Acta Tropica xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Review

Methods for the detection of *Cryptosporidium* and *Giardia*: From microscopy to nucleic acid based tools in clinical and environmental regimes

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ARTICLE INFO

Keywords:
Cryptosporidium
Giardia
PMA
qPCR
Digital PCR
LAMP

ABSTRACT

The detection and characterization of genotypes and sub genotypes of *Cryptosporidium* and *Giardia* is essential for their enumeration, surveillance, prevention, and control. Different diagnostic methods are available for the analysis of *Cryptosporidium* and *Giardia* including conventional phenotypic tools that face major limitations in the specific diagnosis of these protozoan parasites. The substantial advancement in the development of genetic signature based molecular tools for the quantification, diagnosis and genetic variation analysis has increased the understanding of the epidemiology and preventive measures of related infections. The conventional methods such as microscopy, antibody and enzyme based approaches, offer better detection results when combined with advanced molecular methods. Gene based approaches increase the precision of identification, for example, many signatures detected in environmental matrices represent species/genotype that are not infectious to humans.

This review summarizes the available methods and the advantages and limitations of advance detection techniques like nucleic acid-based approaches for the detection of viable oocysts and cysts of *Cryptosporidium* and *Giardia* along with the conventional and widely accepted detection techniques like microscopy, antibody and enzyme based ones. This technical article also encourages the wide application of molecular methods in genetic characterization of distinct species of *Cryptosporidium* and *Giardia*, to adopt necessary preventive measures with reliable identification and mapping the source of contamination.

1. Introduction

Cryptosporidium and *Giardia* are the most commonly occurring enteric protozoan parasites, responsible for gastrointestinal disorders that may lead to nutritional imbalances and severe health problems, particularly among children in developing countries (Thompson and Ash, 2016). They could cause chronic and debilitating illness in immune-compromised individuals (Carmena et al., 2012). Their environmental transmission poses significant risks to human health and these protozoan parasites have repeatedly been recovered in surface waters (Ehsan et al., 2015; Chuah et al., 2016; Kumar et al., 2016) and wastewater (Sroka et al., 2013; Ma et al., 2016). The resistance against chlorine disinfection of *Cryptosporidium* and *Giardia* is a major public health challenge for the water industry (Carmena et al., 2012). *Cryptosporidium* transmitted through water has been the cause of multiple diarrhoeal outbreaks in the United States, Sweden, and United Kingdom as well as in both developed and developing countries (Insulander et al., 2005; Ignatius et al., 2012; Samie and Ntekele, 2014). *Giardia* additionally infects humans and animals and has been reported to be responsible for 2.8×10^8 cases of intestinal infections per annum worldwide (Thompson 2004; Squire and Ryan, 2017).

The specific diagnosis and genetic characterization of *Cryptosporidium* and *Giardia* is a pre-requisite to understand the associated epidemiological risks and is necessary to trace the variants present in a particular population (Thompson and Ash, 2016). Traditional methods based on microscopy, antibodies and enzymes have limitations in the specific diagnosis of protozoan parasites (see detailed reviews by Jex et al., 2008; Koehler et al., 2014). Therefore, further development occurs targeting both speed, sensitivity and specificity, since the conventional methods in addition may be tedious, costly and partly non-species specific. Traditionally, the diagnosis of *Cryptosporidium* and *Giardia* is mainly based on the detection of the typical morphological characteristics of oocysts/cysts in stool specimens, either by using acid-fast staining (Garcia et al., 1983) or by an indirect immunofluorescence assay procedure with monoclonal or polyclonal antibodies (Shams et al., 2016). These traditional methods are used frequently in developing countries, but lack of discrimination between distinct species based on morphology and or host occurrence. This affects the

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https://doi.org/10.1016/j.actatropica.2018.01.011
Received 13 April 2017; Received in revised form 21 December 2017; Accepted 22 January 2018

comparative potential between regions with a need to apply novel discriminatory and rapid analytical methods for fast and accurate identification of these protozoan parasites adaptable both for developed and developing regions and representative for both clinical and environmental samples.

A wider application and advancement of molecular methods would be especially valuable in developing countries, where the health impact due to local poor sanitation and hygiene is of concern. Based on previously published literature, most studies done in developing regions (for example, African countries), were based on conventional techniques for the detection of parasitic protozoans, except a few where advanced molecular methods have been adapted often through cooperation with partners from industrialized countries.

In this review, different methods in general with their pros and cons, their application in developing regions is exemplified but not limited to the African continent.

In African countries, at least 13 species of *Cryptosporidium* and five *G. duodenalis* assemblages in humans have been identified with additional genotypes in animals (Squire and Ryan 2017). Detailed description of different genotypes of *Cryptosporidium* and *Giardia* and their epidemiology and prevalence in the human population, among animals, and in water reservoirs have been described in a recent review by Squire and Ryan, 2017. The most prominent genotypes of these parasitic protozoans are summarized in Table 1 and the most commonly used technologies adopted to detect *Cryptosporidium* and *Giardia,* on the African continent are summarized in Table 2.

According to reports provided by the Global Enteric Multicenter Study (GEMS), *Cryptosporidium* is second only to rotavirus as a causative agent of diarrheal disease during the first 5 years of life in Sub-Saharan Africa (Kotloff et al., 2013). Its impact is seconded by Sow et al. (2016), who estimated that 2.9 million cases related to *Cryptosporidium* infections occurs in children (< 24 months age) in Sub-Saharan Africa. These *Cryptosporidium* attributable infections were found to be associated with mortality (< 2 fold increase) in children in the age group between 12 to 23 months (Kotloff et al., 2013).

In this article, the most commonly used techniques (selected based on published reports) involving microscopy, immunology and advance nucleic acid based approaches are addressed, highlighting their advantages and disadvantages. The review briefly describes the strategies adopted for the detection of viable *Cryptosporidium* ooysts and *Giardia* cysts and emphasizes the wide application of molecular methods for genetic characterization of *Cryptosporidium* and *Giardia* to provide suitable preventative measures.

It was evident from these studies that the conventional methods were mainly used, while the use of advanced molecular tools is applied partly in cooperation with partners outside the African continent. Still the implementation to increase sensitivity, specificity and genetic characterization of the targeted parasitic protozoa is not widespread. Investigations of their occurrence in environmental samples are limited.

2. Conventional techniques

2.1. Microscopic methods

The microscopy based methods have been extensively used for the detection of *Cryptosporidium* oocysts and *Giardia* cysts in environmental water, food, faecal and/or tissue samples (Quintero-Betancourt et al., 2003; Soares and Tasca, 2016). The morphological features of both, but especially in the case of *Cryptosporidium* cannot form the base for identification and differentiation using light microscopy (Fall et al., 2003). Therefore, different staining procedures have been used to aid in the clear distinction of especially *Cryptosporidium* oocysts from co-existing protists and for excluding similarities from the environmental or faecal debris.

2.1.1. Conventional staining methods for the detection of Cryptosporidium and Giardia

Different staining methods such as the traditional Ziehl-Neelsen, “negative staining technique” of Heine, Kinyoun's acid-fast technique, and less traditional applications such as trichome stain of different fluorescent-based stains such as auramine phenol and Safranin methylene blue staining (SMB) have been used for the detection of *Cryptosporidium* (Vohra et al., 2012). Acid-fast staining is usually the method of choice for the clinical microbiology laboratories. Some studies have incorporated dimethyl sulfoxide as a modification of producing oocysts with a brilliant pink to fuchsia colour against a pale green background (Vohra et al., 2012). Variability in stain uptake may occur both due to the stain itself and the age of the oocysts after prolonged

Table 1
Cryptosporidium and *Giardia* species prominent in African Continent (adapted and modified from Zahedi et al., 2015, Thompson and Ash 2016, and Squire and Ryan 2017).

Cryptosporidium Species	Host	Reported in Africa
C. parvum	*Bos taurus* (Cattle)	commonly reported in humans, but also reported in animals
C. hominis	*Homo sapiens* (Human)	most common species in humans but also reported in animals
C. meleagridis	*Meleagris gallopavo* (Turkey)	commonly reported in humans but also reported in animals
C. bovis	*Bos taurus*(Cattle)	reported in animals with rare reports of occurrence in humans
C. viatorum	*Homo sapiens* (Human)	reported in humans
C. felis	*Felis catis* (Cat)	reported in animals with rare reports of occurrence in humans
C. canis	*Canis familiaris* (Dog)	commonly reported in animals but also reported in humans
C. xiaoi	*Ovis aries* (Sheep)	reported in animals with rare reports of occurrence in humans
C. muris	*Mus musculus* (House mouse)	reported mainly in animals but also reported in humans
C. suis	*Sus scrofa* (Pig)	reported mainly in animals but also reported in humans
C. andersoni	*Bos taurus*(Cattle)	reported both in humans and animals
C. cuniculus	European rabbit	reported mainly in animals but also reported in humans
C. ubiquitum	*Bos taurus*(Cattle)	commonly reported in humans and animals
C. ryanae	*Bos taurus*(Cattle)	reported in animals
C. erinacei	*Erinaceus europaeus*(European hedgehog)	reported in animals with rare reports of occurrence in humans
C. baileyi	*Gallus gallus domesticus*(Chicken)	reported in animals

Giardia species	Host	Reported in Africa
G. duodenalis Assemblage A	Human other animals	Animals/humans
G. duodenalis Assemblage B		Animals/humans
G. duodenalis Assemblage C	Dogs and other canids	Animals/humans
G. duodenalis Assemblage D	Dogs and other canids	Animals
G. duodenalis Assemblage E	Cattle and other hoofed animals	Animals/humans
G. duodenalis Assemblage F	Cats	reported in animals with rare reports of occurrence in humans