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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	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)
		tpi primers: AL3543, AL3546, AL3544, AL3545				
		gdh primers: GDHeF, GDHiR, GDHiF, GDHiR				
		SSU rRNA primers: RH11, RH4, GiarF, GiarR				

[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

Patricia Zajaczkowski: Conceptualisation, writing - original draft, writing - review & editing, visualisation, funding acquisition. Rogan Lee: Supervision, writing - review & editing. Stephanie M. Fletcher-Lartey: Supervision, funding acquisition, writing - review & editing. Kate Alexander: Project administration, funding acquisition, writing - review & editing. Abela Mahimbo: Supervision, writing - review & editing. Damien Stark: Funding acquisition, resources, writing - review & editing. John T. Ellis: Supervision, project administration, writing - review & editing. All authors read and approved the final manuscript.

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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