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Real-time nucleic acid sequence-based amplification (NASBA) assay targeting *MIC1* for detection of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts

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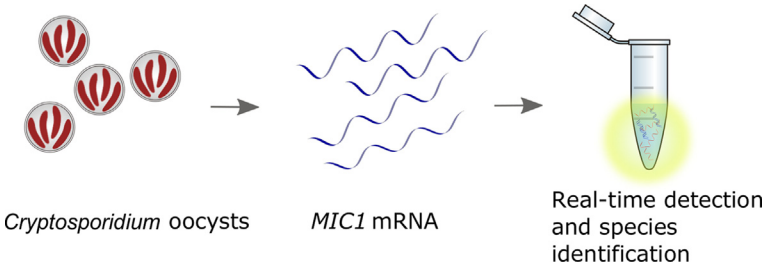
HIGHLIGHTS

- Primer sets targeting *MIC1* in *C. parvum* and *C. hominis* made for real-time NASBA.
- *C. parvum* and *C. hominis* oocysts detected down to 5 oocysts in 10 μl.
- *C. parvum* oocysts distinguished from *C. hominis* oocysts using this NASBA assay.
- *MIC1* transcripts detected in both living and inactivated oocysts.

GRAPHICAL ABSTRACT



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ABSTRACT

Both *Cryptosporidium parvum* and *Cryptosporidium hominis* are often associated with cryptosporidiosis in humans, but whereas humans are the main host for *C. hominis*, *C. parvum* is zoonotic and able to infect a variety of species. The oocyst transmission stages of both species of parasites are morphologically identical and molecular techniques, usually polymerase chain reaction (PCR), are required to distinguish between oocysts detected by standard methods in environmental samples, such as water. In this study, we developed two primer sets for real-time nucleic acid sequence-based amplification (NASBA), targeting the *MIC1* transcript in *C. parvum* (*CpMIC1*) and *C. hominis* (*ChMIC1*). Using these primer sets, we were not only able to detect low numbers of *C. parvum* and *C. hominis* oocysts (down to 5 oocysts in 10 μl, and down to 1 oocyst using diluted RNA samples), but also distinguish between them. One of the primer sets targeted an exon only occurring in *CpMIC1*, thereby providing a tool for distinguishing *C. parvum* from other *Cryptosporidium* species. Although mRNA has been suggested as a tool for assessing viability of *Cryptosporidium* oocysts, as it is short-lived and may have high transcription, this NASBA assay detected *MIC1* mRNA in inactivated oocysts. RNA within the oocysts seems to be protected from degradation, even when the oocysts have been killed by heating or freeze-thawing. Thus, our approach detects both viable and non-viable oocysts, and RNA does not seem to be a suitable marker for assessing oocyst viability.

1. Introduction

The apicomplexan parasite *Cryptosporidium* infects the

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epithelial cells, usually in the intestines, of several animal groups. More than 27 species of *Cryptosporidium* are known (Moore et al., 2016), but *Cryptosporidium parvum* and *Cryptosporidium hominis* are the two species most often involved in human disease (cryptosporidiosis). One major host of *C. parvum* is cattle, but this species is zoonotic and also infects humans. It is very common in calves, affecting up to 100% of them in some instances (Lendner and Daugschies, 2014). *C. hominis* is specialized towards infection of humans (Ryan et al., 2014).

Infection in immunocompetent people is normally self-limiting, but in immunocompromised people and infants, consequences may be severe. Cryptosporidiosis is one of the major reasons for severe diarrhoea in infants and toddlers in low-income countries, and is associated with mortality (Kotloff et al., 2013).

Outside the host, the parasite survives and spreads as oocysts. This lifecycle stage is well protected from the environment by a robust wall, and can survive for long periods, particularly under damp, cool conditions. Transmission occurs through the faecal-oral route, and susceptible hosts are usually infected following ingestion of viable oocyst(s). Community-wide outbreaks of waterborne cryptosporidiosis resulting from contamination of potable supplies still occur commonly throughout the world, including in highly developed countries; e.g. the outbreaks in Östersund and Skellefteå in Sweden in 2010 and 2011 and that resulted in over 45 000 infections (Rehn et al., 2015). In addition, smaller recreational swimming-associated outbreaks of cryptosporidiosis also occur frequently (Briggs et al., 2014). The robustness and size of the oocysts make them a challenge for water treatment; not only can they evade removal by sand filtration, but they can also withstand various chemical treatments (Slifko et al., 2000).

Detection of *Cryptosporidium* oocysts in water samples is usually based on filtration, immunomagnetic separation (IMS), and detection and enumeration by immunofluorescent antibody test (IFAT) following prescribed protocols (e.g., ISO method 15 553; US EPA 1623). These methods do not provide information on the species or infectivity of the parasites, and experienced analysts are needed for successful performance. Molecular methods, based on nucleic acids, have the potential to provide further information. PCR, either for DNA detection (Anceno et al., 2007), or with reverse transcriptase for gene expression (Mauzy et al., 2012; Stinear et al., 1996), microarrays (Zhang et al., 2012), and nucleic acid sequence-based amplification (NASBA) (Baeumner et al., 2001; Connelly et al., 2008) have also been used for analysis of water samples for contamination with *Cryptosporidium* oocysts, but have not been regularly implemented in routine analyses.

NASBA is an isothermal and sensitive tool for amplification of nucleic acids (Compton, 1991). One advantage of NASBA is that it is well suited for RNA amplification, because there is no need for any additional reverse transcriptase step or removal of contaminating DNA. Real-time NASBA can be achieved by using molecular beacons, "nucleic acid probes that recognize and report the presence of specific nucleic acids in homogenous solution" (Leone et al., 1998; Tyagi and Kramer, 1996). The use of NASBA in environmental analyses has recently been discussed (Hønsvall and Robertson, 2017).

Heat shock protein 70 (hsp70) gene transcript has been the target of choice in previous NASBA assays for *Cryptosporidium* (Baeumner et al., 2001; Connelly et al., 2008; Reinholt et al., 2014). NASBA has previously been used to detect *Cryptosporidium* species, but, as far as we know, has not been used to distinguish between *C. hominis* and *C. parvum*. Connelly et al. (2008) developed a NASBA primer pair targeting *hsp70* transcript to detect human pathogenic *Cryptosporidium* species (*C. parvum*, *C. hominis* and *C. meleagridis*), with the oocysts isolated by IMS and then detected by NASBA and lateral flow assay. They reported detection down to one oocyst in 10 μl. Reinholt et al. (2014) used NASBA in a micro total analysis system (μ-TAS). With primers targeting *hsp70* mRNA, they were able to detect down to 30 *C. parvum* oocysts in 150 μl.

Micronemal proteins/adhesins (MICs) are localised in apicomplexan micronemes, organelles that are characteristic of apicomplexan parasites. These proteins are believed to be involved in host cell attachment and invasion. CpMIC1 (*C. parvum* MIC1) is secreted prior to host cell attachment (Putignani et al., 2008). CpMIC1 was previously named thrombospondin-related protein 8 (CpTSP8) (Putignani et al., 2008). It is one of 12 TSP1 domain (thrombospondin-related) proteins in *C. parvum*. An intron was found in CpTSP8 (Deng et al., 2002), which is not common – one other gene in *Cryptosporidium* was known to have intron prior to this finding. Expression of CpTSP8 peaked 48 h after infecting a cell monolayer of HCT-8 cells, normalised against 18S (Deng et al., 2002).

C. parvum is able to infect more species than *C. hominis*, and Webber et al. (2014) hypothesized that CpMIC1 may be involved in this broader host range. The gene transcript in *C. parvum* consist of two exons, while in *C. hominis* (*ChMIC1*) this gene transcript only has the first exon. Webber et al. (2014) were able to distinguish *C. parvum* from *C. hominis* and other *Cryptosporidium* species by reverse transcriptase PCR (RT-PCR) targeting *MIC1*. However, *C. hominis* might not be differentiated from *C. parvum* by PCR (or NASBA) primer sets alone. By combining real-time PCR with melting curve analysis and restriction enzyme, Webber et al. (2014) distinguished *C. hominis* (and other *Cryptosporidium* species) from *C. parvum*.

In this study, our intention was to develop a NASBA-based assay that could be used for detecting *C. parvum* and *C. hominis* oocysts and used for distinguishing *C. parvum* oocysts from oocysts of *C. hominis* and other *Cryptosporidium* species. In addition, we wanted to investigate whether this assay detected only viable oocysts. We hypothesized that primer sets based on the two exons of *MIC1* would be suitable for this work.

2. Materials and methods

2.1. Source and purification of oocysts

C. parvum oocysts were isolated from faeces from infected calves by salt flotation (purified samples coded as NaCl-Cp). For one sample with very high fat content, ethyl acetate sedimentation was used prior to salt flotation, and, as considerable fat quantities remained after flotation, was further purified by IMS (purified sample coded as IMS-Cp) (Dynabeads® GC-Combo, Applied Biosystems™). Prior to, during, and after isolation, oocyst quantities were enumerated by drying 5 μl of sample, or a faeces smear, onto a glass slide, and, after methanol fixation, using IFAT for enumeration (AquaGlo G/C direct, Waterborne Inc. New Orleans, LA; USA). Sample examination was by fluorescence microscope (Leica DM LB, Ortomedic, Oslo, Norway). The purified oocysts (NaC-Cp and IMS-Cp) were stored at 4 °C.

C. hominis oocysts were kindly provided by the UK Cryptosporidium Reference Unit (Singleton Hospital, Swansea, UK).

2.2. Viability assessment

Oocyst viability was determined based on the standard 4′,6-diamidino-2-phenylindole (DAPI)/propidium iodide (PI) viability assay (Campbell et al., 1992).

2.3. Dilutions of oocysts

Dilutions of oocysts were used to investigate the sensitivity of the NASBA assay. Stock suspensions of oocysts with 1% Triton X-100 (VWR) were prepared to ensure dispersion of the oocysts. From the