A review of *Cryptosporidium* spp. and their detection in water

Eman M. Hassan, Banu Örmeci, Maria C. DeRosa, Brent R. Dixon, Syed A. Sattar and Asma Iqbal

**ABSTRACT**

*Cryptosporidium* spp. are one of the most important waterborne pathogens worldwide and a leading cause of mortality from waterborne gastrointestinal diseases. Detection of *Cryptosporidium* spp. in water can be very challenging due to their low numbers and the complexity of the water matrix. This review describes the biology of *Cryptosporidium* spp. and current methods used in their detection with a focus on *C. parvum* and *C. hominis*. Among the methods discussed and compared are microscopy, immunology-based methods using monoclonal antibodies, molecular methods including PCR (polymerase chain reaction)-based assays, and emerging aptamer-based methods. These methods have different capabilities and limitations, but one common challenge is the need for better sensitivity and specificity, particularly in the presence of contaminants. The application of DNA aptamers in the detection of *Cryptosporidium* spp. oocysts shows promise in overcoming these challenges, and there will likely be significant developments in aptamer-based sensors in the near future.

**Key words** | antibody, *Cryptosporidium*, detection, DNA aptamers, monitoring, PCR

**HIGHLIGHTS**

- The review discusses *Cryptosporidium* and methods available for their detection.
- Microscopy, immunology, nucleic acid-based methods and DNA aptamers are discussed.
- Aptamer-based detection is developing and has higher sensitivity and specificity.
- DNA aptamers also perform better in environmental samples with PCR inhibitors.

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INTRODUCTION

Originally described by Tyzzer in 1907 (Tyzzer 1907, 1912), Cryptosporidium spp. are intracellular, extra-cytoplasmic protozoan parasites with a monoxenous (single host) life cycle (Ghazy et al. 2015). The members of the genus Cryptosporidium were first recognized as human pathogens in 1976 (White 1976). They invade the microvillus border of the gastrointestinal epithelium of humans as well as many wild and domestic animals (De Graaf et al. 1999). Cryptosporidium spp. belong to the phylum Apicomplexa (O’Donoghue 1993). They have lost the apicoplast organelle, as well as genomes for both the plastids and the mitochondria (Ryan & Hijjawi 2015), but the genes associated with apical complex organelles (a group of organelles found at the apical end of the organism) are present (Sanderson et al. 2008). Cryptosporidium spp. have many unique features that distinguish them from other protozoa, including: (a) unusual location within the host cell, sequestered between the cell cytoplasm and cell membrane, (b) ability to initiate self-infection, and (c) innate resistance to disinfectants (Ghazy et al. 2015).

Nearly 30 species of Cryptosporidium and more than 40 genotypes are now known to cause infections in mammals, birds, reptiles, and fish (O’Donoghue 1995; Xiao et al. 2004; Ryan et al. 2014). The classification depends on factors such as the size and morphology of the oocysts, the life cycle, and host specificity (Khan et al. 2018). In humans, C. hominis and C. parvum are the most common cause of cryptosporidiosis, representing approximately 90% of infections. However, numerous other species, including C. meleagridis, C. ubiquitum, C. felis, and C. canis have also been reported (Guyot et al. 2001; Xiao et al. 2001; Ryan et al. 2014; Thompson & Ash 2016). The distribution of C. hominis and C. parvum varies by region (Xiao 2016). Both species are common in Europe (Zintl et al. 2009). C. parvum is predominant in the Middle East (Meamar et al. 2007), while C. hominis is more common in Australia and
North America (Morgan et al. 1998). Moreover, C. parvum outbreaks are generally associated with rural areas, while C. hominis is associated with more densely populated areas (Xiao & Feng 2008).

Cryptosporidiosis is the fourth leading cause of death among children under the age of 5, with 1.3 million deaths globally in 2015, accounting for 12.1% of worldwide deaths in children under the age of 5 (GBD Diarrhoeal Diseases Collaborators 2017). In the USA, members of the genus Cryptosporidium are considered the second highest cause of diarrhea and death after retroviruses (GBD Diarrhoeal Diseases Collaborators 2017). In sub-Saharan Africa, 2.9 million Cryptosporidium-attributable cases occur annually in children under 24 months, and infection is associated with a higher than two-fold increase in mortality in children aged 12–23 months (Sow et al. 2016; Squire & Ryan 2017). Cryptosporidiosis is the primary cause of chronic diarrhea among immunocompromised patients and is considered an AIDS-defining illness because of its high association with mortality (Inungu et al. 2000; White 2010).

Despite that healthy individuals may recover from cryptosporidiosis without specific drug treatment, and diarrhea can be managed by drinking fluids to prevent dehydration and, in some cases, taking anti-diarrheal medication, the treatment of cryptosporidiosis is still limited, and many compounds have failed in clinical trials (Hayley et al. 2015). However, nitazoxanide, which is available in the USA, showed success in the treatment of diarrhea caused by Cryptosporidium in immunocompetent individuals (Cabada & White 2010). Nevertheless, laboratory diagnosis is essential to confirm cases of cryptosporidiosis prior to any specific treatment (Hayley et al. 2015).

This review describes the biology of Cryptosporidium spp. and the current methods used in the detection of these parasites, with a focus on the methods used to detect C. parvum and C. hominis. It is necessary to critically examine the pros and cons of existing and emerging methods in order to improve the sensitivity and specificity of Cryptosporidium oocyst detection, especially in more challenging samples.

**LIFE CYCLE**

As the life cycles of all Cryptosporidium spp. are similar, those of C. parvum and C. hominis will be used as examples here. Typically, the infection begins with the ingestion of sporulated oocysts containing four sporozoites (Current & Garcia 1991; Khan et al. 2018). The median infectious dose is 312 oocysts (DuPont et al. 1995); however, as few as 10 oocysts can cause infection in humans (Okhuysen et al. 1999). The motile sporozoites inside each oocyst are released in the gastrointestinal tract through a suture in the oocyst wall by a process called excystation (Figure 1(b)) (Reduker et al. 1985; Bouzid et al. 2015). This process is believed to be triggered upon ingestion by factors such as body temperature, gastric pH, pancreatic enzymes, and bile salts (Fayer & Leek 1984; Reduker et al. 1985; Hijjawi et al. 2001). The released sporozoites glide over the surface of the intestinal cells, releasing proteins to help in the attachment and subsequent invasion of the host cells (Wanyiri 2006).

Sporozoites, in general, are crescent-shaped, with an apical complex containing micronemes, a single rhoptry, and dense granules (Figure 2) (O’Hara & Chen 2011). Each one of these structures is believed to have a role in the attachment to and invasion of the host cells (O’Hara & Chen 2011). The contents of micronemes are involved in adherence to host cells through the secretion of thrombospondin-related anonymous protein, and the rhoptry, possibly involved in host cell invasion and parasitophorous vacuole membrane (PVM) formation, releases its contents subsequently (Spano et al. 1998; Soldati et al. 2001). Dense granules likely release their contents subsequent to PVM formation and presumably modify this structure to help invade the host cells (Tetley et al. 1998). With the help of the rhoptry and micronemes, the parasite invades the host cell, including the cell membrane, to enclose the parasite in the PVM (Figure 1(c)) (Bouzid et al. 2015). The PVM is distinctive in that it remains extracytoplasmic but is considered intracellular as it keeps its position within the host-derived PVM on top of epithelial cells (Tzipori & Ward 2002). In the PVM, the parasite is protected from the hostile gut environment and is supplied with energy and nutrients by the host cell through a unique and highly invaginated membrane, the feeder organelle, which forms between the parasite and the host cytoplasm during internalization (Tzipori & Ward 2002).

Inside the PVM, each sporozoite develops into a spherical trophozoite, which reproduces asexually by a process called merogony to form a Type 1 meront containing eight merozoites (Figure 1); the meronts are morphologically similar to sporozoites (Current & Reese 1986; Bjorneby et al. 1990). When mature, they then invade neighboring intestinal epithelial cells. Type I meronts can either develop other Type I meronts, rapidly increasing the severity of the infection, or they can continue development to Type II meronts that contain four merozoites (Figure 1(f)) (Bouzid et al. 2015).
These merozoites, when released, attach to other epithelial cells in the intestinal tract. Instead of forming more meronts, Type II merozoites initiate a process called gametogony to complete the sexual stage of development (Göbel & Brändler 2013). Each merozoite produces either male or female equivalent sexual reproductive stages, known as microgamonts or macrogamonts, respectively (Figure 1(g) and 1(h)) (Göbel & Brändler 1982). Up to 16 microgametes are formed by the nuclear division of microgamonts, which, when released from the PVM, locate and fertilize a unicellular macrogametocyte that has developed from a macrogamont, forming a zygote (Figure 1(i)) (Current & Reese 1986). The diploid zygote undergoes a process similar to meiosis (sporogony) resulting in four haploid sporozoites within a sporulated oocyst (Figure 1) (Current & Reese 1986).

The resulting oocysts are either thick- or thin-walled and contain four sporozoites. The thick-walled oocysts are environmentally stable and are released into the environment with the host’s feces, allowing for the transmission of infection from one host to another, while the thin-walled oocysts remain in the intestine and autoinfect the same host. Autoinfection occurs when sporozoites are released from the thin-walled oocyst, and the cycle starts again (Smith & Rose 1998). Autoinfection and the formation of Type I meronts (asexual replication) provide an explanation for persistent chronic infection and make cryptosporidiosis a frequent cause of protracted acute diarrhea in some populations (DuPont et al. 1995; Okhuysen et al. 1999; Khan et al. 2018).
PARASITE TRANSMISSION AND OUTBREAKS

As a result of the life cycle of the parasite, substantial numbers (1 × 10^6 to 2 × 10^6 oocysts/g feces) of infective thick-walled oocysts are released in the host’s feces. Oocysts are immediately infectious, meaning they do not need to mature (sporulate) in the environment, as is the case with some other important protozoan parasites (Power et al. 2005). Humans may become infected through direct contact with infected persons (person-to-person transmission) or animals (zoonotic transmission), or indirectly through the ingestion of contaminated food (foodborne transmission) or water (waterborne transmission). Based on the numbers of cases and outbreaks, person-to-person and waterborne transmission are the most common means of spread (Donnelly & Stentiford 2011; Carmena 2013). A total of 936 illness outbreaks due to waterborne protozoa were reported between 1946 and 2016 worldwide (Toledo et al. 2017). Cryptosporidium was responsible for 58% of these outbreaks, in which oocysts either passed through filtered or unfiltered drinking water systems, or contaminated water supply systems in both small and large communities (Toledo et al. 2017). It is important to highlight here the resistance of the oocysts to chlorine and other chemical disinfectants conventionally used in water treatment processes (Chauret et al. 2001; Betancourt & Rose 2004). Moreover, the resistance of the oocysts to environmental factors and the occurrence of zoonotic species extends the range of reservoirs, leading to the contamination of wastewater resources (Smith & Rose 1998). In addition, oocysts may reach groundwater supplies by infiltration of contaminated surface waters (Rose et al. 2000). Oocyst concentrations commonly reported are in the range of 0.01–150/L of surface water, but higher concentrations have been found in agricultural run-off and urban wastewater effluents (Carmena 2010). The largest drinking water-related outbreak of cryptosporidiosis was recorded in 1993 in Milwaukee, Wisconsin (USA), affecting an estimated 403,000 people (out of a population of about 800,000), with 4,400 hospitalizations and more than 100 deaths (Mac Kenzie et al. 1994). Many other recorded outbreaks were associated with contaminated recreational water. Swimming in contaminated rivers and lakes or swimming pools is considered an important transmission route for Cryptosporidium (Lemmon et al. 1996).

There are fewer recorded foodborne outbreaks than waterborne ones. This may be due to the lack of standard detection tools for oocysts in food, and because food contamination may occur less frequently than water (Dawson 2005). Nevertheless, contamination of food may occur during production, harvest, processing, transport, or preparation. The presence of Cryptosporidium spp. oocysts has been documented in many foods such as raw milk, meat, apple cider, fermented milk, salads, and raw vegetables (Ryan et al. 2018).
The Safe Drinking Water Act (1996) of the US Environmental Protection Agency (USEPA) evaluates the risk to public health posed by drinking water contaminants, including Cryptosporidium spp. To implement the Act, the USEPA developed Method 1623 (USEPA 2005) to detect Cryptosporidium spp. and Giardia duodenalis, another important waterborne parasite, in raw surface waters used as source waters for drinking water treatment plants.

Currently, there is a lack of understanding of the importance of the sources and transmission routes of Cryptosporidium spp. Further research in this area will improve understanding of the significance of Cryptosporidium spp. on human and animal health. Moreover, the development of new and novel methods to detect and identify these organisms accurately will improve risk assessments and control methods (Messner et al. 2001).

**DETECTION METHODS FOR CRYPTOSPORIDIUM SPP.**

Due to the very low concentration of Cryptosporidium spp. oocysts present in water samples, and the lack of enrichment methods available, their recovery and detection rely upon filtration or centrifugation of the water sample, followed by selective concentration of oocysts. Water samples are routinely tested using a standardized method, USEPA Method 1623 (USEPA 2005), which involves filtration (using membrane or cartridge filters), or continuous flow centrifugation, of large volumes of water (minimum 10 L) to concentrate suspended particles, followed by selective concentration of Cryptosporidium oocysts and Giardia cysts using immunomagnetic separation (IMS), and staining with fluorescently labeled monoclonal antibodies, as well as 4',6-diamidino-2-phenylindole (DAPI), followed by fluorescence microscopy.

While Method 1623 is the most widely used, the three main steps in analysing water samples for Cryptosporidium and Giardia, i.e., particle concentration, selective concentration (separation) of target organisms, and detection, identification and enumeration, can be also be accomplished using a variety of alternative methods and technologies (Efstratiou et al. 2017). For example, flotation can be used instead of the costly IMS concentration of oocysts and cysts, and PCR (polymerase chain reaction)-based methods can be used for detection, allowing for the differentiation of parasite species and assemblages/genotypes, which is not possible using microscopy.

Several methods are available for the detection, identification, and research of Cryptosporidium spp, in laboratory and field studies. These methods have their pros and cons, such as detection limit, sensitivity, specificity, cost, processing time, level of difficulty and equipment requirement; not every method would be appropriate for routine quantification of Cryptosporidium spp. in water bodies.

**Microscopy-based methods**

Microscopy is one of the most widely used methods for the detection of Cryptosporidium oocysts in water, food, and fecal samples (O’Donoghue 1995). However, the identification of the different species of Cryptosporidium based on light microscopy alone is unreliable and not specific enough, because many species of Cryptosporidium share similar morphological characteristics (Fall et al. 2005). Moreover, the microscopic method does not allow for the determination of oocyst viability (Fall et al. 2005).

Several staining techniques have been employed to help in the differentiation (on glass slides) of Cryptosporidium oocysts from other protists and environmental or fecal debris. The Ziehl–Neelsen stain, also known as the acid-fast stain, is the most popular direct stain used in clinical microbiology laboratories to stain Cryptosporidium oocysts (Henriksen & Pohlenz 1981). In stool samples, dimethyl sulfoxide can be incorporated in the acid-fast procedure to produce pink oocysts to distinguish them from the stool debris (Vohra et al. 2012). Other negative stains such as malachite green (modified Heine staining technique) are used to stain the background material on slides and leave Cryptosporidium oocysts unstained (Elliot et al. 1999).

There are many limitations to using staining methods for the detection and identification of Cryptosporidium spp. oocysts, including (a) the variability in stain uptake by the oocysts, which depends on the stain itself and the age of the oocysts in the case of acid-fast staining, (b) the methods lack the required sensitivity and specificity, particularly for samples containing small numbers of oocysts and due to the fact that most Cryptosporidium oocysts, including C. parvum and C. hominis, are morphologically indistinguishable, (c) the need for a well-trained microscopist for the interpretation of the results, and (d) the method is very slow, tedious and subjective compared to other methods (Table 1) (Jex et al. 2008).

**Immunology-based methods**

Immunological methods offer higher specificity and sensitivity than microscopy in the detection of Cryptosporidium
oocysts in diverse types of samples since they use antibodies that target the wall of the oocyst (Table 2) (Jex et al. 2008).

**Table 1** | Comparison of stains commonly used in the detection of Cryptosporidium spp. oocysts

<table>
<thead>
<tr>
<th>Staining method</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Sensitivity and specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid-fast staining</td>
<td>Low cost, direct use, screening of large number of samples</td>
<td>Variation in the results due to the stain used and the age of the oocysts; time-consuming</td>
<td>70% sensitivity and 78% specificity</td>
<td>Shams et al. (2016)</td>
</tr>
<tr>
<td>Dimethyl sulfoxide modified acid-fast staining</td>
<td>Fast and simple; shows internal morphology without the need for fluorescence or phase-contrast microscopy</td>
<td>Variability in stain uptake; lacks sensitivity and specificity due to the interference from fixatives</td>
<td>Higher sensitivity than traditional acid-fast stain</td>
<td>Shams et al. (2016); Vohra et al. (2012)</td>
</tr>
<tr>
<td>Negative stains (malachite green, methylene blue, and crystal violet)</td>
<td>Useful for screening samples; rapid and inexpensive; good contrast between oocysts and background</td>
<td>Smears may be thick, affecting the visibility of the parasites</td>
<td>Sensitivity can be increased using phase contrast microscopy or examination at 400×</td>
<td>Khanna et al. (2014)</td>
</tr>
</tbody>
</table>

Direct and indirect immunofluorescence assay using monoclonal antibodies (mAbs)

Direct fluorescent antibody (DFA) assay uses a fluorescein isothiocyanate-conjugated anti-Cryptosporidium monochlonal antibody (FITC-C-mAb) to recognize surface epitopes on oocysts (McLauchlin et al. 1987; Arrowood & Sterling 1989; Jex et al. 2008; Smith & Nichols 2010). Fluorescing oocysts are then generally detected using immunofluorescence microscopy. Most commercially available FITC-C-mAbs are immunoglobulin M (IgM), which are used regularly for detecting and enumerating Cryptosporidium oocysts in environmental or fecal samples (Smith & Rose 1998). Monochlonal antibody-based DFA assays differ in their specificity and sensitivity according to many factors, including the purity of the Cryptosporidium antigen used originally to raise the mAb, the avidity and class/subclass of the antibody, the enzyme or fluorochrome-conjugated to the antibody, and the detection system (Shimizu 1997; Johnston et al. 2003). For instance, IgG1 antibodies were shown to have a better diagnostic specificity compared with IgG3 or IgM for the detection of Cryptosporidium oocysts in environmental samples due to the fact that IgG antibodies have a higher affinity to oocyst surface antigens compared to IgM antibodies in water samples (Ferrari et al. 1999; Weir et al. 2000).

Commercial DFA kits are available, such as the widely used MERIFLUOR® Cryptosporidium/Giardia test for stool samples from Meridian Biosciences (Table 3) (Johnston et al. 2003). This kit has recorded high specificity and improved sensitivity (96–100%), and requires less time and skill to apply. Moreover, MERIFLUOR enhanced the identification of the organism by minimizing the background as a result of using high-quality regents (Vohra et al. 2012). The reported specificity and sensitivity against Cryptosporidium using this kit are 99.8–100% (Johnston et al. 2003). Even though most commercially available DFA kits reported high specificity and sensitivity, none of them can specifically identify Cryptosporidium species pathogenic to humans or livestock (Jex et al. 2008). Since mAbs have been produced using a limited number of C. parvum oocyst isolates, it is likely that these antibodies cannot recognize oocysts representing different species and/or genotypes (because of variation in the molecular profile between isolates). Therefore, the intensity of fluorescence using C. parvum-derived FITC-C-mAbs is affected and requires further evaluation and improvement (Jex et al. 2008).

Indirect immunofluorescence assay requires additional steps compared to the DFA assay. The primary antibody in the indirect assay is not conjugated; instead, a second fluorophore-conjugated antibody is used to act against the primary antibody for the detection of Cryptosporidium oocysts. The indirect assay involves additional incubation time, making the process longer. Additionally, the use of the indirect assay produces more non-specific binding as a result of using a second antibody (Iacovski et al. 2004).

Immunomagnetic separation

Immunomagnetic separation (IMS) uses magnetic beads coated with an antibody raised against a particular species of Cryptosporidium, such as C. parvum. This method is often used to isolate oocysts from environmental samples (Smith & Grimason 2003). In this method, the recovery of
<table>
<thead>
<tr>
<th>Immunological method</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Type of sample tested</th>
<th>Sensitivity and specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct and indirect immunofluorescence assays using</td>
<td>Specifically bind to the targeted epitope; less background than traditional staining methods</td>
<td>Non-specific binding might occur when using a secondary antibody; long incubation times; requires expertise; antibodies have a short shelf life and batch-to-batch variations</td>
<td>Feces and water</td>
<td>99.8% sensitivity and 100% specificity as reported by MERIFLUOR® kit</td>
<td>Iacovski et al. (2004); Johnston et al. (2003)</td>
</tr>
<tr>
<td>monoclonal antibodies</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Immunomagnetic separation (IMS)</td>
<td>Bind specifically to the target epitope; when coupled to immunofluorescence assay, could detect a low number of oocysts</td>
<td>Very costly; cannot determine infectivity or viability of the oocysts; cannot distinguish between Cryptosporidium species</td>
<td>Surface water and wastewater</td>
<td>High sensitivity; efficiency depends on the filtration method used; recovery efficiency is between 62 and 100%</td>
<td>Allen et al. (2000); USEPA (2005)</td>
</tr>
<tr>
<td>Immunochromatographic lateral-flow assays</td>
<td>Fast and simple method of detection; the end results are visual without specialized equipment; does not require expertise</td>
<td>Possibility of false-negatives and -positives; often used with other methods of detection</td>
<td>Feces and water</td>
<td>97% sensitivity and 100% specificity</td>
<td>Esther et al. (2018); Johnston et al. (2003)</td>
</tr>
<tr>
<td>Enzyme-linked immunosorbent assay</td>
<td>Does not require microscopy</td>
<td>Non-specific binding when using secondary antibody may result in false-positive results; long incubation times</td>
<td>Feces</td>
<td>Variation in sensitivity and specificity (59–100% and 93–100% respectively)</td>
<td>Chalmers et al. (2011); Ghoshal et al. (2018); Vohra et al. (2012)</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>Rapid analysis of large numbers of samples; high sensitivity when combined with IMS</td>
<td>Costly equipment; requires expertise; limitations in the determination of infectivity</td>
<td>Feces, water, sewage</td>
<td>10 times more sensitive than DFA in fecal samples; limit of detection in water samples is 1,000 oocysts per L</td>
<td>Jex et al. (2008); Power et al. (2003)</td>
</tr>
<tr>
<td>Aptamer-based methods</td>
<td>Aptamers specifically bind to the selected target under a wide range of conditions (e.g., pH and temperature); more stable than antibodies; therefore, have longer shelf lives; chemically synthesized; no batch-to-batch variations, can be chemically modified to function in complex media (e.g., wastewater); short sample processing time (&lt;30 min)</td>
<td>Can be affected by nucleases in complex environments, but this can be overcome by adding functional groups to resist nucleases</td>
<td>Feces, water, sewage</td>
<td>Currently used only on food samples; potential for use on feces and water samples (e.g., drinking, surface and wastewater)</td>
<td>Hassan et al. (2021); Iqbal et al. (2015, 2019); Kong &amp; Byun (2015); Toh et al. (2014)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Comparison of immunological methods and aptamer-based methods for the detection of Cryptosporidium spp. oocysts</th>
</tr>
</thead>
</table>

Table 3 | Commercially available kits for Cryptosporidium spp. oocyst concentration and detection

<table>
<thead>
<tr>
<th>No.</th>
<th>Name of kit</th>
<th>Method of detection or concentrationb</th>
<th>Type of sample tested and sample processing time</th>
<th>Name of company</th>
<th>Specificity (%)</th>
<th>Sensitivity (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MERIFLUOR® Cryptosporidium/Giardia</td>
<td>Direct fluorescent antibody</td>
<td>Feces, 30 min</td>
<td>Meridian Biosciences</td>
<td>99.8</td>
<td>100</td>
<td>Meridian-Bioscience, USAa; Johnston et al. (2003)</td>
</tr>
<tr>
<td>2</td>
<td>Dynabeads™ anti-Cryptosporidium</td>
<td>Immunomagnetic separation (IMS)</td>
<td>Water concentrates, &gt;1 h</td>
<td>Invitrogen</td>
<td>NA</td>
<td>NA</td>
<td>ThermoFisher, USAa; Smith &amp; Nichols (2010)</td>
</tr>
<tr>
<td>3</td>
<td>ISOLATE® Cryptosporidium IMS kit</td>
<td>IMS</td>
<td>Water, &gt;1 h</td>
<td>TCS biosciences</td>
<td>NA</td>
<td>NA</td>
<td>tcsBiosciences, UK; Smith &amp; Nichols (2010)</td>
</tr>
<tr>
<td>4</td>
<td>ImmunoCard STAT®</td>
<td>Immunochromatographic lateral-flow assay (ICLFA)</td>
<td>Feces, &lt;10 min</td>
<td>Meridian Biosciences</td>
<td>100</td>
<td>97.3</td>
<td>Meridian-Bioscience, USAa; Johnston et al. (2005)</td>
</tr>
<tr>
<td>5</td>
<td>Crypto-Strip</td>
<td>ICLFA</td>
<td>Feces, &lt;10 min</td>
<td>CORIS (BioConcept)</td>
<td>100</td>
<td>95.7</td>
<td>Corisbio, Belgium; Cheun et al. (2013)</td>
</tr>
<tr>
<td>6</td>
<td>EDI™ Fecal Cryptosporidium parvum Antigen ELISA Kit</td>
<td>Enzyme linked immunosorbent assay (ELISA)</td>
<td>Feces, &gt;2 h</td>
<td>Epitope Diagnostics, Inc. (EDI)</td>
<td>NA</td>
<td>5 ng/mL of C. parvum antigen</td>
<td>Epitope-diagnostics, USA</td>
</tr>
<tr>
<td>7</td>
<td>Cryptosporidium TaqMan RT-PCR Kit</td>
<td>PCR and qPCR</td>
<td>Water, &gt;1 h</td>
<td>NORGEN BIOTEK. CORP</td>
<td>NA</td>
<td>40 fg of C. parvum DNA</td>
<td>Norgenbiotek, Canada; Guy et al. (2003)</td>
</tr>
<tr>
<td>8</td>
<td>TaqMan® MGB probes</td>
<td>qPCR</td>
<td>Water, &gt;1 h</td>
<td>Applied Biosystems™</td>
<td>NA</td>
<td>Higher than TaqMan</td>
<td>ThermoFisher, USAa; Yao et al. (2006)</td>
</tr>
<tr>
<td>9</td>
<td>BD MAX™ Enteric Parasite Panel</td>
<td>PCR</td>
<td>Feces, water and food, &gt;1 h</td>
<td>BD Life Sciences</td>
<td>99.7</td>
<td>100</td>
<td>BD Molecular Diagnostics, USA; Perry et al. (2017)</td>
</tr>
</tbody>
</table>

*aRecovery efficiency: 60 to >95%.

ARecovery efficiency: 60 to >95%.
bAntibody-based kits (1–6) cannot be used to determine genotypes, but nucleic acid-based kits (7–9) can.

NA: not available.
oocysts takes place when large volumes of water are filtered and concentrated to a smaller volume. For example, USEPA method 1623 uses a cartridge to concentrate the oocysts from large volumes of water (e.g., 1,000 L) to smaller volumes (USEPA 2005). Then the elution is added to antibody-conjugated magnetic beads. After applying a magnetic field, oocysts are isolated and studied by other immuno-assays (USEPA 2005; Smith & Nichols 2010). The count and viability of the oocysts are assessed using immunofluorescence microscopy by staining them with DAPI. The internal morphology of the oocysts is determined using differential interference contrast microscopy (USEPA 2005; Smith & Nichols 2010).

The recovery efficiency of IMS has been reported to be between 62 and 100% (Rochelle et al. 1999). Many IMS kits are commercially available and are widely used, including the Dynabeads™ anti-Cryptosporidium kit developed by Invitrogen and ISOLATE® Cryptosporidium IMS kit and reagents developed by TCS Biosciences (Table 3). Both kits have similar antibody-conjugated bead–oocyst complex concentration systems. Each has a different mAb that was raised against C. parvum. Both kits reported high sensitivity and specificity in detecting C. parvum oocysts from water samples (Smith & Nichols 2010). However, these kits are not likely to recognize other Cryptosporidium species such as C. hominis found in water samples (Smith & Nichols 2010). IMS is a valuable technique to increase the sensitivity of oocyst detection in samples where the abundance of oocysts is low or intermediate, such as surface water and wastewater samples (Atwill et al. 2003; Robinson et al. 2008; Coklin et al. 2011). However, as an antibody-based method, IMS does not provide species or genotype identification of the captured microorganisms. Moreover, IMS is incapable of assessing the infectivity of the captured microorganisms (Allen et al. 2000; Simmons et al. 2001). Other challenges are the time-consuming nature of the method and the high cost of the kits (Lechevallier et al. 2003).

**Immunochromatographic lateral-flow assay**

Immunochromatographic lateral-flow assay (ICLFA) is a rapid antibody strip-based test. In this method, antigens of different Cryptosporidium species, or other parasites such as Giardia, are detected in fecal and water samples (Chan et al. 2000). The antibody raised against the targeted parasite is immobilized on a paper-based strip. Then the sample is migrated through capillary action to interact with the antibody. This method is widely used compared to other immunological methods because of its many advantages, including rapid antigen detection (10 min compared to the DFA method, which requires 1–2 h incubation time), simplicity, cost-effectiveness, no requirement for bulky expensive equipment (e.g., microscopes) and highly trained personnel to analyze the data. ICLFA sensitivities of higher than 97%, and specificities of 100% have been demonstrated (Garcia & Shimizu 2000; Johnston et al. 2003; Shams et al. 2016).

Many strip-based commercial kits have been developed (Table 3) (Garcia & Shimizu 2000; Esther et al. 2018). ImmunoCard STAT® developed by Meridian Biosciences is one example (Johnston et al. 2003). This kit is widely used as a quick test to detect C. parvum and Giardia lamblia antigens (Johnston et al. 2003; Esther et al. 2018). ImmunoCard STAT reported high sensitivity (97.3%) and specificity (100%) in detecting C. parvum (Meridian Biosciences, USA). It is a very easy to use, time-saving, screening (presence/absence) test, without the need for expertise to perform the test or analyze the data (Esther et al. 2018).

Despite the high sensitivity of ICLFA kits, many cases of false-positives have also been reported (Garcia et al. 2000; El-Moamly & El-Sweify 2012), and false-negatives have occurred when there are fewer oocysts (Garcia et al. 2003). In many cases, the strip-based positive results obtained from different laboratories in the USA could not be confirmed by immunofluorescence assay methods (Roellig et al. 2017). Therefore, the US Council of State and Territorial Epidemiologists (CSTE) stated that diagnosis with ICLFA is considered probable rather than confirmed until further improvement of these tests (Ryan et al. 2017; Ahmed & Karanis 2018).

**Enzyme-linked immunosorbent assay**

In the enzyme-linked immunosorbent assay (ELISA), an antibody against C. parvum (or any targeted parasite) is immobilized on the surface of a plate, and then the antigen (i.e., the sample) is introduced. Secondary antibodies and enzymes are added to allow for detection by absorbance spectroscopy. ELISA is widely used in the detection of C. parvum in stool samples obtained from humans and animals (Elgung & Koltas 2011). The procedure does not require the concentration of oocysts from fecal samples before processing. Therefore, ELISA allows for rapid examination of large numbers of stool samples (Clark 1999; Ghoshal et al. 2018).

It has been reported that the sensitivity of ELISA is 10 times higher than acid-fast staining, with a detection limit of $10^3$–$10^4$ oocysts/mL (Shams et al. 2016; Ghoshal et al. 2018). However, high variations of sensitivity (59–100%) and specificity (93–100%) of ELISA results have been
reported (Chalmers et al. 2011; Rossle & Latif 2013; Ghoshal et al. 2018), due to the use of different commercial kits (Table 3), different population samples (human/animal), and the use of different references for evaluation (Ahmed et al. 2011; Ahmed et al. 2012). Moreover, when working with ELISA, longer processing times (1–3 h) and the potential for false-positive results should be considered. Detection of C. parvum, or any other targeted parasite, in water samples is not reliable using ELISA as these samples may be complex in their constituents and the number of oocysts may be very low. ELISA is, therefore, used mainly for testing fecal samples (Esther et al. 2018).

Flow cytometry

Flow cytometry (FC) is an automated technique that has become popular due to its many advantages, including the analysis of large number of cells (or other particles) in a short time, and its high sensitivity and specificity. In FC, the sample containing the cells (or other particles) is suspended in a fluid and directed past a laser beam one cell at a time. The light scattered is picked up by detectors and is characteristic of the cells and their components (Esther et al. 2018).

Cryptosporidium oocysts were first detected by Vesey et al. (1991) using this approach. In that study, Cryptosporidium oocysts were spiked in water and sewage samples after staining with FITC-mAb. The results showed a limit of detection as low as 1,000 oocysts per L (Vesey et al. 1991). Similar studies have shown the detection of Cryptosporidium oocysts in fecal samples with limits of detection around 2 × 10³ oocysts/mL (Bárbarosa et al. 2008). Other studies showed that flow cytometric analysis of stool samples from persistently infected mice is 10 times more sensitive than that of DFA followed by immunofluorescence microscopy (Shams et al. 2016).

Recent studies have combined FC with IMS to increase the recovery of the oocysts from water samples and, therefore, increase the sensitivity of the method (Power et al. 2003; Hsu et al. 2005). However, limitations of the specificity of the current IMS antibodies prevent the differentiation of oocysts which are infective to humans from those that are not. Moreover, the use of the FC method is infrequent in the diagnostic parasitology laboratory because of the cost of the instrumentation and the need for technical expertise (Jex et al. 2008).

Cell culture immunoﬂuorescence assay

None of the methods mentioned above determine the viability or infectivity of Cryptosporidium oocysts. Therefore, new methods that culture the oocysts in vitro have emerged. The principle behind the in vitro cultivation of C. parvum oocysts is to simulate the in vivo environment to allow the oocyst to complete the life cycle outside the host and to eliminate the use of animal models (Villacorta et al. 1996; Arrowood 2002). In addition to the detection of viable oocysts, cell culture can also be used to generate continuous supplies of fresh live oocysts which can facilitate the detailed knowledge of the pathophysiology of C. parvum and can, therefore, be useful in studies on control methods and drug treatment (Arrowood 2002; Karanis 2018).

The cell culture immunoﬂuorescence assay method uses cell lines that are susceptible to infection with low levels of C. parvum oocysts. Then, they are monitored for the production of infectious oocysts and different stages of C. parvum development using specific staining methods by fluorescence microscopy (Johnson et al. 2012). The first successful in vitro culture of C. parvum was performed by Rosales and colleagues in Madin-Darby Canine Kidney (MDCK) cells (Rosales et al. 1993). Many other cell lines have since been used to culture C. parvum in vitro, with the most common one being HCT8 (human colon adenocarcinoma cell line) (Hijjawi et al. 2001; Arrowood 2002; Sifuentes & Di Giovanni 2007). HCT-8 was successfully used for determining the infectivity of C. parvum oocysts in water samples (Slifko et al. 1997). However, the method was incapable of determining the infectivity in samples where the oocyst count was low (under 10 oocysts) (Lai 2009). Moreover, studies have shown that C. parvum-infected HCT-8 does not produce enough infective oocysts to maintain the infected culture, which raises questions regarding the suitability of HCT-8 for the study of Cryptosporidium biology (Müller & Hemphill 2013).

The major limitations with in vitro cultivation methods for C. parvum are the failure of long-term propagation (i.e., maintaining the infected culture) and the low yields of infective oocysts representing any asexual and sexual developmental stages (Karanis 2018). To overcome these limitations, a recent study by Miller and colleagues showed that the use of a new cell line, COLO-680N, produced infective C. parvum oocysts (after 2 weeks of initial infection) 40-fold higher than HCT-8 and other cell lines (Miller et al. 2018). This was confirmed using many methods including fluorescence microscopy. However, further studies on this cell line are required (Miller et al. 2018).

Nucleic acid-based methods

Nucleic acid-based methods for the detection of Cryptosporidium spp. and other infectious parasites offer many
advantages over microscopy and the immunological methods. In addition to being highly sensitive and specific, they allow for the identification of the host species, genotype, and sub-genotype, and they permit the identification of the source and the severity of infection in the case of an outbreak situation (Figure 3).

Conventional PCR

PCR is used to amplify a portion of a target gene into several million detectable copies in a short time, and the product is detected after the amplification ends. This type of PCR is called conventional PCR and it is a well-established method. PCR-based assays have been used extensively to detect Cryptosporidium oocysts and to determine the species and genotypes in various types of environmental and clinical samples (Jex et al. 2008; Ahmed & Karanis 2018; Prystajecky et al. 2018; Smith & Nichols 2010).

Nested PCR

Nested PCR uses two sets of primers, where the first primer set binds to sequences outside of the target DNA, and the product serves as a template for the second pair of primers. Nested PCR increases sensitivity and specificity by decreasing the non-specific binding resulting from the first set of primers. Nested PCR has been used to detect Cryptosporidium oocysts in surface water, wastewater, and in both human and animal fecal samples (Monis & Saint 2001; Osaki et al. 2015; Prystajecky et al. 2014; Mirashemi et al. 2015; Ulloa-Stanojlović et al. 2016; Koehler et al. 2017).

Quantitative PCR

Quantitative PCR (qPCR) is widely used in the detection of different types of pathogens in environmental and clinical samples (Singh et al. 2010, 2016; Hanabarà & Ueda 2016). Unlike conventional PCR, qPCR allows real-time monitoring of DNA amplification over a wide dynamic range genetic target.

qPCR offers rapid, cost-effective, and sensitive identification and quantification of Cryptosporidium species. qPCR has been used to detect C. parvum, and C. hominis isolates separately or in a mixture, with no cross-reaction with other genera (Yang et al. 2015). qPCR was also effectively used to detect and distinguish between C. hominis and C. parvum in human fecal samples by targeting the small subunit (SSU) ribosomal RNA (rRNA) gene (Hadfield et al. 2011). Moreover, qPCR was used to detect C. hominis and C. parvum in environmental and sewage samples by using TaqMan™ primers to target the Cryptosporidium oocyst wall protein (COWP) gene (Guy et al. 2003; Anceno et al. 2007). Other studies have reported a detection limit of 2.5–59 Cryptosporidium oocysts per 100 L of river water (Ohgaki 2006). The presence of inhibitors in fecal and environmental samples can affect the PCR and lower the sensitivity of the method (Le Govic et al. 2016). PCR inhibitors such as bile salts, complex polysaccharides, and sample fixative can inhibit Taq polymerase and block the DNA extraction columns (Clark 1999; Gasser 2006; Wells et al. 2016). To overcome this issue, proper controls should be considered. For example, negative controls containing the substances that are believed to inhibit PCR should be applied along with the sample but in a separate PCR reaction. Furthermore, choosing the proper set of primers can improve the specificity of the assay. For example, the use of TaqMan™ MGB (minor groove binder) probes resulted in higher specificity than standard TaqMan™ primers (Table 3) (Yao et al. 2006).

Multiplex qPCR assays that detect multiple parasites simultaneously have been developed (Guy et al. 2003; Nurminen et al. 2015). Many of them are used to detect Cryptosporidium along with other parasites (Ryan et al. 2017). The sensitivity of these multiplex assays ranged from 95 to 100% and specificity from 99.6 to 100% (Ryan et al. 2017). For example, BD MAX™ Enteric Parasite Panel detects many parasites, including C. hominis and C. parvum, and has a sensitivity ranging from 91.6 to 100% and specificity of 98.9% (Table 3) (Parčina et al. 2018).

Droplet digital PCR

Droplet digital PCR (ddPCR) was recently developed to improve the quantification of nucleic acid for different pathogens in complex environments (Baker 2012; Pinheiro et al. 2012; Pavšič et al. 2016). Unlike traditional qPCR, ddPCR allows DNA quantification without the need for standard calibration curves, which are difficult to generate due to the lack of reference standard curves (Majumdar et al. 2015). The use of ddPCR has not been explored extensively to detect Cryptosporidium. However, recent studies reported the use of ddPCR and qPCR in the quantification of Cryptosporidium in water and fecal samples by targeting the 18S rRNA and actin gene loci (Yang et al. 2014; Zahedi et al. 2016). The precision of ddPCR was higher than qPCR for both loci as measured by the relative standard deviation, but the precision of ddPCR decreased with decreasing DNA concentration, while qPCR was unaffected.
Nucleic acid-based methods used for the detection of Cryptosporidium parvum and Cryptosporidium hominis in fecal or water samples. After oocyst purification from the sample, various nucleic acid-based methods can be applied, including (a): next generation sequencing (NGS), (b) PCR-based methods such as qPCR, nested and droplet digital PCR, (c) fluorescence in situ hybridization (FISH), (d) DNA fingerprinting.
PCR-restriction fragment length polymorphism

PCR-restriction fragment length polymorphism (PCR-RFLP) is a technique that exploits variations in a genome, utilizing specific primer pairs for the selective amplification of the variable region (genetic loci), followed by digestion of the amplified segment with restriction enzymes and analysis by gel electrophoresis to determine the species or genotype. Nested PCR is usually followed by RFLP to increase the sensitivity of the assay (Quintero-Betancourt et al. 2002). This method is used to determine the genotype of Cryptosporidium by the detection of many different genetic loci, including SSU rRNA genes and spacers, COWP, the 70 kDa heat shock protein (HSP70), 18S rRNA and the 60 kDa glycoprotein (gp60) gene (Spano et al. 1997; Nichols et al. 2003; Xiao et al. 2004). Using a multi-locus approach in characterizing Cryptosporidium isolates increases accuracy and confidence in the diagnosis. The 18S rRNA loci was used to determine Cryptosporidium genotypes, while the COWP and HSP70 loci detected and confirmed the presence of C. hominis, C. parvum and C. meleagridis in various samples including water samples (Smith & Nichols 2010). PCR-RFLP at the 18S rRNA and gp60 loci were used in the detection of sub-genotypes of C. parvum in a food-borne outbreak in 2006, indicating the importance of multi-locus PCR-RFLP approaches (Blackburn et al. 2006).

Although PCR-RFLP is a fast and effective tool for distinguishing between very closely related genotypes of Cryptosporidium, the restriction enzymes used only recognize a small number of variable sites (Gasser 2006). Moreover, the assay is more expensive than random amplification of polymorphic DNA (RAPD) (described below) and requires longer times and is more hands-on (Berg 2012). PCR-RFLP is gradually being replaced by the low-cost DNA sequencing technologies currently available (Dwivedi et al. 2017).

DNA sequencing

C. parvum and C. hominis genome sequencing were completed in 2004 using a shotgun Sanger sequencing method (Abrahamsen et al. 2004; Xu et al. 2004). Simply, this process starts with the extraction of whole genomic DNA from purified oocysts, then plasmid libraries were prepared and amplified in Escherichia coli, from which many clones were sequenced and used as reference in the detection of both parasites from different samples (Abrahamsen et al. 2004; Xu et al. 2004). This discovery revolutionized our understanding of C. parvum and C. hominis and transformed the molecular detection methods of these two parasites.

DNA sequencing is considered the gold standard approach in the detection of mutations and the rapid discovery of single nucleotide polymorphisms as indicators of genetic variations (Dwivedi et al. 2017). To date, DNA sequencing has enabled the classification of more than 34 species and 40 genotypes of Cryptosporidium by sequencing the SSU rRNA gene (Koehler et al. 2017). This has enabled researchers to compare their genetic materials and phenotypic differences (Mazurie et al. 2013). It has also allowed for the evaluation of genomic variation within and among species of Cryptosporidium (Stu & Figure 2014). Recently, gp60 gene-based sequencing of both C. parvum and C. hominis enabled the screening of genomic variation at the sub-genotype level (Feng et al. 2017; Sikora et al. 2017).

DNA sequencing has the limitation of providing accurate sequences from only one amplicon because amplicons are produced from an isolate representing a population of oocysts rather than amplification of DNA from only one oocyst, which is not practical. Therefore, a large number of isolates should be analyzed. DNA sequencing includes multiple steps, and is expensive and time-consuming, especially when large numbers of samples are analyzed (Bankier et al. 2005; Gasser 2006). Despite these limitations, DNA sequencing is an established procedure in many laboratories for whole-genome sequencing of Cryptosporidium isolates from clinical and environmental samples (Hadfield et al. 2015; Honsvall & Robertson 2017; Koehler et al. 2017).

Recently, next-generation sequencing platforms have been used to produce new whole genome sequences for C. parvum and C. hominis (Guo et al. 2015). For example, the Illumina MiSeq, combined with multiplex technologies, offers complete sequencing of Cryptosporidium genomes by providing vast amounts of data (100-fold more in-depth than Sanger sequencing) for less than US$100 per sample (Stu & Figure 2014).

C. parvum and C. hominis isolates from water samples have been detected via Illumina sequencing by using as little as nano-gram amounts of genomic DNA (Moreno et al. 2018). Whole genome sequencing of Cryptosporidium spp. has also been accomplished using this technology on stool samples (Hadfield et al. 2015; Kaupke et al. 2017).
DNA fingerprinting

Fingerprinting is the screening of the genome(s) for variation in sequence and organization (Nichols et al. 2006). RAPD is a PCR method which uses short random primers (8–12 nucleotides) to amplify fragments of a genome, and subsequent separation of the amplicons by polyacrylamide gel electrophoresis, therefore, finding a fingerprint for some parasite isolates (Welsh & McClelland et al. 1990). RAPD was used to determine the fingerprint of different isolates of Cryptosporidium species (Morgan et al. 1996). Moreover, RAPD provided information regarding the role of genetic exchange in the population structure and diversity of C. parvum and C. hominis (Jex et al. 2008). The advantage of this method is that no prior knowledge of the genome sequence is required. However, the fingerprint represents a population of organisms rather than an individual profile. Therefore, all individuals in a population might not be represented (Hadrys et al. 1992). Other similar methods such as amplified fragment length polymorphism, satellite DNA (microsatellites and mini-satellites) and multi-locus satellite analysis were coupled with PCR to fingerprint the Cryptosporidium species (C. parvum and C. hominis) (Mallon et al. 2003; Grinberg et al. 2008).

Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) is a molecular technique that uses fluorescent probes (labeled oligonucleotides) that detect and localize specific RNA DNA sequences in the genome with a high degree of sequence complementarity. The probes used in FISH can detect RNA-specific sequences as well, including messenger RNA or rRNA. Currently, most FISH probes available for the detection of Cryptosporidium oocysts hybridize and bind to a variable region of the SSU of the nuclear rRNA (Deere et al. 1998a, 1998b; Bednarska et al. 2007). The SSU rRNA is abundant (i.e., present in high copy numbers) in the cell and has, therefore, been used as the targeted sequence by FISH (Wallner et al. 1993; Vesey et al. 1998). Moreover, it was reported that only viable oocysts would be detected because RNA would be degraded by RNases upon cell death and, accordingly, not be detectable in dead oocysts (Vesey et al. 1998).

FISH probes have been successfully used in the specific identification and viability determination of C. parvum and C. hominis in human fecal and water samples, with higher sensitivity than methods that use mAbs, since FISH probes (RNA oligonucleotides) provide an indication of viability and are species-specific (Lemos et al. 2005; Alagappan et al. 2009). A high concentration of oocysts is frequently required to enhance the sensitivity of the method. Therefore, FISH can be combined with IMS in some cases (Esther et al. 2018). The sensitivity of the technique relies on the decay rate of SSU rRNA, which can be affected by many factors such as environmental conditions (i.e., temperature, pH, salinity and/or RNase contamination), and the degradation of rRNA as a result of sample processing which can produce false-negative FISH results (Smith et al. 2004). Therefore, optimization of the FISH technique is essential, with hybridization time, temperature, permeabilization and fixation conditions, and probe specificity all being considered before using this technique.

Aptamer-based methods

The successful detection of C. parvum using the majority of the methods mentioned previously depends on the separation and concentration of the oocysts from the sample (recovery of the oocysts), and this ultimately depends on the affinity of the ligand used. The recovery of oocysts from water and food samples is a challenge due to many factors. First, the presence of other pathogens and non-pathogens: water and food samples contain many other microorganisms along with C. parvum (e.g., Giardia species), which may lead to false-positives as a result of non-specific binding. Second, the number of oocysts found in these samples may be low (Smith & Nichols 2010), requiring a detection method of high sensitivity. Third, the complexity of the sample: water and food samples contain constituents that could mask the oocysts or interfere with the downstream processing (i.e., DNA extraction and amplification). Therefore, improving the sensitivity and specificity of detection is crucial in accurately assessing the risks and applying more effective preventative measures.

Improving the ligand used in detection methods could improve the sensitivity and reliability of the assay. Aptamers, synthesized molecular recognition probes, represent one approach. Aptamers are single-stranded (ss) DNA or RNA oligonucleotides that bind to their targets with high affinity and selectivity with a unique three-dimensional interaction (Ellingto & Szostak 1990). They are produced by an in vitro process called Systematic Evolution of Ligands by EXponential enrichment (SELEX) (Yüce et al. 2015; Jijakli et al. 2016). SELEX enriches target-binding sequences from randomized large ssDNA or RNA libraries. In each round of SELEX, target-binding sequences are eluted from target molecules, amplified, and used as the library for subsequent rounds (Yüce et al. 2015; Jijakli et al. 2016; Bayat et al. 2018;
Soldevilla et al. 2018). This process results in an aptamer with high affinity and specificity for the target (Figure 4).

Aptamers can replace antibodies due to their similar target recognition properties (Table 2). Aptamers are selected and synthesized at a relatively low cost, without the use of cell lines or animals, which are required for antibody production (Kong & Byun 2013). Furthermore, antibodies have batch-to-batch variations, whereas aptamers do not (Toh et al. 2014). This could affect the reproducibility of many commercially-available mAbs for the detection of C. parvum. Aptamers also have a longer shelf-life than antibodies due to their chemical and thermal stability (Ku et al. 2015). However, unmodified DNA aptamers can be recognized by endonucleases and therefore might have limitations in complex environments. To overcome this, aptamers when synthesized allow for the introduction of functional groups on specific sites without the disruption of their structure or function (e.g., adding polyethylene glycol on the 5’ end of the aptamer for resisting renal clearance, and adding fluorine on the sugar ring for nuclease resistance). This allows them to be used under a wide range of conditions and in complex media (Gao et al. 2016). Furthermore, aptamers can be conjugated to magnetic beads (Modh et al. 2018) and can be used to concentrate the oocysts in a much smaller volume. Therefore, aptamers are powerful candidates for the direct detection of C. parvum.

Figure 4 The process of systematic evolution of ligands by exponential enrichment (SELEX). A random ssDNA library (or ssRNA) is incubated with the target of interest (positive selection B). Unbound sequences are washed off and binding sequences are eluted off the target. The eluted sequences are amplified by PCR, and the rounds are then repeated. A counter selection step (A) may be applied whereby the ssDNA library is incubated with a target similar to that of interest, then un-bound sequences to the counter target serve as the initial library for the positive selection (B).
and other Cryptosporidium species in food and fecal samples, and they have great potential to be used to recover (concentrate) the oocysts from various water and wastewater samples (Table 2).

A DNA aptamer (R4-6) for the detection of C. parvum in food samples was developed by Iqbal et al. (2015). In this study, a group of aptamer candidates was selected against the oocyst wall of C. parvum from an ssDNA library. Then, the selected aptamers were screened for their affinity for the oocysts. An electrochemical aptasensor was developed to detect the parasite in spiked food samples. This proof-of-concept study demonstrated that the R4-6 aptamer could detect as few as 100 oocysts in 25 g fruit samples, indicating that this method is promising for the detection of C. parvum oocysts compared to conventional methods (Table 2) (Iqbal et al. 2015).

In addition to having high sensitivity and specificity, aptamers may also have the advantage of performing better in surface water and wastewater samples. The detection of C. parvum and C. hominis typically requires filtration and concentration of large volumes of water prior to quantification (USEPA 2005). Many constituents, including PCR inhibitors and nucleases, are present in surface water and, particularly, in wastewater. Since the filtration and concentration do not entirely remove these constituents, and the number of oocysts present in these samples is often very low, a potent ligand capable of resisting these interferences and having a high affinity to the oocysts is needed. Due to its ability to be chemically modified with several functional groups, aptamers are promising in this respect. Moreover, aptamers can be selected to bind to their targets with high affinity and selectivity in other complex samples (e.g., plasma and blood) (Hassan et al. 2017).

DNA-aptamer-based aptasensors, coupled with magnetic beads, were developed to detect C. parvum oocysts in drinking and recreational water samples (Iqbal et al. 2019). A sensitive and specific electrochemical aptasensor (5’-biotinylated R4-6 aptamer) was used as a secondary ligand to bind the streptavidin-coated magnetic beads. The aptamer-coated probe could selectively bind to C. parvum oocysts and had a detection limit of 50 oocysts. Hassan et al. (2021) rationally truncated R4-6 DNA aptamer into two minimer aptamers (Min_Crypto1 and Min_Crypto2), conjugated to micro-magnetic beads, and tested them in increasingly challenging water matrices (phosphate buffer, river water and wastewater). Both minimers showed a superior affinity and specificity toward C. parvum oocysts compared to the parent R4-6, and they did not exhibit non-specific binding to Giardia duodenalis cysts. Min_Crypto2 aptamer, which had the shorter truncated sequence (32 nucleotides), could detect five oocysts in 300 μL wastewater, which was the most challenging sample in the study. Min_Crypto2 performed equally well in wastewater when it was used free in wastewater or conjugated to magnetic beads.

The technology of aptamer selection was developed more than 30 years ago, and in spite of the promising aspects of aptamer-based detection of pathogens, progress so far has been rather slow. Issues limiting the progress of aptamers include the time- and labour-intensive process of aptamer generation, rapid degradation of aptamers by nucleases in biological samples, aptamer cross-reactivity, and lack of standardized protocols (Lakhin et al. 2015). Recent advances in aptamer research have already addressed many of these issues alongside the decreasing cost of chemical synthesis and progress in automation of aptamer generation. Aptamers are likely to find widescale application in diagnostics, therapy, and biosensing in the near future.

**CONCLUSION**

This review discusses the waterborne parasite Cryptosporidium and the methods currently available for its detection with particular reference to C. parvum and C. hominis. Microscopy-based methods are unreliable and not specific in the identification of Cryptosporidium species. Moreover, they require tedious work and a high level of expertise. Immunological methods are sensitive and specific, but they cannot be used to identify the parasite to the genotype/sub-genotype level, and do not allow for the determination of viability or infectivity. While the latter can be achieved through in vitro culture, many challenges also exist with this technology. Similarly, nucleic acid-based methods are very sensitive and specific in the detection of C. parvum and other Cryptosporidium species, and they also offer the advantage of genotype/sub-genotype identification. However, these molecular approaches require specialized expertise and equipment, and they cannot be used to determine viability.

There are many challenges faced when testing water samples for the presence of Cryptosporidium spp. First, there is usually a low number of oocysts in water samples. Second, water samples, and especially wastewater samples, contain organic substances and other contaminants that can interfere with the detection of oocysts and hinder the
sensitivity and specificity of the detection methods. Apta-
mers have been recently used for detecting *C. parvum* in
food samples and have advantages over immunology-based
methods and other nucleic acid-based methods. For
example, aptamers can be chemically modified to minimize
the interference from water constituents that could affect the
sensitivity of the detection, and they can be conjugated to
magnetic beads to concentrate oocysts. The development
of aptamers and aptamer-based sensors for detecting *Cryp-
tosporidium* spp. is in the early stages, and significant
developments are expected in this emerging field.

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**DATA AVAILABILITY STATEMENT**

All relevant data are included in the paper or its Supplemen-
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**REFERENCES**

Abrahamsen, M. S., Templeton, T. J., Enomoto, S., Abrahante,
J. E., Zhu, G., Lancto, C. A., Deng, M., Liu, C., Widmer, G.,
Tzipori, S., Buck, G. A., Xu, P., Bankier, A. T., Dear, P. H.,
Konfortov, B., Spriggs, H. F., Iyer, L., Anantharaman, V.,
Aravind, L. & Kapur, V. 2004 Complete genome sequence of the

Ahmed, S. A. & Karanis, P. 2018 Comparison of current methods
used to detect *Cryptosporidium* oocysts in stools. *Int. J. Hyg.
ijeh.2018.04.006.

Alagappan, A., Bergquist, P. L. & Ferrari, B. C. 2009 Development
of a two-color fluorescence in situ hybridization technique
for species-level identification of human-infectious

https://doi.org/10.1002/j.1551-8833.2000.tb09005.x

Anceno, A. J., Katayama, H., Houpt, E. R., Chavalitshewinkoon-
Petmitr, P., Chuluun, B. & Shipin, O. V. 2007 IMS-free DNA
extraction for the PCR-based quantification of
*Cryptosporidium parvum* and *Giardia lamblia* in surface and
doi.org/10.1080/09603120701372573.

Arrowood, M. J. 2002 In vitro cultivation of *Cryptosporidium*
15.3.390.

Arrowood, M. J. & Sterling, C. R. 1989 Comparison of conve-
tional staining methods and monoclonal antibody-

Atwill, E. R., Hoar, B., Das Graças Cabral Pereira, M., Tate, K. W.,
Rulofson, F. & Nader, G. 2003 Improved quantitative
estimates of low environmental loading and sporadic
periparturient shedding of *Cryptosporidium parvum* in adult


Bankier, A. T., Spriggs, H. F., Fartmann, B., Konfortov, B. A., Madera,
Integrated mapping, chromosomal sequencing and sequence

Barbosa, J. M. M., Costa-de-Oliveira, S., Rodrigues, A. G., Hanscheid,
T., Shapiro, H. & Pina-Vaz, C. 2008 A flow cytometric protocol

Bayat, P., Nosrati, R., Aliboland, M., Rafatpanah, H., Abnous, K.,
Khedri, M. & Ramezani, M. 2018 SELEX methods on the
road to protein targeting with nucleic acid aptamers.
2018.09.001.

Bednarska, M., Bajer, A., Sinski, E., Girouard, A. S., Tamang, L. &
Graczyk, T. K. 2007 Fluorescent in situ hybridization as a tool
to retrospectively identify *Cryptosporidium parvum* and
*Giardia lamblia* in samples from terrestrial mammalian
1007/s00436-006-0276-y.

Berg, H. 2002 Restriction fragment length polymorphism analysis
of PCR-amplified fragments (PCR-RFLP) and gel
electrophoresis – valuable tool for genotyping and genetic
org/10.5772/37724.

Betancourt, W. Q. & Rose, J. B. 2004 Drinking water treatment
processes for removal of *Cryptosporidium parvum* and

Bjorneby, J. M., Mazurek, J. M., Hlavsa, M., Park, J., Tillapaw,
M., Parrish, M. K., Salehi, E., Franks, W., Koch, E., Smith, F.,
Xiao, L., Arrowood, M., Hill, V., Da Silva, A., Johnston, S. &
Jones, J. L. 2006 Cryptosporidiosis associated with ozonated
3201/cid1204.050796.


Hsu, B. M., Wu, N. M., Jang, H. D., Shih, F. C., Wan, M. T. & Kung, C. M. 2005 Using the flow cytometry to quantify the...


Stu, I. & Figure, S. 2014 Cryptosporidium: Current State of Genomics and Systems Biological Research. Springer, Vienna, Austria.


