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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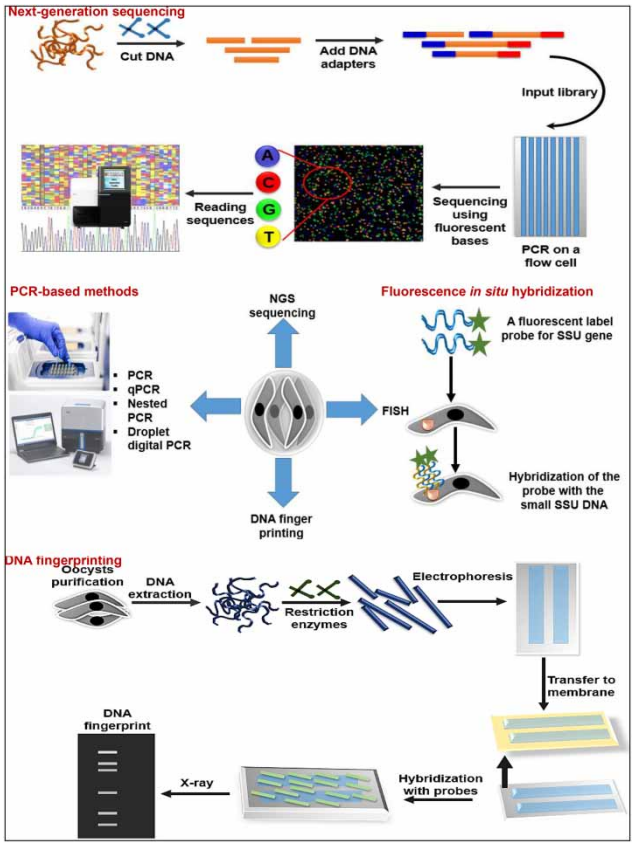
GRAPHICAL ABSTRACT



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 2010). 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 1995). 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 2010). 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, nitazoxinide, 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 C*ryptosporidium* 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.* 2013). 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.* 2013). 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.*

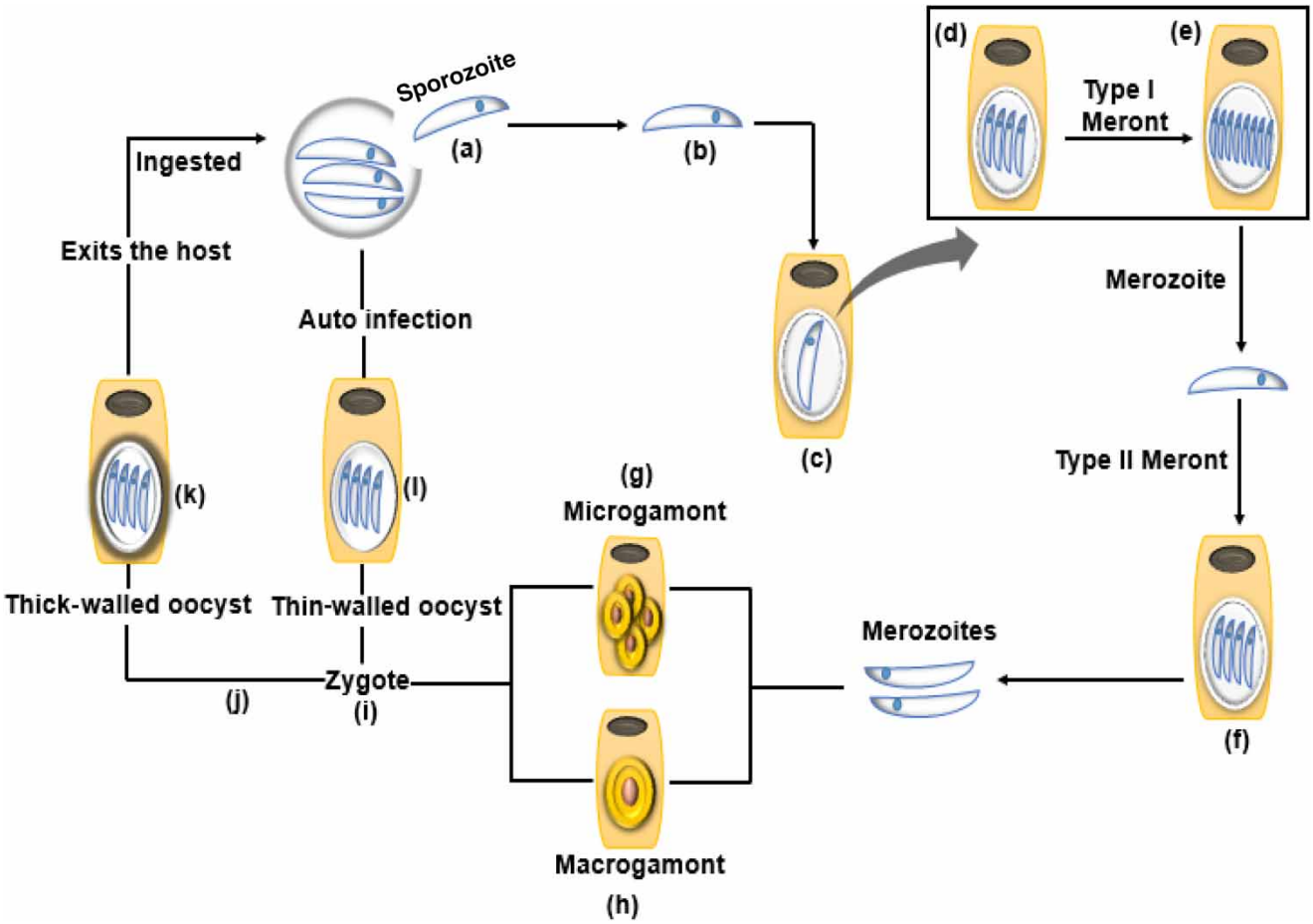


Figure 1 | Schematic diagram of the complete life cycle of *Cryptosporidium parvum*. Sporozoites (a) are released from an ingested oocyst, and begin to enter the intestinal tract lumen (b). They encapsulate into the parasitophorous vacuole membrane (PVM) inside the mucosal epithelium of the intestinal cells (c). Inside the PVM they develop into trophozoites (d) and undergo asexual replication to form Type I meronts containing eight merozoites (e). Merozoites invade other nearby epithelial host cells and they either develop into additional Type I meronts or form Type II meronts containing four merozoites (f). Type II meronts merozoites start a sexual replication process by developing either microgamonts (g) or macrogamonts (h). The zygote (i) forms as a result of the fertilization between a microgamete (from microgamonts) and macrogamete (from macrogamonts). Then the zygote divides asexually (sporogony) (j) into either thick-walled oocysts (k) or thin-walled oocysts (l). The thick-walled oocysts are released in the feces to be ingested by another host. These oocysts are environmentally resistant. The thin-walled oocysts maintain the infection in the same host by autoinfection. (Adapted and modified from Bouzid *et al.* (2013) with permission.)

2013). 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 1982). 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).

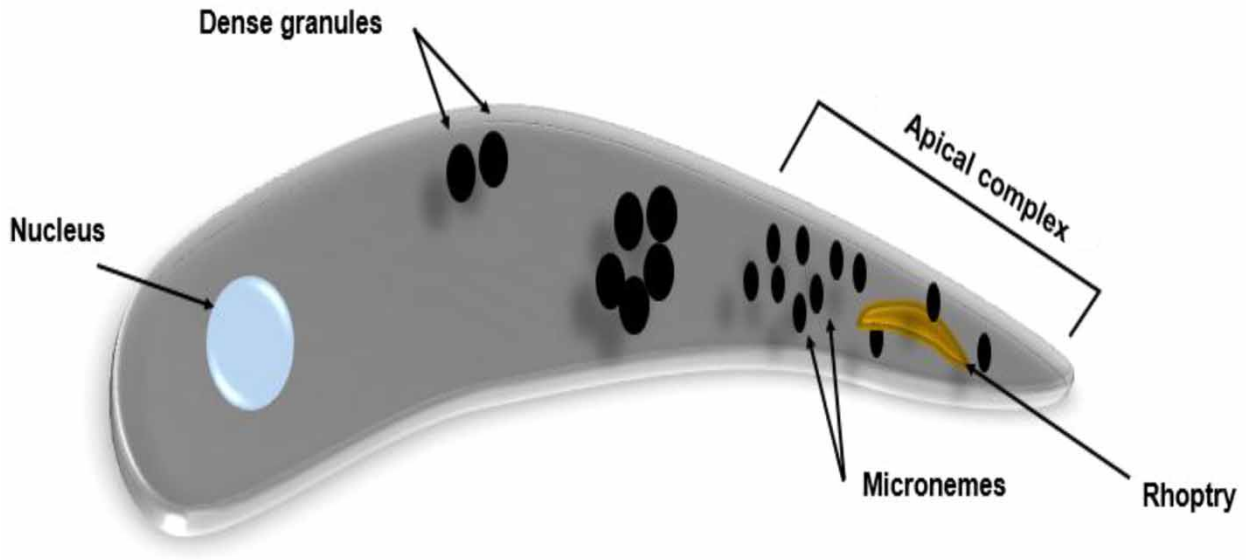


Figure 2 | The sporozoite structure of *Cryptosporidium parvum*. The sporozoite stage of *C. parvum* shares a similar structure with all apicomplexan organisms. The cell is crescent shaped and has an apical complex region which contains a single rhoptry and micronemes. The dense granules are spread throughout the cell. The plasma membrane of *C. parvum* consists of an outer membrane and inner membrane complex composed of two distinct membranes with an underlying array of subpellicular microtubules. (Adapted from O'Hara & Chen (2011).)

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 1997; Carmena 2010).

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.* 2003). Moreover, the microscopic method does not allow for the determination of oocyst viability (Fall *et al.* 2003).

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*

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

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

oocysts in diverse types of samples since they use antibodies that target the wall of the oocyst (Table 2) (Jex *et al.* 2008).

Direct and indirect immunofluorescence assay using monoclonal antibodies (mAbs)

Direct fluorescent antibody (DFA) assay uses a fluorescein isothiocyanate-conjugated anti-*Cryptosporidium* monoclonal 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). Monoclonal 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 2 | Comparison of immunological methods and aptamer-based methods for the detection of *Cryptosporidium* spp. oocysts

Immunological method	Advantages	Disadvantages	Type of sample tested	Sensitivity and specificity	Reference
Direct and indirect immunofluorescence assays using monoclonal antibodies	Specifically bind to the targeted epitope; less background than traditional staining methods	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	Feces and water	99.8% sensitivity and 100% specificity as reported by MERIFLUOR® kit	Iacovski *et al.* (2004); Johnston *et al.* (2003)
Immunomagnetic separation (IMS)	Bind specifically to the target epitope; when coupled to immunofluorescence assay, could detect a low number of oocysts	Very costly; cannot determine infectivity or viability of the oocysts; cannot distinguish between *Cryptosporidium* species	Surface water and wastewater	High sensitivity; efficiency depends on the filtration method used; recovery efficiency is between 62 and 100%	Allen *et al.* (2000); USEPA (2005)
Immunochromatographic lateral-flow assays	Fast and simple method of detection; the end results are visual without specialized equipment; does not require expertise	Possibility of false-negatives and -positives; often used with other methods of detection	Feces and water	97% sensitivity and 100% specificity	Esther *et al.* (2018); Johnston *et al.* (2003)
Enzyme-linked immunosorbent assay	Does not require microscopy	Non-specific binding when using secondary antibody may result in false-positive results; long incubation times	Feces	Variation in sensitivity and specificity (59–100% and 93–100% respectively)	Chalmers *et al.* (2011); Ghoshal *et al.* (2018); Vohra *et al.* (2012)
Flow cytometry	Rapid analysis of large numbers of samples; high sensitivity when combined with IMS	Costly equipment; requires expertise; limitations in the determination of infectivity	Feces, water, sewage	10 times more sensitive than DFA in fecal samples; limit of detection in water samples is 1,000 oocysts per L	Jex *et al.* (2008); Power *et al.* (2003)
Aptamer-based methods	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 (<30 min)	Can be affected by nucleases in complex environments, but this can be overcome by adding functional groups to resist nucleases	Currently used only on food samples; potential for use on feces and water samples (e.g., drinking, surface and wastewater)	R4-6 aptamer could detect as few as 100 oocysts in 25 g fruit samples	Hassan *et al.* (2021); Iqbal *et al.* (2015, 2019); Kong & Byun (2013); Toh *et al.* (2014)

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

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

[a]Recovery efficiency: 60 to >95%.

[b]Antibody-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 (Elgun & 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 & Karanis 2018). 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 2×10^3 oocysts/mL (Barbosa *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; Esther *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.* 2013; 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; Hanabara & 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.* 2013). 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

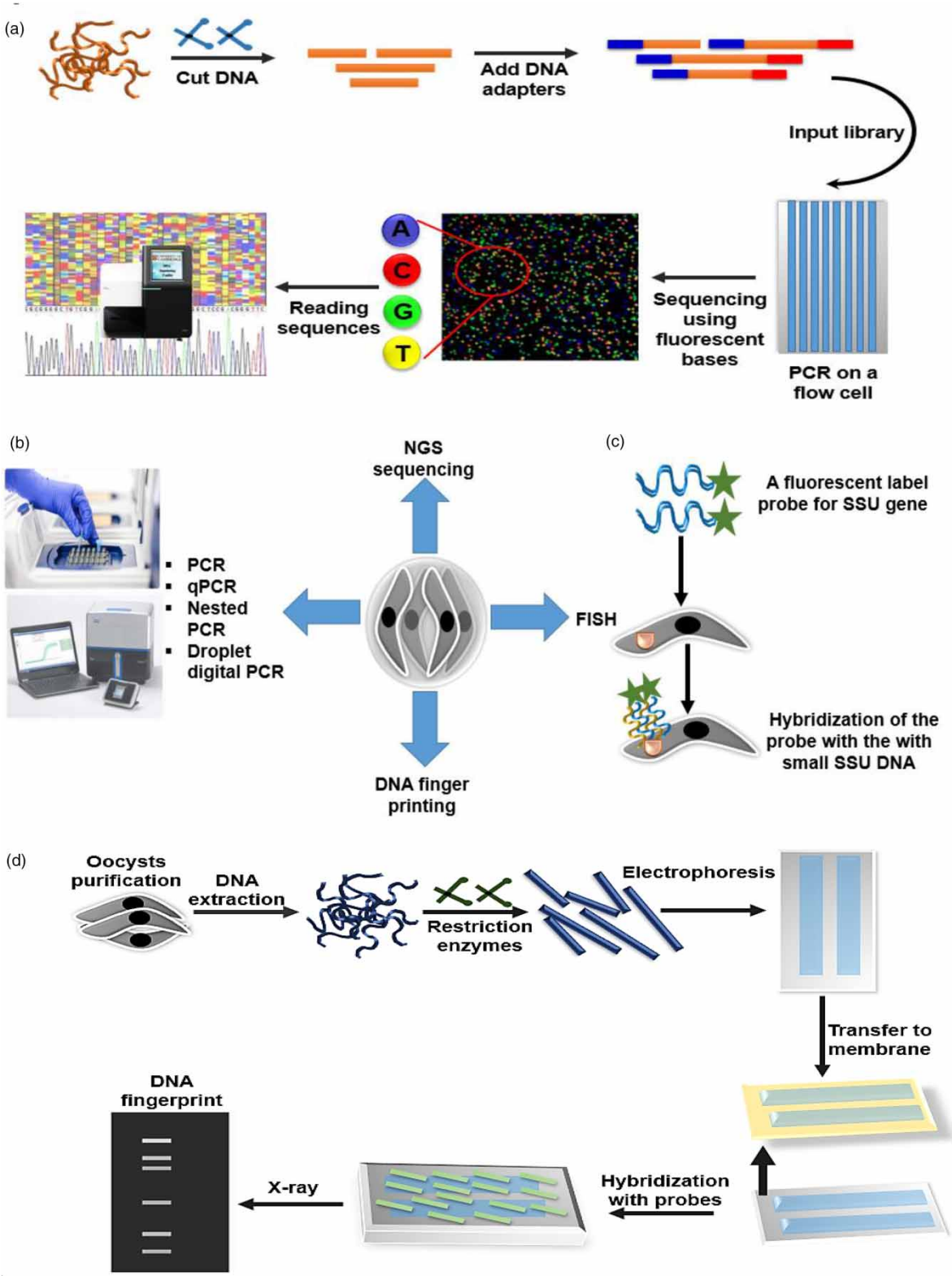


Figure 3 | Nucleic acid-based methods used for the detection of *Cryptosporidium parvum* and *Cryptosporidium hominis* in fecal or water samples. After oocyst purification from the sample, various nucleic acid-based methods can be applied, including (a): next generation sequencing (NGS), (b) PCR-based methods such as qPCR, nested and droplet digital PCR, (c) fluorescence *in situ* hybridization (FISH), (d) DNA fingerprinting.

by template concentration. Moreover, it was found that the presence of PCR inhibitors affected qPCR but not ddPCR. The total cost of ddPCR was reported to be twice that for qPCR, which limits the use of ddPCR in countries in need (Rački *et al.* 2014; Yang *et al.* 2014; Zahedi *et al.* 2016).

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 foodborne 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 propagated 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.* 2003; 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; Hønsvall & 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 1990; Williams *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 minisatellites) 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 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 (Ellington & 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*

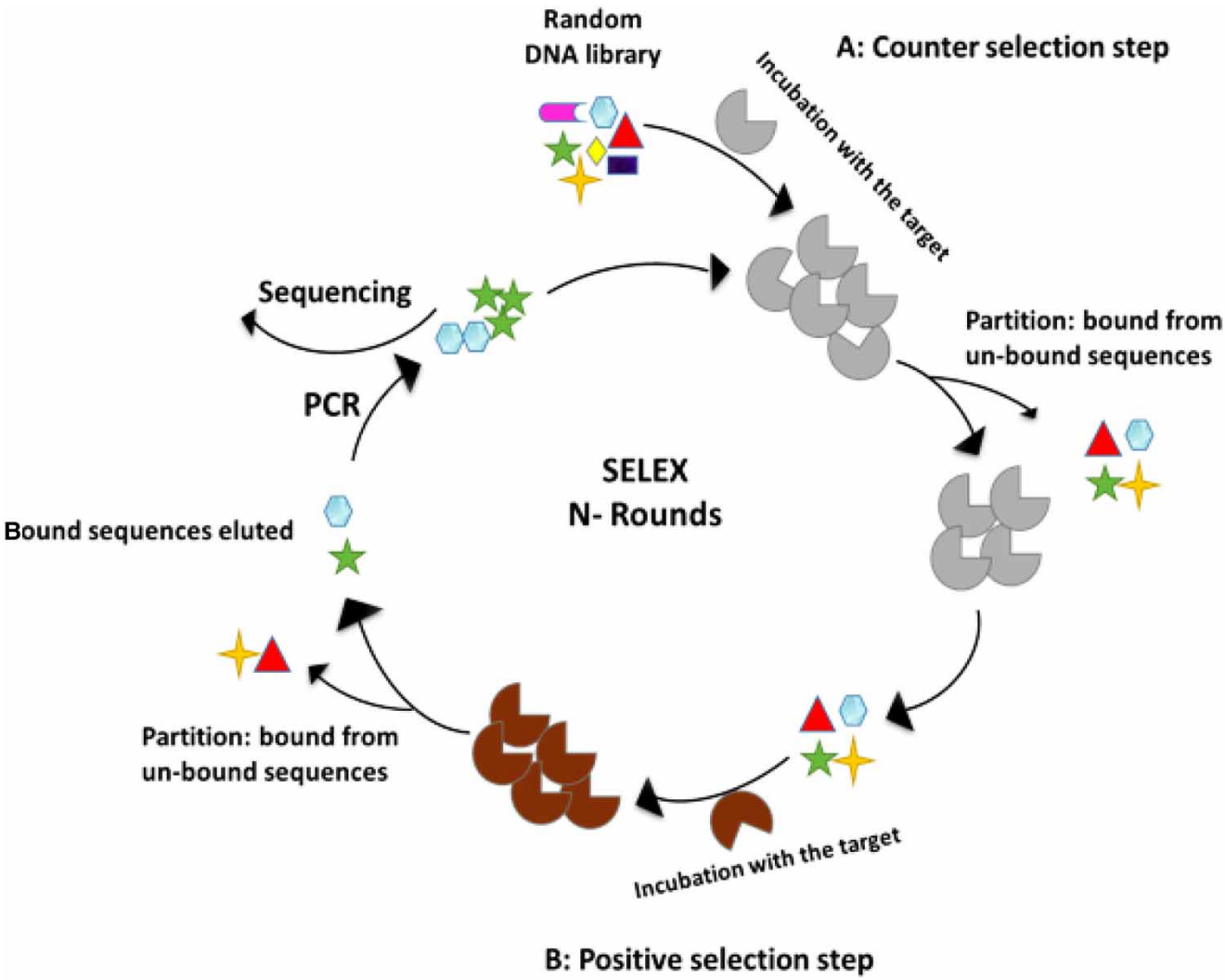


Figure 4 | The process of systematic evolution of ligands by exponential enrichment (SELEX). A random ssDNA library (or ssRNA) is incubated with the target of interest (positive selection (B)). Unbound sequences are washed off and binding sequences are eluted off the target. The eluted sequences are 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 their 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 (3′-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.* 2013). 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. Aptamers 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 *Cryptosporidium* 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 Supplementary Information.

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