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ORIGINAL PAPER

Development of an immunomagnetic bead separation-coupled quantitative PCR method for rapid and sensitive detection of *Cryptosporidium parvum* oocysts in calf feces

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Abstract Cattle feces are the environmental vehicle for the zoonotic *Cryptosporidium* oocysts, but there are drawbacks associated with reliability of the existing methods for the detection of oocysts in the feces. Quantification of the immunomagnetic bead separation (IMS) coupled with real-time TaqMan PCR (qPCR) was accomplished by comparing the fluorescence signals obtained from the calf fecal samples of *Cryptosporidium parvum* oocysts with those obtained from standard dilutions of *C. parvum* oocysts. TaqMan qPCR assays were developed for the detection of *C. parvum* based on 18S rDNA gene. This IMS-qPCR assay allowed a reliable quantification of *C. parvum* oocysts over seven orders of magnitude with a baseline sensitivity of 8.7 oocysts. The newly developed IMS-qPCR technique proved specific as confirmed by negative reactivity against a wide panel of non-*parvum Cryptosporidium* oocysts. As a field application, experimentally infected calves (15 infected and 9 non-infected) were screened for oocysts shedding on 16, 18, and 21 days postinfection. Acid-fast staining microscopy of infected calves revealed oocysts in the feces of 11, 7, and 4 calves, respectively, compared to 15, 15, and 12 in case of screening by IMS-qPCR. Taken together, the proposed IMS-qPCR method significantly improved the diagnostic capacity for *C. parvum* infection in calves, making the technique a useful, sensitive, reliable, and time-saving.

Keywords *Cryptosporidium parvum* oocysts · Immunomagnetic bead separation · Quantitative real-time PCR · 18S rDNA

Abbreviations

IMS	Immunomagnetic bead separation
qPCR	quantitative real-time TaqMan PCR
IMBs	immunomagnetic beads

Introduction

Cryptosporidium is a ubiquitous, obligatory protozoan parasite that infects the gastrointestinal epithelium of a wide range of vertebrate hosts (Plutzer and Karanis 2009; Fayer 2010), resulting in gastroenteritis manifested as diarrhea of varying severities. The infection is acquired by fecal-oral route either via direct contact with infected hosts or ingestion of contaminated food or water (Karanis et al. 2007; Chalmers and Davies 2010; Baldursson and Karanis 2011; Lange et al. 2013). In humans, the highest impact is on immune-compromised individuals such as AIDS patients, those receiving chemotherapy and/or radiotherapy, and individuals lacking of CD40 ligand as well as infants (Hunter and Nichols 2002; Chalmers and Davies 2010). Cryptosporidiosis is especially common in developing countries, creating additional challenges for the poorly supported public health infrastructure (Kotloff et al. 2013; Verbyla et al. 2013). Treatment of cryptosporidiosis is usually limited to supportive therapy, as

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most chemotherapeutic agents have little or no efficacy against the parasite (Fayer 1997; Griffiths 1998).

Several *Cryptosporidium* species are commonly found in humans and farm animals (Xiao et al. 2004; Santin 2013). Nevertheless, *Cryptosporidium hominis* and *Cryptosporidium parvum* in general are responsible for the majority of human *Cryptosporidium* infections (Xiao 2010). Four *Cryptosporidium* species are commonly identified in cattle, including *C. parvum*, *Cryptosporidium bovis*, *Cryptosporidium ryanae*, and *Cryptosporidium andersoni* (Slapeta 2013). Among them, *C. parvum* is the only species with a major zoonotic potential (Xiao 2010). Farm animals, particularly neonatal calves, have been identified as a major reservoir of zoonotic cryptosporidiosis (Plutzer and Karanis 2009; Xiao 2010). The high prevalence and the large numbers of oocysts shed by calves play a important role as direct source of infection (Fayer et al. 2006, 2007; Santin 2013; Amer et al. 2013a, b) and environmental contamination including rivers (Sischo et al. 2000; Tsushima et al. 2003). Infected hosts may excrete several millions to billions of sporulated oocysts in the feces (Fayer et al. 1998; Nydam et al. 2001; Xiao et al. 2001), which may survive and persist in the feces and environment for extended periods, ranging from several weeks to many months (Robertson et al. 1992; King and Monis 2007).

Because of the high demand for animal protein, intensive animal farming is a common practice, making zoonosis a major public health concern. Monitoring of *Cryptosporidium* spp. in farm animals and environment is usually challenged by differentiation between zoonotic and non-zoonotic species. In recent years, molecular biologic analyses have been used widely in characterizing *Cryptosporidium* spp. of various animals at both species and subtype levels (Elsafi et al. 2013; Fayer et al. 2013; Helmy et al. 2014). However, the complexity of molecular analysis and the variable of biological matrix are challenging for diagnostic application of PCR in the feces (Homem et al. 2012). Immunomagnetic bead separation (IMS) method has proven to be effective for separating various types of pathogenic microorganisms, including *Escherichia coli* O157 (Cubbon et al. 1996; Shelton et al. 2004), *Helicobacter pylori* (Enroth and Engstrand 1995), hepatitis A virus (Abd el-Galil et al. 2005), and adenovirus (Haramoto et al. 2010), *Giardia duodenalis* and *Cryptosporidium* spp. (Coklin et al. 2011; Jiang et al. 2005; Schets et al. 2013) from water, food, feces, and clinical materials. In addition, quantitative real-time TaqMan PCR (TaqMan qPCR) technique using fluorescent TaqMan technology has recently been shown to be a good method for target sequence quantitation (Liang and Keeley 2011). It is proved to be a rapid, accurate, and reliable detection method for many pathogens including *C. parvum* (De Waele et al. 2011; Jothikumar et al. 2008). In addition, Hwang et al. (2007) have shown that IMS combined with qPCR is an effective and sensitive detection method for enteroviruses in environment samples. In this study, we used IMS to improve the TaqMan qPCR detection rate for *C. parvum* oocysts in calf feces. We evaluated the sensitivity and the specificity of the newly developed IMS-qPCR technique for the detection of *C. parvum* in calf fecal samples.

Materials and methods

Isolation of *C. parvum* oocysts

Oocysts of *Cryptosporidium* were originally obtained from the feces of a naturally infected calf at a dairy farm in the province of Henan, China. The oocysts were isolated by sugar flotation technique followed by cesium chloride purification (Arrowood et al. 1996). The isolate was genotyped and subtyped by PCR-sequence analysis of a small-subunit (SSU) ribosomal RNA (rRNA) gene and gp60 gene fragments, respectively, as described previously (Feng et al. 2009). Genotyping results indicated that the obtained isolate was *C. parvum*, whereas subtyping at gp60 locus revealed that the isolate belongs to subtype IId A19G1 with a complete identity with the sequence HQ009809 reported by Wang et al (2011a, b). The generated sequence of gp60 was deposited in the GenBank under accession number KJ011899. Purified oocysts were suspended in phosphate-buffered saline solution (PBS), at the concentration of 2×10^8/ml and stored at 4 °C (Fontaine and Guillot 2003a). Viability of oocysts was assessed by propidium iodide (PI) dye and in vitro excystation assays (Robertson et al. 1998).

Preparation of fecal samples

Ten-fold serial dilutions of *C.parvum* oocysts in PBS were prepared from the purified stock suspension of oocysts. Negative fecal matter collected from a 1-month-old calf was confirmed by acid-fast staining and PCR-amplification of small-subunit (SSU) rRNA (Feng et al. 2009). One gram of negative fecal samples was spiked by 100 μl aliquots of each dilution to yield fecal samples with oocysts numbers between 10^7 and 1/g.

Preparation of immunomagnetic beads and IMS technique optimization

Streptavidin Particles Plus-DM immunomagnetic beads (BD Biosciences, USA) were prewashed three times with Dilute BD™ IMag Buffer (BD Biosciences, USA). Twenty microliters of polyclonal anti-immunodominant antigen Cp23 antibodies (Beijing Protein Innovation Co., Ltd., Beijing, China) at 1 μg/μl were added to the washed beads and allowed to bind for 30 min with gentle shaking to avoid sedimentation of the beads. The beads were collected against the wall of an Eppendorf-type tube (Safe lock; 3 ml) using magnetic

separator (BD Biosciences, USA). Antibody-coated beads were washed thrice with the dilute BD™ IMag Buffer (BD Biosciences, USA) to remove the unbound antibodies. The coated immunomagnetic beads (IMBs) were then suspended in PBS containing 0.1 % bovine serum albumin (BSA) and stored at 4 °C until use.

Each spiked fecal sample was suspended in 5 ml PBS and mixed to produce a homogeneous suspension. The slurry was centrifuged at 300 rpm for 5 min to remove the coarse particles, and the supernatant was transferred to a tube containing the IMBs and incubated for 30 min at 37 °C with gentle mixing by inverting the tube three to four times every 10 min. After incubation, the immuno-captured *C. parvum* oocysts were magnetically harvested and washed three times in PBS in the magnetic separator, and finally suspended in 400 μl of ddH_2O.

DNA extraction from *C. parvum* oocysts

DNA used in the cloning and construction of the internal positive control and *C. parvum* 18S rDNA gene was extracted from the immuno-captured *C. parvum* oocysts from the fecal samples. Total DNA from the oocysts was released by 5 cycles of freezing and thawing (liquid nitrogen for 5 min, 56 °C for 5 min). DNA was extracted and purified from the oocysts using a TIANamp Genomic DNA Kit (Tiangen Biotech, Co., Ltd., Beijing, China). After extraction, the DNA was qualitatively evaluated on a 1.8 % agarose gel and quantified in a spectrophotometer (260/280 nm wavelength). The DNA concentration was calculated as described by Sambrook et al. (2001).

C. parvum 18S rDNA gene (Cp18S rDNA) nested-PCR primers and TaqMan qPCR

Given the large database and homology of *C. parvum*18S rDNA sequences for different genotypes, it is considered as a good candidate for a specific probe (Higgins et al. 2001; Fontaine et al. 2003a). Primers of Cp18S rDNA nested PCR were designed based on the published sequence (accession AF093489, Fayer et al 2000). The first PCR utilized Cp18S rDNA P1, 5'-GAAAGCATTTGCCAAGGATGT-3' (nucleotides 901–921) as forward and reverse Cp18S rDNA P2, 5'-GTCTGGACCTGGTGAGTT-3' (reverse complement of nucleotides 1,155–1,172) to amplify a 272 bp fragment. The 144 bp fragment of the amplified 18S rDNA gene was further amplified with the following primers: forward Cp18S rDNA P3, 5'-TACCGTCGTAGTCTTAAC-3' (nucleotides 961–978) and reverse Cp18S rDNA P4, 5'-TTCCGTCAATTC CTTTAAG-3' (reverse complement of nucleotides 1,086–1,104). The sequence of the Cp18S rDNA TaqMan probe was FAM-5'-TCAGCCTTGCGACCATACTCC-3'-TAMRA (reverse complement of nucleotides 1,063–1,084). Cp18S rDNA primers and the TaqMan probe used for qPCR were both positioned inside a specific 272-bp DNA fragment. In these experiments, FAM acted as the reporter dye, while TAMRA acted as the quencher dye. Primers and probes were provided by TAKARA (Takara Biotechnology Co., Ltd., Beijing, China).

Nested-PCR and TaqMan qPCR detection

Nested-PCR reactions (50 μl) consisted of 25 μl of 2×*Ex Taq* Master Mix (Cowin Biotech Co., Ltd., Beijing, China), 2 μl of each primer (10 μM, concentration was optimized in the first step of the experiments), 2 μl of DNA, and 19 μl of ddH_2O. Cycling parameters included initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C (Cp18S P1, Cp18S P2) or 55 °C (Cp18S P3, Cp18S P4) for 45 s, and extension at 72 °C for 45 s and a final extension at 72 °C for 5 min. Nested-PCR products were analyzed by electrophoresis on 1.8 % agarose gels. PCR products were purified using TIAN quick Midi Purification Kit (Tiangen Biotech, Co., Ltd., Beijing, China).

TaqMan qPCR reactions were carried out using the Premix *Ex Taq*™ (Probe qPCR) system (Takara) in reaction volume of 20 μl consisting of 10 μl of 2×Premix *Ex Taq* (Probe qPCR), 0.4 μl of each primer (10 μM), 0.8 μl of the fluorescent probe (10 μM), 0.4 μl of 50×ROX reference dye II, 1 μl of DNA, and 7 μl of RNA-free water. The amplification conditions were 1 cycle at 95 °C for 30 s, 40 cycles at 95 °C for 5 s, and 60 °C for 34 s, on an Agilent Mx3000P QPCR System (Agilent Technologies). Negative (ddH_2O) controls were included in every assay. Fluorescence signals were normalized by dividing the reporter dye emission (FAM) by the emission of the passive reference dye (TAMRA). The threshold (Ct) cycle parameter defined as the fractional cycle number at which the fluorescence of the reporter moiety generated by cleavage of the probe crosses an arbitrarily defined threshold was defined as 0.1 in all of our reactions. The Ct values of each dilution were amplified in triplicate by TaqMan qPCR. From the slope(s) of the standard curve, the amplification efficiency (E) was estimated by the formula $E=10^{-1}/s\text{-}1$ (Ibekwe and Grieve 2003).

Construction of Cp18S rDNA standard curves

The 272-bp Cp18S rDNA fragment amplified with the Cp18S rDNA P1 and P2 primers was cloned into a pEASY-T1 vector using the pEASY-T1 Cloning Kit (TransGen Biotech, China), linearized with the *Hind*III restriction enzyme (Takara). The resultant plasmid DNA, calculated to be 4.2 kb in length, is equivalent to 2.77×10^{6} Da; hence, multiplying via the conversion factor 1.650×10^{-24} gives 4.57×10^{-18} g. Therefore, in these experiments, 1 ng of plasmid DNA contained 3.15×10^{8} copies of the cloned insert. Ten-fold plasmid dilutions ranging from 3.15×10^{7} to 3.15×10^{3} copies of Cp18S rDNA in purified suspensions were made to construct the standard curves for TaqMan qPCR detection. Samples of known initial

concentration were used to calculate the standard curves. And the test samples were quantified by determining their Ct values and through the use of standard curves to deduce their starting copy numbers. Serial plasmid dilutions (three replicates) were used for TaqMan qPCR. Standard curves and data analysis were conducted using MxPro QPCR software.

Calculation of the number of oocysts

DNA was extracted from 2×10^4 oocysts with equal efficiency of 20 μl of Tris-EDTA buffer; hence, the amount of DNA used as a template for TaqMan qPCR (1 μl) is equivalent to 1×10^3 oocysts. With the standard curve, the relationship between DNA concentration and the number of oocysts based on Ct values was established as described by Higgins et al. (2001).

Evaluation of specificity and sensitivity of the IMS-qPCR

To evaluate the specificity of the assay, genomic DNA samples from previously genotyped *Cryptosporidium fayeri*, *C. andersoni*, *C. bovis*, *C. ryanae*, *C. hominis*, *C. galli*, *C. macropodum*, *C. cuniculus*, *C. molnari*, *C. suis*, and *C. wrairi* were used in TaqMan qPCR experiments. The sensitivity of the assay was tested using two replicates with ten-fold plasmid dilutions of the Cp18S rDNA (3.15×10^3 copies to 3.15×10^0 copies per reaction). Data analysis was conducted using MxPro QPCR software.

Modified acid-fast staining and IMS-qPCR for the detection of *C. parvum* oocysts from experimentally infected fecal samples

As field application, this experiment was designed to compare the sensitivity of IMS-qPCR compared to the conventional acid-fast staining microscopy in experimentally infected calves. Twenty four 6-month-old calves, proved negative for *Cryptosporidium* by acid-fast staining and conventional ssRNA PCR, were divided into infected group (15 animals) and control negative group (9 animals). Each animal of infected group was orally inoculated with 25-ml PBS solution

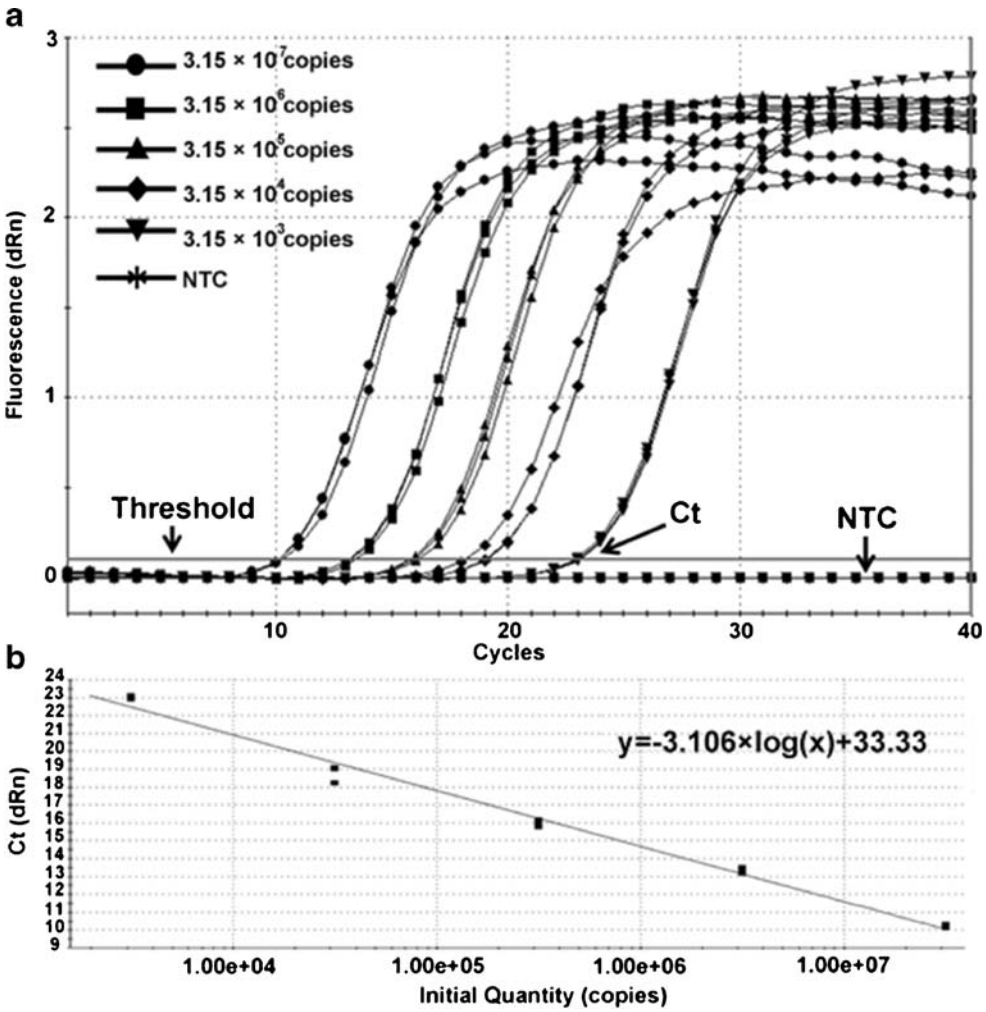

Fig. 1 Standardization of the TaqMan qPCR assay. **a** The TaqMan qPCR amplification of Plasmid dilutions containing 3.15×10^7 to 3.15×10^3 copies of cloned Cp18S rDNA gene fragments from *C. parvum*. The preset threshold fluorescence value, or fluorescence value at which the threshold cycle was determined, was 0.1. Ct threshold cycle or cycle number at which the threshold fluorescence was reached. *NTC* no template control. ΔRn values were plotted against number of cycles. Plots with endpoint ΔRn values above the threshold *horizontal black line* in mid-graph are considered positive. **b** Standard curve generated from the data plotted in **a**

containing 1×10^5/ml *C. parvum* oocysts by means of gastric tube. Apart from the peak period of oocyst shedding (10–14 days), where all the infected calves were positive both by acid-fast staining microscopy and IMS-qPCR, fecal samples were collected late on the latency period to include calves who resolved the infection and those still shedding oocysts even in low number. Therefore, fecal samples were collected from each experimental calf at 16, 18, and 21 days postinfection. The fecal samples were tested by the IMS-qPCR and modified acid-fast staining.

Statistical analyses

Data are presented as the mean±standard deviation (SD). Statistical analyses were carried out using the student *T test*, with $p<0.05$ was considered significant.

Results

TaqMan qPCR standard curve

The Ct values obtained for the current standards were as follows: 10 ng=10.15 cycles, 1 ng=13.26 cycles, 100 pg=16.15 cycles, 10 pg=19.08 cycles, and 1 pg=23.09 (Fig. 1a). The standard regression curve showed an efficiency of 109.9 %, with a slope of *n*-3.106 (Fig. 1b). According to Pfaffl (2001), the efficiency of a TaqMan qPCR assay should be 100 ±20 %, with a corresponding slope of between −3.6 and −3.1. The linearity of the standard curve ($r^2=0.99$) obtained herein showed that the TaqMan qPCR assay is suitable for quantification of *C. parvum* oocysts. Moreover, the standard curve obtained from the purified oocyst suspensions allows the quantification of the samples containing unknown amounts of DNA to be amplified simultaneously.

Strong positive signals were obtained in TaqMan qPCR reaction containing 1×10^3 oocysts (Fig. 2). The Ct values for the three replicates were 22.06, 22.06, and 22.11 cycles, which approximates to 4.215×10^3 copies Cp18S rDNA based on the standard curve. Thus, a detection of one oocyst per reaction was equal to 4.215 copies Cp18S rDNA.

Specificity and sensitivity of TaqMan qPCR

No amplification was obtained using DNA from a wide panel of other *Cryptosporidium* species proving specificity of the applied technique (Fig. 2). To assess the sensitivity of the TaqMan qPCR, two replicates of serial dilutions of Cp18S rDNA were tested. Cp18S DNA quantitation ranged from 3.15×10^3 copies to 3.15×10^0 copies per reaction. The amplification curves (Fig. 3) showed that the Ct values ranged from 22.17 (3.15×10^3 copies) to 33.65 (3.15 copies),

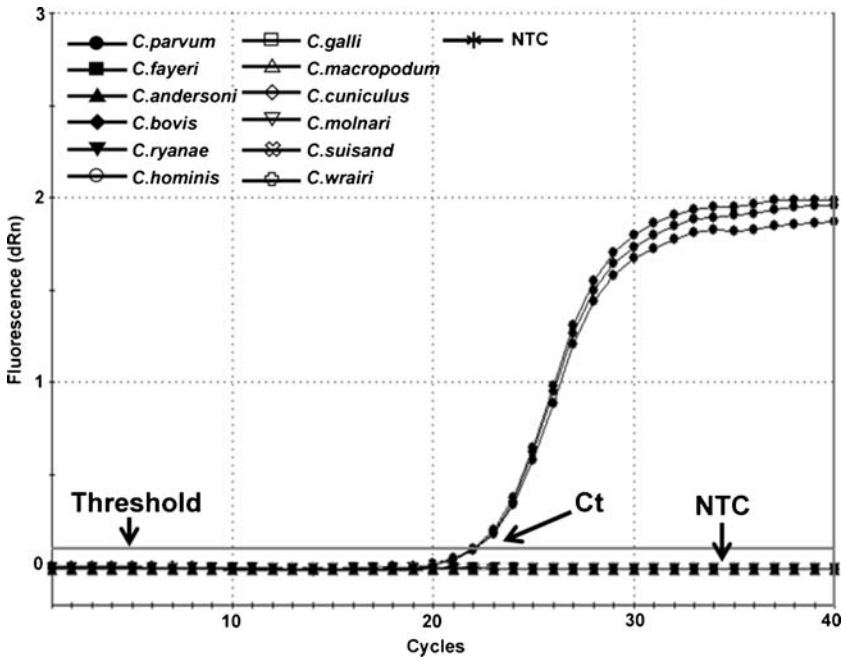

Fig. 2 Calculate the number of oocysts by TaqMan qPCR assay and the specificity of TaqMan qPCR. The template (1 μl) contained the DNA extracted from 1×10^3 *C.parvum* oocysts, and genomic DNA samples extracted from *C fayeri*, *C. andersoni*, *C. bovis*, *C. ryanae*, *C. hominis*, *C. galli*, *C. macropodum*, *C. cuniculus*, *C. molnari*, *C. suis*, and *C. wrairi.* The control sample was ddH_2O. The preset threshold fluorescence value, or fluorescence value at which the threshold cycle was determined, was 0.1. Ct threshold cycle or cycle number at which the threshold fluorescence was reached. *NTC* no template control. ΔRn values were plotted against number of cycles. Plots with endpoint ΔRn values above the threshold *horizontal black line* in mid-graph are considered positive

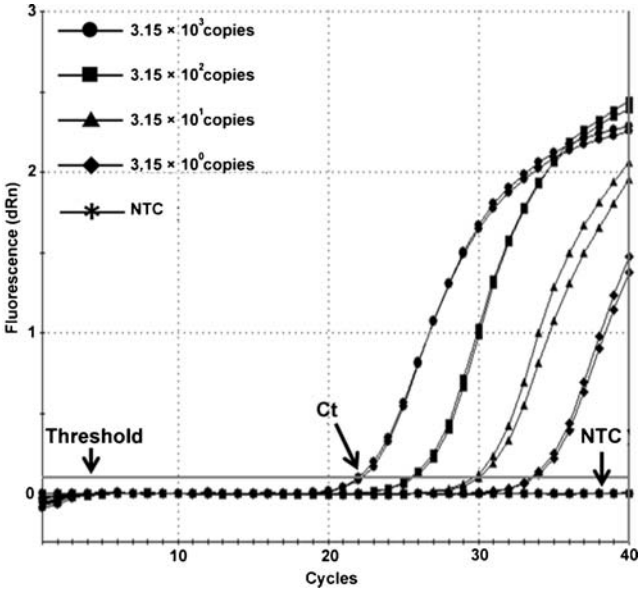

Fig. 3 Sensitivity of TaqMan qPCR. The TaqMan qPCR amplification of plasmid dilutions containing 3.15×10^3 to 3.15×10^0 copies of cloned Cp18S rDNA gene fragments from *C. parvum*. The preset threshold fluorescence value, or fluorescence value at which the threshold cycle was determined, was 0.1. Ct threshold cycle or cycle number at which the threshold fluorescence was reached. *NTC* no template control. ΔRn values were plotted against number of cycles. Plots with endpoint ΔRn values above the threshold *horizontal black line* in mid-graph are considered positive

indicating the sensitivity of the reaction up to 3.15 copies/reaction. No Ct value was obtained in case of negative control.

IMS-TaqMan qPCR detection of *C. parvum* oocysts from fecal samples

IMS-qPCR generated amplification products from the DNA obtained from *C. parvum* oocysts from the spiked fecal samples. Results indicated that the present protocol attained a detection limit up to 8.7 oocysts per gram (Ct value of 29.64) in the spiked fecal samples, with no nonspecific amplification in the negative controls (Fig. 4).

As a field application, experimentally infected calves (15 infected and 9 non-infected) were screened for oocysts shedding on 16, 18, and 21 days postinfection. Acid-fast staining microscopy of infected calves revealed oocysts in the feces of 11, 7, and 4 calves, respectively, compared to 15, 15, and 12 in case of screening by IMS-qPCR, indicating the high sensitivity of the developed method. None of the non-infected calves shed oocysts in both techniques.

Discussion

Accurate detection and burden estimation of medically significant cryptosporidia are the cornerstone of the control and prevention measures. The present study has established adequate methodology to precisely monitor oocysts of *C. parvum* in the feces of cattle and to avoid overestimation due to the presence of other non-zoonotic species. Although the acid-fast staining microscopy is the golden standard for detection of cryptosporidia, it is cannot differentiate the medically important species from the morphologically identical non-zoonotic

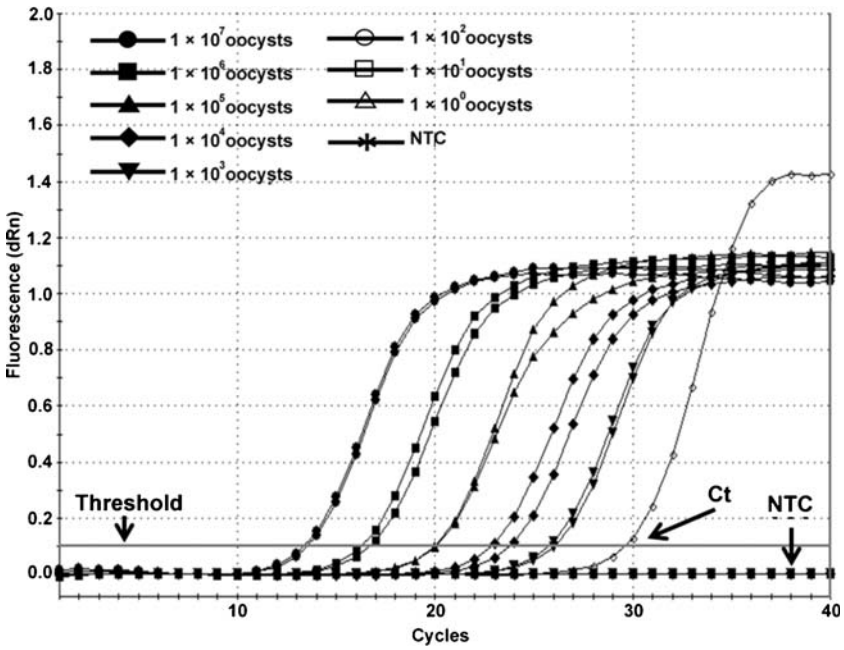

Fig. 4 TaqMan qPCR amplification of Cp18S rDNA of fecal samples. DNA obtained from fecal samples spiked with purified *C.parvum* oocysts after IMS by using the TIANamp Genomic DNA Kit. DNA extracted from fecal samples containing 10^7 to 10^0 oocysts. The preset threshold fluorescence value, or fluorescence value at which the threshold cycle was determined, was 0.1. Ct threshold cycle or cycle number at which the threshold fluorescence was reached. *NTC* no template control. ΔRn values were plotted against number of cycles. Plots with endpoint ΔRn values above the threshold *horizontal black line* in mid-graph are considered positive

species (Xiao 2010), and false positive results may result from unspecific staining of spores of yeast and fungi. The detection limit of acid-fast staining microscopy is low compared with the molecular tools. Results reported herein prove that IMS-qPCR is more reliable than microscopy for the detection of less than 10 oocysts per gram of fecal sample.

DNA quality is a detrimental factor in the efficiency of qPCR (Hoorfar et al. 2003). PCR inhibitors in the sample matrix have hampered the implementation of the tests based on the nucleic acids (Skotarczak 2009) including qPCR assays for *Cryptosporidium* (Yang et al. 2013). In the present study, IMS was used to overcome these difficulties, ensuring good quality start materials. In the present study, IMS was used to magnetize the *Cryptosporidium* oocysts via binding with anti-*Cryptosporidium* antibody-coated beads. The same approach was used in USEPA Method 1623 (2005) for separation and microscopy enumeration of *Cryptosporidium* oocysts. Also, IMS purification is an efficient pre-PCR step for separation and isolation of *Cryptosporidium* oocysts from water and feces (Jiang et al. 2005; Coklin et al. 2011). The main privilege of IMS separation is that it specifically captures the target organism from the bulk materials and removes inhibitory substances from such samples (Abd el-Galil et al. 2004; Wang et al. 2011a, b; Zhang et al. 2013). A variety of qPCR assays have been developed for the detection of *Cryptosporidium* in both fecal and environmental samples (Fontaine and Guillot 2002; Tanriverdi et al. 2002; Ochiai et al. 2005; Alonso et al. 2011; Rolando et al. 2012; Mary et al. 2013; Yang et al. 2013). The present study targeted a gene locus with high copy number, utilizing the TaqMan probe. Ct values generated from Cp18S rDNA TaqMan qPCR showed that the amplification limit was 3.5 copies. This detection limit is comparable to that reported previously (Fontaine and Guillot 2002) for *C. parvum* by TaqMan qPCR assay. Recently, Yang et al. (2013) developed a quantitative qPCR assay using minor groove binder (MGB)-probes targeting *Cryptosporidium*-specific protein-coding gene, that directly detects, quantitates, and identifies *C. hominis* and *C. parvum* in environmental and fecal samples. Being of high copy number, 18S-based assays proved superior in terms of sensitivity (Burnet et al. 2013; Mary et al. 2013) over those based on one-copy gene assays. Also, 18S proved its high ability to differentiate between species and different genotypes of a species (Fontaine et al. 2003b). The protocol described in the present study showed high sensitivity (8.7 oocysts per gram) compared to the recently developed real time-PCR technique (300 oocysts per gram) based on 18S gene locus (Mary et al. 2013). The differences may be attributed to the extraction methods. IMS separation followed by DNA extraction may be better than direct extraction as described by Mary et al. (2013). No cross reactivity was detected when the current technique was tested against a wide panel of *Cryptosporidium* species, indicating the specificity and that only the zoonotic *C. parvum* was detected.

In conclusion, the IMS-qPCR assay allows reliable detection and quantitation of *C. parvum* oocysts from fecal samples and it may be applicable for environmental samples. The technique is specific, sensitive, and time efficient. Results were obtained within 5 h of sample collection (i.e., 2–2.5 h for IMS, 1 h for DNA extraction, and 2 h for TaqMan qPCR).

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