily infected by assemblages A and B, the remaining assemblages C to H are specific to canids (C, D), domestic and wild hoofed animals (E), cats (F), rodents (G) and marine vertebrates (H) (Table 5. 1). Isolates from assemblages A and B have been successfully cultured *in vitro*, and whole genome sequencing (WGS) analyses support the theory that both assemblages should be regarded as two separate species of *Giardia* [35–37]. More recently, an alternative naming system has also been proposed by Wielinga *et al*. (2023) who performed a taxonomic review of the *G. intestinalis* species complex. Most notably, the study proposes renaming assemblage AI as *G. duodenalis*; AII as *G. intestinalis* and assemblage B as *G. enterica* [35,38–40] (Table 5. 1).

Table 5. 1. Summary of *Giardia* species complex assemblages A to H, the major host species, and proposed species naming system.

Assemblage	Proposed nomenclature[a]	Major Host(s)	References
AI	*G. duodenalis*	Humans, cats, dogs, horses, marsupials, non-human primates, pigs, wild and domestic ruminants, wolves	[41–52]

AII	*G. intestinalis*	Humans, cats, ruminants	[17,53–55]
AIII	*G. cervus*	Ruminants	[16,41,56]
B	*G. enterica*	Humans, non-human primates, rabbits, horses, ruminants, pigs, captive rodents, dogs, wolves, marsupials	[41–43,47,50,51,57–59]
C	*G. canis*	Coyotes, dogs, raccoon dogs	[16,43,60–62]
D	*G. lupus*	Coyotes, dogs, raccoon dogs	[16,60–64]
E	*G. bovis*	Humans, horses, pigs, wild and domestic ruminants	[64–66,53,67,68]
F	*G. cati*	Cats	[16,44,62,64]
G	*G. simoni*	Domestic and wild rodents	[16,43,64]
H	*G. pinnipedis*	Seals, gull	[69]

[a]An alternative naming system proposed [35,38-40]

5.4.1. Common molecular epidemiologic tools

While the use of PCR-based methods is gaining prevalence in clinical laboratories, most methods involving genotyping *G. intestinalis* are limited to research laboratories. There are several molecular markers that have been developed for use in genotyping *G. intestinalis* by PCR, although the most common are the *β*-giardin (*bg*), glutamate dehydrogenase (*gdh*) and the triosephosphate isomerase (*tpi*) genes [70–74]. Other studies also partially sequence the small subunit ribosomal DNA (SSU-rDNA) which is a particularly sensitive marker due to its multicopy nature, and highly specific due to strong sequence conservation [74]. However, genotyping *G. intestinalis* isolates at a single locus has proven to be unreliable. Several studies have reported inconsistent results in data generated from analysis using a single marker [17,75], and erratic assignment of assemblages by different markers. This is in part due to the test sensitivity and specificity of targeted loci, as well as potential mismatches in the binding regions of the primer sets used [67,76,77]. To combat this issue, a multi-locus sequence typing (MLST) methodology is recommended to type *G. intestinalis* isolates at three or more genetic markers (*bg*, *gdh*, *tpi* and SSU rRNA) [9].

Mixed-assemblage infections are also known. These infections are difficult to discriminate with a single-locus PCR, often leading to the preferential amplification of an assemblage depending on the marker being used, and the quantity of the assemblage present [74,78]. In a previous review, Zajaczkowski *et al*. noted that the use of assemblage-specific *tpi* primers allowed for the detection of more mixed-assemblage infections in comparison to the standard primers for PCR [45,74,79]. This was confirmed in a genotyping study based in Australia that found almost 30.0% of cases had mixed-assemblage infections after typing at the *tpi* locus (Zajaczkowski *et al*., Chapter 3). In contrast, the same study identified only 8.0% mixed-assemblage infections when typing the same isolates using SSU-rDNA.

Analyses of nucleotide sequences have further identified genetic variation within *G. intestinalis* assemblages and differentiated them into phylogenetic clusters known as ‘sub-assemblages’ (Supplementary Table S5. 1). These sub-assemblages have been reported in assemblage A (AI, AII and AIII) and assemblage B (BIII and BIV) and appear to differ in host preference [16]. Sub-assemblages AII, BIII and BIV are regarded as anthroponotic whereas AI is predominant in humans, livestock, and companion animals [18]. Sub-assemblage AIII was identified from wild ruminants such as deer [80].

To further confuse matters, sequence heterogeneity was also documented within each sub-assemblage [18]. These genetic variants are referred to as ‘sub-types’ and often differ from one

another by a single point mutation [16]. A number of these sub-types have been identified with the use of multi-locus sequence typing (MLST) analyses targeting the beta-giardin (*bg*), glutamate dehydrogenase (*gdh*) and triose-phosphate isomerase (*tpi*) loci [18]. Within sub-assemblage AI there are a total of four sub-types (A1, A5, A8 and A9), and within sub-assemblage AII there are six sub-types reported (A2 – A4, A7, A11 and A12). Sub-assemblage AIII currently has one sub-type documented (A6) (Supplementary Table S5. 1). Assigning sub-types to assemblage B isolates remains difficult due to its considerably higher sequence diversity and lower phylogenetic resolution at commonly used loci [74].

5.4.2. Spectrum of clinical manifestations

It was suggested that a large proportion of *G. intestinalis* infections represent asymptomatic cases [81,82]. However, those without symptoms are difficult to detect as they are also less likely to attend their general practitioner for a diagnosis. A recent genotyping study by Zajaczkowski *et al*. Chapter 3 noted that asymptomatic *G. intestinalis* cases were often identified inadvertently during routine health screening for immunocompromised individuals and recently arrived refugees, as well as for household members residing with an already confirmed *G. intestinalis* positive case. Although often asymptomatic, *G. intestinalis* infections can lead to a variety of self-limiting symptoms, the most common being acute or chronic diarrhoea, abdominal pain, bloating, nausea, vomiting, weight loss and fatigue (Zajaczkowski *et al*., Chapter 3). Despite not being a life-threatening disease, some cases of *G. intestinalis* infection can become chronic and persistent. If not treated adequately, reinfections can occur, and symptoms can have long-term complications including reactive arthritis, irritable bowel syndrome (IBS), malnutrition and a slowing of physical and mental development in children [83–87]. Immunocompromised or immunosuppressed individuals such as those with human immunodeficiency virus and/ or acquired immunodeficiency syndrome (HIV/AIDS) can not only develop life-threatening symptoms, but are also at a greater risk of refractory giardiasis and chronic gastrointestinal complications [88–90].

In developed countries, first-line treatment options include the commonly prescribed drugs: metronidazole, tinidazole and furazolidone (5-nitroimidazoles) as well as albendazole (benzimidazole). These drugs are highly effective against *G. intestinalis* and readily available as prescription-only medicine, however negative side effects and clinical relapses do occur and can result in treatment failures. Children are more susceptible to treatment failure and subsequent reinfections, as they are more likely to be less compliant in completing the whole course of drugs [91]. Drug resistant isolates of *G. intestinalis* have also been reported and specifically for metronidazole which is the most frequently used drug [92]. To combat this resistance, alternative

anti-parasitic drugs are employed including albendazole, mebendazole, nitazoxanide, and paromomycin. Fumagillin, an antimicrosporidiosis drug, has also shown promise as an anti-*Giardia* drug although clinical trials have yet to be completed before it can be introduced as an alternative option for treatment [93].

5.4.3. Molecular pathogenesis

The broad spectrum of symptoms exhibited by *G. intestinalis* infection may be attributed to a variety of mechanisms including infection dose, host-parasite factors, host immune and nutritional status, as well as host age and gender [94]. The pathogenesis of *G. intestinalis* infection is still not well understood; however, several studies have employed *in vitro* and *in vivo* models to identify the processes involved in triggering specific symptoms [95,96]. Currently it is recognised that *G. intestinalis* trophozoites cause damage to the human duodenum surface, as well as atrophy of the microvillus brush-border of the intestinal epithelial cells [94,97]. This can explain the typical symptoms of malabsorption and maldigestion. It has also been suggested that clinical symptoms are determined by the variable genetics of the parasite and the impact *G. intestinalis* assemblages have on the host's small intestine [74]. This is not surprising, as assemblages A and B have been found to differ in growth rates, generation time and drug sensitivity [94].

Studies that have attempted to identify links between clinical symptoms of *G. intestinalis* infection and assemblage type are incredibly limited. Pavanelli *et al.* compared the effects of infection caused by sub-assemblage AII and BIV on the response of the small intestine, and behavioural parameters in mice [98]. Overall, sub-assemblage AII was found to be more aggressive than BIV, not only causing a greater number of tissue lesions but inducing changes in the intestinal microbiota. These results were confirmed by a number of epidemiological studies that have identified significant associations between assemblage A infection and serious clinical symptoms [8,73,99–102]. Despite this, assemblage B has been implicated as the cause of persistent and chronic infection, as well as more serious symptoms [71,103–107]. In addition, a recent study in Australia identified no associations between either assemblage type and severity of symptoms (Zajaczkowski *et al*., Chapter 3). This result echoed previous studies reported globally [5,70,108,109]. Although there are various reports from around the world that have attempted to correlate *G. intestinalis* assemblages with symptomatology, the observations remain largely conflicting.

5.5. Prevalence and disease burden of *G. intestinalis* in the global population

Globally, more than 200 million people are at risk of *G. intestinalis* infection [2] although prevalence of human giardiasis is higher in developing countries and regions of low socio-economic status [18]. Studies have reported infection rates between 0.9% to 22.9% in Asian and Pacific countries [4,43,55,67,99,109–112], 0.4% to 48.7% in European nations [76,113–115], 5.5% to 24.2% in the Middle-east [5,77,116,117], 14.9% to 27.6% in South America [84,118–123] and 2.1% to 67.2% in African nations [71,124–133] (Figure 5. 1). Most of these studies focused on children under the age of 15 years who attended municipal day-care centres or primary schools [84,113,115,116,118,130]. Other studies enrolled symptomatic adults presenting at local hospitals and health care centres, as well as livestock farmers in rural or remote regions [43,55,99,124,125]. The incidence of *G. intestinalis* infection is lower in developed countries, and this can be attributed to better access to water, sanitation, and hygiene infrastructure. Instead, the risk factors of giardiasis in developed countries appear to be related to socioeconomic, lifestyle and behavioural determinants. Recent studies in industrialised nations show infection rates of 4.0% in Belgium [134], 2.9% to 4.6% in Canada [135,136], 4.3% in Germany [49], 1.8% in Italy [137], 0.6% in Japan [138], 4.3% in the Netherlands [139], 7.6% in New Zealand [140], 3.7% to 6.8% in Portugal [70,141], 15.6% in Spain [142], 0.5% in South Korea [143], 1.3% to 2.3% in the United Kingdom [144,145], 1.4% in the United States [146] and 1.9% to 7.6% in Australia [7–9] (Figure 5. 1).

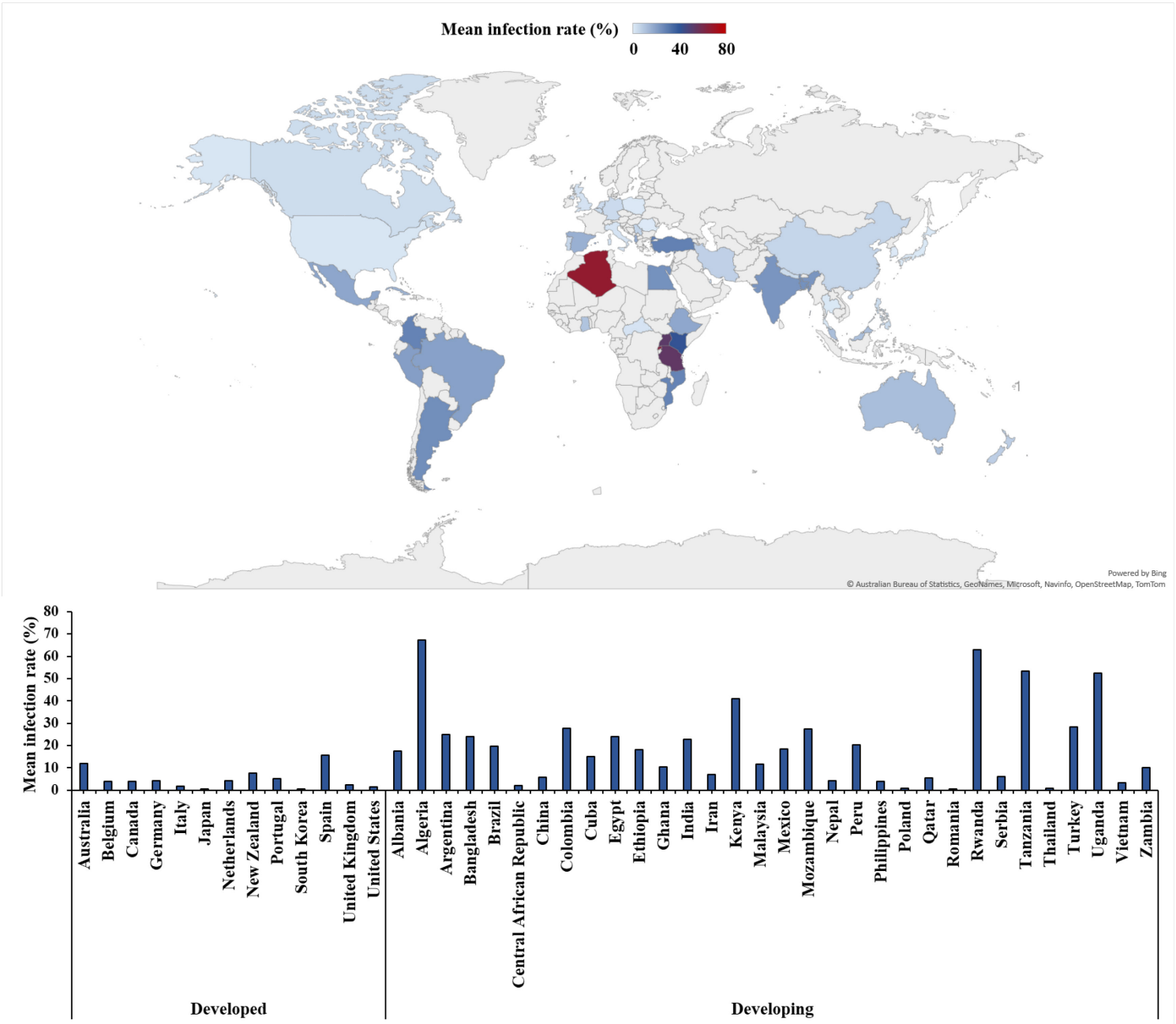


Figure 5. 1. Global mean infection rates (%) of human *G. intestinalis* infection. The mean infection rates (%) of *G. intestinalis* infection across the developed and developing world. For developed countries, the infection rates ranged from 0.5% to 15.6%. Spain reported the highest infection rate in comparison to other developed countries. For developing countries, the infection rates ranged from 0.4% to 67.2%. Algeria reported the highest infection rate in comparison to other developing countries.

The prevalence of *G. intestinalis* infections also varies within Australian states and territories, suggesting that differences in population characteristics and socioeconomic determinants are directly linked to the incidence rates of giardiasis. While rates of infection were similar in Queensland (2.0%) [7] and Victoria (2.5%) [9], there was an almost three times higher rate seen in children living in Western Australia (7.6%) [8]. This is not a surprising result as children are often implicated as the main source of person-to-person transmission of giardiasis [147]. Indeed, children residing in remote Indigenous communities in the Northern Territory were reported as having a prevalence of *G. intestinalis* infection at almost 70.0% [148]. However, care should be taken when comparing prevalence rates between regions, as the study population, sampling strategies and diagnostic methods used can vary between studies. Molecular studies based in these states and territories also found a predominance of assemblage B infections as opposed to assemblage A [7,8,148] (Table 5. 2). A more recent genotyping study in Australia's most populous state, NSW, similarly identified a higher prevalence of assemblage B, however they also detected a high number of mixed-assemblage A and B infections, and only a small number of assemblage A cases (Zajaczkowski *et al*., Chapter 3). It was suggested that the increased rate of cyst shedding seen in assemblage B infections in comparison to assemblage A would account for its increased incidence [108]. Indeed, meta-analyses of several molecular typing studies have reported a higher prevalence of assemblage B infections in developed and developing countries worldwide [72,107,135,149].

Table 5. 2. Occurrence of *G. intestinalis* assemblages and/or sub-assemblages in humans in Australia.

State/ Territory	Study population and sample size	Age group (years)	*Giardia*-positive samples (*n*)	Total number of samples successfully genotyped (*n*)	Methodology	Genetic locus targeted	*G. intestinalis* assemblage *n* (%)				Sub-assemblage(s) (*n*)	Reference
							A	B	A+B	E		
New South Wales	*Giardia*-positive samples were collected from various private pathology clinics and public hospitals (*n* = 169)	All ages	169	147	Assemblage-specific PCR	*tpi*	27 (18.4)	80 (54.4)	40 (27.2)	-	-	(Zajaczkowski *et al*., Chapter 3)
				136	PCR sequence analysis	SSU-rDNA	25 (18.4)	100 (73.5)	11 (8.1)	-	-	
New South Wales	Clinical samples positive for *Giardia* were collected from pathology laboratories (*n* = 243)	All ages	243	165	PCR-RFLP	*gdh*	21 (12.7)	142 (86.1)	2 (1.2)	-	-	[150]
New South Wales	Clinical samples positive for *Giardia* were collected from pathology laboratories (*n* = 73)	Not reported	73	72	T-RFLP	*gdh*	-	-	-	-	AI (1/72); AII (10/72); BIII (3/72); BIV (54/72); BIII/BIV (4/72)	[151]

REF analysis: restriction endonuclease fingerprinting analysis; T-RFLP: terminal-restriction fragment length polymorphism

Table 5. 2. Continued.

State/ Territory	Study population and sample size	Age group (years)	*Giardia*-positive samples (*n*)	Total number of samples successfully genotyped (*n*)	Methodology	Genetic locus targeted	*G. intestinalis* assemblage *n* (%)				Sub-assemblage(s) (*n*)	Reference
							A	B	A+B	E		
Northern Territory	Faecal samples were collected as part of a separate ivermectin study from children living in a remote community (*n* = 87)	< 15	54	45	PCR sequence analysis	SSU-rDNA	11 (24.4)	34 (75.6)	-	-	-	[148]
				32	T-RFLP	*gdh*	-	-	-	-	AII (8/32) BIII (3/32) BIV (12/32) BIII/BIV (9/32)	
Queensland	Faecal samples positive for *Giardia* were collected from patients in rural and urban communities (*n* = 88)	All ages	88	88	Assemblage-specific PCR	*tpi*	44 (50.0)	34 (38.6)	4 (4.6)	6 (6.8)	-	[66]
				30	PCR sequence analysis	*gdh*	-	-	-	6 (20.0)	AII (11/30) BIV (13/30)	
Queensland	Human outpatients with clinical histories of gastrointestinal symptoms (*n* = 695)	All ages	13	13	PCR sequence analysis; REF analysis	*tpi*	4 (30.8)	9 (69.2)	-	-	-	[7]

REF analysis: restriction endonuclease fingerprinting analysis; T-RFLP: terminal-restriction fragment length polymorphism

Table 5. 2. Continued.

State/ Territory	Study population and sample size	Age group (years)	*Giardia*-positive samples (*n*)	Total number of samples successfully genotyped (*n*)	Methodology	Genetic locus targeted	*G. intestinalis* assemblage *n* (%)				Sub-assemblage(s) (*n*)	Reference
							A	B	A+B	E		
Western Australia	Sporadic human cases collected from a diagnostic pathology laboratory (*n* = 124)	0 - 70	124	124	PCR sequence analysis	SSU-rDNA	31 (25.0)	93 (75.0)	-	-	-	[152]
Western Australia	Stool samples were collected from children attending day-care centres (*n* = 353)	< 5	27	23	PCR sequence analysis	SSU-rDNA	7 (30.4)	16 (69.6)	-	-	-	[8]

REF analysis: restriction endonuclease fingerprinting analysis; T-RFLP: terminal-restriction fragment length polymorphism

5.6. *Giardia intestinalis* in Australia: current and future challenges

Giardia cysts are not only highly infectious but incredibly resilient to environmental exposures. As a result, infection with *G. intestinalis* is commonly associated with ingesting contaminated food or water sources, as well as through direct person-to-person transmission [11,153]. Sexual transmission of *G. intestinalis* cysts has also been reported albeit rarely [154,155]. In the developed world, *G. intestinalis* infection is increasingly regarded as a re-emerging disease due to its contribution to outbreaks of diarrhoeal diseases within day-care and aged-care facilities [156]. It also accounts for the majority of reported waterborne outbreaks in industrialised nations [157,158] and has been implicated as one of the most important parasitic causes of 'traveller's diarrhoea' [159]. As cases of endemic giardiasis continue to rise in Australia, it is essential that we consider the extent to which current and future global changes will have on risk factors associated with *G. intestinalis* infection. Although most developed countries have implemented surveillance systems and preventative strategies to control the spread of giardiasis, it is speculated that issues of over-population, rapid urbanisation, and climate change will introduce new sources of infection and bolster current transmission pathways.

5.6.1. Population growth and overcrowding

Rapid rates of urbanisation in Australia and the increase in populations residing in crowded, urban dwellings have created new opportunities for the re-emergence of enteric protozoan diseases. Increasing fertility and longevity rates, as well as the rise of Australia's net overseas migration are the main factors driving this growth in population numbers. More than 70.0% of Australia's current population is concentrated in major cities, with the remainder residing in inner and outer regional areas [160]. It is therefore no surprise that recent geospatial studies have found metropolitan areas are a high risk for giardiasis (Zajaczkowski *et al*., Chapter 4).

It is largely suggested by Zajaczkowski *et al*. that the dissemination of *G. intestinalis* in Australian urban areas is facilitated by children aged 5 years old or under attending child-care facilities and preschools. In Australia, there is an increasing demand for child-care centres which is in part due to rapid population growth, as well as changing workforce trends. Indeed, the Australian Bureau of Statistics (ABS) reported that nearly half (46.4%) of children aged 0-5 years old attended formal child-care in 2021 [161], an increase of 20.3% since 2011 [162]. As transmission of *G. intestinalis* in humans is mainly driven by faecal-oral contamination, young children are at a greater risk of acquiring the infection through poor hygiene and sanitation behaviours [163]. A molecular analysis

conducted by Zajaczkowski *et al*. reported that children aged under 5 and adults at parental age maintained a higher prevalence of *G. intestinalis* assemblage B in comparison to assemblage A, suggesting that child-care workers, caregivers, and family members are at risk of exposure to giardiasis via infected toddlers (Zajaczkowski *et al*., Chapter 3). A previous longitudinal study of child-care centres in Western Australia also found that children infected with isolates of *G. intestinalis* were more likely to be infected with assemblage B [8]. Assemblage B infections have previously demonstrated a higher level of cyst shedding, which would facilitate a faster spread within institutional settings and areas where children frequent [108]. Likewise, there is a potential for reinfection with the same assemblage type, particularly in facilities where there is a greater risk of person-to-person transmission between children.

5.6.2. Giardiasis in a developed world – is it endemic or imported?

In the past two decades, rapid changes in international travel have led to a surge in low-cost carriers and an increased demand for international travel. This increased global connectivity has provided the means for infectious diseases to spread across international populations. Indeed, international travel has long been implicated as a significant factor in the acquisition and dissemination of giardiasis in developed countries, and has often been referred to as a 'traveller's disease' [164]. Zajaczkowski *et al*. designed the first case-control study in Australia to examine travel history amongst giardiasis cases [11]. It reported that international travel was a significant factor for infection in both univariate and multivariable analyses [11]. Additionally, the study found that those travelling to South and Southeast Asia, Central Asia, North Africa, and Oceania had a 20 times greater risk of *G. intestinalis* infection [11]. This result echoed a previous global study that had observed a total of 25,867 returned travellers and concluded that the rates of giardiasis were highest in individuals travelling to African, Asian and South Pacific regions of the world [165]. Nevertheless, these studies had not investigated further the potential transmission pathways and sources of infection within the countries visited, and so it is difficult to ascertain how travellers are initially infected. There is a consensus that inadequate sanitation and hygiene, and poor drinking water systems are major risk factors for *G. intestinalis* infections in developing regions [3,4]. It is speculated that person-to-person contact and ingesting contaminated water are the greatest risks to international travellers frequenting developing countries.

In recent years, it has become apparent that cases of domestic *G. intestinalis* are being underreported in industrialised countries [74]. This is particularly true as patients with travel histories are more likely to be routinely screened for *G. intestinalis* infection [166]. Indeed, an audit of Scottish diagnostic microbiology laboratories determined that less than 20.0% of stools tested for

G. intestinalis would originate from domestic cases [166]. Moreover, a study in Australia found that more than 90.0% of *G. intestinalis*-positive individuals did not travel overseas prior to illness onset, suggesting that most of the cases were acquired through endemic transmission and domestic risk factors (Zajaczkowski *et al*., Chapter 3). Studies based in Germany [167] and Spain [168] also determined that the vast majority of individuals with giardiasis were domestic citizens with no record of travelling abroad.

Very few studies have molecularly characterised *G. intestinalis* in travel associated cases. A study based in London observed that the majority of international travellers were infected with assemblage B, although those who visited the Far East were predominantly infected with assemblage A [100]. This is not a surprising result, as assemblage B tends to have the highest prevalence rates worldwide. Interestingly, molecular typing analyses in Australia found that that individuals with a history of overseas travel were six times more likely to be infected with mixed assemblages A and B (Zajaczkowski *et al*., Chapter 3). As the occurrence of mixed-assemblage infections appears to be higher in developing countries as opposed to developed regions of the world [169], it may be that these mixed infections are being picked up by travellers and directly imported into Australia. Although not statistically significant, Zajaczkowski *et al*. determined that travel-associated infections did not harbour any single assemblage A (Zajaczkowski *et al*., Chapter 3). While this does suggest that assemblage A infections are autochthonous in Australia, more studies are needed to confirm if *G. intestinalis* assemblages differ in their transmission pathways and infectious sources.

5.6.3. Zoonotic risk

Giardia intestinalis is often observed in domesticated and wild animals as well as livestock, although the majority of global prevalence studies focus on the cattle industry [18]. Overall, the global, pooled prevalence of *G. intestinalis* in cattle is estimated to be 16.0% to 24.0% depending on the methodology used for detection [170] Reported prevalence rates in livestock are often higher in younger animals, whilst older livestock animals demonstrate lower and persistent prevalence rates [74]. These notable differences in the reported infection rates are also related to variances in study design, sampling size, techniques employed for diagnosis as well as farming practices regarding the animals.

There are emerging concerns that livestock animals may operate as reservoirs for sporadic giardiasis in humans [74]. Grazing animals have the potential to cause widespread environmental contamination with infectious cysts, and particularly in countries with traditional animal husbandry

systems where cattle are free-roaming and have direct access to rivers, streams, or other water supplies. Younger animals, such as calves, have been reported to excrete significant quantities of cysts into the environment, posing a greater risk of infection to humans, wildlife, and other grazing livestock [171]. On a global scale, livestock animals are predominantly infected with *G. intestinalis* assemblage E – a genotype commonly isolated from cloven-hoofed, grazing animals [170,172]. Multiple studies in Australia agree that assemblage E is the dominant assemblage in infected livestock (Table 5. 3)., and this is echoed in reports from Europe and North America [16,18,79]. While this may indicate that the public health risk of zoonotic giardiasis is minimal, it cannot be ignored that the human-specific assemblage A is increasingly isolated from livestock animals [172].

In New South Wales, Australia, 32.0% of cattle grazing close to drinking water catchments were found to be infected with assemblage A using assemblage-specific primers that targeted the *tpi* locus [173]. Subtyping a subset of these *G. intestinalis* assemblage A sequences identified the zoonotic sub-assemblage AI and the human-specific sub-assemblage AII [173]. Likewise, a longitudinal study by Yang *et al*. across four states in Australia, reported that sheep were predominantly infected with assemblage E and sub-assemblage AII – a sub-assemblage that has been identified in humans in Australia previously [54,152].

In Australia, *G. intestinalis* has been reported in domestic dogs and cats with prevalence rates of 9.4% and 2.0%, respectively [174]. Molecular epidemiological studies of *G. intestinalis* in dogs have determined that they are almost exclusively infected with the host specific assemblages C and D, while cats are predominantly reported with assemblage F (Table 5. 1). Domestic animals in Australia have also been reported with human-specific assemblages A and B albeit with low prevalence rates (Table 5. 3). Interestingly, it has been proposed that the spread of potentially zoonotic assemblages between humans and their pets is favoured in domestic households where there is a greater chance of interaction between both hosts [74]. Read *et al*. utilised PCR-RFLP and characterised nine samples originating from dogs living alongside humans in a Western Australian community [75]. One sample was identified as the zoonotic sub-assemblage AI, whilst two dogs were found to harbour the anthroponotic sub-assemblage BIV [75], overall suggesting that domestic pets might pose a higher zoonotic risk depending on if there is an established transmission source between owner and pet. However, the discovery that similar assemblages and sub-assemblages exist in many host species, is not, by itself, proof that zoonotic transmission is occurring [74]. Rather, the circulation of potentially zoonotic *G. intestinalis* cysts in the environment can lead to indirect infections reported in animals. Humans may be considered as major reservoirs of giardiasis for animals living on the outskirts, or in, urban areas. Indeed, this calls for further investigations into

the dynamics of reverse zoonotic transmission or ‘zooanthroponosis’ across hosts that share living spaces and environments.

Table 5. 3. Occurrence of *G. intestinalis* assemblages in wildlife, livestock, and domestic pets in Australia.

State/ Territory	Host origin	Total number of samples genotyped (*n*)	Methodology	Genetic locus targeted	*G. intestinalis* assemblage (%)								Reference
					A	B	C	D	E	F	A+E	Other	
New South Wales	Rabbit	16	PCR sequence analysis	*tpi*	-	16 (100.0)	-	-	-	-	-	-	[173]
New South Wales	Kangaroo	47	PCR sequence analysis	*tpi*	33 (70.2)	13 (27.7)	-	-	1 (2.1)	-	-	-	[173]
New South Wales	Sheep	39	PCR sequence analysis	*tpi*	8[a] (20.5)	-	-	-	31 (79.5)	-	-	-	[173]
New South Wales	Cattle	78	PCR sequence analysis	*tpi*	25[b] (32.0)	2 (2.6)	-	-	51 (65.4)	-	-	-	[173]
New South Wales	Cattle	29	PCR-RFLP	*gdh*	-	9 (31.0)	-	-	20 (69.0)	-	-	-	[150]
New South Wales	Wallaby	12	PCR sequence analysis	SSU-rDNA	7 (58.3)	5 (41.7)	-	-	-	-	-	-	[175]

[a] Further sub-typing at the *gdh* locus identified sub-assemblage AI; *n* = 2

[b] Further sub-typing at the *gdh* locus identified sub-assemblage AII; *n* = 5 and sub-assemblage BIV; *n* = 2

Table 5. 3. Continued.

State/ Territory	Host origin	Total number of samples genotyped (*n*)	Methodology	Genetic locus targeted	*G. intestinalis* assemblage (%)								Reference
					A	B	C	D	E	F	A+E	Other	
New South Wales	Sheep (weaning, post-weaning, and pre-slaughter)	73	PCR sequence analysis	*tpi*	32[c] (43.8)	-	-	-	41 (56.2)	-	-	-	[54]
Northern Territory	Water Buffalo (wild)	6	PCR sequence analysis	*gdh; bg*	-	-	-	-	6 (100.0)	-	-	-	[176]
Queensland	Deer (wild)	3	PCR sequence analysis	*tpi*	2 (66.7)	-	-	-	1 (33.3)	-	-	-	[173]
Queensland	Cattle	147	PCR sequence analysis	*tpi*	34[d] (23.1)	35 (23.8)	-	-	78 (53.1)	-	-	-	[173]
South Australia	Alpaca	1	PCR sequence analysis	*tpi*	1[e] (100.0)	-	-	-	-	-	-	-	[177]
South Australia	Sheep (weaning, post-weaning, and pre-slaughter)	88	PCR sequence analysis	*tpi*	26[f] (29.5)	-	-	-	62 (70.5)	-	-	-	[54]

[c] Further sub-typing at both the *gdh* and *bg* loci identified sub-assemblage AII; *n* = 2
[d] Further sub-typing at the *gdh* locus identified sub-assemblage AI; *n* = 2, AII; *n* = 5, BIII; *n* = 4 and BIV; *n* = 3
[e] Further sub-typing at the *tpi* locus identified sub-assemblage AI; *n* = 1
[f] Further sub-typing at both the *gdh* and *bg* loci identified sub-assemblage AII; *n* = 4

Table 5. 3. Continued.

State/ Territory	Host origin	Total number of samples genotyped (*n*)	Methodology	Genetic locus targeted	*G. intestinalis* assemblage (%)								Reference
					A	B	C	D	E	F	A+E	Other	
Victoria	Alpaca	1	PCR sequence analysis	*tpi*	1[g] (100.0)	-	-	-	-	-	-	-	[177]
Victoria	Wombat	1	PCR sequence analysis	*tpi*	1 (100.0)	-	-	-	-	-	-	-	[178]
Victoria	Deer (wild)	10	PCR sequence analysis	*tpi*	10 (100.0)	-	-	-	-	-	-	-	[178]
Victoria	Sheep (weaning, post-weaning, and pre-slaughter)	98	PCR sequence analysis	*tpi*	32[h] (32.7)	-	-	-	66 (67.3)	-	-	-	[54]
Western Australia	Marsupial[i]	12	PCR sequence analysis	*bg;* SSU-rDNA	2 (16.7)	-	3 (25.0)	-	1 (8.3)	-	-	6[k] (50.0)	[179]
Western Australia[j]	Cattle (pre-weaned)	75	PCR sequence analysis	SSU-rDNA	-	1 (1.3)	-	-	71 (94.7)	-	1 (1.3)	2[l] (2.7)	[180]

[g] Further sub-typing at the *tpi* locus identified sub-assemblage AI; *n* = 1
[h] Further sub-typing at both the *gdh* and *bg* loci identified sub-assemblage AII; *n* = 4
[i] Marsupials included the quenda and common planigale (or "marsupial mouse")
[j] Samples collected from five different farms in Western Australia and one farm from New South Wales
[k] Six samples were identified as the novel *G. peramelis* species (originally referred to as the 'quenda' genotype)
[l] One sample was a mixed A, B and E infection; the other sample was the novel *Giardia peramelis* species (originally referred to as the 'quenda' genotype)

Table 5. 3. Continued.

State/ Territory	Host origin	Total number of samples genotyped (*n*)	Methodology	Genetic locus targeted	*G. intestinalis* assemblage (%)								Reference
					A	B	C	D	E	F	A+E	Other	
Western Australia	Dog	9	PCR-RFLP	*gdh*	1 (11.1)	2 (22.2)	4 (44.5)	2 (22.2)	-	-	-	-	[75]
Western Australia	Cat	18	PCR-RFLP	*gdh*	6 (33.3)	2 (11.1)	2 (11.1)	7 (38.9)	1 (5.6)	-	-	-	[75]
Western Australia	Cat	7	PCR sequence analysis	SSU-rDNA	1 (14.3)	-	-	-	-	6 (85.7)	-	-	[181]
Western Australia	Sheep (weaning, post-weaning, and pre-slaughter)	214	PCR sequence analysis	*tpi*	16 (7.5)	-	-	-	190 (88.8)	-	8 (3.7)	-	[54]

5.6.4. Waterborne giardiasis

The cysts of *G. intestinalis* are not only resistant to environmental conditions but may remain viable for months in surface water. In addition, they are well known to have mild resistance to chlorine which poses serious challenges for water treatment authorities. These factors have established giardiasis as one of the most common causes of waterborne transmission across the globe. In the United States, water exposure made up almost 30.0% of all recorded outbreaks from 2012 to 2017 [181]. Tap water systems were the main source of infection in these outbreaks, followed by contact with contaminated outdoor freshwater and recreational pools. In Australia, *G. intestinalis* only gained notoriety as a waterborne pathogen during the 1998 Sydney water crisis [182]. During this incident, low levels of *Cryptosporidium* and *Giardia* were detected in the Greater Metropolitan Sydney water supply, and notices to boil tap-water were issued across the state of NSW. Although no symptomatic cases of cryptosporidiosis and giardiasis were reported during this incident, the event amassed strong media attention and generated major public alarm.

As water is a common vehicle for the transmission of *G. intestinalis* cysts, drinking water infrastructure such as rainwater harvesting (RWH) systems, bore water and wells have been implicated as major risk factors of giardiasis in Australia. RHW systems have become popular in both rural and urban regions in Australia, and it has been reported that more than one million households currently own a rainwater system [183]. Quantitative microbial risk assessment analyses based on RWH systems in urban Queensland, Australia found that compared to households with municipal water supply, the users of RWH had a higher risk of giardiasis [184]. This is not surprising as most studies agree that roof-collected rainwater may easily become contaminated following rainfall events, where bird and animal faecal matter found on the roof can be washed into the rainwater tank via runoff [184,185]. However, this was only true for those drinking rainwater contaminated with *G. intestinalis* cysts, and ultimately those using the tank water as non-potable water had a lower risk of infection [184]. Likewise, an epidemiological study in NSW, Australia found no significant association between giardiasis and those using water sourced from alternative supplies such as roof-harvested rainwater systems, tank water or bore wells [11]. It has been reported that only a small number of Australians (10.0%) will use RWH systems as a major source of their drinking water [186]. It is also suggested that the addition of filtration systems to alternative water supplies has contributed to the lower risks of infection seen in Australians.

In Australia, recreational exposure to pools, rivers, lakes, streams, and other bodies of water have long been implicated as major sources of waterborne outbreaks of gastrointestinal disease [158]. A recent epidemiological study on sporadic *G. intestinalis* cases in Australia had initially found that those who reported swimming were significantly associated with giardiasis [11]. However, when including confounding variables (i.e., age and sex) in the multivariable model, this significance was lost. This was not a surprising result as recreational swimming in Australia is a popular and frequent pastime and particularly amongst school-aged children who may take part in standard school swimming activities and educational swimming classes. While public pool waters are commonly disinfected with low doses of chlorine, *G. intestinalis* cysts have shown resistance to chemical disinfection [187]. Rather it is suggested that a combination of coagulation and high-rate sand filtering is best used to remove *G. intestinalis* cysts from pool water, however the majority of backyard pools do not employ these tactics putting pool-owners at risk [188].

5.6.5. Potential impact of climate change on G. intestinalis infection

Seasonal peaks of giardiasis in late summer and early autumn have been reported in Australia (Zajaczkowski *et al*., Chapter 3), Canada [189], and the United States [190]. These seasonal incidences of giardiasis are often reflective of underlying human behaviours and activities. For example, Australian agricultural practices such as calving and lambing will predominantly take place between spring through to autumn, suggesting that young livestock are significant facilitators of *G. intestinalis* infection in cattle farmers. Such a proposal requires that young livestock shed zoonotic *Giardia* assemblages. Warmer months also lead to an increased use of recreational water sources, particularly in urban areas as suggested in a recent spatio-temporal analysis in Australia (Zajaczkowski *et al*., Chapter 4). There is further evidence indicating that *G. intestinalis* infection rates are related to climatic factors such as heavy rainfall events and subsequent flooding disasters [191–193]. Although *G. intestinalis* cysts are incredibly hardy and can survive for prolonged periods of time in the environment, the viability and infectivity of these cysts is temperature dependent [18]. Hot and arid conditions are known to reduce the survivability of *G. intestinalis* cysts, particularly in soil [194]. However prolonged dry periods followed by intense rainfall is likely to flush cyst-contaminated soil into local waterways, reservoirs, or water supplies (Zajaczkowski *et al*., Chapter 4).

This seasonal variability of giardiasis suggests that global climate changes will have a direct impact on the frequency and intensity of cases in Australia. Current climate change observations have seen temperatures in Australia increase by each decade since 1950, and trends of record-breaking heatwaves and severe droughts are growing [195]. As a result, water shortage supplies for commercial, domestic, and agricultural purposes are common in across Australia, and this is particularly true for regional and remote areas which have no access to municipal piped-water supplies. To combat this growing water shortage crisis, the use of RWH systems and bore water wells have become a significant source of drinking water throughout regional Australia. However, this approach has led to an increased risk of human exposure to waterborne bacterial and protozoal pathogens [185]. Rising global temperatures have also been suggested to adversely impact climatic phenomena such as the *El Niño*-Southern Oscillation (ENSO), which often drives precipitation and drought events over Australia [196,197]. The impact of global warming is expected to cause strong and prolonged weather events such as severe droughts, bushfires and megafires during the drier *El Niño* years, and excessive rainfall, damaging storms, and catastrophic flooding events during the wetter *La Niña* years [198]. These shifting weather events are a threat to existing water infrastructure and water sources in Australia by indirectly influencing disease transmission pathways and facilitating exposure to these contaminated sources. Indeed, with greater climate variability and ongoing weather events, the frequency of enteric diseases will likely grow, and the burden of giardiasis may increase not only within Australia, but globally.

5.7. Addressing barriers to the diagnosis, treatment, and prevention of *G. intestinalis* infection in humans

As with most enteric pathogens, there are several surveillance steps that must occur before a laboratory-confirmed case can be ascertained. In Australia, a diagnosis requires an initial clinical appraisal of the patient followed by stool sample collection, testing and subsequent reporting and registration of the positive *G. intestinalis* case to a state-wide surveillance system. However, this sequence of events is entirely dependent upon the experience of the healthcare provider and upon whether a laboratory diagnostic of a stool specimen is requested [199]. In the United States, paediatricians in general practices lacked an overall awareness of giardiasis and were more likely to suspect viral causes in patients with diarrhoeal illness [200]. In addition, a survey of 455 clinical laboratories in the United States found that nearly 90% of laboratories did not routinely test faecal samples for enteric parasites, suggesting that

parasitic diseases such as giardiasis are likely to be underdiagnosed [201]. In Australia, the true burden of giardiasis is also underrepresented across low-socioeconomic urban communities and remote and/or rural regions where there is increased difficulty accessing primary healthcare services. This was confirmed by a recent geospatial analysis, wherein Zajaczkowski *et al*. observed significantly lower rates of *G. intestinalis* cases in the suburban regions of Western Sydney and South-Western Sydney; these being two of the most disadvantaged local government areas in NSW, Australia (Zajaczkowski *et al*., Chapter 4). It remains important to address the cost barriers that may affect individuals living in areas of socio-economic disadvantage. For example, it has been previously noted that general practitioners (GPs) working with socioeconomically disadvantaged patients are more likely to prescribe medications to cases of suspected disease rather than ordering diagnostic tests [202]. Additionally, GPs with strong clinical experience are more likely to be recruited in metropolitan health-care practices that are financially lucrative than a comparative practice in a disadvantaged, remote community (Zajaczkowski *et al*., Chapter 4). To establish a well-balanced system of affordable health care, there is an urgent need for affordable GPs and diagnostic laboratories to be employed within disadvantaged areas. However, until this issue can be addressed, the proportion of individuals unable to access health care will continue to grow along with the ever-increasing Australian population.

With the combined use of molecular typing and geospatial analyses, epidemiologists and policy makers can effectively target areas of high-risk and effectively control giardiasis by applying infection control principles, preventative practices, and public health policy management. Health education and training must be aimed towards members of the community most at risk of direct or indirect contact with infected individuals. Child-care workers, nursing home attendees, workers in diagnostic laboratories and GPs should be made aware of how *G. intestinalis* infection spreads, what the common symptoms and risk factors are, as well as the reporting requirements for their relevant public health unit. Likewise, healthcare workers should be notified that they cannot attend work while suffering from gastrointestinal symptoms, and must wait a minimum of 24 hours after symptoms have resolved before attending their workplace [203]. There is a pressing need to introduce and standardise newer molecular techniques such as MT-PCR in Australian diagnostic laboratories. In fact, the application of epidemiological research combined with novel PCR-based laboratory tools for *G. intestinalis* typing can facilitate a better understanding of the transmission pathways of this parasite as well as help to detect endemic outbreaks,

particularly those that have no obvious point source. Ultimately these tools not only simplify the process of routine screening for *G. intestinalis* infection, but aid in developing future prevention strategies and clinical guidelines for managing giardiasis in human populations.

The safety of drinking water in Australia is maintained as part of the Australian Drinking Water Guidelines (ADWG), and via separate state and territory legislation that enforces water suppliers to comply with the ADWG framework [204]. As *G. intestinalis* cysts can remain viable in moist environments and most water sources, there are several recommended standards for water supply systems. The combined use of chlorination, chloramination and ultraviolet irradiation is highly effective in inactivating protozoa such as *G. intestinalis* [204].

Basic hygiene, such as hand washing, must be promoted for all high-risk individuals, particularly as *G. intestinalis* is often transmitted between humans via the faecal-oral route. Recent behavioural studies undertaken in England during the first 6 months of the COVID-19 outbreak suggest that improved hygiene practises and social distancing measures coincided with a decreasing trend of gastrointestinal infections [205]. Similarly, the Australian Government introduced several public health measures to contain and control transmission of the COVID-19 virus [206]. These measures included a nationwide lockdown, social distancing, improved hand hygiene, closures of most community premises, and travel restrictions for international visitors. As a result, there was a drastic reduction in the number of human cases of giardiasis between 2020 to 2021 (Supplementary Figure S5. 1.), suggesting that the popular person-to-person transmission routes for *G. intestinalis* infection were disrupted during this period.

5.8. Concluding remarks

Over the last 20 years, public health reporting systems have seen a marked resurgence of G. intestinalis infections across Australia. Indeed, a broad consensus is that current and future global changes are promoting the emergence and spread of infectious diseases. In Australia, endemic outbreaks of giardiasis have been increasingly identified in child-care centres, pre-schools, and kindergartens, further aided by changing workforce trends and rapid population growths in urban city centres. Increased global interconnectivity and mass migrations to Australia have also been considered as major facilitators for imported G. intestinalis cases. Further concerns have been raised regarding global climate changes and the ongoing emission of greenhouse gases that will not only continue to intensify climatic hazards but may exacerbate the frequency of protozoan diseases such as giardiasis.

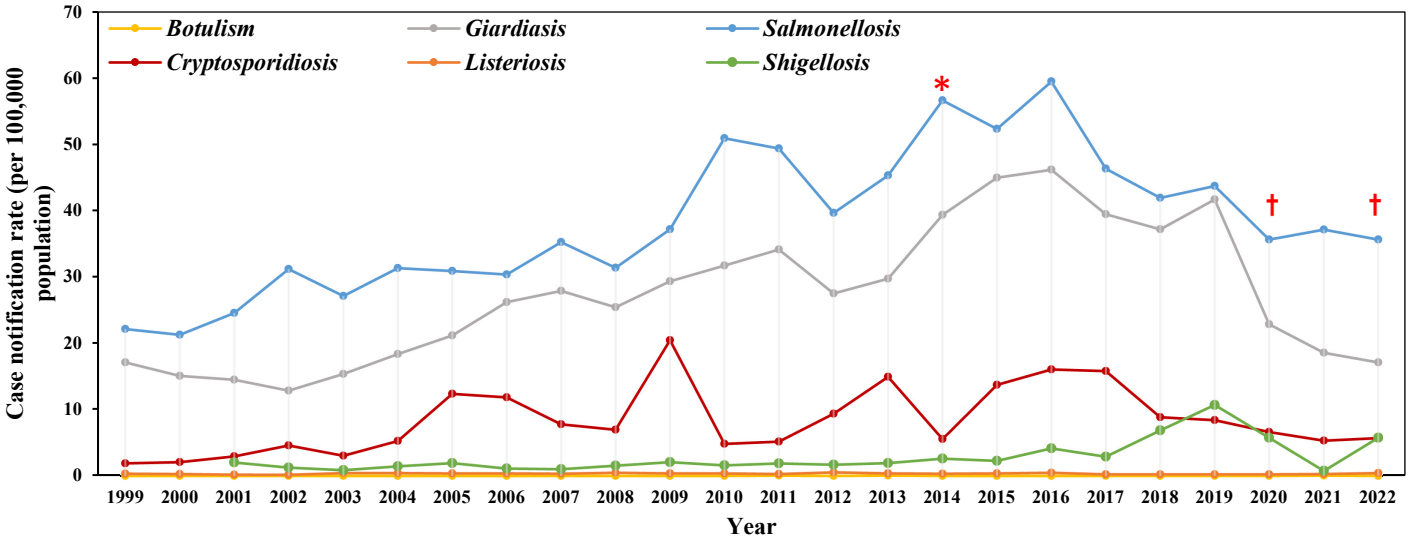
Data from molecular epidemiological studies of human giardiasis have significantly improved our understanding of the distribution, transmission, and host dynamics of *G. intestinalis* assemblages. The introduction of WGS comparative analyses and the development of alternative PCR-based tools has further provided evidence that *G. intestinalis* exists as a 'species complex.' Genetic diversity has been observed at the sub-assemblage and sub-type levels, and these findings have allowed for greater differentiation between the host-specific assemblages of *G. intestinalis*. Reliable classification of *G. intestinalis* from human infections is essential for public health as it helps to answer fundamental questions concerning parasite transmission and host dynamics. Effectively combining molecular epidemiological techniques with geospatial analyses and geographic information system (GIS) mapping also provides the means for developing proper disease control programs. Such tools have been utilised to generate information on the geographical distribution of *G. intestinalis* assemblages across populations, determine disease progression and changing frequencies of infection rates over a set period, and identify high-risk areas of disease clustering.

5.9. Supplementary data

Supplementary Table S5. 1. Overview of *G. intestinalis* sub-assemblages, sub-types, MLG type and the major host species.

Assemblage[a]	Sub-assemblage	Sub-type	MLG type[b]	Major host(s)[c]	References
A1	-	A1; A5; A8; A9	AI-1 – AI-8	**Humans**, **cats**, **dogs**, marsupials, non-human primates, pigs, rodents, **wild and domestic ruminants**	[17,45,53,58,175,207]
AII	-	A2; A3; A4; A7; A11; A12	AII-1 – AII-11	**Humans**, cats, ruminants	[17,53,54,208]
AIII	-	A6	AIII-1	Humans, cats, **ruminants**	[16,41,50,56,68,80]
B	BIII	-	-	**Humans**, non-human primates, rabbits, rodents, ruminants	[16,17,53,64]
	BIV	-	-	**Humans**, marsupials, non-human primates, rabbits, wild and domestic rodents	[16,62,175,207]

[a] An alternative naming system proposed [35,38–40]
[b] MLG; a multi-locus typing scheme defined by Caccio *et al*. [17]
[c] Bolded text represents the predominant host(s) for each sub-assemblage



Supplementary Figure S5. 1. Notification rates (per 100,000 population) of Giardiasis, Cryptosporidiosis, Botulism, Salmonellosis, Listeriosis and Shigellosis reported in humans in NSW, Australia from January 1999 – December 2022. Note that notification data for Shigellosis cases was only available from January 2021 – December 2022. An increasing trend of *Giardia* cases is noticeable, with an overall average of 2,000 cases annually. Cases in NSW peaked in 2016 with 3,455 cases (46.2 cases/100,000). COVID-19 restrictions in 2020 to 2021 led to a decline in cases, which followed into 2022.

*An additional private laboratory was included in the notification data from January 2014 [209]
†COVID-19 restrictions were put in place from March 2020 – October 2022

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5.11. Ethical approval

Not applicable.

5.12. References

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