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Giardia/giardiasis — A perspective on diagnostic and analytical tools

Anson V. Koehler [a], Aaron R. Jex [a], Shane R. Haydon [b], Melita A. Stevens [b], Robin B. Gasser [a,c,*]

[a] Faculty of Veterinary Science, The University of Melbourne, Parkville, Victoria 3010, Australia
[b] Melbourne Water Corporation, Victoria, Australia
[c] Institute of Parasitology and Tropical Veterinary Medicine, 14163 Berlin, Germany

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ABSTRACT

Giardiasis is a gastrointestinal disease of humans and other animals caused by species of parasitic protists of the genus *Giardia*. This disease is transmitted mainly via the faecal–oral route (e.g., in water or food) and is of socioeconomic importance worldwide. The accurate detection and genetic characterisation of the different species and population variants (usually referred to as assemblages and/or sub-assemblages) of *Giardia* are central to understanding their transmission patterns and host spectra. The present article provides a background on *Giardia* and giardiasis, and reviews some key techniques employed for the identification and genetic characterisation of *Giardia* in biological samples, the diagnosis of infection and the analysis of genetic variation within and among species of *Giardia*. Advances in molecular techniques provide a solid basis for investigating the systematics, population genetics, ecology and epidemiology of *Giardia* species and genotypes as well as the prevention and control of giardiasis.

Contents

* Corresponding author. Tel.: +61 3 97312283.
E-mail address: robinbg@unimelb.edu.au (R.B. Gasser).

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1. Introduction

Species of *Giardia* (Metamonada) are important parasitic protists that are transmitted by the faecal–oral route and cause gastroenteritis in vertebrates, including mammals, birds, reptiles and fishes (Adam, 2001; Filice, 1952; Thompson and Monis, 2004, 2012). In mammals, including humans, giardiasis is mainly caused by *Giardia duodenalis* (syn. *Giardia intestinalis* and *Giardia lamblia*) (Thompson, 2004). This disease is usually transmitted from human-to-human (anthroponotic) or animal-to-human (zoonotic) (Xiao and Fayer, 2008; Feng and Xiao, 2011). In agricultural animals, for instance, giardiasis can lead to morbidity and economic losses (Olson et al., 2004), although asymptomatic infections are common (Geurden et al., 2010b). In humans, giardiasis is usually a self-limiting illness, characterised by diarrhoea, colic, headache, dehydration, malabsorption, weight loss and/or wasting (Buret and Cotton, 2011). This disease particularly affects children and immuno-deficient or -compromised people (Muhsen and Levine, 2012; Stark et al., 2009; Thompson, 1994). Nonetheless, asymptomatic infections are common in apparently immuno-competent individuals, particularly in developing countries (e.g., Farthing et al., 1986; Feng and Xiao, 2011; Haque et al., 2005; Mason and Patterson, 1987). Chemotherapeutics, such as metronidazole, fenbendazole and febantel, are commonly used to treat clinical cases, while other drugs employed include albendazole, nitazoxanide, furazolidone, tinidazole, quinacrine and ornidazole (Escobedo and Cimerman, 2007; Gardner and Hill, 2001; Huang and White, 2006).

Outbreaks of human giardiasis are commonly associated with child day-care centres, contaminated drinking water or swimming pools (Baldursson and Karanis, 2011; Karanis et al., 2007). *Giardia* cysts are quite resistant to disinfectants, such as chlorine, routinely used for water treatment (Betancourt and Rose, 2004). Waterborne outbreaks of giardiasis have been reported in developed countries, including the USA, Canada and, more recently, Norway (e.g., Baldursson and Karanis, 2011; Karanis et al., 2007; Nygård et al., 2006), the most notable being the outbreak in Portland, Oregon, USA, in 1954, which resulted in ~50,000 human cases (Karanis et al., 2007). Additionally, *Giardia* is a common etiological agent of traveller's diarrhoea (Ross et al., 2013). Given the relative resilience of cysts in water and the environment (Olson et al., 1999), the cost of chemotherapeutic compounds or regimens for treatment or vaccination in animals (O'Handley and Olson, 2006) and the socioeconomic impact of giardiasis, *Giardia* is recognised as a key waterborne pathogen impairing health and development, and hindering socioeconomic improvement in developing countries (Savioli et al., 2006; WHO, 2011b).

The identification and characterisation of *Giardia* is central to investigating and understanding the epidemiology of giardiasis. However, there are significant limitations in detection or diagnosis using conventional microscopic, biochemical, immunological and serological techniques (Dixon et al., 1997; Thompson, 2004), such that there has been a need for reliable and practical molecular methods. Using genetic methods, seven recognised species and eight genotypes, called assemblages A–H, have been reported from various vertebrate host groups (Thompson and Monis, 2012), although assemblage H requires further verification. These species and genotypes cannot be distinguished based on host origin or parasite morphology (Feng and Xiao, 2011; Thompson and Monis, 2012). In the present article, we provide an account of some key microscopic and immunological methods used for the detection or identification of *Giardia*, and review nucleic acid-based approaches for the diagnosis of giardiasis and analysis of genetic variation within and among species of *Giardia*. We also describe the advantages and disadvantages of some techniques, and emphasise the benefits of using molecular tools to achieve a better understanding of the systematics, epidemiology and population genetics of members of the genus *Giardia*, underpinning the prevention and control of giardiasis in animal and human host populations. Topics that are beyond the scope of this paper can be found in key review articles of *Giardia*/giardiasis (Ankarklev et al., 2010; Appelbee et al., 2005; Feng and Xiao, 2011; Fletcher et al., 2012; Gardner and Hill, 2001; Huang and White, 2006; Monis et al., 2009; Ryan and Cacciò, 2013; Smith and Paget, 2007; Thompson, 2004; Thompson and Monis, 2012; Xiao and Fayer, 2008).

2. Classical diagnostic methods

2.1. Microscopy

Conventionally, the detection of *Giardia* cysts in duodenal, faecal, tissue, environmental and/or water (= biological) samples is achieved mainly by microscopic examination (e.g., Behr et al., 1997; de Souza et al., 2004; Dixon et al., 1997; Garcia, 2009; Goka et al., 1990; Huang and White, 2006; Mank et al., 1997; Sauch, 1985; Schuurman et al., 2007) (Fig. 1). A number of morphological features of the trophozoite, including median body shape and location, shape, ventrolateral disc length, length of caudal flagella, and number and shape of nuclei (Adam, 2001; Kulda and Nohynkova, 1978; Thompson and Monis, 2004), can be used for the identification of *Giardia*, but it is not possible to identify trophozoites or cysts to species by light microscopy (Thompson and Monis, 2004). Staining techniques can be employed to aid the detection of these stages of *Giardia*, and their differentiation from other microorganisms, protists and faecal or environmental debris (Fig. 1). The simplest stains include iodine (Smith and Paget, 2007; Wolfe, 1990) and iron-haematoxylin (Garcia, 2007), Giemsa (Ament, 1972; Wolfe, 1990) or trichrome (Thornton et al., 1983) (Fig. 1). Cysts can be concentrated using various methods which employ, for example, formalin-ether or formalin-ethyl acetate (Smith and Paget, 2007). Motile trophozoites can be detected by direct microscopic examination of fresh samples (smears prepared immediately with warm [37 °C] saline), while dead trophozoites can be detected in air-dried faecal smears stained, for instance, with Giemsa (Smith and Paget, 2007). Multiple, successive faecal samples should be taken and examined over a period of 1–2 weeks, because of the intermittent nature of cyst excretion (Garcia, 2009; Smith and Paget, 2007). Electron microscopy might be useful for the identification of some *Giardia* species (Adam, 2001), but is not applicable for routine use.

2.2. Immunological tools

The use of immunological methods (Fig. 1) can be advantageous over light microscopy for the detection of *Giardia* cysts or trophozoites in biological samples. For instance, the use of fluorescence microscopy and the direct fluorescence antibody (DFA) test using a fluorescein isothiocyanate-conjugated anti-*Giardia* monoclonal antibody (e.g., FITC-*G*-mAb), which recognises surface epitopes on cysts, has been reported to achieve relatively high specificity (99.8–100%) and sensitivity (93–100%) for the detection of cysts in faecal smears and environmental samples (Alles et al., 1995; Baig et al., 2012; Garcia and Shimizu, 1997; Geurden et al., 2008a; Grigoriew et al., 1994; Johnston et al., 2003; Lemos et al., 2005; Mekaru et al., 2007; Riggs et al., 1983; Rimhanen-Finne et al., 2007; Zimmerman and Needham, 1995).

The detection of *Giardia* antigens in faecal samples (i.e., coproantigens) is another approach. Various enzyme-linked immunoassays (including ELISAs) have been used (Addiss et al., 1991; Aldeen et al., 1995, 1998; Aziz et al., 2001; Behr et al., 1997; Chan et al., 2000; Elsafi et al., 2013; Fedorko et al., 2000; Garcia and Shimizu, 1997; Goka et al., 1986; Green et al., 1985; Johnston et al., 2003; Katanik et al., 2001; Knisley et al., 1989; Maraha and Buiting, 2000; Mekaru et al., 2007; Nash et al., 1987; Rosenblatt et al., 1993; Rosoff et al., 1989; Scheffler and Van Etta, 1994; Schunk et al., 2001; Schuurman et al., 2007; Stibbs et al., 1988; Ungar et al., 1984; Weitzel et al., 2006; Yolken and Ungar, 1985; Zimmerman and Needham, 1995) and have achieved specificities of 87–100% and sensitivities of 63–100%; in addition, immuno-chromography (IC) tests (Costache et al., 2009;

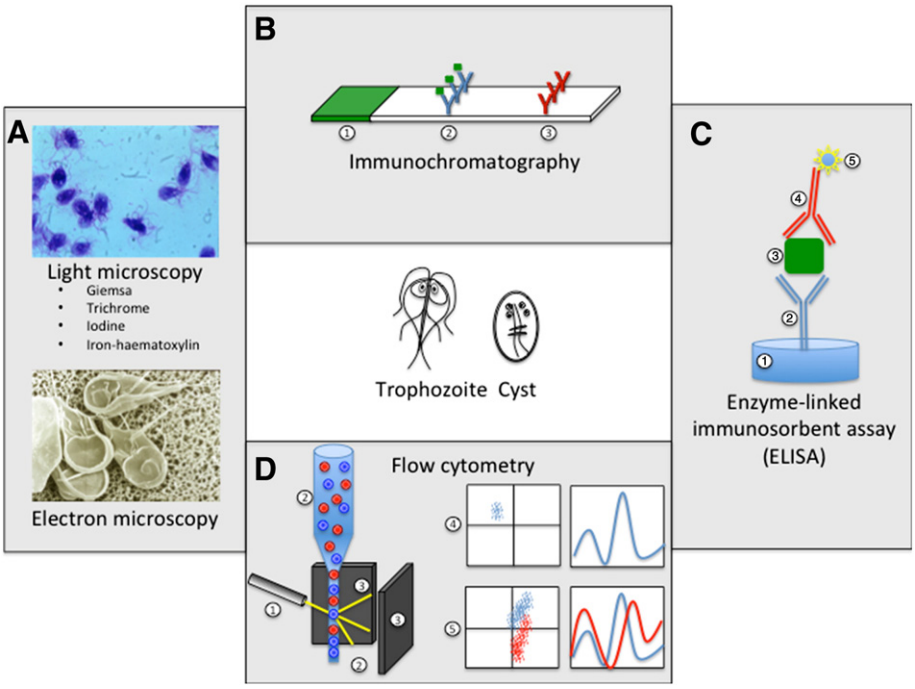

Fig. 1. Some key approaches used for the detection, recovery or enumeration of *Giardia* in faecal or water samples. (A) Conventional microscopic identification of cysts or trophozoites, and electron microscopic analysis of these stages; (B) Immuno-chromatography: (1) sample loading area; (2) positive-control antibody for the assay; (3) antibody for the specific antibody-based detection of *Giardia* antigens; (C) Immunoassay for the detection of cyst or trophozoite antigens: (1) ELISA well; (2) capture antibody; (3) *Giardia* antigen; (4) specific secondary antibody; (5) enzyme-labelled conjugate for colorimetric detection; (D) Flow cytometry (FC) for the recovery or enumeration of cysts: (1) laser emitter; (2) sample containing fluorescently labelled cysts; (3) light detectors; (4) display of results using one colour; (5) two-colour FC.

Elsafi et al., 2013; Elwakil and Ahmed, 2008; Garcia and Garcia, 2006; Garcia et al., 2003; Geurden et al., 2008a, 2010a,b; Goñi et al., 2012; Gutiérrez-Cisneros et al., 2011; Johnston et al., 2003; Nguyen et al., 2012; Oster et al., 2006; Papini and Cardini, 2006; Pillai and Kain, 1999; Regnath et al., 2006; Van Kerkhoven et al., 2006; Weitzel et al., 2006) have also found application, achieving specificities of 79–100% and sensitivities of 26–100%, depending on study. However, the sensitivity of coproantigen detection assays can be lower than microscopic approaches (Johnston et al., 2003). Variation in diagnostic performance of different assays can be due to cross-reactivity (affecting specificity) and intermittent shedding of cysts and *Giardia* antigens, or the use of formalin as a fixative (reducing sensitivity). Decreased sensitivity might also relate to low numbers of cysts in faeces (Strand et al., 2008) or trophozoites in the host intestine. A disadvantage of some kits is that they rely on a visual inspection of staining intensity, which can lead to a subjective interpretation of test results (Garcia and Shimizu, 1997; Johnston et al., 2003). Advantages that coproantigen detection methods offer over microscopy are that they have the capacity to detect (prepatent) infections prior to the excretion of cysts in host faeces (Nash et al., 1987), and can be employed for the cost-effective and rapid screening of large numbers of faecal samples (Garcia et al., 2003; Geurden et al., 2008b; Johnston et al., 2003). However, like other immuno- or sero-diagnostic techniques, they do not allow the identification of the species or genotype of *Giardia* infecting a host.

2.3. Methods to recover or concentrate cysts

Various methods have been evaluated for the recovery of *Giardia* cysts from water, faecal or environmental samples for subsequent microscopic, immunological or genetic testing. Current methods for water, recommended, for example, by the American Society for Testing and Materials (ASTM, 1991), the United States Environmental Protection Agency (US-EPA) (US-EPA, 1996, 2012), and adopted by the World Health Organization (WHO, 2011a), can be coupled to immunofluorescence microscopy. These techniques rely on filtration, immunomagnetic separation (IMS) and complementary detection by direct fluorescent antibody (DFA) and/or Nomarski differential interference contrast (DIC) microscopy. Diagnostic procedures include the "Information Collection Rule Protozoan Method for Detecting *Giardia* and *Cryptosporidium* Oocysts in Water by the Fluorescent Antibody Procedure" or the "ICR method" (US-EPA, 1996) and modified approaches, such as Method 1623.1 (US-EPA, 1999, 2012). These techniques rely on mechanical filtration, followed by cyst purification and DFA. Method 1623.1 provides increased cyst recovery rates and appears to be more efficient, in terms of speed and ease of use, than previous approaches, such as the ICR method (cf. DiGiorgio et al., 2002; Hsu and Huang, 2000; McCuin and Clancy, 2003). Alterations to some steps (e.g., centrifugation, filters, IMS procedure and/or staining) in Method 1623.1 have led to increased recovery rates of *Giardia* cysts (e.g., Ferguson et al., 2004; Hill et al., 2009; Hu et al., 2004; Kimble et al., 2012; McCuin and Clancy, 2003; Quintero-Betancourt et al., 2003; Zarlenga and Trout, 2004; Zuckerman and Tzipori, 2006).

Flow cytometry (FC) is another approach for the recovery of *Giardia* cysts (e.g., Vesey et al., 1994) (Fig. 1) for subsequent immunofluorescent staining, microscopic examination and/or enumeration. In addition, it also seemed possible, using nucleic acid staining with propidium iodine (PI), to reliably distinguish between viable and dead cysts (Barbosa et al., 2008; Schupp and Erlandsen, 1987). However, a study by Sauch et al. (1991) showed that PI could only detect viability in cysts inactivated by heat or a quaternary ammonium compound, but not chlorine, suggesting that cysts inactivated by chlorine did not take up PI and, thus, would be overestimating viability. Although, initially, FC appeared to be promising (Dixon et al., 1997), the large number of false-positive results and the low recovery of cysts in spiked samples in some studies (Keserue et al., 2011; Zarlenga and Trout, 2004) raises questions as to the suitability of this approach for routine use. Ferrari and Veal (2003) employed a "two-colour" staining method for the FC-based analysis of water samples for *Giardia* cysts, with the aim of

detecting a single cyst within 10–100 l of water. In this latter study, an IMS approach was employed prior to the use of an independent monoclonal antibody (mAb) and a novel peptide nucleic acid (PNA) probe for detection. The addition of the PNA-probe resulted in increased fluorescent intensities (by a factor of four), thereby reducing the number of false-positive test results to zero. However, the use of IMS resulted in a low recovery rate (39%) (Ferrari and Veal, 2003). Nonetheless, a recent study (Keserue et al., 2011) showed that micro-bead technology can result in 90% recovery, with no false-positive test results, raising the prospect for a method for routine application.

3. Nucleic acid techniques for diagnosis or genetic analysis

Since phenotypic methods (Section 2) cannot be used to distinguish different species or assemblages of *Giardia*, various nucleic acid-coupled methods have been developed to identify and detect genetic variation within and among members of this genus within clinical and environmental samples (Fig. 2). Although some methods rely on the specific in situ hybridisation of probes to particular genetic loci within the genome of *Giardia* cysts (Subsection 3.1.), most techniques rely on the specific amplification of one or more loci from small amounts of genomic DNA by the polymerase chain reaction (PCR). The application of PCR has led to enhanced knowledge and understanding of the systematics, biology, epidemiology, ecology and population genetics of *G. duodenalis* in particular (Adam, 2001; Ankarklev et al., 2010; Cacciò and Ryan, 2008; Cooper et al., 2007; Feng and Xiao, 2011; Kutz et al., 2009; Monis et al., 1999; Smith and Paget, 2007; Thompson, 2004; Thompson and Monis, 2004, 2012; Thompson et al., 1998), and supports the prevention and control of giardiasis.

3.1. Fluorescent in situ hybridisation (FISH)

FISH uses fluorescently-labelled probes or oligonucleotides to specifically detect target sequences within RNA or DNA (Amann et al., 1990, 1995) (Fig. 2). Presently, most FISH assays for the detection of *Giardia* cysts have relied on the hybridisation to RNA rather than to DNA, targeting a variable region of the small subunit (*SSU*) of the nuclear ribosomal RNA (rRNA) gene (Bednarska et al., 2007; Dorsch and Veal, 2001; Erlandsen et al., 1994, 2005; Ferrari and Veal, 2003; Lemos et al., 2005). *SSU* rRNA was identified as a suitable and abundant target, because it has a high copy number within the cell (Dorsch and Veal, 2001; Wallner et al., 1993). The latter authors hypothesised that only viable cysts would be detected, because RNA would be degraded by RNAses upon cell death, and thus would not be detectable in dead cysts (Bednarska et al., 2007; Dorsch and Veal, 2001; Lemos et al., 2005). Bednarska et al. (2007) used FISH to retrospectively test faecal samples that had been stored in either potassium dichromate or ethanol, and found that, while the method could be used for the identification of *Cryptosporidium parvum* oocysts, it was less reliable for *Giardia* cysts due to rRNA degradation. Obviously, the utility of such FISH probes for studies of cyst viability is dependent on the rate of target decay. Although it has been assumed that this decay is rapid upon cell death, this has not been assessed quantitatively for the rRNAs evaluated to date, and, it is likely that the rate of degradation (both within and outside of cysts) will vary depending on different environmental conditions (i.e., temperature, pH, salinity and/or RNase contamination present). Irrespectively, FISH can be employed to detect cysts, but most nucleic probes reported to date do not allow for a reliable distinction among species or assemblages. Nonetheless, one study (Erlandsen et al., 2005) designed probes in *SSU* that could distinguish among *G. lamblia* (= *G. duodenalis*), *Giardia muris* and *Giardia ardeae*.

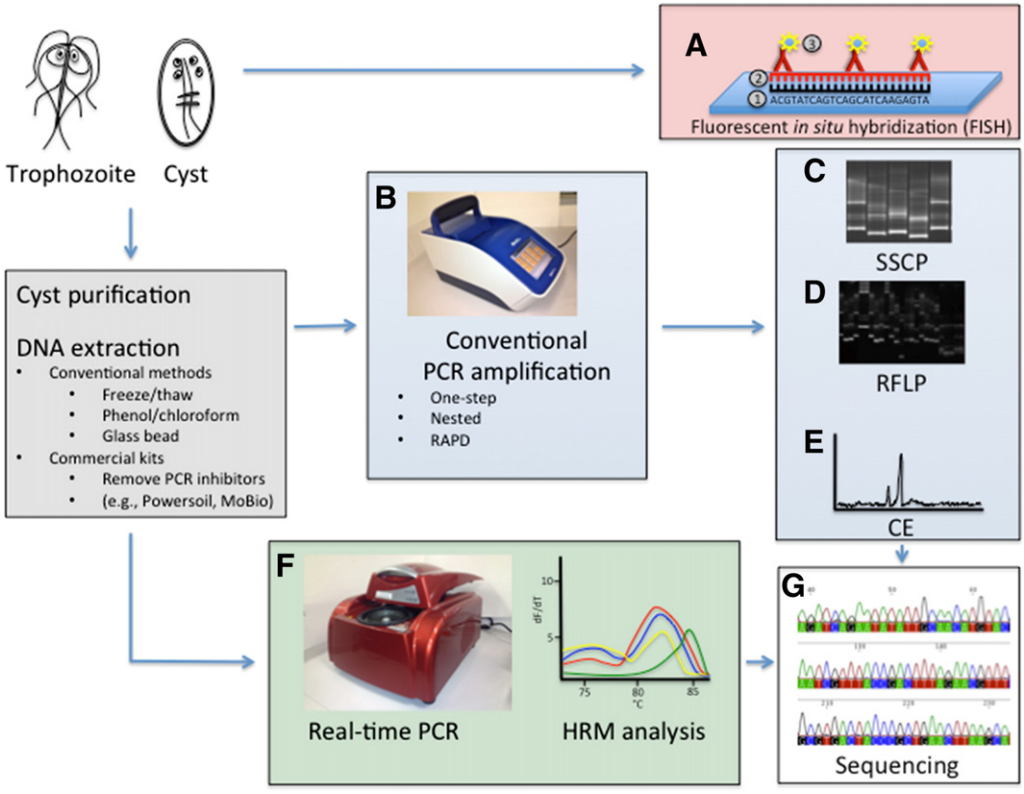

Fig. 2. Summary of principal nucleic acid-based methods used for the specific, assemblage or sub-assemblage identification of *Giardia* or the diagnosis of giardiasis. (A) Fluorescent in situ hybridisation (FISH): (1) target locus (e.g., small subunit of the nuclear ribosomal RNA gene), (2) hybridised nucleic acid segment; (3) fluorescently labelled oligonucleotide probe; (B) Polymerase chain reaction (PCR) approaches, including one-step, nested and random amplification of polymorphic DNA (RAPD) methods; (C) Gel-based single-strand conformation polymorphism (SSCP), (D) Restriction fragment length polymorphism (RFLP) or (E) Capillary electrophoresis (CE) analysis of PCR products; (F) Real-time PCR combined with high resolution melting-curve analysis (HRM); (G) Sanger sequencing of PCR products.

3.2. Cyst viability

Some methods have relied on assessing cyst viability and/or infectivity to mice (e.g., Cole et al., 1989; Schupp and Erlandsen, 1987; Schupp et al., 1988). However, not all *Giardia* species and genotypes are infectious to rodents, and such approaches have been considered as too expensive, labour-intensive and time-consuming for routine application, and usually require relatively large numbers of cysts for analysis. Animal ethics concerns must also be considered. Other methods reliant on vital dye uptake (e.g., PI) or the excystation of cysts (e.g., Barbosa et al., 2008; Bertrand et al., 2009; Cole et al., 1989; Sauch et al., 1991; Schupp and Erlandsen, 1987; Smith and Smith, 1989; Thiriat et al., 1998) have been employed, but appear to be relatively unreliable or irreproducible (Sauch et al., 1991). Microscopic approaches assisted by the use of nucleic acid probes, such as FISH (Subsection 2.1), may have had limitations in that they may lack specificity for assemblage-level identification (Amann and Ludwig, 2000; Monis et al., 2005). There had been a significant focus on developing a method for the assessment of cyst viability by specific amplification of messenger RNA from *Giardia* cysts by enzymatic amplification of the beta-giardin (*bg*) (Kaucner and Stinear, 1998) or heat shock protein-70 (*hsp-70*) gene (Abbaszadegan et al., 1997; Lee et al., 2009). Recently, Baque et al. (2011) also established an amplification method that can simultaneously evaluate cyst viability and discern *G. duodenalis* assemblages A and B using *bg*.

3.3. PCR-based methods for diagnosis and/or genetic analysis

PCR enables the specific amplification of DNA regions from complex genomes. Many different PCR methods are available (e.g., Gasser, 2006), and some key methods applied to *Giardia* (Fig. 2) are reviewed in the following sub-sections. Central to the effective application of PCR are reliable techniques for the isolation of nucleic acids from biological or environmental samples, which also effectively and consistently remove constituents that are inhibitory to PCR (cf. Gasser, 2006). Numerous methods have been assessed (e.g., Adamska et al., 2010; Anceno et al., 2007; Asher et al., 2012a; Babaei et al., 2011; Deng and Cliver, 1999; Feng and Xiao, 2012; Guy et al., 2003; Nantavisai et al., 2007; van Zanten et al., 2011; Yu et al., 2009) and include the use of sonication, freeze/thaw cycling, glass beads and/or phenol/chloroform extraction, followed by ethanol precipitation, and/or commercial extraction kits, such as QIAamp (Qiagen, USA). However, DNA isolation and purification methods require critical evaluation for each distinct application and biological matrix being tested, in order to ensure that PCR-inhibition is minimised.

3.3.1. DNA fingerprinting

PCR-coupled fingerprinting methods rely on the screening of a genome for variation in organisation and sequence length. The advantage of some of them is that no prior knowledge of the genome or genes is required for a parasite to be characterised. A disadvantage is that the genetic fingerprint of, for example, *Giardia* cyst or trophozoite isolate represents a population of organisms rather than an individual. Random amplification of polymorphic DNA analysis (RAPD) (Welsh and McClelland, 1990; Williams et al., 1990) was used for the purpose of detecting variation within and among populations of a particular species. This approach has been applied to *Giardia* (Deng and Cliver, 1999; McRoberts et al., 1996; Morgan et al., 1993; Paintlia et al., 1999; Pelayo et al., 2003; Rocha et al., 2003; Šedinová et al., 2003; van Belkum et al., 1993). Although originally considered useful, because of its ability to amplify from small amounts of genomic DNA and its capacity to rapidly screen genomes for variation without requiring prior sequence information (Hadrys et al., 1992), there were significant problems with the specificity and reproducibility of RAPD due to the low stringency often used in PCR (Ellsworth et al., 1993; MacPherson et al., 1993; Perez et al., 1998).

3.3.2. Restriction fragment length polymorphism, specific PCR and sequencing

PCR-based techniques employing specific primer pairs for the selective amplification of different genetic loci, followed by enzymatic cleavage or sequencing, have been used to characterise and classify *Giardia* species, "genotypes" and/or assemblages (reviewed by Wielinga and Thompson, 2007; Cacciò and Ryan, 2008; Feng and Xiao, 2011, 2012). Some key gene markers (loci) include the *bg* (cf. Baker et al., 1988), elongation factor 1 alpha (*ef1α*) (Hashimoto et al., 1994), glutamate dehydrogenase (*gdh*) (Yee and Dennis, 1992), triose-phosphate isomerase (*tpi*) (Baruch et al., 1996) and variable surface protein (*vsp*) (Nash and Mowatt, 1992a) genes, and the *G. lamblia* open reading frame-C4 (GLORF-*C4*) (Nash and Mowatt, 1992b), nuclear ribosomal RNA genes and DNA spacers (Weiss et al., 1992). *SSU* provides useful genetic markers for the specific identification of *Giardia*, having relatively low intraspecific and relatively high interspecific sequence variation (Feng and Xiao, 2011, 2012; Hopkins et al., 1997; Sogin et al., 1989; van Keulen et al., 1995; Weiss et al., 1992; Wielinga and Thompson, 2007). Additional markers achieving assemblage or sub-assemblage identification, based on specific PCR or sequencing are in the *tpi*, *gdh* and *bg* genes (reviewed by Monis et al., 1996; Lu et al., 1998; Monis et al., 1999; Amar et al., 2002; Wielinga and Thompson, 2007; Geurden et al., 2009; Feng and Xiao, 2012; Ryan and Cacciò, 2013). Each of these latter genes has been utilised, sometimes together with *SSU*, in systematic investigations of *Giardia*, providing the basis for the current classification of members within the genus (Cacciò and Ryan, 2008; Feng and Xiao, 2011, 2012; Thompson and Monis, 2011, 2012). Also the internal transcribed spacers (ITS) of ribosomal DNA are useful for assessing genetic variability within *Giardia* species (Beck et al., 2011a,b, 2012; Cacciò et al., 2010; Hunt et al., 2000), because their sequences have higher intraspecific variation than the ribosomal RNA gene regions examined thus far (Cacciò et al., 2010) (Table 1).

PCR-based restriction fragment length polymorphism (PCR-RFLP) analysis has been employed for the 'genetic' classification of *Giardia* isolates (e.g., Amar, 2003; Read et al., 2004; Robertson et al., 2007; Sousa et al., 2006; van Keulen et al., 2002; Yong et al., 2002). Although useful, this analytical approach does not detect all sequence and length

Table 1
Genetic markers commonly used for the identification of *Giardia* to the specific, assemblage and/or sub-assemblage levels.

Genetic marker or locus	Gene copy number	Unique to *Giardia*	Relative degree of sequence variability	Level of identification and/or differentiation
Small subunit (*SSU*) of nuclear ribosomal RNA (18S gene)	High	No	Low	Species and assemblage
Second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA	High	No	Moderate	Assemblage and sub-assemblage
Elongation factor 1 alpha (*ef1α*)	Low	No	Moderate	Species, assemblage and sub-assemblage
Beta giardin (*bg*)	Low	Yes	Moderate	Species, assemblage and sub-assemblage
Giardia lamblia open reading frame-*C4* (GLORF-*C4*)	Low	Yes	Moderate	Assemblage and sub-assemblage
Glutamate dehydrogenase (*gdh*)	Low	No	Moderate	Species, assemblage and sub-assemblage
Variable surface protein (*vsp*)	Low	Yes	Moderate	Sub-assemblage
Triose phosphate isomerase (*tpi*)	Low	No	High	Species, assemblage and sub-assemblage

variations within or among amplicons, because the endonuclease(s) used only recognise a small number of potentially variable sites. Possible limitations of this approach can relate to incomplete enzymatic digestion of amplicons and/or sequence variation within individual amplicons, which can lead to misinterpretations of RFLP profiles. Direct DNA sequencing and comparative analyses of sequence data remain the "gold-standard" for detecting genetic variation or polymorphism, and can be applied to single-copy and multi-copy genes (provided that there is sequence homogeneity among copies of the latter). DNA sequence data have the added value of being suitable for direct, comparative genetic and phylogenetic investigations at any time. Provided sufficient starting template is available, multilocus genotyping (MLG) (Cacciò et al., 2008, 2010; Feng and Xiao, 2011) appears to have significant advantages for molecular characterisation studies. Terminal-restriction fragment length polymorphism (T-RFLP) (Asher et al., 2012b) might also serve as an effective tool for the analysis of genetic variation within *Giardia*, given its high throughput potential.

3.3.3. Mutation detection

Limitations in the analysis of sequence variability both within and among amplicons can be circumvented by mutation scanning (Gasser, 2006; Gasser et al., 2006). Single-strand conformation polymorphism (SSCP) (Orita et al., 1989) is a useful and relatively universal approach (Gasser et al., 2006); it relies on the principle that the electrophoretic mobility of a single-stranded DNA molecule in a non-denaturing gel is highly dependent on its secondary structure and size, and is capable of detecting single point mutations in amplicons of <500 bp (Gasser et al., 2006). For amplicons of >500 bp, the mutation detection rate of this approach can decrease significantly (Gasser et al., 2006). Restriction endonuclease fingerprinting (REF) (Liu and Sommer, 1995), also an SSCP-based approach, can overcome this limitation and allows the accurate scanning of long fragments (≤2 kb) for mutations (Warren et al., 1997). For REF, large amplicons are digested with one or more restriction endonucleases, denatured and the amplicon fragments scanned for nucleotide variation by SSCP.

Although various genetic loci (e.g., *bg*, *ef1α*, *C4*, *gdh*, *SSU*, *tpi* and *vsp*) allow the specific and/or genotypic identification of *Giardia* (Feng and Xiao, 2011; Wielinga and Thompson, 2007), a gene with considerable promise for use in epidemiological investigations, for which sub-assemblage identification is required, is *tpi* (Wielinga and Thompson, 2007). This gene is particularly suitable for REF, because it is present as a single-copy in the genome, thus reducing the likelihood of sequence polymorphism among *Giardia* individuals within populations from individual host animals, thereby enabling direct sequence analyses following nested PCR. Recently, we explored, for the first time, the genetic composition of *Giardia* in faecal samples from various animals living within nine water catchment areas in Victoria, Australia (Nolan et al., 2013). Reservoirs in these areas provide drinking water to the entire human population of the city of Melbourne and environs. In these catchments, samples were collected over a period of two-years, in order to genetically classify genotypes, assess their host/geographical distributions and zoonotic potential. For this purpose, a REF-based sequencing approach was used to assess nucleotide variability within *tpi* among *Giardia* samples (isolates). The analysis identified *G. duodenalis* assemblage A in 68 of 2009 (3.4%) individual faecal samples. Using direct sequencing, 28 distinct *tpi* sequence types were defined, two of which had been reported previously, whereas all others were new records.

In addition, a molecular epidemiological investigation of *Giardia* in pre- and post-weaned calves from eight locations in New Zealand was also conducted using a REF-based analysis of *tpi* (Abeywardena et al., 2012). Of 180 individual faecal samples, two contained assemblage A, and nine samples contained assemblage E; this was the first record of assemblage E in cattle in New Zealand. This study indicated that dairy calves on the South Island harbour 'zoonotic' genotypes of *Cryptosporidium* and *Giardia*, a finding which is likely to have significant public health implications. Since assemblages A and B of *G. duodenalis* are recognised to present the greatest zoonotic risk, given their presence in humans, livestock and companion animals (Monis et al., 2003; Xiao and Fayer, 2008), investigating genetic variation within and among *Giardia* isolates and genetically classifying them are pivotal to inferring the zoonotic potential of distinct genotypes within *G. duodenalis*. The results are consistent with those of previous studies (Geurden et al., 2008c; Monis et al., 1999; Sulaiman et al., 2003, 2004) and indicate that *tpi* is robust for defining *G. duodenalis* assemblages from animals and the environment. However, accurate 'molecular tracking' requires extensive epidemiological information and continual monitoring of the genetic structure of parasite populations (preferably using multiple genomic markers) in the geographical region under investigation. PCR-based mutation scanning, combined with targeted sequencing, provides an accurate and cost-effective tool for 'tracking' and large-scale molecular epidemiological studies of *Giardia*.

3.3.4. Moderate to high throughput PCR-based approaches

Real-time PCR (Higuchi et al., 1992) allows the monitoring of DNA amplification as it occurs in vitro, which has practical advantages over conventional PCR. This PCR can be linked to HRM analysis. The rate of dissociation of the dsDNA template in HRM can be measured by a reduction in fluorescence over time. Such data are used to identify the temperatures that cause the greatest rate of change in fluorescence (corresponding to the period at which dsDNA dissociation is greatest) and to infer the melting temperature of an amplicon or a domain within the amplicon. Some studies have used real-time PCR for the specific detection of *G. duodenalis* (see Bertrand et al., 2004; Haque et al., 2007) or assemblages (Alonso et al., 2010, 2011; Baque et al., 2011; Guy et al., 2003; Ng et al., 2005; Zhang et al., 2012), and Almeida et al. (2010) used markers in the *C4*, *gdh* and *tpi* genes to differentiate assemblages A and B of *G. duodenalis*.

A possible limitation of HRM analyses is that they may not always be able to delineate multiple, distinct types of sequence (e.g., paralogues) within a sample, particularly if the melting temperatures or melting profiles of these types are similar, since the peaks might merge, and the combined profile can become uninterpretable. However, the high-throughput potential and relatively low cost of HRM analysis as well as the ability to rapidly differentiate isolates based on melting profile data using advanced bioinformatic software, appears to offer a suitable tool for the identification/differentiation of *Giardia* species and population variants from environmental, water and/or faecal samples, provided suitable genetic markers are used.

Both multiplexed tandem-PCR (MT-PCR) (Jex et al., 2012; Stark et al., 2011) and Luminex (Li et al., 2010; Taniuchi et al., 2011) approaches also have considerable potential for medium to large-scale epidemiological studies of *Giardia* and other waterborne pathogens. In particular, Luminex technology (MagPix®) should allow the establishment of a high-throughput genotyping assay for temporal and spatial surveys, and the monitoring of changes in water and the environment in, for example, water catchments. This technology uses micro-beads for large-scale and customisable genotyping. High-density digital coding of micro-beads allows the tracking not only of the target organisms but also of critical identifiers, such as sample code and type, geographical origin (coordinates), collection date, laboratory code and reagent kits. This technology could provide a robust detection approach for multiplex assays with outstanding precision, accuracy and speed, which should allow for the design and implementation of effective, integrated monitoring and surveillance programmes for the ongoing safeguarding of water and the environment. In addition, methods for the simultaneous detection of multiple pathogens are now available. In particular, the *Taq*Man array card (TAC) system (Liu et al., 2013) uses a 384-well real-time PCR format to detect 19 enteropathogens, including *Giardia*; primers and probes are spotted on to microfluidic cards, and achieve diagnostic specificity and sensitivity of 96% and 98%, respectively.

4. Conclusions

Advances have been made in the development of specific and sensitive molecular tools for the identification and genetic characterisation of *Giardia*, and the diagnosis of giardiasis. In particular, the identification and classification of *Giardia* species and assemblages using genes, such as *bg*, *C4*, *ef1α*, *gdh*, *tpi* and/or *vsp*, in combination with more or less variable loci in ribosomal DNA (e.g., ITS and *SSU*), have assisted in elucidating the systematics, population genetics and epidemiology of *Giardia*, and in confirming and monitoring outbreaks in humans and animals. In the future, genome-wide sequencing (Franzen et al., 2009; Jerlstrom-Hultqvist et al., 2010; Morrison et al., 2007; Perry et al., 2011; Upcroft et al., 2010) of *Giardia* and the definition of a broad range of genetic markers for use in specific and sensitive diagnostic tools should offer unique opportunities to address questions regarding the complex network of biological and ecological factors involved in the interactions among *Giardia*, host(s) and the environment. The integrated use of advanced molecular (-omic) and bioinformatic tools will be crucial to underpin investigations of the systems biology of *Giardia* and giardiasis, on a scale never before possible, and could provide unprecedented prospects for the design of entirely new diagnostic and intervention methods.

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