J Trop Med Parasitol. 2014;37:20-34.

REVIEW

Available online at http://www.tm.mahidol.ac.th/ptat

Cryptosporidium Viability Testing: A Review

Aongart Mahittikorn, Khuanchai Koompapong, Hirotake Mori, Yaowalark Sukthana

Department of Protozoology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

ABSTRACT

Cryptosporidium has emerged as a significant cause of water-borne parasitic outbreaks, making it an important priority for water-resource management. It is challenging to detect the parasite and to determine whether oocysts are viable since non-viable oocysts in environmental samples do not cause infections and are therefore of little concern for public health, making accurate viability testing a crucial step in monitoring and controlling this parasite. The ideal test for defining viability is testing the ability of parasite in causing disease in humans, which is not feasible. This has led to alternative procedures to determine whether oocysts are capable of causing infection in susceptible hosts. We reviewed existing articles concerning methods used in determining *Cryptosporidium* viability, the advantages and disadvantages of each technique, and proposed a method of choice by rating and weighting criteria based on five indicators: (i) reliability; (ii) applicability; (iii) technical expertise required; (iv) time consumed; and (v) cost. Based on these criteria, fluorescence *in situ* hybridization (FISH) and vital dye staining are classified as excellent methods, while RT-PCR, animal inoculation, *in vitro* excystation, and cell culture are considered good. Good and excellent methods were then evaluated in multiple sample types. FISH was the most appropriate technique for clinical specimens due to its high tolerance to inhibitors, which affect the results obtained by other methods. Viability studies in environmental samples, such as drinking water, wastewater, ground water, soil, and sludge, are difficult, and need special consideration because of low levels of oocyst contamination. FISH, vital dye staining, and *in vitro* excystation are possible methods for use with these kinds of samples, while animal infectivity is the most appropriate technique for chemical or physical inactivated samples. The information presented here could be a guideline for selecting the appropriate technique, or for developing new methods for viability testing that may benefit public health, and overcome common obstacles.

Keywords: *Cryptosporidium*, viability, animal infectivity, cell culture, *in vitro* excystation, vital dye staining, reverse transcription-PCR, fluorescence *in situ* hybridization, FISH

INTRODUCTION

Cryptosporidium spp. are enteric protozoa responsible for a number of water-borne outbreaks of human cryptosporidiosis worldwide [1]. They cause diarrhea in humans, with some causing severe debilitating illness, especially in immunosuppressed persons with HIV/AIDS. The true global burden of cryptosporidiosis is not known due to an under-appreciation of the frequency and severity of the disease in immunocompetent patients, and difficulties

Correspondence: *Yaowalark Sukthana*
E-mail: yaowalark.suk@mahidol.ac.th

in quantifying the impact of an infection that causes acute illness with long-term sequelae [2]. However, estimates of the prevalence of human cryptosporidiosis in the general population have ranged from 2.6-21.3% in African countries, 3.2-31.5% in Central and South America, 0.1-14.1% in Europe, 0.3-4.3% in North America, and 1.3-13.1% in Asia [3,4]. In developed countries, cryptosporidiosis is more common during the warm, rainy months, and has a bimodal age distribution, with a high number of cases in children 1-9 years of age and adults 25-39 years of age [2]. Risk factors associated with sporadic infection include contact with patients and cattle, history of travel abroad, and anal intercourse among homosexuals. Outbreaks in childcare centers and public pools are also common, and can result in spread to the larger community [5]. In developing countries, peaks tend to occur during the warm and rainy months, as well. The disease is more prevalent in young children, who are often infected by the age of two [6]. Breastfeeding during the first three months of life and partial breastfeeding appears to afford some protection [7]. Although highly active antiretroviral therapy (HAART) has been available for HIV-infected patients in many parts of the world, *Cryptosporidium* prevalence rates remain high among this group, with significant associated morbidity. High prevalence can also be observed in HIV/AIDS patients in developing countries. For example, up to 30.0% of HIV/AIDS patients in Thailand are positive for *C. hominis* [8] and as many as 81% have been found positive in India [9].

To date, approximately 30 *Cryptosporidium* species have been identified as parasites of mammals, birds, reptiles, and amphibians [10]. Among these, *C. hominis, C. parvum, C. meleagridis*, *C. cuniculus*, *C. canis*, *C. felis* and *C. suis* are recognized as zoonotic transmitters of which the first three species are of major public health concern [11,12]. *C. parvum* and *C. hominis* are responsible for more than 90 percent of human cryptosporidiosis worldwide [13]. It has been suggested that in developing countries, human *C. parvum* infections are mainly transmitted from person to person, while in developed countries, infections tend to occur from environmental contamination [14].

The largest outbreak of cryptosporidiosis occurred in 1993 in Milwaukee (WI, USA), where approximately 403,000 people contracted the disease by consuming the municipal water supply contaminated with *Cryptosporidium* oocysts [15]. One hundred and twelve people died, and 85% of deaths occurred among elderly or immunocompromised patients [16]. MacKenzie *et al,* demonstrated that the contamination occurred when oocysts passed through the water-filtration system of a water treatment plant [17]. Other reports of *Cryptosporidium* outbreaks and sporadic infection occurred in childcare centers, recreation centers, and drinking-water reservoirs [18-22].

Oocysts are able to survive in the environment for long periods due to their robust and small structure. They are resistant to most disinfectants used in water treatment systems, and environmental stresses [23]. Although the number of *Cryptosporidium* oocysts present in the water supply is generally low, the infective dose of the parasite is as small as 1-10 oocysts [3,18]. A high number of oocysts are shed with feces (up to 10^9 per time), which can be transmitted from one person to another and potentially infect immediately after contact [2]. These contribute to the water-borne transmission of *Cryptosporidium*, a serious global issue in drinking-water safety. *Cryptosporidium* are able to infect and multiply in a wide range of wildlife and domestic animals, which become potential sources of oocyst shedding into the environment, and risk establishing infection in humans. Therefore, many methods for detecting oocysts in environmental samples have been developed, ranging from conventional staining to immunological and molecular techniques. The standard recovery and detection method involves three basic steps: (i) concentration of the sample (e.g. filtration, centrifugation) to recover the low numbers of parasites typically found in

the environment; (ii) purification (e.g. density gradients, specific antibodies); and (iii) detection of the parasites by means of immunofluorescent staining, which enhances our ability to detect oocysts microscopically in filtered sample concentrates, or through molecular techniques (e.g., PCR, real-time PCR) [24]. However, a challenge with the detection and disinfection of *Cryptosporidium* is the difficulty in determining whether a parasite is viable. It is important to assess the viability status of the organisms to determine whether they pose a threat to public health, to provide appropriate and effective prevention measures, and to determine the efficiency of the inactivation technique used. It is also important to ensure that public-health decisions are based on accurate information, since the presence of dead parasites in finished water or other environmental samples is of little concern for disease transmission. The ideal test for defining viability is assessing the ability of parasite in causing disease in the human population. Clearly, this is not feasible, and classical methods for the determination of *Cryptosporidium* viability rely on animal infectivity, which is time-consuming, difficult, and expensive, and unsuitable for normal laboratory analysis in water management systems. Under such circumstances, alternative methods for determining viability and infectivity have been developed. At present, six methods are used in *Cryptosporidium* survival studies: (i) animal infectivity, (ii) cell culture, (iii) *in vitro* excystation, (iv) vital dyes, (v) reverse transcription-polymerase chain reaction (RT-PCR) and (vi) fluorescent *in situ* hybridization (FISH). Each method has advantages and limitations, and can assess the viability at various temperatures, pH conditions, and sample types (clinical or environmental; contamination levels). Therefore, an in-depth review of the available literature on viability studies of this parasite was conducted to compare different methods of viability testing and to propose criteria for selecting a method for general application. The sensitivity, specificity, results obtained, and limitations of each method are also discussed.

Cryptosporidium viability assays

(I) Animal infectivity

For a decade, human volunteer studies and animal models have been used to evaluate viability by determining the infectivity or reduced infectivity of *Cryptosporidium* following exposure of oocysts to disinfectants and environmental stressors [25]. Due to ethical concerns and potential adverse health effects, animal models became the more practical methods, which also provided reliable results. The lack of sufficient human volunteers was another obstacle that made human volunteer studies less popular [26].

Hamsters, macaques, pigs, lambs, and opossums have been used for *Cryptosporidium* viability/infectivity studies, but the most common animal model is neonatal mice. Infectivity in mice is recognized as the "gold standard" method, since it appears to be the only method that can measure the infectivity of oocysts. Therefore, the neonatal mouse model system is the reference standard with which new *in vitro* methodologies are compared. Several strains of mice have been used, and gerbils, BALB/C mice, and neonatal CD-1 mice aged 5-7 days are among the most preferred [27-29].

The principle of the animal model is to infect the mouse with sufficient numbers of purified oocysts through the digestive tract for seven days, then remove the small intestine of the inoculated mouse to check for infectivity and parasite-intestinal cell interactions [30,31]. This technique reveals both the actual infectivity of the oocyst and information about host-parasite interaction. However, the gold standard technique is subject to ethical concerns and is impractical for use on a routine water industry inspection, since it is time-consuming and expensive (animal based laboratory setting, staff expertise, maintenance and license fee) [32]. Moreover, not all mouse strains are susceptible to infection with the parasite and the use of a variety of strains and animal species has likely contributed to the variability in experimental data. The animal model technique also requires high oocyst numbers and relatively clean lab and equipment. At least 1,000 oocysts per mouse are required to induce 100% infection

[26], so that this method is unsuitable for routine water or other environmental sample testing, since these sample types tend to contain low numbers of oocysts. These samples are also limited by their turbidity and unclean nature.

(II) Cell culture

To overcome these difficulties in animal models, significant efforts have been made to develop cell culture techniques to test the viability of *Cryptosporidium* oocysts. Over 20 cell lines have been used, such as human ileocecal adenocarcinoma (HCT-8), Caco-2, and Madin-Darby canine kidney cell (MDCK). Oocyst inoculation number, growth conditions, and assay format, depend on *Cryptosporidium* isolates and cultured cell lines [25]. Among available cell lines, HCT-8 provides the best results for *C. parvum* culture and infectivity testing [33-36]. Rochelle *et al,* [25] revealed that a 50% infective dose (ID_{50}) of *C. parvum* oocysts for the HCT-8 cell line ranged between 27-106 oocysts. Jenkins *et al,* showed that oocysts stored at 15°C for seven months remained viable and infectious to the HCT-8 cell [34].

The principle of cell culture on *Cryptosporidium* infection is to provide a suitable environment for parasite growth, mimicking that of the host. The cell line is cultured in media and maintained in tissue culture flasks in certain conditions until a monolayer ready for infection appears. The oocysts are normally pretreated with reagents, such as 5.25% sodium hypochlorite and 0.75% sodium taurocholate, to induce sporozoite rupture from the oocysts; the suspension containing the infectious sporozoites is inoculated onto cultured epithelial cells [25,33]. Sporozoites invade the cells and proceed to replicate within the intracellular environment of the cell. The intracellular stage, an infectivity indicator of the parasite, can be harvested after 24 - 48 hour post-inoculation and detected by immunofluorescence assay or PCR [35,68].

The advantage of cell-culture assays is that the initial establishment of the parasites can be determined. The assay is less time-consuming, lower in cost, and involves fewer ethical issues than the animal model. However, this technique has several weak points. The sensitivity of the cell culture is low (usually < 10% of viable parasites become established), expensive due to tissue culture, and prone to contamination problems. Therefore, it requires great care and high levels of technical expertise. Although some studies have shown correlated results between cell culture and animal model [25,34,35], considerable variation in the susceptibility of different cell lines to *C. parvum* remains, making comparisons with animal infectivity difficult. In addition, only *C. parvum*, *C. hominis*, *C. meleagridis*, *C. andersoni* and *C. muris* can be cultured in cell lines [37].

(III)*In vitro* excystation

Since live sporozoites can grow and split themselves, *in vitro* excystation was introduced as a viability-assessment technique [38]. This is a process by which oocysts are exposed to the host's gastrointestinal tract, which induces destabilization of the oocyst wall suture and sporozoite excystation [36]. *In vitro* excystation can be performed in the laboratory by mimicking conditions similar to those in the gut of the host, at the proper temperature. By microscopically determining the ratio of totally/partially excysted oocysts to the total number of enumerated oocysts, oocyst viability can be assessed quantitatively. The release of motile sporozoites can be observed and the ratio of sporozoites to excysted, or partially excysted, oocysts can be calculated. It is one of many choices for testing the efficiency of oocyst inactivation in chemical compounds [39-41]. Many reagents have been used to induce oocyst excystation, such as sodium hypochlorite, trypsin, sodium taurocholate, and extracted bile. The optimum pH is about 7.6 and temperature 37°C [38]. Maximum excystation can occur after 30 minutes' incubation [42].

The main advantage of this technique is that all *Cryptosporidium* spp. can be tested, the cost is low, and it is not time-consuming. The results show good correlation with the fluorogenic vital dye staining technique [43]. However, *in vitro* excystation provides less reliable results and tends to overestimate infectivity, as shown

in the viability assessment of *C. parvum*. This technique is not generally applicable for analyses in which oocysts are associated with matrix materials [44,45]. Neumann *et al*. reported that excysted and unexcysted sporozoites cannot infect neonatal CD-1 mice, whereas the study by Hou *et al*. revealed that unexcysted or intact oocysts can infect neonatal CD-1 mice [46,47]. High numbers (10^4 - 10^5) of purified oocysts and a concentrated suspension are required for *in vitro* excystation [48]. Some inhibitors can block excystation, such as 1,10-phenanthrolene, amastatin, H-boronorleucine (pinacol), and saliva [38,49]. Moreover, an expert microscopist is needed to examine the excysted sporozoites, since this is a non-reproducible technique [32].

(IV) Vital dye staining

Vital dye staining or fluorogenic dye staining was developed in 1992, and because of its simplicity, reliability, and speed, it later became one of the most commonly used techniques for assessing the viability of *Cryptosporidium* oocysts. [50]. This technique relies on the permeability/integrity of the oocyst wall and sporozoite cytoplasmic and nuclear membranes to these vital dyes.

Many dyes have been used, such as 4', 6-diamidino-2-phenylindole (DAPI), propidium iodide (PI), SYTO-9, SYTO-59 and hexidium. DAPI and PI are most commonly used for assessing the viability of *Cryptosporidium* oocysts (> 90% of published papers from 1992 - 2013). In viable oocysts, intact membranes are permeable to DAPI but impermeable to PI; therefore, oocysts selectively accumulate DAPI within the DNA of sporozoites contained within an oocyst, but cannot accumulate PI. Non-viable oocysts accumulate both DAPI and PI non-selectively.

The results of DAPI and PI staining show very good correlations with *in vitro* excystation, with 0.99 coefficient [43,51]. However, staining overestimates non-viable oocysts compared with the animal model [52]. Other vital dyes, SYTO-9 and SYTO-59, have been developed and show good correlations with infectivity in animals, but not with the *in vitro* excystation method [46,53]. As a result, these dyes are not commonly used for DAPI and PI staining. A brief protocol of vital dye staining begins with concentrating oocysts in isotonic buffer incubated with the vital dye (i.e. 250µM of SYTO-9, 5µM - 150µM of PMA, 10µl of 2 $mg.ml^{-1}$ in methanol of DAPI, 10µl of 1 $mg.ml^{-1}$ in 0.1M PBS of PI) for 30-90 min at 37°C. To improve the visibility of the oocysts, FITC should be added and incubated for a further 30 min in a dark at room temperature [54]. Finally, the samples can be examined under a fluorescence microscope at a certain wave length of light source according to the staining dye being used.

Vital dye staining can also be used with PCR to assess the viability of *Cryptosporidium* oocysts. Brescia *et al*. treated heat-killed oocysts and viable oocysts with propidium monoazide (PMA) prior to PCR analysis [37]. Since PMA can only penetrate dead oocysts and blocks amplification of their DNA, only viable oocysts were amplified. This method was recognized as CryptoPMA-PCR, an attractive approach to detect the species/genotypes of viable oocysts.

Among the tests discussed here, vital dye staining is the most popular for assessing the viability of *Cryptosporidium* oocysts in environmental samples, since it is the cheapest, easiest, and fastest (20 min) method to perform. Moreover, the method provides useful information for investigating environmental factors, regardless of oocyst numbers and purification [55,56]. However, vital dye staining requires expensive equipment and often overestimates non-viable oocysts compared with the gold-standard animal infectivity test, and must be optimized for each disinfectant tested [52,55,56].

(V) Reverse transcription-PCR (RT-PCR)

The first PCR combined with the excystation technique pre-DNA extraction was developed in 1995, and allowed differentiation between live and dead *C. parvum* [57]. Later, with advancements in molecular technology, a more practical technique, RT-PCR, became a popular method for assessing oocyst viability in many sample types, particularly environmental samples, since the technique

provided fast, sensitive, and reliable results. The principle of this method is to amplify and detect mRNA or rRNA of the viability-indicating gene of the oocysts. Both metabolic (amyloglucosidase, AG) and non-metabolic (heat shock protein 70, b-tubulin, 18s rRNA, *Cryptosporidium* oocyst wall protein: COWP and CP2, a membrane protein) genes were used as viability markers [58-62]. Among these, the first and most commonly used was hsp70, because this gene was the first and most replicated (1,000-10,000 fold synthesis) in heat shock conditions at 45°C for 20 min, which increases detection sensitivity to as much as 10 oocysts [48,63-65]. However, high replication of the hsp70 gene can sometimes cause the overestimation of viable oocyst numbers in RT-qPCR and cross reactivity with *Toxoplasma gondii* [58].

Other than hsp70, 18s rRNA, COWP, β-tubulin, and CP2 genes were also used as viability markers. However, the 18s rRNA is stable in heat killed conditions, which can overestimate viability, while COWP and β-tubulin genes disappear in heat-kill conditions, but give lower sensitivity than the CP2 gene. The CP2 gene seems to be suitable for calculating viable oocysts, since the gene is stable in heat-shock conditions, degenerates rapidly in heat-kill conditions, and yields high detection sensitivity [58,66].

RT-PCR is an advanced technique in terms of sensitivity, speed, reliability, and specificity to species level [59,67]. It is also useful for the direct detection of viable *C. parvum* in water containing concentrated oocysts [68]. However, it requires a molecular-lab setup, expensive equipment, reagents, and expertise. Care must be taken, since some inhibitors (i.e. fulvic acid, humic acid, and heavy metals) can interfere with the reaction. Cross-reaction with other pathogens may occur if primers are not well-designed, and residual RNA or RNase(s) present in the samples can dramatically decrease RT-PCR sensitivity [63].

(VI) Fluorescence *in situ* hybridization (FISH)

FISH was developed for assessing *C. parvum* viability in 1998 [62], and has been used with many sample types [69-72]. This molecular assay aims to detect 18S rRNA, which is present in high copy numbers in viable oocysts, but has a short half-life with a rapid decline in copy numbers in non-viable cells. Detection is done by using specific hybridization probes, labeled with differently colored fluorescents, i.e. Cy3, Texas Red (TR), and fluorescein isothiocyanate (FITC). These target a specific sequence in the 18S rRNA and can be examined under a fluorescence microscope [34]. Viable oocysts are fluorescent, while dead oocysts and organisms other than *C. parvum* are not. Many probes have been designed for FISH, such as Cry-1, Cry-2, Cpar677, and Chom253 [68].

The result of viability testing by FISH has correlated well with animal infectivity, cell culture, and *in vitro* excystation techniques [34,62]. The Cry-1 probe had been the most commonly used in FISH until the Cpar677 was developed specifically to detect *C. parvum* and differentiate it from *C. hominis,* and showed a good correlation (coefficient of 0.994) with the PCR-RFLP assay [70]. Recently, the *C. hominis* species-specific probe, Chom253, was designed and the two probe (two-color) system based on the previously published Cry-1 probe was developed for simultaneously detecting *C. hominis* and *C. parvum* [73].

FISH staining is a highly sensitive and relatively simple method that can overcome several of the obstacles inherent in other viability assays. It is very useful for environmental samples with low oocyst concentrations, facilitates the calculation of viable oocysts, and may provide information on species identification. The protocol is practical, fast (within 3 hours) and does not require expensive equipment or reagents. However, RNase can interfere with the results of FISH and great care must be taken to preserve target rRNA during sample processing procedures. Smith *et al*. reported that exogenous RNase(s) did not affect FISH results if the resuspend was neutralized before permeabilization [74]. They also found that vanadyl ribonucleoside complex (VRC) can extend the rRNA half-life of heat-permeabilized oocysts up to 155 hours. In some cases, heat-killed oocysts can be detected by FISH for up to

9 hours, which can result in an overestimation of viable oocysts [34,74]. So far, FISH can only detect *C. parvum* and *C. hominis*, whereas 5 other species presenting health risks to humans may be missed. Additionally, autofluorescent algae and mineral particles can mimic the brightness of the fluorescence hybridization probe and caution must be exercised in interpreting positive FISH results [62].

Selecting the "best" method for studying *Cryptosporidium* viability

Each method of studying *Cryptosporidium* viability has advantages and limitations. To select a preferred method, we propose 5 selection criteria: 1) reliability, 2) applicability in various sample types, 3) technical expertise required, 4) time consumed, and 5) cost. The rating for each criterion ranges from 1 to 4 (Table 1). For instance, a method with reliable results, or which could used with any type of sample would be rated 4, while procedures that are time-consuming, expensive, and require specific training, would be rated 1. Although simple and inexpensive methods are generally required, a high degree of reliability, and wide application in many types of samples, are more important. Therefore, the reliability indicator was weighted at 40, applicability at 30, and technical expertise required, time consumed, and cost, weighted at 10 each (Table 2). A method with a score of ≥75 was considered "excellent", a score of ≥62.5 was "good", and a score of ≥50 was "fair".

The six *Cryptosporidium* viability assays mentioned above have been put into consideration based on the selection criteria in Table 1. The result is shown in Table 2. Animal infectivity testing scores 65, because it provides strongly reliable results and is flexible, with 2 or 3 sample types (physical/chemical inactivation or clinical specimens). However, specific individual training is needed and it takes at least 72 hours to get a result. In addition, it is the most expensive compared with the other tests. Although animal infectivity is known as the gold standard, it is classified as a "good", not an "excellent", test using our criteria.

Table 1 Rating indicators for existing methods used to study *Cryptosporidium* viability.

Indicators	Rating Description			
	1 (Poor)	2 (Fair)	3 (Good)	4 (Excellent)
1. Reliability (result obtained)	Unreliable/ subjective	Equivocal	Reliable	Strongly reliable/ objective
2. Applicability (application in various sample types)	Very specific	Flexible 2-3 sample types	Widely used in >3 sample types, but not all	All types
3. Technical expertise	Special or specific training	Basic plus experimental laboratory training	Basic training	No training needed
4. Time to result (hour)	Very slow >72	Slow 49-72	Wait up to 24-48	Fast <24
5. Cost per test (US$)	Very expensive >30	Expensive 20-30	Reasonable 5-20	Cheap <5

Other good methods are RT-PCR with a score of 72.5, and *in vitro* excystation with 67.5. RT-PCR is fast (<24 hours), has acceptable cost, with a reliable outcome, but requires personnel with specific training. *In vitro* excystation is also fast, cheap, and simple to use, although its results are inaccurate ("dead" parasites excyst).

Cell culture, a fair technique with a score of 50, is time consuming (24-48 hours), not very accurate, expensive, has low infectivity, and requires special training. Moreover, frequent culture contamination is a major problem.

Vital-dye staining and FISH are excellent methods, with scores of 75, due to their wide application to many sample types, speed, low cost, and the necessity for only basic lab training. Nevertheless, the results obtained from the inclusion/exclusion of vital dyes in staining are sometimes unclear and inconsistent.

Appropriate method selection for various sample types

Different sample types (from patients, environments, or physical or chemically treated samples) have different natures of *Cryptosporidium* contamination. In general, environmental samples, either water or soil, contain low numbers of oocysts. For these, one should not only consider the excellent/good scores awarded above for *Cryptosporidium* viability studies, but also other factors, such as contamination levels and inhibitor(s). Inhibitors containing physical/chemical or biological compounds can decrease or blind the expected results, even when the most reliable method is employed. Therefore, before selecting any viability test, the appropriate and satisfactory yield method should be considered.

Table 3 presents a description of the contamination level of oocysts and inhibitor(s) found in each type of sample. Among methods rated "excellent" or "good" from Table 2, FISH is the most appropriate for *Cryptosporidium* viability studies in clinical samples, while vital dye staining, RT-PCR, animal infectivity and *in vitro* excystation are less than optimal, because biological inhibitors in clinical specimens always influence these methods, but do not affect FISH. Cell culture is also not recommended for use with clinical specimens, again due to contamination of other organisms, which may result in false positives.

Due to the low numbers of oocysts normally found in soil and water samples, viability studies in water and soil are difficult and need special consideration. FISH, vital dye staining, and *in vitro* excystation are possible procedures while RT-PCR, cell culture, and animal inoculation are not recommended.

Animal infectivity provides the most reliable results in samples containing sufficient clean oocysts. Mouse infectivity has been reported to be the best choice for determining *C. parvum* inactivation (using ozone, chlorine dioxide, UV, etc.) as it is more sensitive than *in vitro* assays for determining levels of oocyst inactivation [31,77,78]. However, there are limitations when examining samples from the environment or after water treatment. The number of oocysts recovered from these samples is almost always too low to cause infection in an animal. If infection can be established, it can only be concluded that infectious oocysts were present in the sample. It is not possible to estimate the proportions of viable and non-viable oocysts from an individual environmental sample.

Existing *in vitro* assays offer several advantages over mouse infectivity, in that they are simple, have an acceptable cost, and do not require ethical considerations or specialized facilities. Furthermore, *in vitro* assays enable determination of the viability status of individual oocysts, making them a user-friendly alternative to mouse infectivity assays. Unfortunately, these assays demonstrate poor correlation with mouse infectivity following oocyst treatment with disinfectants, such as UV light or ozone. The potentially high levels of chemical/physical inhibitors present in environmental samples usually hamper the reaction of RT-PCR and interfere with cell-culture results.

In vitro cell culture and molecular techniques,

Table 2 Weighting and rating of the 6 methods used in *Cryptosporidium* viability study, according to 5 criteria.

Method	Indicators (weighting score)																				Total Score
	Reliability (40)				Applicability (30)				Technical expertise required (10)				Time consumed (hour) (10)				Cost (US$) [reference] (10)				
Rating	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	
Animal infectivity				Strongly reliable		Flexible sample types			Specific training need				>72					20-30 [67]			65
Cell culture		Equi-vocal				Flexible sample types			Specific training need						24-48			20-25 [25]			50
In vitro excystation		Equi-vocal					General use			Basic and lab training						<24				<1 [52]	67.5
Vital dye staining		Equi-vocal						Any type		Basic and lab training						<24				<1 [75]	75
Reverse transcription-PCR (RT-PCR)			Re-liable				General use		Specific training need							<24			10 -15 [76]		72.5
Fluorescent in-situ hybridization (FISH)			Re-liable				General use			Basic and lab training						<24			5.5 [75]		75

Excellent method, indicated by a score of ≥75; Good method, indicated by a score of ≥62.5; Fair method, indicated by a score of ≥50

Table 3 Different sample types exhibit different levels of oocyst contamination and inhibitor(s), and affect the preferred method of choice when studying *Cryptosporidium* viability.

Sample type	Nature of sample	Method of choice
Clinical specimens • Stool • Bile	• Moderate oocyst contamination • High level of biological inhibitors found, contaminating sample with other organisms • Chemical and physical inhibitors may be found in some circumstances	Most appropriate 1. FISH Acceptable 2. Exclusion/Inclusion vital dye staining 3. RT-PCR 4. Animal infectivity 5. *In vitro* excystation Inappropriate 6. Cell culture
Environmental samples • Water • Soil	• Very low level of oocyst contamination • Chemical and physical inhibitors may be found but not many biological inhibitors	Most appropriate • None Acceptable 1. FISH 2. Exclusion/Inclusion vital dye staining 3. *In vitro* excystation Inappropriate 4. RT-PCR 5. Cell culture 6. Animal infectivity
Physical or chemical treated samples	• High oocyst contamination (due to evaluating conditions of the efficacy of physical or chemical treatment) • Chemical, physical and biological inhibitors can be controlled	Most appropriate 1. Animal infectivity 2. FISH 3. RT-PCR 4. Cell culture Acceptable 5. *In vitro* excystation 6. Exclusion/Inclusion vital dye staining

FISH and RT-PCR, are good when samples contain high numbers of oocysts. The other two possible methods are vital dye staining and *in vitro* excystation. However, vital dye staining is superior because it is less affected by inhibitor components when compared with *in vitro* excystation.

CONCLUSION

In conclusion, this article has evaluated the 6 available techniques capable of determining *Cryptosporidium* viability (Tables 2, 3). We rated and weighted selection criteria for choosing the preferred method and compared their advantages and limitations. There is no perfect

test for *Cryptosporidium* viability, and selecting an appropriate method is important and must be considered depending on the type of sample, contamination level of oocysts, and the presence of inhibitors in the sample. Such factors will determine the test that is likely to be used for rapid testing, which will, in turn, greatly influence test performance. The selected technique will need to be reliable, applicable to a variety of different sample types and a diversity of matrices, and be able to establish simple, fast, cost-effective, and accurate results for evaluating prevention measures for identifying viable cells, and more precise risk assessment. Finally, the issue of sample preparation, beyond the scope of this review, is critical to delivering enriched oocyst samples with high recovery rates.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Anootnara Talkul Kuster, Faculty of Public Health, Khon Kaen University for critical review of this article.

Conflicts of interest

The authors declare that there are no conflicts of interest associated with this paper.

REFERENCES

1. Chalmers RM. Water-borne outbreaks of cryptosporidiosis. Ann Ist Super Sanita. 2012;48:429-46.
2. Shirley DA, Moonah SN, Kotloff KL. Burden of disease from cryptosporidiosis. Curr Opin Infect Dis. 2012;25:555-63.
3. Fayer R. *Cryptosporidium*: a water-borne zoonotic parasite. Vet Parasitol. 2004;126:3 7-56.
4. Xiao L, Fayer R. Molecular characterisation of species and genotypes of *Cryptosporidium* and *Giardia* and assessment of zoonotic transmission. Int J Parasitol. 2008;38:1239-55.
5. Baldursson S, Karanis P. Water-borne transmission of protozoan parasites: review of worldwide outbreaks - an update 2004-2010. Water Res. 2011;45:6603-14.
6. Ayuo PO. Human cryptosporidiosis: a review. East Afr Med J. 2009;86:89-93.
7. Bilenko N, Ghosh R, Levy A, Deckelbaum RJ, Fraser D. Partial breastfeeding protects Bedouin infants from infection and morbidity: prospective cohort study. Asia Pac J Clin Nutr. 2008;17:243-9.
8. Saksirisampant W, Prownebon J, Saksirisampant P, Mungthin M, Siripatanapipong S, Leelayoova S. Intestinal parasitic infections: prevalences in HIV/AIDS patients in a Thai AIDS-care centre. Ann Trop Med Parasitol. 2009;103:573-81.
9. Desai NT, Sarkar R, Kang G. Cryptosporidiosis: an under-recognized public health problem. Trop Parasitol. 2012;2:91-8.
10. Slapeta J. Cryptosporidiosis and *Cryptosporidium* species in animals and humans: a thirty colour rainbow? Int J Parasitol. 2013;43:957-70.
11. Chalmers RM, Katzer F. Looking for *Cryptosporidium*: the application of advances in detection and diagnosis. Trends Parasitol. 2013;29:237-51.
12. Xiao L, Feng Y. Zoonotic cryptosporidiosis. FEMS Immunol Med Microbiol. 2008;52: 309-23.
13. Xiao L, Ryan UM. Cryptosporidiosis: an update in molecular epidemiology. Curr Opin Infect Dis. 2004;17:483-90.
14. Xiao L. Molecular epidemiology of cryptosporidiosis: an update. Exp Parasitol. 2010;124:80-9.
15. Karanis P, Kourenti C, Smith H. Water-borne transmission of protozoan parasites: a worldwide review of outbreaks and lessons learnt. J Water Health. 2007;5:1-38.
16. Hoxie NJ, Davis JP, Vergeront JM, Nashold RD, Blair KA. Cryptosporidiosis-associated mortality following a massive water-borne outbreak in Milwaukee, Wisconsin. Am J Public Health. 1997;87:2032-5.
17. Mac Kenzie WR, Hoxie NJ, Proctor ME, Gradus MS, Blair KA, Peterson DE, *et al*. A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. N Engl J Med. 1994;331:161-7.

18. Caccio SM, Thompson RC, McLauchlin J, Smith HV. Unravelling *Cryptosporidium* and *Giardia* epidemiology. Trends Parasitol. 2005;21:430-7.
19. Creek TL, Kim A, Lu L, Bowen A, Masunge J, Arvelo W, *et al*. Hospitalization and mortality among primarily nonbreastfed children during a large outbreak of diarrhea and malnutrition in Botswana, 2006. J Acquir Immune Defic Syndr. 2010;53:14-9.
20. Cabada MM, White AC, Jr. Treatment of cryptosporidiosis: do we know what we think we know? Curr Opin Infect Dis. 2010;23:494-9.
21. Collinet-Adler S, Ward HD. Cryptosporidiosis: environmental, therapeutic, and preventive challenges. Eur J Clin Microbiol Infect Dis. 2010;29:927-35.
22. Lee MB, Greig JD. A review of gastrointestinal outbreaks in schools: effective infection control interventions. J Sch Health. 2010;80:588-98.
23. Smith HV, Rose JB. Water-borne cryptosporidiosis. Parasitol Today. 1990;6:8-12.
24. Quintero-Betancourt W, Peele ER, Rose JB. *Cryptosporidium parvum* and *Cyclospora cayetanensis*: a review of laboratory methods for detection of these water-borne parasites. J Microbiol Methods. 2002;49:209-24.
25. Rochelle PA, Marshall MM, Mead JR, Johnson AM, Korich DG, Rosen JS, *et al*. Comparison of *in vitro* cell culture and a mouse assay for measuring infectivity of *Cryptosporidium parvum*. Appl Environ Microbiol. 2002;68:3809-17.
26. Carey CM, Lee H, Trevors JT. Biology, persistence and detection of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocyst. Water Res. 2004;38:818-62.
27. Korich DG, Marshall MM, Smith HV, O'Grady J, Bukhari Z, Fricker CR, *et al*. Inter-laboratory comparison of the CD-1 neonatal mouse logistic dose-response model for *Cryptosporidium parvum* oocysts. J Eukaryot Microbiol. 2000;47:294-8.
28. Chen F, Huang K, Qin S, Zhao Y, Pan C. Comparison of viability and infectivity of *Cryptosporidium parvum* oocysts stored in potassium dichromate solution and chlorinated tap water. Vet Parasitol. 2007;150:13-7.
29. Kvac M, Kvetonova D, Salat J, Ditrich O. Viability staining and animal infectivity of *Cryptosporidium andersoni* oocysts after long-term storage. Parasitol Res. 2007;100:213-7.
30. Current WL, Reese NC. A comparison of endogenous development of three isolates of *Cryptosporidium* in suckling mice. J Protozool. 1986;33:98-108.
31. Finch GR, Black EK, Gyurek L, Belosevic M. Ozone inactivation of *Cryptosporidium parvum* in demand-free phosphate buffer determined by *in vitro* excystation and animal infectivity. Appl Environ Microbiol. 1993;59:4203-10.
32. Bukhari Z, Marshall MM, Korich DG, Fricker CR, Smith HV, Rosen J, *et al*. Comparison of *Cryptosporidium parvum* viability and infectivity assays following ozone treatment of oocysts. Appl Environ Microbiol. 2000; 66:2972-80.
33. Slifko TR, Friedman D, Rose JB, Jakubowski W. An *in vitro* method for detecting infectious *Cryptosporidium* oocysts with cell culture. Appl Environ Microbiol. 1997;63:3669-75.
34. Jenkins M, Trout JM, Higgins J, Dorsch M, Veal D, Fayer R. Comparison of tests for viable and infectious *Cryptosporidium parvum* oocysts. Parasitol Res. 2003;89:1-5.
35. Joachim A, Eckert E, Petry F, Bialek R, Daugschies A. Comparison of viability assays for *Cryptosporidium parvum* oocysts after disinfection. Vet Parasitol. 2003;111:47-57.
36. King BJ, Hoefel D, Daminato DP, Fanok S, Monis PT. Solar UV reduces *Cryptosporidium parvum* oocyst infectivity in environmental waters. J Appl Microbiol. 2008;104:1311-23.
37. Brescia CC, Griffin SM, Ware MW, Varughese EA, Egorov AI, Villegas EN. *Cryptosporidium* propidium monoazide-PCR, a molecular biology-based technique for genotyping of viable *Cryptosporidium* oocysts. Appl Environ Microbiol. 2009;75:6856-63.

38. Fayer R, Leek RG. The effects of reducing conditions, medium, pH, temperature, and time on *in vitro* excystation of *Cryptosporidium*. J Protozool. 1984;31:567-9.
39. Kniel KE, Sumner SS, Pierson MD, Zajac AM, Hackney CR, Fayer R, *et al*. Effect of hydrogen peroxide and other protease inhibitors on *Cryptosporidium parvum* excystation and *in vitro* development. J Parasitol. 2004;90:885-8.
40. Hommer V, Eichholz J, Petry F. Effect of antiretroviral protease inhibitors alone, and in combination with paromomycin, on the excystation, invasion and *in vitro* development of *Cryptosporidium parvum*. J Antimicrob Chemother. 2003;52:359-64.
41. Anthony JP, Fyfe L, Stewart D, McDougall GJ, Smith HV. The effect of blueberry extracts on *Giardia duodenalis* viability and spontaneous excystation of *Cryptosporidium parvum* oocysts, *in vitro*. Methods. 2007;42 339-48.
42. Robertson LJ, Campbell AT, Smith HV. *In vitro* excystation of *Cryptosporidium parvum*. Parasitology. 1993;106(Pt 1):13-9.
43. Campbell AT, Robertson LJ, Smith HV. Viability of *Cryptosporidium parvum* oocysts: correlation of *in vitro* excystation with inclusion or exclusion of fluorogenic vital dyes. Appl Environ Microbiol. 1992;58: 3488-93.
44. Bukhari Z, McCuin RM, Fricker CR, Clancy JL. Immunomagnetic separation of *Cryptosporidium parvum* from source water samples of various turbidities. Appl Environ Microbiol. 1998;64:4495-9.
45. Black EK, Finch GR, Taghi-Kilani R, Belosevic M. Comparison of assays for *Cryptosporidium parvum* oocysts viability after chemical disinfection. FEMS Microbiol Lett. 1996;135:187-9.
46. Neumann NF, Gyurek LL, Finch GR, Belosevic M. Intact *Cryptosporidium parvum* oocysts isolated after *in vitro* excystation are infectious to neonatal mice. FEMS Microbiol Lett. 2000;183:331-6.
47. Hou L, Li X, Dunbar L, Moeller R, Palermo B, Atwill ER. Neonatal-mouse infectivity of intact *Cryptosporidium parvum* oocysts isolated after optimized *in vitro* excystation. Appl Environ Microbiol. 2004;70:642-6.
48. Hallier-Soulier S, Guillot E. An immunomagnetic separation-reverse transcription polymerase chain reaction (IMS-RT-PCR) test for sensitive and rapid detection of viable water-borne *Cryptosporidium parvum*. Environ Microbiol. 2003;5:592-8.
49. Okhuysen PC, Chappell CL, Kettner C, Sterling CR. *Cryptosporidium parvum* metalloaminopeptidase inhibitors prevent *in vitro* excystation. Antimicrob Agents Chemother. 1996;40:2781-4.
50. Peng X, Murphy T, Holden NM. Evaluation of the effect of temperature on the die-off rate for *Cryptosporidium parvum* oocysts in water, soils, and feces. Appl Environ Microbiol. 2008;74:7101-7.
51. Robertson LJ, Campbell AT, Smith HV. Survival of *Cryptosporidium parvum* oocysts under various environmental pressures. Appl Environ Microbiol. 1992;58:3494-500.
52. Vergara-Castiblanco CA, Freire-Santos F, Oteiza-Lopez AM, Ares-Mazas ME. Viability and infectivity of two *Cryptosporidium parvum* bovine isolates from different geographical location. Vet Parasitol. 2000;89:261-7.
53. Belosevic M, Guy RA, Taghi-Kilani R, Neumann NF, Gyurek LL, Liyanage LR, *et al*. Nucleic acid stains as indicators of *Cryptosporidium parvum* oocyst viability. Int J Parasitol. 1997;27: 787-98.
54. Jenkins MB, Anguish LJ, Bowman DD, Walker MJ, Ghiorse WC. Assessment of a dye permeability assay for determination of inactivation rates of *Cryptosporidium parvum* oocysts. Appl Environ Microbiol. 1997;63:3844-50.
55. Gomez-Couso H, Fontan-Sainz M, McGuigan KG, Ares-Mazas E. Effect of the radiation intensity, water turbidity and exposure time on the survival of *Cryptosporidium* during simulated solar disinfection of drinking water. Acta Trop. 2009;112:43-8.
56. Gomez-Couso H, Fontan-Sainz M, Sichel C,

Fernandez-Ibanez P, Ares-Mazas E. Efficacy of the solar water disinfection method in turbid waters experimentally contaminated with *Cryptosporidium parvum* oocysts under real field conditions. Trop Med Int Health. 2009;14:620-7.

57. Wagner-Wiening C, Kimmig P. Detection of viable *Cryptosporidium parvum* oocysts by PCR. Appl Environ Microbiol. 1995;61:4514-6.
58. Lee SU, Joung M, Ahn MH, Huh S, Song H, Park WY, *et al*. CP2 gene as a useful viability marker for *Cryptosporidium parvum*. Parasitol Res. 2008;102:381-7.
59. Jenkins MC, Trout J, Abrahamsen MS, Lancto CA, Higgins J, Fayer R. Estimating viability of *Cryptosporidium parvum* oocysts using reverse transcriptase-polymerase chain reaction (RT-PCR) directed at mRNA encoding amyloglucosidase. J Microbiol Methods. 2000;43:97-106.
60. Garces-Sanchez G, Wilderer PA, Horn H, Munch JC, Lebuhn M. Assessment of the viability of *Cryptosporidium parvum* oocysts with the induction ratio of hsp70 mRNA production in manure. J Microbiol Methods. 2013;94:280-9.
61. Widmer G, Orbacz EA, Tzipori S. beta-tubulin mRNA as a marker of *Cryptosporidium parvum* oocyst viability. Appl Environ Microbiol. 1999;65:1584-8.
62. Vesey G, Ashbolt N, Fricker EJ, Deere D, Williams KL, Veal DA, et al. The use of a ribosomal RNA targeted oligonucleotide probe for fluorescent labelling of viable *Cryptosporidium parvum* oocysts. J Appl Microbiol. 1998;85:429-40.
63. Stinear T, Matusan A, Hines K, Sandery M. Detection of a single viable *Cryptosporidium parvum* oocyst in environmental water concentrates by reverse transcription-PCR. Appl Environ Microbiol. 1996;62:3385-90.
64. Kaucner C, Stinear T. Sensitive and rapid detection of viable *Giardia* cysts and *Cryptosporidium parvum* oocysts in large-volume water samples with wound fiberglass cartridge filters and reverse transcription-PCR. Appl Environ Microbiol. 1998;64:1743-9.
65. Nam S, Lee G. A new duplex reverse transcription PCR for simultaneous detection of viable *Cryptosporidium parvum* oocysts and *Giardia duodenalis* cysts. Biomed Environ Sci. 2010;23:146-50.
66. Bajszar G, Dekonenko A. Stress-induced Hsp70 gene expression and inactivation of *Cryptosporidium parvum* oocysts by chlorine-based oxidants. Appl Environ Microbiol. 2010;76:1732-9.
67. Alum A, Rubino JR, Khalid Ijaz M. Comparison of molecular markers for determining the viability and infectivity of *Cryptosporidium* oocysts and validation of molecular methods against animal infectivity assay. Int J Infect Dis. 2011;15:e197-200.
68. Kothavade RJ. Potential molecular tools for assessing the public health risk associated with water-borne *Cryptosporidium* oocysts. J Med Microbiol. 2012;61:1039-51.
69. Graczyk TK, Grimes BH, Knight R, Da Silva AJ, Pieniazek NJ, Veal DA. Detection of *Cryptosporidium parvum* and *Giardia lamblia* carried by synanthropic flies by combined fluorescent *in situ* hybridization and a monoclonal antibody. Am J Trop Med Hyg. 2003;68:228-32.
70. Alagappan A, Tujula NA, Power M, Ferguson CM, Bergquist PL, Ferrari BC. Development of fluorescent *in situ* hybridization for *Cryptosporidium* detection reveals zoonotic and anthroponotic transmission of sporadic cryptosporidiosis in Sydney. J Microbiol Methods. 2008;75:535-9.
71. Sunderland D, Graczyk TK, Tamang L, Breysse PN. Impact of bathers on levels of *Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts in recreational beach waters. Water Res. 2007;41:3483-9.
72. Bednarska M, Bajer A, Sinski E, Girouard AS, Tamang L, Graczyk TK. Fluorescent *in situ* hybridization as a tool to retrospectively identify *Cryptosporidium parvum* and *Giardia lamblia* in samples from terrestrial mammalian wildlife. Parasitol Res. 2007;100:455-60.

73. Alagappan A, Bergquist PL, Ferrari BC. Development of a two-color fluorescence *in situ* hybridization technique for species-level identification of human-infectious *Cryptosporidium* spp. Appl Environ Microbiol. 2009;75:5996-8.
74. Smith JJ, Gunasekera TS, Barardi CR, Veal D, Vesey G. Determination of *Cryptosporidium parvum* oocyst viability by fluorescence *in situ* hybridization using a ribosomal RNA-directed probe. J Appl Microbiol. 2004;96:409-17.
75. Boxell A, Hijjawi N, Monis P, Ryan U. Comparison of various staining methods for the detection of *Cryptosporidium* in cell-free culture. Exp Parasitol. 2008;120:67-72.
76. Morgan UM, Pallant L, Dwyer BW, Forbes DA, Rich G, Thompson RC. Comparison of PCR and microscopy for detection of *Cryptosporidium parvum* in human fecal specimens: clinical trial. J Clin Microbiol. 1998;36:995-8.
77. Korich DG, Mead JR, Madore MS, Sinclair NA, Sterling CR. Effects of ozone, chlorine dioxide, chlorine, and monochloramine on *Cryptosporidium parvum* oocyst viability. Appl Environ Microbiol. 1990;56:1423-8.
78. Belosevic M, Craik SA, Stafford JL, Neumann NF, Kruithof J, Smith DW. Studies on the resistance/reactivation of *Giardia muris* cysts and *Cryptosporidium parvum* oocysts exposed to medium-pressure ultraviolet radiation. FEMS Microbiol Lett. 2001;204:197-203.