

## PROGRESS IN THE MOLECULAR METHODS FOR THE DETECTION AND GENETIC CHARACTERIZATION OF *CRYPTOSPORIDIUM* IN WATER SAMPLES

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**Abstract:** *Cryptosporidium*, the protozoan parasite, has several transmission routes, including anthroponotic and zoonotic transmission, as well as the foodborne way, but mainly by water. The oocysts, the resistant stage produced by *Cryptosporidium*, are remarkably stable, and can survive for weeks or even months in the environment. Furthermore, the infective dose is low, probably even a single oocyst can cause infection. The *Cryptosporidium* genus includes at least 16 species; nevertheless, only a few can cause cryptosporidiosis, an intestinal disease in human and domestic mammals. Thus, the genetic characteristics of different *Cryptosporidium* species became fundamental in the diagnosis, monitoring, prevention and control of infections caused by this pathogen. Unfortunately, the traditional phenotypic techniques meet with difficulties in the specific diagnosis of cryptosporidiosis, therefore the new molecular tools must be applied. The RT-qPCR method can be used to differentiate viable and dead *Cryptosporidium* oocysts, and the LAMP assays have advantages for detection of organisms at relatively low concentration in environmental samples; however, the NASBA assay specifically detects as few as one oocyst of a viable human pathogenic *Cryptosporidium* species. Reverse line blot hybridization (RLB) has been successfully used for specific identification and for differentiation of *Cryptosporidium* species. Described techniques are the most promising methods for the sensitive and accurate detection, but require a considerable selection of appropriate tools, genetic markers and analytical techniques for interpretations of database. However, the applicability of most of these methods to detect *Cryptosporidium* species or genotypes from environmental samples needs to be evaluated and standardized.

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### INTRODUCTION

The protozoan *Cryptosporidium* parasite has a complex series of transmission routes, including anthroponotic and zoonotic transmission, as well as foodborne, but mainly by water. A few species from this genus can cause cryptosporidiosis, an intestinal disease in human and domestic mammals. A biotope of *Cryptosporidium* is the mucous of the digestive system (mainly the mucous of small intestine, stomach, appendix, colon, rectum, cholecyst, pancreatic duct). There have also been some described cases of localization of the parasite in respiratory tract mucous. In immunocompetent people, it can occur in a latent form or

cause short-lived, self-restrained diarrhea [49], whereas in people with immunosuppression the diarrhea is insistent, often causing death as a result of dehydration, considerable weight decrease, and bad absorption syndrome [3, 9]. Taking into account the socioeconomical factor (especially in the population with HIV/AIDS), the World Health Organisation classifies *Cryptosporidium* as a reference pathogen in a global estimation of water quality [37].

*Cryptosporidium* genus belongs to *Apicomplexa* type, which includes at least 16 species: *C. andersoni*, *C. baileyi*, *C. bovis*, *C. canis*, *C. felis*, *C. galli*, *C. hominis*, *C. meleagridis*, *C. molnari*, *C. muris*, *C. parvum*, *C. saurophilum*, *C. scophthalmi*, *C. serpentis*, *C. suis* and *C. wrairi* [55, 65].

*C. hominis* (previously known as a *C. parvum* human genotype or genotype I) exceptionally can infect humans, while *C. parvum* (previously known as a *C. parvum* cattle genotype or genotype II) infects humans, ruminants and other animals [41, 46]. Moreover, species have been detected in humans such as *C. meleagridis*, *C. suis*, *C. felis* and *C. canis*, which indicates the risk of zoonotic transmission of these pathogens [10, 33, 64, 65]. Because oocysts of all *Cryptosporidium* spp. are morphologically similar and have the potential to be present in water, sensitive and specific detection and typing of *Cryptosporidium* oocysts in water in order to determine both the species and the genotype are essential for source water management and risk assessment.

Cases of cryptosporidiosis occur all over the world as a result of contamination of different sources and also concern developed countries in Europe [4, 7, 14, 21, 22], as well as the USA and Canada. Most of these cases occurred in 1993 in Milwaukee, Wisconsin, where over 400,000 people were infected and over 100 died after drinking water from the municipal water system [62]. In August 2005, in Seneca Lake State Park in Geneva, New York, about 4,000 people who drank chlorinated water were infected with this protozoan. Estimations show that in the USA there are about 300,000 cases of cryptosporidiosis annually, and on average 66 people die [36]. In Poland thus far, no outbreaks of waterborne or foodborne cryptosporidiosis have been reported, but this may be due to lack of monitoring at a national level. In Poland tap, raw and reclaimed waters are not routinely monitored for the presence of parasite oocysts of *Cryptosporidium*. A limited number of studies monitoring the distribution of this protozoan in water and food using standardized methods have been carried out in several regions of the country. *Cryptosporidium* oocysts were detected in the great majority of water samples taken from surface waters in the Poznań area [44, 54] and in the Tri-city area [57]. The occurrence of *Cryptosporidium* infections in humans in Poland were found in high numbers of patients among the high-risk groups (with primary and secondary immunodeficiencies [4, 31, 35]). On the basis of literature, data analysis concerning human infections, domestic and wild animals and environmental samples, Bajer [4] concludes that *Cryptosporidium* is currently widely distributed in Poland, and the overall risk of outbreaks of cryptosporidiosis in our country is assessed as relatively high.

Oocysts from these organisms are extremely robust and capable of surviving in the environment for extended periods of time. The commonly used method of water conditioning by chlorination is not effective in prevention of oocysts transmission of this protozoan [14, 22]. Despite the number of oocysts detected in water, contamination is often very low, the available studies show that 10 oocysts can cause the illness in healthy people [12], and the US Food and Drug Administration (FDA) suggests that as few as one oocyst could cause infection. Thus, the effective and specific detection, and species characteristics of *Cryptosporidium* requires a very sensitive diagnostic method.

The precise identification of a parasite at species and/or genotype level has great consequences for various aspects of human and veterinary parasitology, including taxonomy, diagnosis, treatment and control. The advent of molecular techniques, in particular those based on the *in vitro* amplification of nucleic acids, has improved the ability to detect infections caused by parasites. However, the majority of diagnostic methods used in clinical practice has limited application regarding to the detection of protozoans in water samples. The most important restriction is lower concentration of cysts or oocysts in water in comparison with material collected from patients. For example, the levels of oocyst of *Cryptosporidium* and *Giardia* cysts in surface waters are very low, ranging from 0.5–5,000 organisms in 100 liters of water [45]. Detection of such low numbers of organisms is difficult and requires very sensitive techniques. In order to improve the monitoring of oocysts of *Cryptosporidium* in water, the US Environmental Protection Agency (USEPA) introduced the 1622 [59] method and then 1623 [60], which were designed for the concentration and detection of *Cryptosporidium* oocysts and *Giardia* cysts in water samples.

Additionally, in the environmental sample, a much wider range of protozoans than may potentially be found in the studied environment, should be taken into consideration, as well as the presence of unknown interfering factors that can hamper the course of many molecular reactions. Detection of the presence of cysts and oocysts with the molecular methods, in such material as environmental samples, is often hampered by the occurrence of organic and inorganic substances that can potentially be inhibitors in test based on the nucleic acids [17, 51].

### THE POLYMERASE CHAIN REACTION (PCR)

To date, no species identification of isolates has been carried out on a routine study of cryptosporidiosis; only identification to confirm which of the *Cryptosporidium* species has significance in the epidemiology of this illness in humans. Application of molecular methods makes also possible the estimation of the zoonotic potential of *Cryptosporidium* spp. and sources of infection for human, which constitutes a basis for the characteristics of transmission dynamics on the endemic and epidemic areas. Different varieties of PCR amplifying DNA from the purified oocysts are the most widely used molecular technique for the detection and genotyping of *Cryptosporidium*. This technique was developed in the mid-80s of the 20<sup>th</sup> century and within a dozen years it became a basic research tool in many laboratories. It is based on the specific amplification of nucleic acids, and thanks to using the specific starters, thermostable polymerase, deoxyribonucleotides, it enables to copy the selected DNA fragments in million of copies during a few hours. The most often used molecular markers for the detection of *Cryptosporidium* in clinical

material, as well as in environmental samples, are genes encoding 18 small ribosomal RNA subunit (18S) [42, 48, 53, 63]; the *Cryptosporidium* 60-kDa glycoprotein (*gp60*) [1]; the *Cryptosporidium* oocyst wall protein (COWP) [2, 52], the *Cryptosporidium* heat shock protein (HSP)-70 [40] and beta-tubulin [58] and microsatellite locus 1 (ML1) and 2 (ML2) [11, 23, 32].

Identification down to the species, assemblage, genotype or subgenotypes level was accomplished by targeting highly variable genes or genes that were unique. "Variable" genes, such as the *gp60* and microsatellite locus 1 (ML1) and 2 (ML2), are used for the identification and classification of *Cryptosporidium* species and population variants (i.e. genotypes or subgenotypes). The gene of the small ribosomal RNA subunit (SSU) provides a useful genetic marker for the specific identification of *Cryptosporidium*. It has a relatively low intraspecific and relatively high interspecific sequence variation, and has assisted significantly in elucidating transmission patterns and cryptosporidiosis outbreaks in animals and humans [24, 65].

Although these PCR methods continue to offer a powerful tool for epidemiology and transmission studies of the parasite, their sensitivity and specificity are limited by several factors, including the quantity and quality of DNA preparations [5]. For environmental samples, the efficiency of DNA extraction methods is determined by the DNA recovery rate and PCR inhibitor reduction during DNA extraction. Many studies have shown that PCR inhibitors present in water samples suppress or reduce PCR amplification [25, 26, 34]. Environmental samples (water, soil, and food) are rich in PCR inhibitors, such as humic acids, potassium dichromate, and formaldehyde which could be coextracted with DNA during the DNA isolation and purification process and which could interfere with PCR amplification. Numerous direct DNA commercial extraction methods have been tested for the reduction or removal of PCR inhibitors in the preparation of DNA from *Cryptosporidium* spp. oocysts [51].

The main limitation of PCR is that it does not provide data on the viability and infectivity of e.g. the *Cryptosporidium* oocysts. To obtain additional information on these very important aspects, indirect methods, such as the Reverse-Transcriptase PCR (RT-PCR), must be used. Since RT-PCR is based on mRNA, which usually has a very short half-life, its use provides a more closely correlated indication of viability status compared to DNA-based methods.

### **POLYMERASE CHAIN REACTION WITH THE ANALYSIS IN REAL TIME (REAL TIME PCR, qPCR, r-t PCR)**

R-t PCR method can be used for monitoring of amplicons in the PCR reaction and for estimation of the first concentration of DNA template in a sample. This method has been successfully used for the estimation of the genomes number and for the diagnostic search of the DNA of para-

sitic protozoans such as *Plasmodium* or *Toxoplasma gondii* [8, 47]. The classic polymerase chain reaction is a quality method, which constitutes a big disadvantage in the monitoring of infections. In this technique, the intensity of the obtained product depends mainly on the number of cycles, and to a lesser degree, on the initial number of template copies. Theoretically, the quantity of the product after the end of reaction should be exponentially proportional to the number of templates, whose sequences are analyzed in the investigated sample (doubled in every cycle). But there are big differences in practice; therefore, a technique enabling the precise quantity analysis has been developed. This allows the monitoring of the quantity of DNA product in every cycle of the PCR reaction. This technique has been called the DNA polymerase chain reaction with the analysis in real time. It is commonly called "real-time PCR" (r-t PCR, q PCR).

The other important advantage of r-t PCR is that it does not require electrophoretic division of PCR products, which is time-saving, as well as saving the chemical reagents intended for classic PCR with electrophoresis. In general, qPCR and amplified product detection are completed in an hour or less, which is considerably faster than conventional PCR detection methods. On the other hand, amplification and detection steps are performed in the same closed vessel, and the risk of releasing amplified nucleic acids into the environment is negligible. Moreover, the r-t PCR technique provides the possibility to carry out multiplex PCR, which is economically beneficial. It also allows for a quantity measurement of RNA molecules after copying the RNA sequence on cDNA with the use of reverse transcriptase.

In the real-time PCR technique, the basis of the measurement of increase speed of the number of analyzed DNA molecule is the measurement of fluorescence, and therefore requires the use of a special thermocycler, for example, coupled with a spectrofluorimeter, which enables the measurement of fluorescence during the amplification. Many methods for enabling a precise estimation of the number of selected DNA fragment copies creating during this PCR reaction have been developed. One of these methods depends on the measurement of fluorescence coming from the fluorochrome molecule bound with the twostrands DNA molecule. An other method requires the use of special probes complementary to fragments of amplified DNA sequences. Such probes are equipped with a molecule of fluorescent reporter, which emits the light in definite conditions. A variation of this method, called the Taqman method, depends on the hydrolysis of probes, and fluorescence occurs only during the elongation stage in every cycle of the PCR reaction. A single probe is used in this method, equipped with a fluorochrome, and the molecule suppressing the fluorescence is hydrolyzed by *Taq* polymerase during the elongation stage. Degradation of the probe makes the fluorochrome separate from the suppressor and enables the emission of fluorescent light.

**qPCR protocol has been developed for the detection and identification of *Cryptosporidium* oocysts species/genotypes for different materials and matrices.** Using 18S rRNA as a target, the detection sensitivities of real-time PCR and nested-PCR systems were compared by Sunnotel *et al.* [56]. A sensitivity analysis of this assay determined that it was routinely capable of detecting three oocysts. A multiplex real-time PCR (qPCR) assay for simultaneous detection of *G. lamblia* and *C. parvum* was adapted by Guy *et al.* [20], and for the simultaneous detection of *E. histolytica*, *G. lamblia*, and *C. parvum* in faecal samples by Verweij *et al.* [61]. However, qPCR has revolutionized clinical microbiology laboratories diagnose of many human microbial infections [13, 16].

**A variation of this method is a reverse transcription quantitative real-time PCR.** Garces-Sanchez *et al.* [18] used this method to differentiate viable and injured *C. parvum* oocysts. Methods that estimate viable oocysts include *in vitro* excystation, vital staining (dye exclusion, FISH), infectivity (*in vitro* or *in vivo*) and reverse transcriptase (RT)-PCR. Each of these methods has limitations including *in vivo* infectivity which requires animal testing, is time-consuming, and the most costly. Furthermore, excystation and vital staining overestimate oocysts viability and do not respond well with animal infectivity. This inconvenience is solved by the RT-qPCR, especially in environmental analysis when rapid responses are needed and when there is a risk of contamination of water, food or food sources with *C. parvum* oocysts, e.g. due to manure spreading on agricultural land, or overflows from wastewater treatment facilities. Garces-Sanchez *et al.* [18] tested two mRNA extraction methods targeting heat induced *hsp70* mRNA in combination with RT-qPCR. In the opinion of the authors, this can be used for the screening of *C. parvum* oocysts in environmental samples to provide fast data on the presence and viability of these organisms, and is very useful for hygiene controls during waste water treatment processes.

#### REVERSE LINE BLOT HYBRIDIZATION (RLB)

RLB was initially developed as a reverse dot blot assay for the diagnosis of sickle cell anaemia, but the basis of both techniques is the hybridization of PCR products to specific probes immobilized on a membrane in order to identify differences in the amplified sequences [19]. This tool was initially developed for the identification of *Streptococcus* serotypes [30], followed by an RLB for *Mycobacterium tuberculosis* strain differentiation [27]. RLB is not a new method in the diagnostics of parasitic protozoans, because in the end of the 90s of the last century it was adapted for the detection and marking of *Babesia* and *Theileria* species [19], but its application for *Cryptosporidium* diagnostics is still in the development stage.

Negatively loaded nylon membranes are used in the RLB technique. After activation, they gain the carboxylic

groups binding covalently with positive amin groups joined with oligonucleotide probes. The PCR products created as a result of using a starter marked with biotin are placed on the membrane, which was earlier bound with probes, and they are hybridized [19]. Next, the streptavidin-peroxidase complex joins with the hybridization products bound with biotin. The visualization of chemiluminescence begins after adding the specific substrate for peroxidase (luminol), what enables the reaction of its oxidation, the side product of which is light registered on photographic film [30], or analyzed in the compact flow cytometer [6].

Hybridization with the RLB technique enables simultaneous detection of many species and strains of investigated organisms on one membrane, which has the character of a macromatrix. The membrane with the covalently linked species-specific oligonucleotides can be used at least 20 times, thus reducing costs for screening pathogens [19]. Binding the PCR product with the probe occurs only when there is 100% complementarity between the probe and the amplicon. Therefore, accurately designed probes enable the interspecies differentiation, and additionally provide the possibility to detect microheterogenetic differences within the species, the so-called inner-species variability of investigated organisms. Thanks to these features, the RLB technique enables identification of organisms from the genus, through species, up to specific strain.

The advantage of RLB is the possibility to use it for samples, where there is a probability of the presence of a bigger number of investigated organisms; for example, in the case of mixed infections with related species of parasites, or in environmental samples.

Because *C. hominis* and *C. parvum* are morphologically identical, they have distinct epidemiologies and different transmission cycles, identification to the species level is important for determination of the source of the infection. Bandyopadhyay *et al.* [6] developed RLB, methods that do not require DNA sequencing analysis of PCR amplicons as an additional step after PCR, which have been successfully used for specific identification and for differentiation of *Cryptosporidium* species. The assay was based on species-specific probes linked to carboxylated Luminex microspheres that hybridize to a *Cryptosporidium* microsatellite-2 region (ML-2), where *C. hominis* and *C. parvum* differ by one nucleotide substitution. The two capture probes were covalently bound to spectrally distinct populations of fluorescent microspheres that reacted with streptavidin-phycoerythrin and analyzed in a unique, compact flow cytometer, the Luminex 100. PCR was performed using the primer pair M15/M16 (CAATGTAAGTTTACTTATGATTAT and C GACTATAAAGATGAGAGAAG) which amplifies a specific fragment from the ML-2 locus, of the *Cryptosporidium* genome. The reverse primer M16 was labeled with biotin at the 5' extremity to allow detection of hybridized amplicons with streptavidin-phycoerythrin. The assay was validated by testing a total of 143 DNA samples extracted from clinical specimens, environmental

samples, or samples artificially spiked with *Cryptosporidium* oocysts. In the opinion of the authors, this assay is fast (the entire procedure can be performed within 5 h) and is less expensive than sequencing of PCR amplicons. The total cost of one RLB test was less than \$0.16, compared with the traditional DNA sequencing method, which costs at least \$4.00 per reaction. Although this technique was 100% specific and more sensitive than the direct fluorescent antibody test, it cannot differentiate *C. parvum* from *C. hominis*. In conclusion, this rapid, inexpensive, and relatively simple method may prove to be a very useful diagnostic tool for the rapid identification of *C. hominis* and *C. parvum*.

### METHODS OF AMPLIFICATION IN ISOTHERMAL CONDITIONS

**Loop-mediated isothermal amplification of DNA (LAMP).** LAMP has recently been as a better method for amplification of DNA which exceeds the classic PCR in its effectiveness and in its relative insensitivity to contamination of DNA.

Notomi *et al.* [43] developed a method called the loop-mediated amplification of DNA, which amplifies DNA with high specificity, efficiency and speed in isothermal conditions. According to Notomi *et al.* [43], one of the advantages of the LAMP method is that the DNA amplification in isothermal conditions proceeds without any influence of the DNA coexisting in a sample. This method requires only the DNA polymerase and a set of four specially designed starters which recognize six different sequences in the DNA template. The inner starter, including sequences of the sense and antisense strand of template DNA, begins the LAMP reaction. Next, there is a synthesis of separated DNA strands carried out by the outer starters, which releases the one-strand DNA. This becomes the template for synthesis of DNA carried out by the other inner and outer starters, which hybridizes with the other end of target DNA, which in consequence gives a stem-loop structure ("stem with loop"). In the next LAMP cycle, one inner cycle hybridizes with the loop on the product (the structure of "stem with loop") and initiates the synthesis of separated strands giving an original structure of stem-loop, plus a new one, twice as long. The cycle of reactions continues, and in less than an hour, 109 copies of the template DNA are accumulated. The final products are the stem-loop structures with several inverted repeats of target sequences, as well as the cauliflower-like structures with multiple loops made by elongation between, in turn, inverted repeats of target sequences on the same strand.

The product of the LAMP is a mixture of different length DNA fragments, having cauliflower-like structure with numerous loops induced by annealing between inverted repeats of template sequences on the same strand; these are later simply and easily detected, the same as in the mechanism of cooperation between multivalent antigen and anti-

body. Another advantage of the LAMP method is its high specificity towards the template sequences. This is caused by recognizing the template sequences by 6 independent sequences in the initial stage, and by 4 independent sequences during later stage of LAMP reaction – partly reducing the main problem that accompanies all methods of amplification [43]. Moreover, the simplicity of this method comes from the facility of preparing starters, and from the fact that there are only 4 starters, the DNA polymerase, and laboratory water bath or heating block (amplification occurs in the isothermal conditions). One more advantage is the possibility of using this method for highly efficient amplification of RNA, if it is joined with reverse transcription.

A survey of the literature shows that the LAMP method has already been developed and applied for the detection of over 100 different pathogens [28]. This method seems to be promising for species identification and species differentiation. However, development of its application depends on the attainability of species specific DNA sequences (e.g. in the GeneBank). Currently, the DNA sequences that may be the basis for marking the *Cryptosporidium* species with the LAMP method are available only in a limited range [5]. Karanis *et al.* [29] described the first application of the LAMP for detecting *Cryptosporidium* on the basis of amplification of the *gp60* gene of the *C. parvum*.

Bakheit *et al.* [5] described application of LAMP to identify the *Cryptosporidium* species obtained from different breeding animals on the basis of HSP-70 gene, based on targeting the S-adenosyl-methionine synthetase (SAM) gene and *gp60* gene, and validation of the LAMP test based on the sequenced amplicons of these genes. Verification was facilitated by an innovative modification of the LAMP primers to incorporate restriction sites to permit target product excision. Results of the detection were compared with those obtained from nested-PCR with starters complementary to 18S rRNA gene of *Cryptosporidium*. All the samples investigated with the nested-PCR method were negative, while in the LAMP test one third of these samples were positive. The authors emphasize that the LAMP test is simple, fast and not expensive, thus very useful in diagnostics. Moreover, it is advantageous in the detection of organisms in environmental samples when their concentration is low. The use of 3 starters set makes this procedure highly specific for an imprecisely isolated DNA template from a sample containing foreign biological material. This test turned out to be insensitive to commonly occurred interference, restrictive for the application of PCR.

### Nucleic acid sequence-based amplification (NASBA).

In this method, the initial template is RNA and there are 3 enzymes and 2 starters engaged. One starter and reverse transcriptase amplify a single cDNA strand, the second enzyme, RNA-ase H degrades RNA from the created RNA-DNA hybrid, and finally, the second starter (with added T7 promoter sequence) attaches to the cDNA and the transcrip-

tion begins with the third enzyme and RNA polymerase. Each transcript is a template for creating another cDNA with T7 promoter, which is again a template for RNA transcripts. According to Mens *et al.* [38], this method, based on rRNA in the detection of blood parasites, predominates over r-t PCR (reverse-transcription PCR), because it is independent of the presence of DNA, which could occur in an inaccurately cleaned sample. The NASBA method is very sensitive because it detects rRNA, the number of copies of which occurring on the parasites genome is significantly higher than the rDNA, on which r-t PCR is based. Because NASBA is a method based on the isothermal reaction occurring at a temperature of 41°C, and does not require denaturation, it prevents amplification of the genome DNA in the case of contamination. However, many authors think that this low temperature for the amplification of a selected DNA fragment is a factor that worsens the specificity of the NASBA [43]. Because of this low specificity in the selection of target sequence, it requires a precise amplification tool and well-constructed method for detecting amplified products. Despite the simplicity, the requirement of high precision of the thermocycler for PCR limit the application of this method in the wide range, for example, as a routine diagnostic tool in private laboratories [43]. Another restriction is probably that individual preparation of the chemical reagents mixture is difficult and commercial kits are expensive [39]. Application of NASBA for monitoring in real-time with molecular probes (quantity amplification in real-time based on sequences of nucleic acid, q-t NASBA) has enhanced interest in this method, and when used in the detection of *Plasmodium falciparum*, it proved to be very sensitive, with a limitation of detection up to 20 protozoans/ml of blood [50].

Connelly *et al.* [15] used the NASBA method for specific detection of viable human pathogenic *Cryptosporidium* species, *C. parvum*, *C. hominis*, and *C. meleagridis*. Oocysts were isolated from water samples. The amplified target sequence employed was *hsp70* mRNA, production of which was stimulated via a brief heat shock. The NASBA product was detected in a nucleic acid hybridization lateral flow sandwich assay. It proved the extreme sensitivity of this assay using flow-cytometer counted samples of *C. parvum*, and the assay can detect all human-pathogenic *Cryptosporidium* species. In the opinion of the authors, even one oocyst can be detected from among large numbers of common waterborne microorganisms and packed pellet material filtered from environmental water samples with this method.

## CONCLUSIONS

The risk of waterborne transmission of *Cryptosporidium* is a serious global problem in drinking water safety. Oocysts from these organisms are extremely long-lived in

the environment. Among the numerous species belonging to *Cryptosporidium* oocysts, in principle, only 3 are pathogenic for humans. Because oocysts of all *Cryptosporidium* spp. are morphologically very similar and potentially present in water, the sensitive and specific detection and typing of *Cryptosporidium* oocysts in water to determine the species and genotype are crucial for source water management and risk assessment. This review shows that some progress has been made in the development of molecular methods for the identification, genetic characterization and differentiation of *Cryptosporidium* species. The most extensively used molecular techniques for the detection and genotyping of *Cryptosporidium* are different varieties of PCR amplifying DNA from the purified oocysts. However, the RT-qPCR method provides information about viability and can be used to differentiate viable and injured *Cryptosporidium* oocysts, which may be very useful for e.g. hygiene controls during waste water treatment processes. The loop-mediated isothermal amplification of DNA (LAMP) assays have advantages for the detection of organisms at relatively low concentration in environmental samples. It is a highly specific procedure for an imprecisely isolated DNA template from a sample containing foreign biological material. This test is insensitive to commonly occurred interference limiting the PCR application.

The NASBA method is able to discriminate between the 3 human pathogenic species and several nonpathogenic *Cryptosporidium* species, and can distinguish between viable and non-viable oocysts. NASBA amplification and a nucleic acid hybridization lateral flow sandwich assay, specifically detects as few as one oocyst of a viable human pathogenic *Cryptosporidium* species. NASBA has potential for adoption as a diagnostic tool for environmental pathogen. Reverse line blot hybridization (RLB) has been successfully used for specific identification and for differentiation of *Cryptosporidium* species. However, applicability of most of these methods to detect *Cryptosporidium* species or genotypes from environmental samples needs to be evaluated and standardized.

The choice of molecular method by laboratories is determined mainly by the high cost of equipment needed for its realization. Using molecular assay to characterize genetic diversity does not mean that more traditional methods of discrimination are no longer valuable. Traditional phenetic techniques have had major imperfections for the specific diagnosis of cryptosporidiosis, but molecular characterization is not always possible in some field situations. However, it can be used to demonstrate whether phenotypic feature, particularly morphologic, have a genetic base.

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