Water Science & Technology

IWA PUBLISHING

Water Science & Technology Vol 88 No 3, 615 doi: 10.2166/wst.2023.229

Biosensors as early warning detection systems for waterborne *Cryptosporidium*

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ABSTRACT

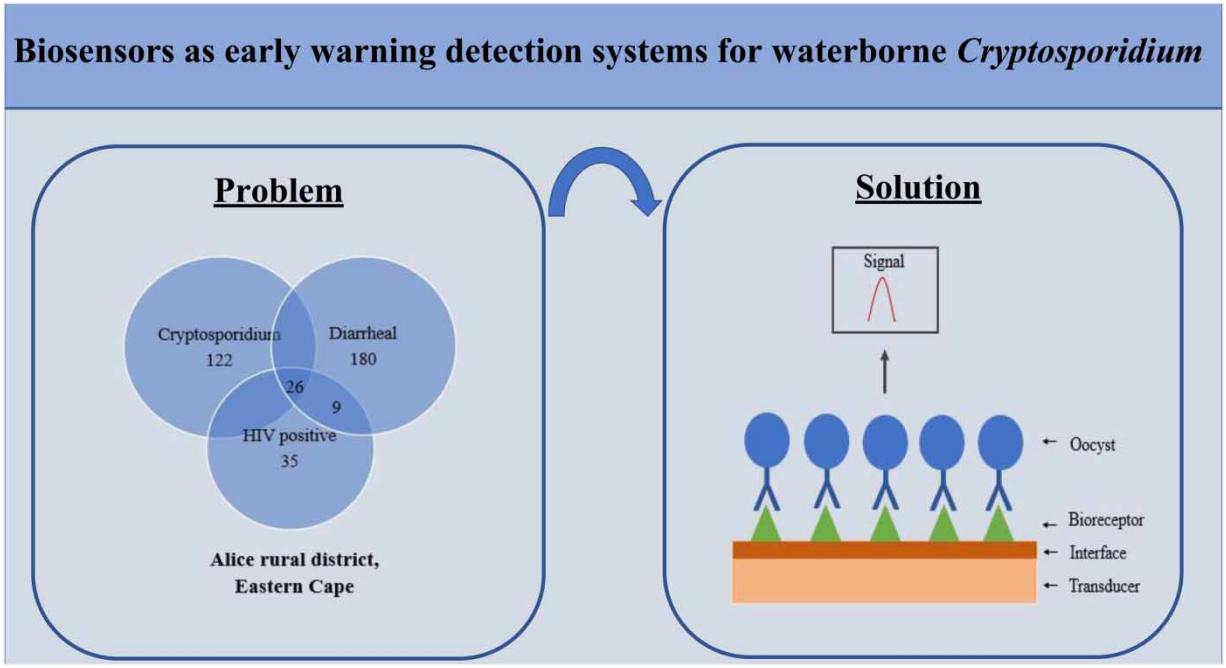
Waterborne disease is a global health threat contributing to a high burden of diarrhoeal disease, and growing evidence indicates a prospective increase in incidence coinciding with the profound effects of climate change. A major causative agent of gastrointestinal disease is *Cryptosporidium*, a protozoan waterborne parasite identified in over 70 countries. *Cryptosporidium* is a cause of high disease morbidity in children and the immunocompromised with limited treatment options for patients at risk of severe illness. The hardy nature of the organism leads to its persistence in various water sources, with certain water treatment procedures proving inefficient for its complete removal. While diagnostic methods for *Cryptosporidium* are well-defined in the clinical sphere, detection of *Cryptosporidium* in water sources remains suboptimal due to low dispersion of organisms in large sample volumes, lengthy processing times and high costs of equipment and reagents. A need for improvement exists to identify the organism as an emerging threat in domestic water systems, and the technological advantages that biosensors offer over current analytical methods may provide a preventative approach to outbreaks of *Cryptosporidium*. Biosensors are innovative, versatile and adaptable analytical tools that could provide highly sensitive, rapid, on-site analysis needed for *Cryptosporidium* detection in low-resource settings.

Key words: biosensors, *Cryptosporidium* detection, sensors, waterborne disease

HIGHLIGHTS

- The impact *Cryptosporidium* has on the developing world, using South Africa as a case study.
- The use of conventional methods of *Cryptosporidium* detection in the South African research and testing sphere.
- A comprehensive assessment of the new developments in biosensor fabrication for the detection of *Cryptosporidium* instead of the use of conventional methods.

GRAPHICAL ABSTRACT



1. INTRODUCTION

A major threat to global health is waterborne disease, which is responsible for significant morbidity, mortality and economic damage in developing countries and developed countries alike. Water is the most important finite resource, however, freshwater sources comprise only 2.5% of water on the planet (Stephens *et al.* 2020). Water misuse, freshwater loss from climatic shifts, rising demand from agricultural and industrial sectors, and overpopulation are all contributing factors to the growing issue of water scarcity. Without adequate quality control measures, the release of effluent water into freshwater sources may compromise human health and safety, with UN estimates approximating that 80% of wastewater released into the environment is inefficiently treated for disease-causing microbial contaminants, including bacteria, viruses, protozoa and helminths (United Nations Water 2020).

The apicomplexan protozoan parasite *Cryptosporidium* is the etiological agent of the potentially life-threatening waterborne gastrointestinal disease cryptosporidiosis, and is a leading contributor to the global waterborne disease burden. *Cryptosporidium* was first identified in humans in 1976, and infects a wide variety of vertebrate animals, with genotype names designated after the strain's host specificity (Nime *et al.* 1976). Infection in humans is most commonly caused by *C. hominis* or the cattle genotype *C. parvum*, although other species such as *C. meleagridis, C. felis* and *C. canis* occasionally cause human infection (Leitch & He 2012). In its thick-walled oocyst life stage, *Cryptosporidium* is especially hardy, and decontamination of this organism from water sources can be arduous. This challenge is due to the resistance of the organism to many water treatment techniques such as chlorine treatment, high rate filtration and activated sludge (Nasser 2016).

Transmission of *Cryptosporidium* occurs mainly through the faecal-oral route by ingestion of contaminated water or foods, direct person-to-person contact or zoonotic spread. Studies have shown that most drinking water sources are contaminated by oocysts before treatment, and contaminated drinking water from surface or ground waters has been attributed to most major outbreaks of cryptosporidiosis (Huang & White 2006). Sewage contamination, failed water treatment and leakage into distribution systems are potential causes of such outbreaks. The largest recorded waterborne cryptosporidiosis outbreak occurred in Milwaukee, Wisconsin in 1993 where 403,000 people from a potentially exposed population of 1.6 million were affected and at least 54 died (MacKenzie *et al.* 1994). The causative species of the Milwaukee outbreak was *C. hominis*, however, *C. parvum* has been associated with other waterborne outbreaks due to water contamination with agricultural runoff.

Cryptosporidiosis is a major cause of childhood diarrhoeal disease globally and particularly in low-resource settings. Studies have identified *Cryptosporidium* as the second most predominant cause of moderate to severe diarrhoea in children

under the age of 5 years in the developing world (Troeger *et al.* 2017; Khalil *et al.* 2018). In the Global Burden of Diseases, Injuries, and Risk Factors study (GBD), cryptosporidiosis was associated with greater childhood morbidity and mortality than diarrhoeal disease from other causes and identified that younger ages are associated with a higher risk of infection (Troeger *et al.* 2017). The Global Enteric Multicentre Study (GEMS) investigated the burden of paediatric diarrhoeal disease in sub-Saharan Africa and estimated that 2.9 million *Cryptosporidium* cases occur annually in children under the age of 2 years, with mortality increasing two-fold in children aged between 1 and 2 years (Kotloff *et al.* 2013). HIV status has also been identified as a significant risk factor for the host, and HIV-positive children are more likely to experience *Cryptosporidium* infection than HIV-negative children (Squire & Ryan 2017).

The burden of disease is high among the immunosuppressed and immunodeficient (Checkley *et al.* 2015). In immunocompetent hosts, diarrhoea is acute and self-limiting, generally resolving in less than 2 weeks. On the other hand, infection is persistent and reoccurring in immunocompromised hosts and can lead to life-threatening malabsorption and dehydration, resulting in high morbidity and mortality. The global burden of cryptosporidiosis is believed to be highly underestimated as diagnostic tests are underused and have poor sensitivity, and treatment options are limited for patients at high risk of serious illness (Abubakar *et al.* 2007).

The GEMS study has identified the four main pathogens most attributable to moderate-to-severe diarrhoeal cases in children in developing countries: rotavirus, *Cryptosporidium*, enterotoxigenic *Escherichia coli* producing heat-stable toxin and *Shigella* (Kotloff *et al.* 2013). Despite being identified as one of these four key pathogens, *Cryptosporidium* is the only one of them without an effective vaccine or treatment. This lag in pharmaceutical development is due in part to the lack of accessible animal infection models and *in vitro* culture methods that can reproduce the entire *Cryptosporidium* life cycle (Karanis 2018). Hence, public health approaches such as thorough sanitation measures and frequent testing of drinking water supplies are crucial for disease prevention over treatment in the absence of efficacious therapeutics.

Cryptosporidium is a ubiquitous intestinal parasite with global distribution, and cryptosporidiosis infection has been reported in over 70 countries (Dong *et al.* 2020). At least 44 species of *Cryptosporidium* exist, and of these over 19 species and genotypes have been reported in humans (Ryan *et al.* 2021). As clinical testing usually is restricted to patients who are severely ill enough to seek treatment and testing, cryptosporidiosis is substantially underdiagnosed worldwide, and the global burden of the disease is not yet well understood. Even in countries such as the USA where modern diagnostics are readily available, it is estimated that only 1% of cases are diagnosed and reported (Scallan *et al.* 2011). A recent meta-analysis study on the global burden of *Cryptosporidium* identified that high-income countries have the lowest prevalence of infection, and that prevalence is significantly lower in urban areas regardless of national income due to better hygiene and access to treated water. The overall global prevalence of cryptosporidiosis was estimated to be 7.6%, with a prevalence disparity of 4.3% in developed nations and 10.4% in developing nations (Dong *et al.* 2020).

The global health impact of *Cryptosporidium* is significant, however preventative water testing for the organism is underutilized due to the inconvenience and high costs associated with conventional testing methods. Biosensors offer technological advantages over current analytical methods, and recent research suggests a shift towards biosensors as a future tool for *Cryptosporidium* detection. The aim of this review is therefore to explore the shortcomings of conventional testing methods and expose the viability of biosensors as a rapid, sensitive alternative for *Cryptosporidium* detection.

2. *CRYPTOSPORIDIUM* IN THE DEVELOPING WORLD

The incidence of *Cryptosporidium* is high in developing countries with high prevalence rates of HIV/AIDs. A Zambian study reported a prevalence rate of 9.5% of *Cryptosporidium* infection in HIV-positive individuals, while prevalence has been found to be 8.3% in HIV-infected patients in Mozambique and 9% in Zimbabwe (Gumbo *et al.* 1999; Casmo *et al.* 2018; Sinyangwe *et al.* 2020). Studies indicate that *Cryptosporidium* is emerging as a major human pathogen in various South African communities with prevalence rates ranging from 5.7 to 67.8% in study groups from Kwa-Zulu Natal, Limpopo and Eastern Cape municipalities (Samie *et al.* 2006; Omoruyi *et al.* 2011; Msolo *et al.* 2020).

Little research has been carried out to investigate the possible risk factors for *Cryptosporidium* transmission in South Africa, but factors that have been identified are lower socioeconomic status, domestic animal contact and HIV status (Omoruyi *et al.* 2011). Environmental and demographic risk factors for *Cryptosporidium* infection were assessed in the Alice district in the Eastern Cape to elucidate the role of socioeconomic status, poor water supply and limited sanitation facilities in susceptibility to infection. *Cryptosporidium* ELISA antigen testing of diarrhoeal samples from rural settlements in the

Alice district revealed that 77% of diarrhoeal patients were positive for *Cryptosporidium* infection (Omoruyi *et al.* 2011). In the HIV-positive cohort, the prevalence peak was detected in low-income patients (85.7%) rather than high-income patients (32%) and contact with farm animals was shown to be significant risk factor for infection.

2.1. *Cryptosporidium* and HIV status

HIV endemicity is high in South Africa and is one of the country's most dire health concerns. According to UNAIDs, South Africa has the largest population of HIV afflicted persons a figure of 7.8 million individuals as of 2020 (UNAIDS South Africa 2016). HIV/AIDs status has been shown to be one of the leading risks for developing cryptosporidiosis and also for elevated disease severity, with rates of infection in HIV-positive patients varying with economic status but reaching an overall pooled prevalence of 11.2% worldwide (Ahmadpour *et al.* 2020). The high rates of poverty and HIV in South Africa indicate that *Cryptosporidium* should be of significant concern, however, the subject has achieved limited recognition. Several studies have illustrated the need for this concern, recording high incidence of cryptosporidiosis among HIV-positive populations in various South African provinces (Omoruyi *et al.* 2011; Bartelt *et al.* 2013; Samie *et al.* 2014).

The relationship between HIV status and *Cryptosporidium* infection shown in these studies is presented in Figure 1. A sero-oepidemiological study of HIV-infected adults and healthy student volunteers throughout multiple provincial healthcare sites in Limpopo reported seropositivity of anti-*Cryptosporidium* IgG in 146 of 194 HIV-positive patients (75.3%) and 19 of 58

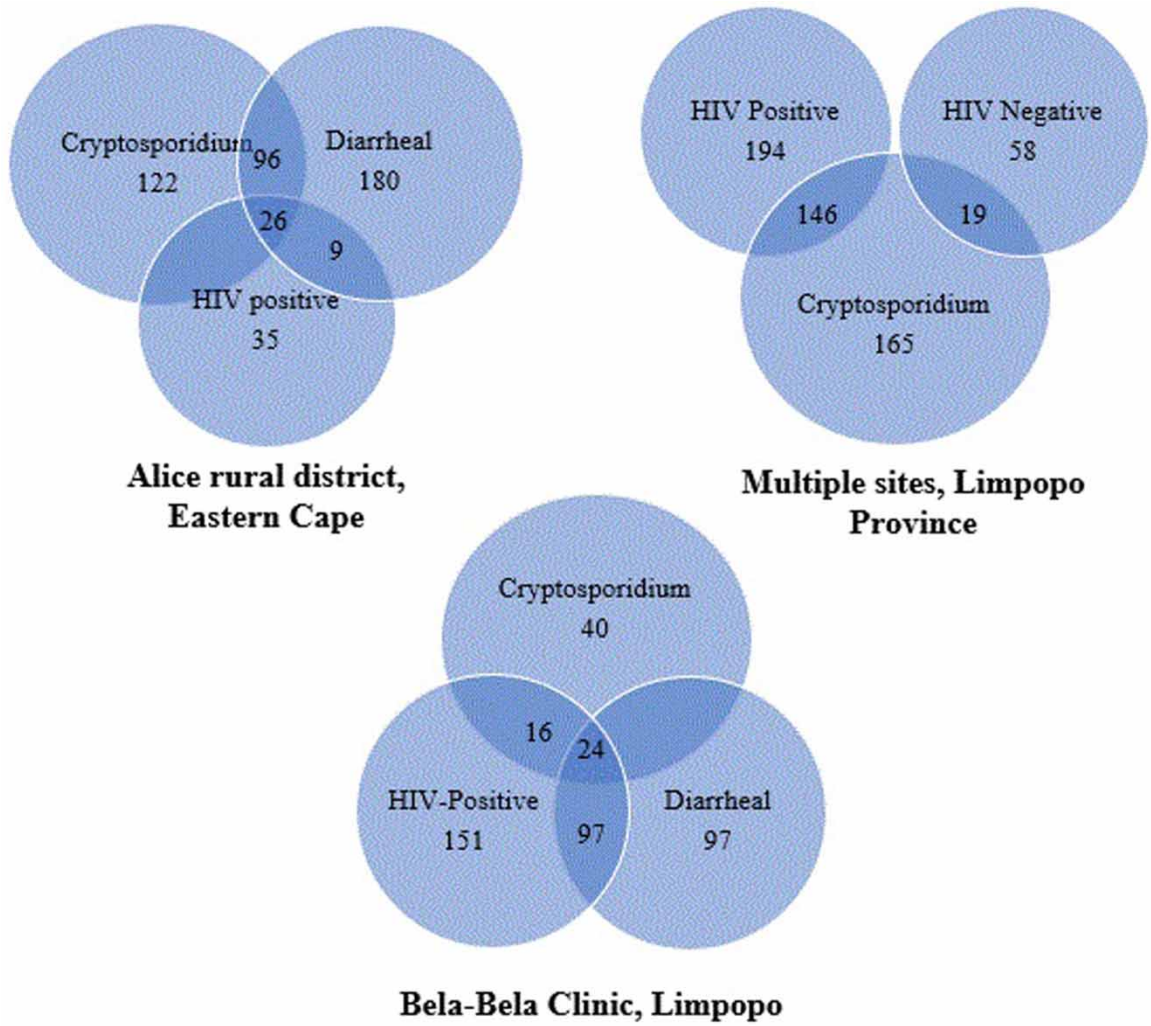


Figure 1 | The relationship between *Cryptosporidium* infection and positive HIV status in various South African testing sites (Omoruyi *et al.* 2011; Bartelt *et al.* 2013; Samie *et al.* 2014).

student volunteers (32.8%) (Bartelt *et al.* 2013). In a separate parasitic infection study in HIV/AIDs patients conducted in Limpopo, microscopy and molecular screening of stool samples collected from patients attending an HIV treatment clinic identified *Cryptosporidium* spp. in 24 of 97 HIV-positive diarrhoeal patients (25%) (Samie *et al.* 2014). In the Alice district of the Eastern Cape, Omoruyi *et al.* found that 26 of 35 HIV-infected diarrhoeal patients (74.3%) were suffering from *Cryptosporidium* infection (Omoruyi *et al.* 2011). These rates are worrying as they significantly exceed *Cryptosporidium* infection rates in HIV patients in other African countries with a high HIV endemicity.

2.2. *Cryptosporidium* in children

Cryptosporidium infection rates are elevated for children under the age of 2 in sub-Saharan Africa and accordingly, high prevalence rates and poor patient outcomes have been documented for cryptosporidiosis in children in South Africa, particularly with an HIV comorbidity. Moodley *et al.* documented a 9% prevalence of *Cryptosporidium* in paediatric gastro-enteritis patients at King Edward VIII Hospital, 89.7% of which were under 2 years of age (Moodley *et al.* 1991). From the cohort, 10% of infected children died and gastro-enteritis was confirmed as the cause of death for 6.3%. A sequence diversity study of *Cryptosporidium* isolates in HIV-positive children under the age of 3 years was carried out in the same hospital 11 years later. The outcomes declined further, with 31.8% of patients in the study dying 8 weeks post-admission (Bartelt *et al.* 2013).

Cryptosporidium has recently been identified by the National Institute for Communicable Diseases (NICD) as a major causative agent of diarrhoea in South Africa under the age of five in 2016 (Diseases 2015). A sentinel surveillance program for rotavirus revealed that *Cryptosporidium* had displaced rotavirus as the causative pathogen of diarrhoea in hospitalized children, with over 95% of diarrhoea stool samples containing pathogenic parasites harbouring *Cryptosporidium* species. Genotyping studies by PCR-restriction fragment length polymorphism and DNA sequence analysis of the 18S rRNA gene have revealed that *C. hominis* is the most predominantly detected species in humans in the country, followed by *C. parvum* and *C. meleagridis* (Samie *et al.* 2006; Samra *et al.* 2013). This confirmation that a human-specific species is the emerging cause of childhood diarrhoea in South Africa points to untreated effluent water and sewage contamination of drinking water as the likely source of infection.

2.3. Trends in diagnostics and water analysis

A paucity of data exists for prevalence rates of *Cryptosporidium* infection in South Africa with only eight studies found for this review being published in the last 16 years. The NICD has identified that the main underlying reason for the underreporting of *Cryptosporidium* as a diarrhoeal agent is the difficulty of detecting oocysts by conventional laboratory methods (UNAIDS South Africa 2016). Hence, clinical diagnosis of *Cryptosporidium* has not been routinely implemented in South African hospitals and pathology laboratories, and available *Cryptosporidium* prevalence data has been generated from independent scientific studies utilizing diverse detection methods.

Figure 2 represents assay types that were used for the determination of *Cryptosporidium* incidence in various South African communities. Included studies were published between 2006 and 2022, and only Samie *et al.* used more than one test type to assess samples, comparing the sensitivity of microscopy and different molecular detection methods. ELISA, qPCR and Ziehl-Neelsen staining were the most commonly used to detect *Cryptosporidium* in stool samples, while LAMP, PCR and lateral flow test strips were less popular choices. The detection of anti-*C. parvum* antibodies in serum were utilized only once in a study assessing the rates of past infections in an HIV patient cohort.

In the absence of routine clinical diagnosis and effective therapeutics for *Cryptosporidium* in South Africa, it is evident that the testing focus should shift from the clinical sphere to water analysis as a means of prevention of disease outbreaks. According to the South African National Standard for drinking water, SANS241:2015, the present standard operational limit of less than 1 oocyst/10 L is acceptable for drinking water in South Africa. Rand Water is the largest bulk water supplier in South Africa and has adapted and optimized the US-EPA Method 1623.1 for *Cryptosporidium* detection. The EPA method 1623.1 method also remains the method of choice for most independent *Cryptosporidium* studies in South Africa (Dungeni & Momba 2010), however, few South African environmental laboratories have implemented routine *Cryptosporidium* analysis due to the high cost of equipment and long processing times required from this method and difficulty of validating and implementing alternative methods.

3. DETECTION METHODS FOR *CRYPTOSPORIDIUM*

A variety of detection methods are available for *Cryptosporidium* identification in stool samples and water sources for clinical and water surveillance purposes respectively, although sensitivity and selectivity vary significantly by method. Clinical

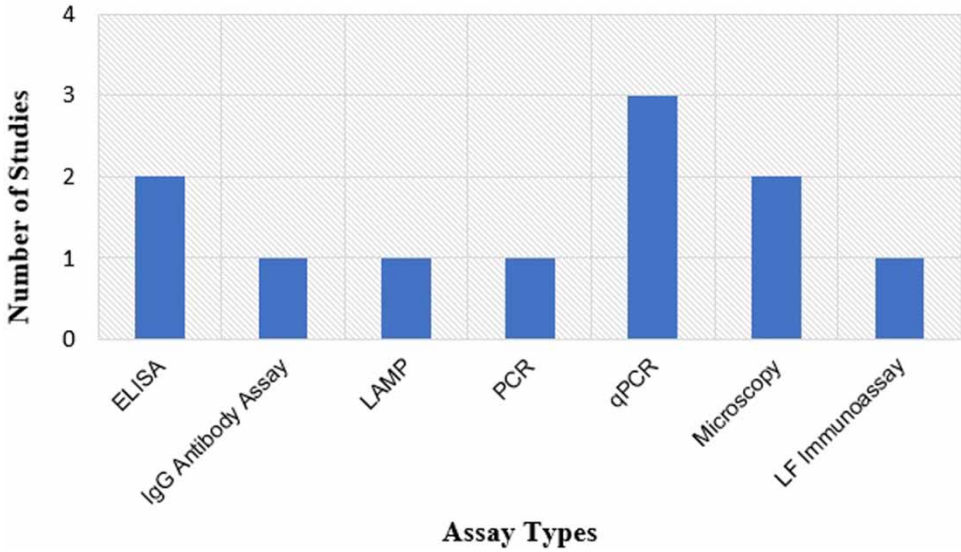


Figure 2 | Diagnostic methods used by studies from 2006 to 2022 assessing *Cryptosporidium* infection rates in South Africa (Samie *et al.* 2006, 2009, 2014; Omoruyi *et al.* 2011; Bartelt *et al.* 2013; Korpe *et al.* 2018; Msolo *et al*. 2020; Ngobeni *et al*. 2022).

detection is simplified by the nature of the disease. Most clinical cases of cryptosporidiosis involve the gastrointestinal tract where oocysts are shed by the millions in patient stool (Chappell *et al.* 1996). Diagnosis is achieved through the detection of shed oocysts or *Cryptosporidium* antigens in a single stool sample. Traditionally, oocysts are examined microscopically and concentration methods such as formalin-ethyl acetate concentration or sugar solution flotation substantially aid diagnosis by improving oocyst yield and removing faecal debris which may interfere in the background (Adeyemo *et al.* 2018). Antigen-based and molecular methods such as ELISA, lateral flow assays and real-time polymerase chain reaction (RT-PCR) have increased in popularity due to their high sensitivity and reduction of hands-on analysis time, and several commercial kits are available for stool analysis.

3.1. Waterborne *Cryptosporidium*

Cryptosporidium detection in water has proven to be more challenging than in the clinical sphere. This is partially due to the large volumes of water that are required by testing schemes. As only 1–10 oocysts need to be ingested to cause disease in healthy adults (Okhuysen *et al.* 1999), current detection protocols call for the processing of at least 10 L of water. This necessitates the use of concentration and filtration steps which can result in loss of oocyst yield, further complicating the task of detecting low parasitic numbers in high volumes and requiring a highly sensitive analytical method to compensate.

Interference from background particles in complex water matrices also complicates analysis. In microscopy-based techniques, background particles and microorganisms may be mistaken for oocysts and result in false positives, hindering selectivity. Alternatively, techniques that may improve the dire issue of sensitivity in waterborne *Cryptosporidium* analysis such as molecular-based methods suffer from the issue of PCR inhibition by environmental contaminants.

Undeniably, water quality studies have indicated the presence of parasitic protozoa in South African water systems and have highlighted the shortcomings of water treatment systems in removing these pathogens. *Cryptosporidium* oocysts have been reported in multiple water sources such as raw sewage, treated effluent and surface and drinking waters. Dungeni and Mbomba assessed the prevalence of oocysts in four wastewater treatment plants in Gauteng and discovered that the mean removal efficiency of *Cryptosporidium* ranged between 67.40 and 98.26%, identifying the failure of effective removal of oocysts by several South African wastewater treatment works (Dungeni & Momba 2010). The oocyst concentration from wastewater effluents was greater than 1 oocyst/L, indicating a risk of *Cryptosporidium* discharge into receiving drinking water bodies due to water scarcity issues in South Africa. As 14% of total freshwater in South Africa stems from return flows such as sewage and purified effluent, this risk is elevated (Haldewang 2009).

Potgieter *et al.* assessed the occurrence of human enteric pathogens in rivers used for drinking water by rural communities in the Vhembe district of Limpopo and found the presence of *Cryptosporidium* oocysts in the Tshihane River (Potgieter *et al.*

2020). The fact that *Cryptosporidium* contaminates both untreated and treated drinking waters in South Africa indicates that infection is a risk for communities across a wide variety of socioeconomic backgrounds. It is essential that the removal efficiency of oocysts is bolstered by effective physical processes such as UV treatment, and that this is reinforced by adequate and frequent screening of water sources for oocyst contamination in accordance with the South African National Standard (SANS) 241:2015, which stipulates that the acceptable limit for *Cryptosporidium* in drinking water is 0 oocysts/10 L.

3.2. The gold standard

In 1996, the lack of reliable standardized detection methods for *Cryptosporidium* prompted the US environmental protection agency (USEPA) to initiate the development of an innovative method for detection, method 1622, which as of 2012 is published as EPA-1623.1 and is currently the gold standard method for *Cryptosporidium* detection (USEPA 2005). The EPA-1623.1 method can be divided into four steps: (1) filtration or continuous flow centrifugation of at least 10 L of water; (2) separation of oocysts by immunomagnetic separation (IMS); (3) staining with fluorescently labelled antibodies and 4′,6-diamidino-2-phenylindole (DAPI) and (4) fluorescence microscopy.

In the filtration step, a cartridge or membrane filter can be used to concentrate oocysts, and portable pump and filter systems options are available for in-field processing. Oocysts are eluted from the filter with an elution buffer through a mechanical wash process and recovery efficiencies (0–93%) have been shown to vary with the type and pore size of the filter membrane used as well as the type of elution buffer (Pavli *et al.* 2016). Raw water typically contains a high number of undesired particles that are retained on the filter along with the oocysts such as soil, bacteria, algae and other protozoans. This necessitates the use of IMS to purify eluted oocysts, reducing the number of false positives due to background particles. IMS kits like the Dynabeads® anti-*Cryptosporidium* kit contain superparamagnetic beads functionalized with anti-*Cryptosporidium* mAbs that bind oocysts, and can be removed with a magnet to improve separation efficiency (Smith & Nichols 2010). The purified oocysts are stained with approved fluorescently labelled anti-*Cryptosporidium* mAbs (MeriFluor® *Cryptosporidium*; Aqua-Glo™ G; Crypt-a-Glo™; EasyStain™C&G) and DAPI and enumerated by fluorescent microscopy and differential interference contract (DIC) microscopy (USEPA 2005).

The EPA 1623.1 procedure is the most commonly used method for *Cryptosporidium* detection in water, however, steps in the EPA method can be substituted by a variety of alternative technologies to enhance sensitivity, allow for species subtyping or reduce labour and costs (Efstratiou *et al.* 2017). The EPA method is costly, suffers from variable recovery efficiencies and requires skilled personnel to interpret fluorescent microscopy results, hence a need exists to provide a more effective *Cryptosporidium* water monitoring system that can preferably be implemented on an on-site and real-time basis. Although the EPA procedure is most commonly used for water quality analysis, the detection step can be substituted with alternative detection methods such as microscopy, molecular methods and flow cytometry to suit the analytical laboratories' capabilities and equipment.

3.3. Microscopy based

Microscopy-based methods have traditionally been relied on for the detection of oocysts, however, there are few distinguishing morphological characteristics for *Cryptosporidium* and identification by light microscopy is unreliable (Fall *et al.* 2003). Staining techniques can be employed to aid in the differentiation of oocyst from other protozoa and environmental debris, the most popular of which is the acid-fast stain, also known as the Ziehl-Neelson stain (Henriksen & Pohlenz 1981). *Cryptosporidium* was shown to have acid-fast properties in 1981 and a modified Ziehl-Neelsen stain for differential staining was developed and became the predominantly used method in clinical laboratories. Although the modified Ziehl-Neelsen technique is relatively inexpensive, it is time-consuming and demands a skilled microscopist to distinguish oocysts from background yeasts, fungus and bacterial spores for accurate identification.

Other staining techniques are useful for oocyst detection, particularly in clinical applications, but there are many limitations such as lack of specificity and sensitivity, and variability in stain uptake (Jex *et al.* 2008). Phase contrast and DIC are microscopy methods that have been implemented to improve oocyst visualization and identification in comparison to light microscopy. These techniques provide higher contrast between the specimen and background and aid in revealing internal parasite morphology and have been incorporated into the US-EPA 1623 method alongside immunofluorescence assays (Efstratiou *et al.* 2017).

3.4. Immunoassay based

Immunological assays for *Cryptosporidium* detection were developed to overcome the limitations of microscopy-based methods and have surpassed the sensitivity and specificity drawbacks of conventional bright-field staining techniques.

Immunofluorescent detection of *Cryptosporidium* has increased in popularity and is the standard detection technique used for the EPA method. Although the utilization of a fluorescent microscope is required, the benefit of a clear distinction between fluorescently marked oocysts and non-fluorescent background increases specificity and reduces the time required for analysis. The direct fluorescent antibody (DFA) assay uses fluorescein isothiocyanate-conjugated anti-*Cryptosporidium* monoclonal antibodies (FITC-C-mAb) that recognize surface epitopes on oocysts and emit a yellow-green fluorescence to allow visualization of the oocyst. DFA has demonstrated high specificities (96–100%) and sensitivities (98.5–100%) in comparative studies of environmental and clinical samples (Jex *et al.* 2008). Nucleophilic fluorescent dyes such as DAPI and propidium iodide are often used in conjunction with DFA, as they actively stain nuclei and facilitate morphological identification (Smith *et al.* 2002).

The specificity and sensitivity of monoclonal antibody-based DFA assays are influenced by various factors, including the purity of the *Cryptosporidium* antigen used to produce the mAb, the avidity and class/subclass of the antibody, the fluorochrome conjugated to the mAb and the detection system (Garcia & Shimizu 1997; Johnston *et al.* 2003). Studies have reported that IgG_1 isotype antibodies demonstrate better diagnostic specificity and increased avidity to oocyst surface antigens than IgG_3 or IgM for *Cryptosporidium* in water samples (Ferrari *et al.* 1999; Weir *et al.* 2000). A limitation to DFA and other immunoassays for *Cryptosporidium* is that commercially produced anti-*Cryptosporidium* mAbs are raised against a limited pool of *C. parvum* isolates, and significant variations in binding affinity may occur to different species or genotypes. Furthermore, the species or genotype of *Cryptosporidium* cannot be determined by DFA.

In contrast to DFA, indirect immunofluorescence assays require primary and secondary antibodies. The primary antibody is unconjugated and binds to the oocyst and the secondary fluorophore-conjugated antibody acts against the primary antibody to enable detection. DFA is generally preferred to the indirect assay as only one tagging step is required, reducing processing time. In addition, more non-specific binding and higher background are associated with the indirect assay as a result of using a second antibody (Hassan *et al.* 2021). Despite this, indirect immunofluorescent has been reported to exhibit similar sensitivity and specificity to DFA.

Enzyme-linked immunosorbent assay (ELISA) offers an ease of use alternative to microscopy-based techniques for *Cryptosporidium* detection in the clinical sphere as it eliminates the need for skilled microscopists, enables batch testing and increases the sensitivity of analysis. In sandwich ELISA, a monoclonal antibody against *Cryptosporidium* is immobilized on a 96-well plate surface. When the sample is added, oocysts are captured by the immobilized antibodies. An enzyme-labelled secondary antibodies coupled to an enzyme such as horseradish peroxidase, alkaline phosphatase or β-galactosidase and a corresponding substrate is added to enable colorimetric detection by absorbance spectroscopy to indicate the interaction between the antibody and oocyst. ELISA is predominantly used for the detection of *Cryptosporidium* in stool samples but is not often employed in water samples due to the complexity of water matrices. Additionally, the concentration of oocysts may be too low for detection via ELISA (Hassan *et al.* 2021).

Immunochromatographic lateral-flow assays have been developed for the rapid detection of *Cryptosporidium* in faecal and water samples and are used frequently due to their simplicity, cost-effectiveness and time effectiveness. In this method, anti-*Cryptosporidium* antibodies are immobilized on a paper-based strip and when is sample applied it migrates across the strip through capillary action and binds to the immobilized antibody. While ELISA and immunochromatographic strips are highly sensitive, they have both been proven to generate false positive results (Table 1).

4. BIOSENSORS – THE PARADIGM SHIFT NEEDED FOR *CRYPTOSPORIDIUM* DIAGNOSTICS?

As most current analytical methods for *Cryptosporidium* detection are costly, time-consuming and require specialized equipment, an urgent need exists to develop user-friendly, rapid testing platforms to detect *Cryptosporidium* species in water sources. Biosensors are analytical devices that can possibly overcome the drawbacks of conventional testing methods due to their sensitivity, cost-effectiveness and real-time analysis capabilities. The composition of a biosensor includes a biological receptor, transducer and detector. The biological receptor interacts with the analyte to generate a signal, a process known as bio-recognition, and the transducer transforms the bio-recognition event into a measurable signal which is measured by a suitable detector (Chambers *et al.* 2002).

A search using keywords '*Cryptosporidium*', 'biosensor' and 'water' was conducted on ScienceDirect and Scopus to assess the global trend of biosensor development for *Cryptosporidium* detection in water. The number of articles with these terms from 2001 to 2021 are represented in Figure 3 and it appears that the focus is shifting from conventional detection methods

Table 1 | Comparison of conventional detection methods for *Cryptosporidium* detection in water

Method	Principle	Advantages	Disadvantages
Microscopy	Morphological identification of oocysts, identification can be improved by staining e.g., modified Ziehl-Neelsen, malachite green.	– Inexpensive – Simple sample preparation	– Unreliable – Low sensitivity – Time-consuming – Requires experienced personnel for identification of oocysts
Direct fluorescent antibody assay	FITC conjugated anti-*Cryptosporidium* mAbs bind to oocysts, enabling fluorescent detection.	– Increased specificity and sensitivity – Commercially available kits	– Variations in binding affinity to different species – Cannot determine genotype – Time-consuming
Lateral flow assay	Binding of oocysts to anti-*Cryptosporidium* mAb immobilized on nitrocellulose membrane.	– Simple – Cost-effective – Rapid detection – Portable – Potential for on-site use	– Low sensitivity – Tendency to produce false positives
ELISA	Immobilized anti-*Cryptosporidium* antibody, enzyme-labelled secondary antibodies for detection.	– High specificity – High throughput – Commercially available kits – Automation ability	– Susceptibility to background interference – Time-consuming due to multiple binding steps – Requires trained personnel
Molecular (PCR, LAMP)	Amplification and detection of target DNA sequence	– High specificity, allows for determination of genotype	– Time-consuming – Expensive equipment and consumables – Requires highly trained personnel – Environmental contaminants interfere in PCR

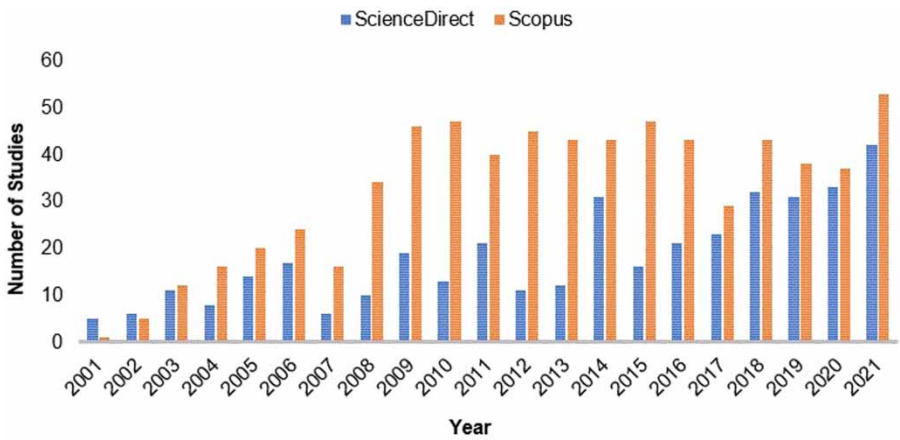


Figure 3 | The trend of *Cryptosporidium* sensor development represented by number of articles on ScienceDirect and Scopus with keywords '*Cryptosporidium*', 'biosensor' and 'water' from 2001 to 2021.

towards portable, sensitive and reliable biosensor technology. As *Cryptosporidium* incidence in drinking waters in South Africa is underreported due to challenges in the detection of the microorganism, testing systems may benefit from a paradigm shift from conventional testing to a suitable biosensor to simplify analysis. Over the past 20 years, advancements have been made in *Cryptosporidium* biosensing, but refinement of these techniques is essential before their commercialization and implementation in routine testing becomes viable. Several *Cryptosporidium* biosensors have been proposed and are summarized in this review according to their signal transduction mechanisms.

4.1. Electrochemical biosensor protocols

Electrochemical biosensors function by reacting with a target analyte to produce an electrical signal and are usually composed of a three electrode system. The small size of *Cryptosporidium* usually necessitates the use of a chemical agent to amplify the electrochemical signal to a detectable level as the organism will cover a small portion of the electrode surface and impart only a minor effect on current changes at the electrode–electrolyte surface. Thiruppathiraja *et al.* developed an electrochemical enzyme amplified sandwich immunosensor using dual-labelled gold nanoparticles and indium tin oxide (ITO) as the electrode for *C. parvum* detection (Thiruppathiraja *et al.* 2011). An anti-oocyst monoclonal antibody was immobilized on a gold nanoparticle functionalized ITO electrode for oocyst capture, and a gold nanoparticle probe labelled with alkaline phosphatase and anti-oocyst Mab was used for detection. The electrochemical signal was generated from the hydrolysis of phosphate ester *p*-nitro-phenol phosphate to the electro-active species *p*-nitro-phenol by alkaline phosphatase. Differential pulse voltammetry was used to measure the electrochemical signal generated when the gold probe bound the oocyst, and a sensitive limit of detection of 3 oocysts/mL was achieved by the gold probe signal amplification system. The stability and sensitivity of this low-cost system show promise for implementation into an environmental monitoring scheme.

Aptamers are promising bioreceptors due to their high affinity and offer higher stability, longer shelf lives and lower production costs in comparison to antibodies and have been widely used in electrochemical sensors. Iqbal *et al.* developed the first electrochemical aptasensor for *C. parvum* detection in fresh produce, a rapid label-free system with a detection limit of around 100 oocysts (Iqbal *et al.* 2015). The sensing interface comprised of a self-assembled monolayer of thiolated-DNA capture probe and anti-*C. parvum* aptamer on a AuNP-modified screen-printed carbon electrode with a ferricyanide/ferrocyanide redox couple as the electrolyte. The redox current increase after oocyst binding to the immobilized aptamer was measured by square wave voltammetry.

The same research group expanded on this work in spiked water matrices, fabricating an electrochemical aptasensor where biotinylated aptamer was bound to streptavidin-coated magnetic beads incorporated into an AuNP-modified screen-printed carbon electrode (Iqbal *et al.* 2019). Square wave voltammetry was used to measure the increase in current and the sensitivity of the aptasensor was greater than the first system, with an LOD of 50 oocysts in buffer. Although aptamer-based assays offer great potential in pathogen detection and these studies show promise for oocyst detection, drawbacks exist in their application. The selection process for aptamers, called systematic evolution of ligands by exponential enrichment (SELEX), requires screening large oligonucleotide libraries through many cycles of selection and amplification (Chai *et al.* 2011). The highly variable and complex structure of the oocyst may influence aptamer performance and improvement of aptamer selection efficiency by SELEX is needed for high specificity of the aptamer to its whole cell or protozoan target (Teng *et al.* 2016).

Cryptosporidium oocysts produce the protein hsp70 in response to heat shock, and the mRNA gene coding for hsp70 is often used as a marker in nucleic acid-based detection methods (Bridle *et al.* 2012). Nugen *et al.* developed a polymethyl methacrylate (PMMA) chip-based electrochemical biosensor for the detection of *Cryptosporidium* nucleic acids (Nugen *et al.* 2009). Standard photolithography was used to fabricate an interdigitated ultramicroelectrode array on the PMMA surface, and the analyte used was hsp70 mRNA extracted and amplified from *C. parvum* oocysts. The detection system was a sandwich hybridization assay based on a magnetic bead/liposome complex captured on the channel by a magnet. The capture probe was coated with superparamagnetic beads and the reporter probe was composed of liposomes entrapping potassium ferro/ferrihexacyanide allowing for amperometric detection of the nucleic acids. The system was highly sensitive and enabled the detection of amplified mRNA from a single oocyst, however, the method does not offer a significant improvement over conventional methods due to expensive labels and the time-consuming preparation, mRNA extraction and amplification steps.

4.2. Spectroscopic biosensor protocols

Raman spectroscopy is a light scattering technique where incident monochromatic light is used to excite molecules to higher vibrational energy levels to emit radiation at a different wavelength, a process called Raman scattering. Raman signals are relatively weak and are enhanced by coating the sensing surface with noble metals. This technique is called Surface-Enhanced Raman

Spectroscopy (SERS), and when coupled to resonance enhancement by adjusting the incident light energy to the molecule electronic transition level it is named Surface-Enhanced Resonance Raman Spectroscopy (SERRS) (Bridle *et al.* 2012).

Grow *et al.* reported the first label-free SERRS chip-based biosensor for *Cryptosporidium* detection, composed of a charge-coupled device array, surface-enhanced biochip and software for analysis (Grow *et al.* 2003). The sensor was reported to theoretically detect a single oocyst, but no LOD was reported due to the low capture efficiency of the system. Rule and Vikesland developed a SERRS biosensor for co-detection of *C. parvum* and *G. lamblia* using immunogold Raman labels (Rule & Vikesland 2009). The labels were composed of gold nanoparticles conjugated to commercial monoclonal antibodies labelled with rhodamine B isothiocyanate and malachite green isothiocyanate, respectively. The sensor specificity and sensitivity were high with no cross-reaction of antibodies, demonstrating the feasibility of a Raman-based method for multi-pathogen detection.

Many drawbacks exist to SERRS-based biosensors that limit their viability in *Cryptosporidium* analysis. Reproducible and quantitative analyses are a challenge, and no *Cryptosporidium* studies reported accurate detection limits (Luka *et al.* 2022). The acquisition time is long, taking up to 15–20 min per oocyst and only highly concentrated low volume samples can be analysed in a single step. Furthermore, oocysts need to be anchored to the surface as movement in the sample over the detection surface can disrupt measurements (Bridle *et al.* 2012).

Surface plasmon resonance (SPR) measures changes in refractive index at the interface between a dielectric material and a planar metal surface. Bio-recognition is detected by coupling photons from a light source to surface plasmons and measuring the change in the incident and reflected light after analyte binding. Kang *et al.* have reported the use of an SPR biosensor with *C. parvum* as an analyte (Kang *et al.* 2006). The system was a flow type biosensor based on a mixed self-assembled monolayer, and a streptavidin interaction used to immobilize biotinylated anti-*C. parvum* monoclonal antibodies to the surface. Due to the low capture efficiency of the surface immobilized antibody, the LOD was 1×10^6 oocysts/mL. The sensitivity was enhanced to an LOD of 100 oocysts/mL by instead labelling the oocysts with biotinylated antibody and taking advantage of the high affinity reaction between the immobilized streptavidin and biotin.

A drawback to this method is that the sample processing requires centrifugation, making it difficult to integrate the detection method into a continuous flow system. As variations in the refractive index of various sample matrices due to differing turbidity will influence the response of the system (Bridle *et al.* 2012), these factors complicate SPR analysis in environmental samples and must be strictly controlled for. If SPR is to be feasible for *Cryptosporidium* detection in a biosensor, an ideal system would include a processing step such as IMS purification to reduce complex matric effects in the biosensor response signal.

Colorimetric and fluorescent biosensors measure the colour or fluorescent signal produced by the biological recognition event, which can be seen by the naked eye or quantified using a simple optical instrument. Esch *et al.* developed a competitive lateral flow chip for the detection of amplified hsp70 coding mRNA (Esch *et al.* 2001b). Amplicons were hybridized with dye-loaded liposomes conjugated to an oligonucleotide probe and biotin and loaded onto a nitrocellulose test strip. A probe complementary to the reporter was immobilized in the control zone, and anti-biotin antibody immobilized in the test zone where liposomes would bind in the presence of target mRNA.

The same group developed a similar microfluidic fluorescence biosensor based on an mRNA detecting sandwich assay (Esch *et al.* 2001b). Reporter probes were conjugated to carboxyfluorescein-loaded liposomes to enhance the detection signal. Both systems allowed for the detection of low concentrations of the mRNA target and several studies have since reported lateral flow sensors for an hsp70 mRNA target (Kozwich *et al.* 2000; Baeumner *et al.* 2004; Connelly *et al.* 2008). However, such systems experience the disadvantages of most nucleic acid-based sensors such as time-consuming pre-treatment and expensive reagents, limiting their application.

4.3. Piezoelectric biosensor protocols

In cantilever-based sensors, binding of the analyte to an antibody-immobilized cantilever results in a decrease in resonance frequency corresponding to the increase in mass. This technique is suitable for targets heavier than molecular targets such as microorganisms that can generate significant signal changes (Jain *et al.* 2019). Piezoelectric-excited millimetre-sized cantilevers (PEMC) are composed of two layers, a piezoelectric layer that acts as an actuator and sensor and a silica or glass layer that is functionalized with a receptor to bind the microorganism. Campbell and Mutharasan fabricated a PEMC IgM antibody functionalized biosensor that could detect *C. parvum* oocysts at 100 oocysts/mL (Campbell & Mutharasan 2008). A study by the same group assessed the detection of *C. parvum* by the PEMC biosensor in deionised water, PBS and milk (Xu & Mutharasan 2010), but testing in raw and drinking water matrices has not been performed and hence the suitability of this system for *Cryptosporidium* detection in relevant water sources has not been assessed (Table 2).

Table 2 | Comparison of biosensors developed for detection of *Cryptosporidium*

Biosensor type	Detection signal	Detection principle	Sensitivity	Ref
Electrochemical enzyme-enhanced immunosensor	Differential pulse voltammetry	Sandwich detection using anti-oocyst mAb and dual-labelled AuNPs (alkaline phosphatase and anti-oocyst mAb). Hydrolysis of phosphate ester *p*-nitro-phenol phosphate to electrochemically active *p*-nitro-phenol by the ALP after oocyst capture produced an electrochemical signal.	3 oocysts/mL	Thiruppathiraja *et al.* (2011)
Electrochemical aptasensor	Square wave voltammetry	Self-assembled monolayer of thiolated-ssDNA capture probe and anti-oocyst aptamer on an AuNP-modified SPCE. A redox current increase of the ferricyanide/ferrocyanide redox couple occurred after oocyst binding to aptamer.	100 oocysts	Iqbal *et al.* (2015)
Electrochemical aptasensor	Square wave voltammetry	Streptavidin-coated magnetic beads bind to biotinylated aptamer specific for *C. parvum* immobilized onto an AuNP-modified SPCE.	50 oocysts	Iqbal *et al.* (2019)
Electrochemical biosensor	Amperometry	Detection of hsp70 mRNA from oocyst by sandwich hybridization assay with capture probe-coated superparamagnetic beads and reporter probe-tagged liposome. Liposomes entrap potassium ferro–ferrihexacyanide to produce an electrochemical signal on an interdigitated ultramicroelectrode array.	Amplified DNA from 1 oocyst was detectable	Nugen *et al.* (2009)
SERS chip-based biosensor	Raman spectrometry	Label-free whole-organism fingerprint collection via capture of oocyst by antibody immobilized on SERS biochip.	No LOD reported	Grow *et al.* (2003)
SERRS biosensor	Raman spectrometry	An AuNP/anti-oocyst antibody conjugate was labelled with SERRS active label rhodamine B isothiocyanate and bound to oocysts for SERRS detection.	No LOD reported	Rule & Vikesland (2009)
SPR biosensor	Surface plasmon resonance	Flow type biosensor based fabricated by mixed self-assembled monolayers. A streptavidin interaction was used to immobilize biotinylated anti-*C. parvum* monoclonal antibodies to the sensor surface.	100 oocysts/mL	Kang *et al.* (2006)
Competitive lateral flow chip	Colour/visual	Detection of the hsp70 amplicon by hybridizing the amplicon with dye-entrapping liposomes with oligonucleotides and biotin on their surface. The amplicon complex migrates to an immobilized anti-sense reporter probe on a nitrocellulose membrane.	80 fmol of amplicon	Esch *et al.* (2001b)
Microfluidic fluorescence biosensor	Fluorescence	Microfluidic chip utilizing sandwich hybridization by hybridizing the hsp70 amplicon between capture probe and a carboxyfluorescein-filled liposomes.	5 fmol of amplicon	Esch *et al.* (2001a)
PEMC immunosensor	Resonance frequency	Detection of oocysts immobilized by IgM on PEMC surface by resonance frequency response.	100 oocyst/mL	Campbell & Mutharasan (2008)

5. CONCLUSION

Cryptosporidium is one of the most commonly occurring protozoan parasites in the world and despite being underdiagnosed in even the most technologically advanced nations, is responsible for significant disease burden globally. Chronic illness and

poor outcomes in children and immunocompromised individuals place strain on health systems, and its environmental transmission and resistance against common disinfection practices in water treatment facilities present a major public health challenge for the water quality sector. The lack of effective treatment and vaccines for *Cryptosporidium* shifts effective disease management to prevention measures such as early pathogen detection in water sources.

Although substantial efforts have been made to develop sensitive techniques for the detection of *Cryptosporidium*, current methods suffer from limitations such as lengthy processing times, multistep analysis, low sensitivity and high cost of reagents and equipment. Maintaining the health and safety of water consumers requires continuous monitoring of water sources, and a dire need exists to simplify screening for *Cryptosporidium* with improved efficiency and reliability. Biosensors are a technological advancement that shows promise in the field of environmental monitoring and may provide a portable, real time, on site and compact solution for *Cryptosporidium* detection in water sources. Sensitive detection of *Cryptosporidium* has been achieved with biosensors and several studies have shown their potential for implementation in environmental monitoring for this parasite. Electrochemical biosensors using aptamers or antibodies as recognition elements have particularly shown promise due to the demonstrated sensitivity of several designs and affordability in comparison to spectroscopic and nucleic-based protocols. However, despite the potential of these preliminary advances, the need remains to improve the functionality of these technologies to enable commercialization.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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First received 16 February 2023; accepted in revised form 10 July 2023. Available online 21 July 2023