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PII: S1567-1348(19)30170-4
DOI: https://doi.org/10.1016/j.meegid.2019.103951
Article Number: 103951
Reference: MEEGID 103951

To appear in: Infection, Genetics and Evolution

Received date: 2 May 2019
Revised date: 30 June 2019
Accepted date: 2 July 2019

Please cite this article as: R.C.A. Thompson and A. Ash, Molecular epidemiology of Giardia and Cryptosporidium infections – What's new?, Infection, Genetics and Evolution, https://doi.org/10.1016/j.meegid.2019.103951

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Molecular epidemiology of *Giardia* and *Cryptosporidium* infections – what's new?

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Abstract

New information generated since 2016 from the application of molecular tools to infections with *Giardia* and *Cryptosporidium* is critically summarised. In the context of molecular epidemiology, nomenclature, taxonomy, *in vitro* culture, detection, zoonoses, population genetics and pathogenicity, are covered. Whole genome sequencing has had the greatest impact in the last three years. Future advances will provide a much better understanding of the zoonotic potential of both parasites, their diversity and how this is linked to pathogenesis in different hosts.

**Keywords:** *Giardia; Cryptosporidium;* taxonomy; detection; molecular epidemiology; zoonoses; wildlife; pathogenicity
1. Introduction

One only has to look at the rising number of publications reporting the application of molecular techniques to studies on *Giardia* and *Cryptosporidium* to gauge what level of interest there is about these two ubiquitous parasites. However, only a small number of recent publications actually deal with the impact of such approaches on the epidemiology of infections caused by *Giardia* and *Cryptosporidium*. Our aim here is to provide an update to our more comprehensive review (Thompson and Ash, 2016), by highlighting new information that has been generated from the application of molecular tools to infections with these two ubiquitous enteric protozoan parasites.

We previously highlighted nomenclature and a lack of standardisation with the use of molecular tools. In addition, we concluded that there should be a move away from documenting ‘new’ genotypes to more analysis of molecular data in terms of better understanding population genetics and its impact on transmission, particularly at local levels. In this respect, perhaps the greatest advance in the last three years has been the accessibility and applicability of whole genome sequencing in molecular epidemiological studies of both *Giardia* and *Cryptosporidium*.

2. Maintenance and amplification in the laboratory

The ability to amplify isolates of *Giardia in vitro* for subsequent molecular characterisation has played an important role in epidemiological studies. Unfortunately, only isolates of *G. duodenalis* and *G. enterica* can be routinely amplified in *in vitro* culture. Host adapted species/assemblages (Table 1), particularly those from domestic dogs have not been established and amplified in *in vitro* culture which has prevented whole genome sequencing of these species.
(Caccio et al., 2018). As such, there is a need to re-visit the culture and amplification of host adapted species of *Giardia* so that material can be obtained for sequencing. It is nearly 10 years since the last genome of a *Giardia* species was completed (Jerlström-Hultqvist et al., 2010). A more sophisticated approach to *in vitro* culture systems for *Giardia* has been advocated, for example involving co-culture with gastrointestinal organoids (Caccio et al., 2018), as recently developed for *Cryptosporidium* (Heo et al., 2018; Morada et al., 2018).

*Cryptosporidium hominis* and *C. parvum* can be grown in culture but significant amplification of isolates has not been achieved. Novel culture platforms have been developed that allow the generation of oocysts in culture (Josse et al., 2019; Miller et al., 2018; Heo et al., 2018; Morada et al., 2018). Of these, those involving organoids (Heo et al., 2018; Morada et al., 2018) coupled with sophisticated imaging techniques offer the greatest potential to study the developmental biology of *Cryptosporidium* in detail. However, no advances have been reported since those of Hijjawi et al. (2004) and Koh et al. (2013, 2014), which allowed the development of the parasite to be studied in detail and the demonstration of novel gregarine-like developmental stages and extracellular development (Aldeyarbi and Karanis, 2016; Clode et al., 2015; Thompson et al., 2016). Such studies were complemented by molecular phylogeny and the re-classification of *Cryptosporidium* as a gregarine (Cavalier-Smith, 2014). These findings, which demonstrate that non-oocyst extracellular developmental stages may survive in the environment, will have a tremendous impact on the water industry (Ryan et al., 2016) since it is now aware attention can no longer focus on the oocyst as the only transmissible stage, thus complicating surveillance. As such, there is a need to refine *in vitro* studies in order to identify what other developmental stages may be transmitted in water, and how best to visualise and detect them. Future *in vitro* studies
should focus on further characterization of novel life cycle stages. Success in this effort represents an essential step towards significant progress in the control of Cryptosporidium infections (Karanis, 2018).

3. Taxonomy and Terminology

Two new species have been described in Giardia, both in wildlife (Table 1). G. peramelis occurs in an Australian marsupial, the quenda (syn. southern brown bandicoot) (Isodon obesulus) (Hillman et al., 2016). It has not been found in cohabiting species and quenda are considered a natural host for Giardia paramelis. Phylogenetic analyses placed G. peramelis external to the ‘Giardia duodenalis species complex’ and Giardia microti (Hillman et al., 2016). Lyu et al. (2018) described Giardia cricetidarum from captive hamsters (Phodopus sungorus), a species phylogenetically distinct but closely related to G. muris. G. cricetidarum is considered to be host specific to the hamsters P. sungorus, P. campbelli and Mesocricetus auratus. However, infected hamsters were all “domestic”, maintained in captivity. It is not known how common G. cricetidarum is in wild free-ranging hamsters nor whether co-habiting mammals are susceptible to infection. The status of the genetically distinct isolate recovered from seals and referred to assemblage H (Table 1) requires additional studies.

It is likely that more new species of both Giardia and Cryptosporidium will be described, principally in wildlife, as awareness of the importance of surveillance of native fauna increases. Novel genotypes are increasingly being reported, for example of both Giardia and Cryptosporidium in wild Tasmanian devils (Sarcophilus harrisii) (Wait et al., 2017), and as additional data is obtained from other species of wildlife, this may result in new species
descriptions. Given the controversial history of past species descriptions based on host occurrences, it has clearly proven to be a good predictor of species recognition and acceptance, particularly in wildlife. However, molecular tools are required to validate species proposals in the absence of morphological differences.

A stable, working, formal terminology for the causative agents responsible for infection is essential in epidemiological studies. It has been controversial for both *Giardia* and *Cryptosporidium*, principally with respect to the recognition of described species. The situation appears to be fairly stable with *Cryptosporidium*, and recognition of *C. pestis* (Šlapeta, 2011) appears not to have received wide favour. However, this is not the case with *Giardia*. As more host-adapted species are described, it is likely that the proposed terminology for all non-zoonotic forms will be accepted (Table 1). The controversy primarily concerns the zoonotic forms, *G. duodenalis* (assemblage A) and *G. enterica* (assemblage B). Isolates of these two species/assemblages can be amplified in *in vitro* culture. As such, whole genome sequencing is considered to support the proposal by Monis et al. (2009) that *Giardia* assemblages should be viewed as different species (Tsui et al., 2018, Xu et al., 2012), with no evidence of genetic exchange between species (Xu et al. 2012). The results from these whole genome sequencing studies have been complemented and supported by proteomic analysis (Emery-Corbin et al., 2018). However, these latter authors unfortunately suggested referring to assemblages A (*G. duodenalis*) and B (*G. enterica*) as different sub-species despite the fact that phylogenetically *G. duodenalis* is closer to *G. cati*, *G. bovis* and *G. simondi* (Assemblages F, E and G) than *G. enterica* (Thompson and Monis, 2012).
Although the species name *G. duodenalis* is the name most commonly used for Assemblage A, *G. intestinalis* and *G. lamblia* are also sometimes used as synonyms. This continues to create confusion and makes it difficult to compare results between published studies. A recent paper by Fantinatti et al. (2018) illustrates this, in which the authors refer to *G. lamblia* genotype A in dogs, and with no discrimination between AI, AII or AIII.

4. Molecular detection

Both *Giardia* and *Cryptosporidium* can be transmitted via the faecal oral route or through ingestion of contaminated soil, food or water and a corresponding number of detection methods exist for this array of sample types (Thompson and Ash, 2016). Diagnostic techniques ranging from traditional microscopy, immunofluorescent antibody tests and the ever-evolving DNA based molecular protocols have been employed; often displaying a range of sensitivity and specificity as noted in many recent reviews (Adeyemo et al., 2016; Efstratiou et al., 2017; Koehler et al., 2014; Ryan et al., 2018, 2019; Soares, 2016). Of these, molecular techniques perhaps have the most promise, particularly as genetic characterization is the only way to determine the species and genotype/subtype of *Giardia* or *Cryptosporidium* isolated; a necessity for epidemiological studies investigating pathogen transmission and pathways.

Multiplex and real time quantitative PCR protocols have been developed both as individual methodologies and those that can be applied in combination (Shin et al., 2018). These techniques provide quantitative, rapid, high throughput assays able to detect multiple pathogens
simultaneously and are of great use for the diagnostic laboratory (Mero et al., 2017; Rogowski et al., 2018; Sánchez et al., 2018; Shin et al., 2018).

Another useful application of molecular tools is the ability to discriminate between viable and non-viable cysts/oocysts using mRNA assays. These techniques detect metabolically functional and active cells, which are therefore considered viable; a necessary requirement to determine the potential infectivity of a given sample set (Alonso et al., 2014; Ma et al., 2016; Rousseau et al., 2018; Travaille et al., 2016). These techniques may also help to elucidate whether unusual host records (Abdel-Moein and Saeed, 2016; Pipikovka et al., 2018) are actual evidence of patent infections or rather mechanical passage from a contaminated environment.

Possibly the most limiting factor for molecular detection is reliable sensitivity. Some studies suggest PCR detection of these protozoa is more sensitive than methodologies such as microscopy and ELISAs (Helmy et al., 2018; Hijawii et al., 2018; Pan et al., 2018), while others have noted a significant lack of sensitivity (Imre et al., 2017; Ma et al., 2016; Mero et al., 2017). This variation in reported sensitivities may be linked to several factors such as DNA extraction methods, the presence of PCR inhibitors, gene region and the variety of sample types being analysed e.g. faecal, soil, water (Gasser 2006; Piggot and Taylor 2003). However, the variation in sensitivity still being reported highlights the lack of a robust methodology available to all laboratory settings, as well as levels of expertise and access to appropriate training.

To-date, a standardized molecular detection method which is rapid, affordable, and both sensitive and specific across a range of sample types remains elusive.
5. Molecular Epidemiology

Studies at a local level looking at intra-species genetic diversity using multilocus techniques have demonstrated the value of such approaches in better understanding the population genetics of Cryptosporidium infections (Feng et al., 2018). For example, Avendano et al. (2019) demonstrated the endemic nature of C. parvum in cattle on dairy farms in central Colombia. They confirmed the genetic distinctiveness of C. parvum in cattle farms in their region but also demonstrated the important role of transmission within farms of genotypes distinct for individual farms. Cryptosporidium genotyping has also been shown to be useful in characterising sources of infection by identifying subtypes of C. hominis (Beser et al., 2017; Mosnier et al. 2018; Ng-Hublin et al. 2018).

Another study looked at the genetic diversity of Cryptosporidium among human populations in a developing country, Tunisia (Essid et al., 2018). The authors showed that the distribution of Cryptosporidium isolates in urban areas of Northern Tunisia was dominated by anthroponotic transmission of C. hominis and the IIc subtype of C. parvum. However, zoonotic transmission of zoonotic subtypes of C. parvum (Ila and IId) and C. meleagridis (IIIb) was still possible. There is a particularly high genetic diversity of both C. hominis and C. parvum in humans in developing countries suggesting that genetic recombination among diverse subtypes could occur more frequently (Feng et al., 2018).

Interestingly, another study which carried out a systematic review of papers (King et al. 2019) identifying the subtype distribution of C. parvum infections in humans globally found that anthroponotic C. parvum IIc predominates, primarily in lower-income countries with poor
sanitation and in HIV-positive individuals. The differing population genetic structure of zoonotic and anthroponotic subtypes of *C. parvum* has been suggested to support their recognition as different subspecies *C. p. parvum* and *C. p. anthroponosum* respectively (Nader et al., 2019). It will be interesting to see whether this leads to a re-evaluation of the taxonomy of human infective *Cryptosporidium*.

Whole genome sequencing has been shown to have great potential in molecular epidemiological investigations of waterborne outbreaks of *Giardia*. Tsui et al. (2018) reported that the A1 assemblage of *G. duodenalis* was highly clonal and that isolates from surface water, human, and veterinary origins from Canada, United States, and New Zealand clustered together with minor variation, consistent with this being a pan global zoonotic lineage. In contrast, isolates of *G. enterica* (assemblage B) were variable and consisted of several clonal lineages relating to waterborne outbreaks and geographic locations (Tsui et al., 2018).

Whole genome sequencing provides a more discriminatory approach in determining the potential for zoonotic transmission and source of outbreaks (Tsui et al., 2018). Sequencing has confirmed that mixed infections with *Giardia* species are common (Ankarklev et al., 2018; Tsui et al., 2018) and also occur with *Cryptosporidium* (Paparini et al., 2017). Mixed infections are more common than previously thought, and will be overlooked by conventional PCR (Tsui et al., 2018).

6. Evidence of zoonotic transmission and new wildlife reservoirs
Zoonotic transmission involving domestic hosts occurs with both *Giardia* and *Cryptosporidium* although, apart from waterborne outbreaks, the frequency of direct transmission requires further studies (Thompson and Ash, 2016). As mentioned above, host occurrence has been and is still a good predictor of species of *Giardia* and *Cryptosporidium*, particularly in wildlife but molecular tools are required for validation in the absence of morphological differences. The situation is complicated by anthropologically driven reverse zoonotic transmission, with infections in wildlife increasingly the consequence of anthropogenic activities (Heyworth, 2016; Xiao and Feng, 2017). As a consequence, reservoirs (amplifiers) may establish in wildlife hosts. Beavers are the classical example of this but more recently, marine mammals, marsupials and non-human primates have been shown to play this role (Brynildsrud et al., 2017; Delport et al., 2014; Hogan et al., 2014; Tangtrongsup et al., 2019; Teichroeb et al., 2009; Tsui et al., 2018).

Captive animal studies can provide useful data on the transmission of *Giardia* and *Cryptosporidium* in zoos and animal sanctuaries, in terms of both host and public health. However, there are an increasing number of reports of the occurrence and molecular characterisation of *Giardia* and *Cryptosporidium* in various species of wildlife in captivity often not related to the clinical or public health significance of the parasites. It is not our intention to provide a critical review of these studies here, but would like to suggest that such studies may be of limited value in terms of parasite ecology and epidemiology in nature.

Care is necessary with the interpretation of results, particularly when unusual species or genotypes are found in faecal samples. For example, Pipikova et al. (2018) found host specific *G. cati* (assemblage F) in three of 333 Slovakian children living in poor environmental
conditions. This is unlikely to be a true infection and probably represents DNA of *G. cati* derived from cysts accidentally consumed from the environment. The results were based on the finding of DNA in faeces and no microscopy was undertaken.

7. Evidence for pathogenicity?

There is little doubt that *Giardia* and *Cryptosporidium* are capable of causing pathogenic changes within the host including acute diarrhoea, malabsorption, nausea and vomiting (Wolf 1992; Hopkins and Juranek, 1991; O’Donoghue, 1995). Conversely, it is equally apparent that many cases are asymptomatic, perhaps more so with *Giardia* infections. A recent large-scale community-based study of ~30,000 children aged 0-24 months reported that *Giardia* infections were not associated with diarrhoea for any age group, site or diarrhoeal syndrome (Platts-Mills et al., 2015). Research has even suggested that *Giardia* infection may have a protective effect against diarrhoea with a study reporting that preschool children infected with *Giardia* had significantly less episodes of acute diarrhoea than those not infected with *Giardia* (Muhsen et al., 2014).

Reasons for this wide range of symptomology appear to be multifactorial with *Giardia* species and/or strain, host immunity, age, nutrition plane, stress and epithelial dysfunction considered significant in the aetiology of giardiasis (Berkman et al., 2002; Buret, 2008; Farthing et al., 1986; Robertson et al., 2010; Sahagun et al., 2008). Perhaps the most interesting and least known factor is the possible interplay between the host microbiome and concomitant pathogens.
Recent studies investigating the microbiome structure of dogs and cats naturally infected with enteric parasites, found an association between microbiome structure and diarrhoea in dogs (Gagné et al., 2013; Šlapeta et al., 2015). *In vitro* studies have reported functional changes in commensal bacteria leading to an increase in pathogenicity when in association with *Giardia* (Gerbaba et al., 2015). Barash et al. (2017) reported a downward shift in commensal microbes in mice infected with *Giardia*, particularly in short-chain fatty acids which are thought to be influential in maintaining gut health. Conversely, in an *in vivo* model of infectious colitis *Giardia* infections were able to attenuate granulocyte infiltration, providing an anti-inflammatory affect (Cotton et al., 2014). These contradictory reports highlight the elusive nature of host/parasite interactions and *Giardia* as a pathogen or as a beneficial microorganism is dependent on a variety of factors potentially linked to the host microbiome. Unravelling this further will no doubt rely heavily on molecular techniques such as next generation sequencing, and sophisticated qPCR protocols able to both catalogue and quantify entire microbiomes within the host.

8. Concluding comments

Perhaps the most interesting advance in the last few years has been the application of whole genome sequencing to both *Giardia* and *Cryptosporidium*. It is no longer a technique out of reach to most researchers due to cost and time constraints, and next generation sequencing (NGS) is becoming more routine in many laboratories. As whole genome sequencing is used more widely, it will provide much more information on the diversity of *Giardia* and *Cryptosporidium*, population genetics, ecology, dynamics of transmission and pathogenicity. For results to be meaningful, care must be taken to develop meaningful sampling strategies so that
results can contribute to a better understanding of the molecular epidemiology of infections with Giardia and Cryptosporidium. For example, as discussed above, it is likely to be much more meaningful to sample free ranging wildlife than animals in captivity.

Research on the in vitro cultivation of Cryptosporidium should put less reliance on indirect measures of growth and more on microscopy in order to advance knowledge on the developmental biology of Cryptosporidium. With Giardia, there is a need to investigate why host-specific species will not multiply in conventional media. The fact that they will not grow serves to demonstrate how different they are to the zoonotic species and at the same time presents a challenge to search for media better tailored for their nutritional requirements.

Conflicts of interest

I declare that I have no conflicts of interest.

9. References


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<table>
<thead>
<tr>
<th>Species</th>
<th>Assemblage</th>
<th>Host(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. duodenalis</em></td>
<td>A</td>
<td>Humans and other primates and a wide range of mammals</td>
</tr>
<tr>
<td><em>G. enterica</em></td>
<td>B</td>
<td>Humans and other primates, dogs, cats, and some species of wild animals</td>
</tr>
<tr>
<td><em>G. canis</em></td>
<td>C/D</td>
<td>Dogs and other canids</td>
</tr>
<tr>
<td><em>G. bovis</em></td>
<td>E</td>
<td>Cattle and other hoofed animals</td>
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<td><em>G. cati</em></td>
<td>F</td>
<td>Cats</td>
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<td><em>G. simondi</em></td>
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<td>Rats</td>
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<td><em>G. muris</em></td>
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<td>Rodents</td>
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<td><em>G. microti</em></td>
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<td>Microtine voles and muskrats</td>
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<tr>
<td><em>G. peramelids</em></td>
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<td>Southern brown bandicoot (<em>Isodon obesulus</em>)</td>
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<tr>
<td><em>G. cricetidarum</em></td>
<td></td>
<td>Hamsters</td>
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<td>Marine mammals</td>
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Highlights:

- New advances in molecular ecology of *Giardia* and *Cryptosporidium*
- Taxonomy and nomenclature issues remain
- Zoonotic and anthroponotic transmission are complex issues
- Whole genome sequencing and parasite diversity