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Molecular epidemiologic tools for waterborne pathogens *Cryptosporidium* spp. and *Giardia duodenalis*

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ABSTRACT

Molecular diagnostic tools have played an important role in improving our understanding of the transmission of *Cryptosporidium* spp. and *Giardia duodenalis*, which are two of the most important waterborne parasites in industrialized nations. Genotyping tools are frequently used in the identification of host-adapted *Cryptosporidium* species and *G. duodenalis* assemblages, allowing the assessment of infection sources in humans and public health potential of parasites found in animals and the environment. In contrast, subtyping tools are more often used in case linkages, advanced tracking of infections sources, and assessment of disease burdens attributable to anthroponotic and zoonotic transmission. More recently, multilocus typing tools have been developed for population genetic characterizations of transmission dynamics and delineation of mechanisms for the emergence of virulent subtypes. With the recent development in next generation sequencing techniques, whole genome sequencing and comparative genomic analysis are increasingly used in characterizing *Cryptosporidium* spp. and *G. duodenalis*. The use of these tools in epidemiologic studies has identified significant differences in the transmission of *Cryptosporidium* spp. in humans between developing countries and industrialized nations, especially the role of zoonotic transmission in human infection. Geographic differences are also present in the distribution of *G. duodenalis* assemblages A and B in humans. In contrast, there is little evidence for widespread zoonotic transmission of giardiasis in both developing and industrialized countries. Differences in virulence have been identified among *Cryptosporidium* species and subtypes, and possibly between *G. duodenalis* assemblages A and B, and genetic recombination has been identified as one mechanism for the emergence of virulent *C. hominis* subtypes. These recent advances are providing insight into the epidemiology of waterborne protozoan parasites in both developing and developed countries.

1. Introduction

Protozoan parasites are important causes of diarrhea and other enteric diseases in humans (Custodio, 2016). They include mostly *Cryptosporidium* spp., *Giardia duodenalis*, *Cyclospora cayetanensis*, *Cystoisospora belli*, *Entamoeba histolytica*, and *Toxoplasma gondii*, although human infections with the latter are usually associated with non-gastrointestinal symptoms. Other protozoa in humans are not considered major pathogens, such as other *Entamoeba* spp., *Blastocystis hominis*, and *Balantidium coli*. As these pathogens use the fecal-oral route to maintain the lifecycle, foodborne and waterborne transmission are important in disease epidemiology (Thompson

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and Ash, 2016). Among them, *Cryptosporidium* spp. and *G. duodenalis* are best known for their potential to cause large waterborne outbreaks of illness (Efstratiou et al., 2017; Moreira and Bondelind, 2017), thus are major targets in recent research on waterborne protozoan pathogens in humans. Earlier waterborne outbreaks of diseases by these pathogens were mostly associated with drinking water, but more recently, recreational water-associated outbreaks of cryptosporidiosis and giardiasis are increasingly reported, and for cryptosporidiosis, they are now responsible for most waterborne outbreaks in the United States (Adam et al., 2016; Hlavsa et al., 2015; Painter et al., 2015).

Molecular diagnostic tools have long been used in studies of the transmission of *Cryptosporidium* spp. and *G. duodenalis* (Thompson and Ash, 2016; Xiao and Fayer, 2008). This is largely due to the existence of a large number of morphologically identical species or genotypes within both group of protozoa, which require the use of molecular diagnostic tools for differentiation. Thus far, over 30 *Cryptosporidium* species and many genotypes of unknown species status have been described (Ryan et al., 2014). Similarly, there are at least eight genotypes (assemblages) of *G. duodenalis* that are likely cryptic species (Feng and Xiao, 2011). Because of the existence of host specificity among *Cryptosporidium* species and *G. duodenalis* genotypes, characterizations of pathogens at the species or genotype level are helpful to the assessment of infection sources in humans and public health potential of parasites in animals and the environment. Advanced characterization of human-pathogenic species or genotypes can be used in case linkage, tracking of virulent subtypes, and assessment of disease burdens attributable to different transmission routes (Ryan and Caccio, 2013; Xiao, 2010). This can be achieved through conventional subtyping based on sequence analysis of individual polymorphic genes, multilocus typing, and comparative genomic analysis. In this report, we will review various molecular diagnostic tools used in the characterization of human-pathogenic *Cryptosporidium* spp. and *G. duodenalis*.

2. Genotyping tools

2.1. Genotyping tools for Cryptosporidium spp.

Accurate identification of *Cryptosporidium* species requires the use of genotyping tools. Currently, most *Cryptosporidium* genotyping tools use PCR targeting the small subunit (SSU) rRNA gene, largely because of the existence of conserved *Cryptosporidium*-specific sequence for designing primers that allow broad specific detection of all *Cryptosporidium* spp. and semi-conserved and hypervariable regions that can be used for the differentiation of various species and genotypes by restriction fragment length polymorphism (RFLP), melting curve, or DNA sequence analyses (Xiao, 2010). PCR tools targeting other genes were used in early *Cryptosporidium* research, but they are now infrequently used in *Cryptosporidium* genotyping because of their narrow detection range; they can only be used for genotyping *Cryptosporidium* species that are closely related to *C. parvum* or *C. hominis* because of the nature of sequences used in primer design (Jiang and Xiao, 2003).

Among the SSU rRNA-based *Cryptosporidium* genotyping tools, the PCR-RFLP tool using nested PCR amplification of a ~830-bp fragment and restriction analysis of the secondary PCR products using enzymes *Ssp*I and *Vsp*I (Xiao et al., 1999) is the most commonly used one. It can be used in genotyping *Cryptosporidium* spp. from both humans and animals. More recently, the use of *Vsp*I has been replaced with *Mbo*II in RFLP analysis of PCR products from *Cryptosporidium* spp. in ruminants (Feng et al., 2007). The technique requires stringent PCR conditions, but when optimized, can be used effectively in detecting single oocysts in water samples (Jiang et al., 2005; Xiao et al., 2006). Alternatively, a PCR assay that targeting a smaller fragment of the SSU rRNA gene can be used (Ryan et al., 2003). The technique is more adaptable to other PCR buffers, although is slightly less specific for *Cryptosporidium* (mostly presenting a minor problem to the analysis of water samples) and requires DNA sequence analysis in *Cryptosporidium* genotype determination.

In recent years, qPCR assays are increasingly used in genotyping human-pathogenic *Cryptosporidium* spp. Several species-specific genotyping tools based on the SSU rRNA and other genes have been developed for *C. hominis*, *C. parvum*, and *C. cuniculus* (Bouzid et al., 2016; Burnet et al., 2012; Hadfield and Chalmers, 2012; Hadfield et al., 2011; Jothikumar et al., 2008; Mary et al., 2013; Staggs et al., 2013; Yang et al., 2013). One major issue is the broad range of *Cryptosporidium* species that can occur in humans, which has limited the wide use of these species-specific qPCR assays. However, some of these tools also include a SSU rRNA-based generic qPCR assay for the detection of all *Cryptosporidium* species, which can be used in conjunction with *C. parvum* and *C. hominis*-specific assays for rapid differentiation of the two dominant *Cryptosporidium* species in human specimens. Recently, using fluorescence resonance energy transfer probes and melt curve analysis, one SSU rRNA-based PCR assays has been developed for rapid genotyping to five common *Cryptosporidium* species in human specimens (Li et al., 2015). In addition to the potential of streamlining the detection and identification of *Cryptosporidium* spp. in human clinical specimens, qPCR-based genotyping assays have been used in quantifying *Cryptosporidium* oocysts in these specimens (Mary et al., 2013; Operario et al., 2015; Yang et al., 2013), although low levels of oocysts that are typically found in drinking water samples cannot be accurately quantified (Staggs et al., 2013).

Next generation sequencing techniques are now increasingly used in *Cryptosporidium* genotyping, especially accurate identifications of mixed *Cryptosporidium* genotypes. Their usage in sequencing SSU rRNA and actin PCR products from several *Cryptosporidium* species of humans and animals was effectively demonstrated (Paparini et al., 2015). Although they are more expensive than traditional Sanger sequencing when analyzing small numbers of specimens, with the use of product indexing, the costs are comparable when specimens are processed in batches. With standardization, these techniques are likely to be used in routine *Cryptosporidium* genotyping in future (Ryan et al., 2017).

The use of genotyping tools in the analysis of clinical specimens has led to the identification of over 20 *Cryptosporidium* species and genotypes in humans (Table 1). With the exception of *C. meleagridis*, which infects both birds and mammals, all other human-pathogenic *Cryptosporidium* species and genotypes are pathogens of mammals. It is likely that some other mammalian *Cryptosporidium*