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ORIGINAL ARTICLE

Evaluation of modified Ziehl–Neelsen, direct fluorescent-antibody and PCR assay for detection of *Cryptosporidium* spp. in children faecal specimens

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Abstract To determine the sensitivity and specificity of routine screening methods for cryptosporidiosis, three methods including conventional modified Ziehl–Neelsen (MZN), direct fluorescent-antibody (DFA) and Nested-PCR assay compared together. To this end, their ability to identify the low concentrations of *Cryptosporidium* spp. oocysts in children fecal samples was evaluated. The sample population of this study was children under 12 years old who had diarrhea and referred to pediatric hospitals in Tehran, Iran. 2,510 stool specimens from patients with diarrhea were screened for *Cryptosporidium* oocysts by concentration method and MZN. To determine sensitivity and specificity, Nested-PCR and DFA were performed on 30 positive and 114 negative samples which previously had been proved by MZN. By using the microscopic method, DFA assay and PCR analysis, a total of 30 (1.2 %), 28 (1.1 %) and 32 (1.27 %) positive samples were detected respectively. According to the results, the sensitivity, specificity, and positive and negative predictive values of the Nested-PCR assay were 100 %, compared to 94, 100, 100, and 98 %, respectively, for MZN and 87.5, 100, 100, and 96 %, respectively, for DFA. Results of the present study showed that the Nested-PCR assay was more sensitive than the other two methods and laboratories can use the Nested-PCR method for precise diagnosis of *Cryptosporidium* spp. However, regarding the costs of Nested-PCR and its unavailability in all laboratories and hospitals, MZN staining on smears has also enough accuracy for *Cryptosporidium* diagnosis.

Keywords *Cryptosporidium* · Modified Zeihl–Neelsen · PCR · Direct fluorescent-antibody

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Introduction

Cryptosporidium is an obligated intracellular protozoan parasite that has been identified as one of the major causes of enterocolitis and waterborne diarrheal disease in human. Food and water are the main sources of infection in humans (Sterling and Arrowood 1986; Barwick et al. 2000; Fayer and Xiao 2008). In immunocompetent individuals, cryptosporidiosis manifests as a self-limited diarrhoea, whereas in immunocompromised patients, it causes severe and chronic diarrhoea, which if untreated, can lead to death (McDonald 1996). Children are more susceptible to the infection due to low immunity. They suffer from severe diarrhoea, which is self-limiting in most of children with a healthy immune system. But regarding the nutritional status, health and other environmental factors which affect child health safety, the treatment appears necessary in some of them (McDonald 1996). Cryptosporidiosis should be considered a major cause of diarrheal disease, but the *Cryptosporidium* oocysts are not easily recognized from other similar artifacts using routine staining procedures (Bialeka et al. 2002). The routine diagnosis methods for *Cryptosporidium* are Ziehl–Neelsen staining and microscopic consideration (Lumb et al. 1985).

Considering the presence of other acid-fast microorganisms in faeces which are similar to *Cryptosporidium* in terms of size or shape (e.g. yeasts, pollens and other protozoa like *Cyclospora*), these methods are arduous and require skill and experience (Lumb et al. 1985). The abovementioned flaws and the necessity of existence of at least 50,000–500,000 oocysts per gram of stool for detection, has led researchers to search for a better diagnostic method that are capable of recognizing a lower parasite rate in the sample (Garcia et al. 1987).

Serological examinations including direct fluorescent-antibody (DFA), enzyme immunoassays (EIAs) and indirect fluorescent antibody (IFA) tests recognise the soluble stool antigens used to diagnose cryptosporidiosis in human. Because of the high sensitivity and specificity, these methods are considered a gold standard in many laboratories (Cole 1997; Barugahare et al. 2011). Species identification and genotyping of human parasites are necessary to identify the transition pattern and disruption of the transmission cycle that conventional methods are not able to achieve (Morgan et al. 1997; Fayer et al. 2000). Today, molecular methods are used for accurate diagnosis of many diseases. Several studies have shown a high sensitivity and specificity of these methods for the detection of *Cryptosporidium* (Morgan et al. 1998; Ziegler et al. 2007; Kaushik et al. 2008; Zaidah et al. 2008; Paul et al. 2009). Despite the drawbacks of staining method and conventional microscopic assessment, today most of the health care centres in developing countries including Iran are using the modified Ziehl–Neelsen (MZN) staining as the gold standard method for diagnosis of this protozoan.

Few comprehensive studies have been conducted to evaluate and compare the diagnostic methods for *Cryptosporidium*, and to our knowledge there is no study conducted in Iran that compared the MZN, DFA and PCR methods to identify *Cryptosporidium*. Therefore, the evaluation and comparison of the standard method (MZN), which is used today for the clinical diagnosis of *Cryptosporidium*, with serological (DFA) and molecular (PCR) methods appears essential.

Materials and methods

Faecal specimens

A total of 2,510 stool samples were collected from laboratories between May 2009 and November 2010. Samples were transferred to the research laboratory of parasitology and mycology department, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. It should be noted that in this study diarrhoea was defined as loose or watery stool.

Research population

This study was carried out on stool samples from children less than 12 years old who had diarrhoea and were referred to Shahid Fahmideh, Mofid paediatric hospital and Mahak Medical Center, Tehran, Iran. To compare the sensitivity and specificity of MZN, DFA and PCR methods, 30 positive and 114 negative samples in terms of the presence or absence of *Cryptosporidium* were assessed (based on statistical consulting and with a prevalence of 1 %, the maximum and confidence level of 95 % and 0.1 errors). To gather 114 negative samples, cluster sampling was conducted on entire samples that had negative results by microscopic examination.

Microscopy

All samples were concentrated using the formalin-ether concentration method. The smears were prepared from sediment and then were stained by MZN staining method. Afterwards, the slides were considered under 100× microscopic observation. Each slide was examined for at least 5 min. To evaluate the sensitivity and specificity of three methods, PCR and DFA were performed on 30 positive and 144 negative samples that previously were confirmed by MZN.

Direct fluorescence-antibody assay (DFA)

The DFA test was implemented using the MeriFluor *Cryptosporidium*/*Giardia* kit (Meridian Bioscience Inc., Cincinnati, OH), according to the manufacturer's instructions. Stool specimens were prepared by formalin ether sedimentation, then 10 µl of the sediment was spread thinly on a DFA well slide and dried at 37 °C for 30 min. The staining process was performed according to the manufacturer's instructions. Thereafter, the slides were examined for *Cryptosporidium* oocysts under fluorescent microscope using a magnification of 40× (excitation filter, 490–500 nm, barrier filter; 510–530 nm, Carl Zeiss, Inc., Germany).

DNA extraction

DNA was extracted from the faecal samples using the QIAamp DNA stool Mini Kit (QIAGEN, Hilden, Germany), according to manufacturer's instructions with five additional 5 min freeze/thaw cycles before the DNA extraction to rupture the *Cryptosporidium* oocysts. Concentration and quality of the extracted DNA was measured by spectrophotometer (Cecile England).

Table 1 Comparison of Nested-PCR versus microscopic detection of *Cryptosporidium*

Method	Examined samples	Positive samples	Negative samples	Sensitivity (%)[a]	Specificity (%)[b]	Positive predictive value (%)[c]	Negative predictive value(%)[d]	Genotyping ability[e]
N-PCR	144	32	112	100	100	100	100	Yes
MZN	144	30	114	94	100	100	98	No
DFA	144	28	116	87.5	100	100	96	No

[a] Sensitivity of the three methods calculated by using both true positives and false negatives as follows: [number of true positive/(number of true positives + numbers false negative)] × 100

[b] Specificity of the methods calculated by using the true negatives and false positives as follows: [number of true negatives/(number of true negatives + numbers false positives)] × 100

[c] Positive predictive value calculated by using the true positives and false positive as follows: [number of true positive/(number of true positives + numbers false positive)] × 100

[d] Negative predictive value calculated by using the true negative and false negative as follows: [number of true negative/(number of true negative + numbers false negative)] × 100

[e] In spite of the fact that MZN and DFA are unable to detect the isolated genotypes of *Cryptosporidium*, PCR assay can identify them precisely

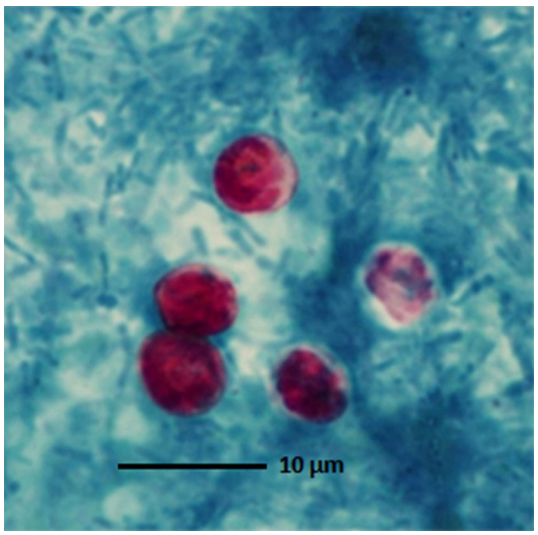

Fig. 1 Oocysts of *Cryptosporidium* stained using the MZN method

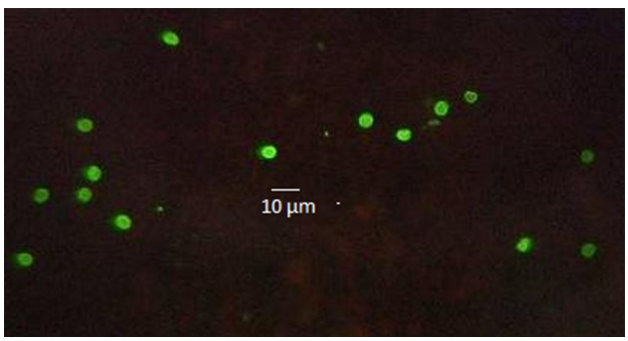

Fig. 2 Oocysts of *Cryptosporidium* stained using the DFA method

Nested-PCR

A nested PCR assay targeting the small subunit rRNA gene was performed using the earlier reported primers (Xiao et al. 2001). Initial primers Cr18PA: (5′-TTCTAGAG CTAA TACATGCG-3′) and Cr18PB: (5′-CCCATTTCC TTCGAAACAGGA-3′) amplified a 1.3 kb sequence of 18S-rRNA gene. The inner primer sets Cr18NA: 5′-GGA AGGGTTGTATTTATTAGATAAAG-3′ and Cr18NB: 5′-CTCATAAGGTGCTGAAGG AGTA-3′ amplified an 826–864 bp sequence of the former amplified sequence. PCR was performed in a final volume of 20 µl prepared in PCR buffer (50 mM KCL, 20 mM Tris–HCL, 2.5 mM $Mgcl_2$, pH 8.4) and contained 0.1 µg/ml BSA, 0.4 µM of the respective primers, a 0.2 mM concentration each of dNTPs, 1.2 U of recombinant *Taq* polymerase and 1 µl of the purified target DNA. For the second round of amplification, the reaction mixture was prepared as described above, except the inner primers and 1 µl of the amplified product from the first PCR was used as a source of DNA. Primary amplification was carried out in 30 cycles, each consisting of 94 °C for 45 s, 50 °C for 45 s and 72 °C for 1 min, with an initial melting at 94 °C for 10 min and a final extension at 72 °C for 10 min. For secondary amplification, 35 cycles were used, with identical temperatures and times. All PCRs were run in a PCR thermocycler (Techne, England). The PCR products were analysed by electrophoresis on 1.5 % agarose gels, stained with ethidium bromide (1 µg/ml) and visualized on a UV transilluminator (Syngene, England). Positive controls including PCR mixture reaction with the *Cryptosporidium* DNA were used in each PCR assay. PCR mixture reactions without DNA were also used as negative controls in each PCR assay.

Statistical analysis

The maximum likelihood method was used to estimate the sensitivity and specificity of the three methods. After setting up the PCR to a fixed protocol, PCR with the same

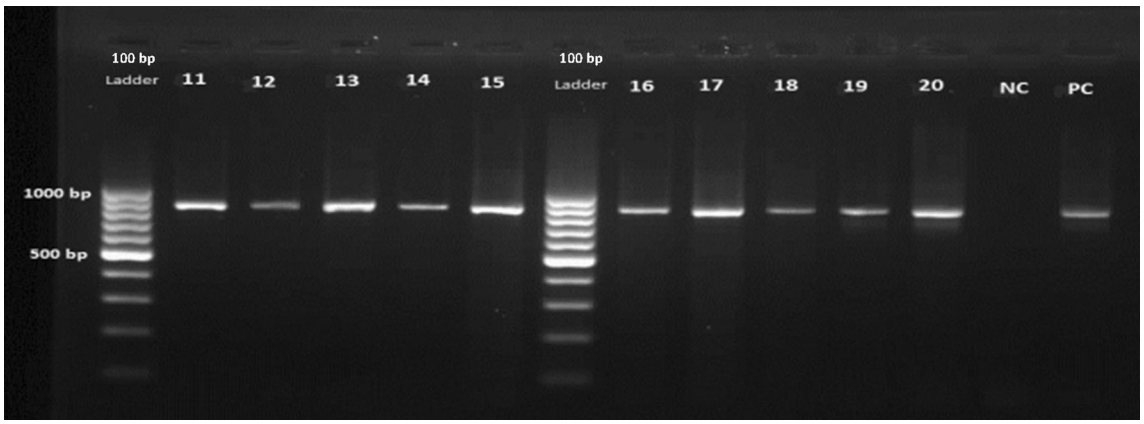

Fig. 3 Revealed positive samples by PCR assay which previously had positive result by staining method. *M* 100 bp molecular-weight marker, *PC* positive control, *NC* negative control, *lanes 11–20* represent ten randomly selected samples from patients which previously had positive result by staining method

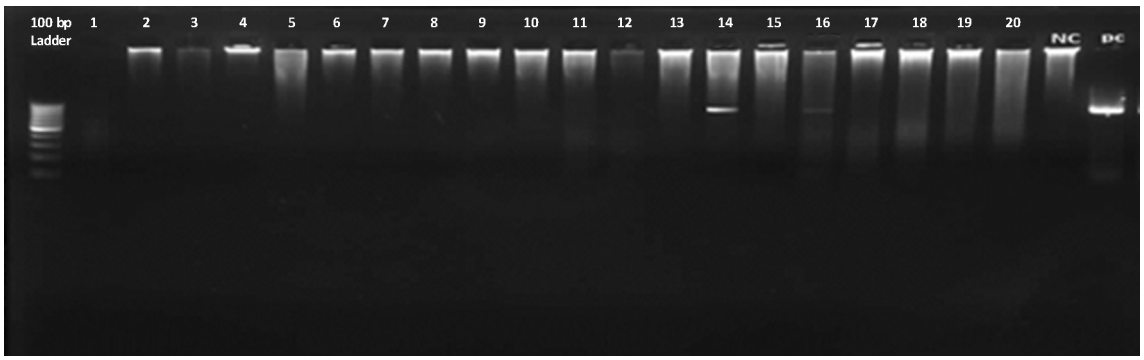

Fig. 4 Revealed two positive samples by PCR assay which previously had negative result by staining method. *M* 100 bp molecular-weight marker, *PC* positive control, *NC* negative control, *lanes 2–20* represent selected samples which previously had negative result by staining method. *Lanes 14 and 16* represent two positive samples by PCR

protocol was performed for all samples. Statistical analysis was done by using the Chi square test and SPSS software version 19.

Results

Of 2,510 children with diarrhoea, 1,353 patients (54 %) were male and 1,157 (46 %) were female. The prevalence of *Cryptosporidium* among these children was 1.27 %. Most infection (0.71 %) was observed in the age group of 2–3 years. 1.4 % of males and 0.95 % of females were positive for *Cryptosporidium* spp. A total of 144 diarrheic faecal samples were screened by microscopy, DFA and PCR methods (Table 1). All 144 samples were spiked with *Cryptosporidium* DNA as a positive control, which amplified the correct sized band indicating that PCR inhibition was not a factor in this trial. A total of 30 (1.2 %) (Fig. 1), 28 (1.1 %) and 32 (1.27 %) positive samples were detected using microscopy, DFA (Fig. 2) and PCR methods (Figs. 3, 4), respectively. DNA amplification of 30 positive samples diagnosed by MZN had 30 positive results (Fig. 3) that indicated there was no false positive result with the MZN method. PCR analysis of the samples that previously were negative by MZN identified two positive samples (Fig. 4) that indicated two false negative results by the MZN method. The DFA method indicated one true positive and four false negative results.

Discussion

Cryptosporidium is one of the important causes of diarrhoea in immunocompromised individuals and children (Fayer and Ungar 1986). Worldwide distribution of this protozoa and increase of immunocompromised persons such as AIDS patients or consumers of immunosuppressive drugs, has led to the increased presence of this parasite. This study was performed to compare the PCR, MZN and DFA assays, the most common methods in *Cryptosporidium* diagnosis. Morgan and his colleagues (Morgan et al. 1998) compared PCR with the staining method for

Cryptosporidium parvum diagnosis in diarrheic samples of human. By using PCR and staining methods 36 and 29 samples were positive, respectively. In their study, the sensitivity and specificity of the staining method was 83.7 and 98.9 %, respectively, and for PCR analysis both were 100 %. According to the ability of PCR in detection of the genotypes of infection causes, they suggested PCR as a useful method in the diagnosis of *Cryptosporidium*, which is different than in our study. Our results revealed the 100 % specificity for MZN method, which is different to Morgan's results. They detected five false positive results, while in our study we had no false positive results. This difference may be created in the process of staining or expertise of technicians. Kaushik et al. (2008) evaluated the four diagnostic methods for *Cryptosporidium*, including Ziehl–Neelsen staining, Safranin Methylene Blue staining, Ag detection and Nested-PCR. Their results revealed 100 % sensitivity for PCR, which was more sensitive than Ziehl–Neelsen staining (41.2 %). This finding is similar to the results obtained in our study.

They also reported 100 % specificity for the two above mentioned methods, which is similar to our results. Zaidah et al. (2008) compared the PCR and MZN method for the detection of *Cryptosporidium* in AIDS patients. Eight out of 59 samples were detected by PCR, and just two samples were detected by the MZN method. Just one sample was detected positive by both methods. Their study revealed the same results that show high sensitivity for PCR in comparison with the MZN, and 100 % specificity for both methods. Their MZN assay was less sensitive, likely because they didn't use any concentrating method. In most studies, the sensitivity and specificity of DFA testing by Merifluor kits have been reported at 96–100 and 99.8–100 %, respectively (Garcia et al. 1992; Scheffler and VanEtta 1994; Zimmerman and Needham 1995; Garcia and Shimizu 1997). However, in the present study the sensitivity of the DFA test was recorded at 87 %. Bialek and colleagues (Bialeka et al. 2002) compared DFA, EIA and PCR methods, and DFA (91 %) was lower than EIA (94 %) and PCR (97 %)in sensitivity, which is in contrast with our study. In the study of Vohra et al. (2012), the sensitivity of DFA (80 %) was significantly higher than MAF (40 %), and specificity of DFA and MAF were 100 and 91.7 %, respectively, which is dissimilar with the results of our study. Ziegler et al. (2007) announced the increased sensitivity by PCR in *Cryptosporidium* diagnosis, which is in line with our results. Paul et al. (2009) revealed that PCR is the most sensitive method for *Cryptosporidium* diagnosis, and the concentration methods are reliable in case the medical laboratories are lacking PCR facilities, which is more common in developing countries such as India.

Results of this study suggest that laboratories of paediatric hospitals, or the hospitals with immunocompromised patients, would be better to use the PCR method for *Cryptosporidium* diagnosis. Nevertheless, regarding to the costs of PCR and its lack of availability in some laboratories and hospitals, MZN staining on smears, prepared by the formalin-ether method, seems useful and has enough accuracy for the detection of *Cryptosporidium*.

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