



RESEARCH ARTICLE

GENOTYPING OF CRYPTOSPORIDIUM ISOLATES FROM CLINICAL SAMPLES

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ABSTRACT

Cryptosporidium is an intracellular protozoan that infects the gastrointestinal tract of human and other animals. Two main species of this parasite are known to infect human *C. hominis* and *C. parvum* with the latter has a zoonotic transmission. This study aimed to genotype the clinical isolates of *Cryptosporidium* from children with diarrhea.

A total of 64 fecal samples were collected from diarrheic children. Cryptosporidial infection was detected using modified acid fast stain. DNA of the parasite was extracted from oocysts of positive fecal samples and nested PCR method was used for TRAP-C2 gene amplification. Restriction fragment length polymorphism (RFLP) was applied for genotyping. Four fecal samples from 64 (0.625%) gave positive result for *Cryptosporidium*. RFLP-PCR suggested that 3 samples of these related to *C. parvum* and 1 sample of mixed infection with both *C. parvum* and *C. hominis*. From these results it can be concluded that most infections in children with cryptosporidiosis are of bovine origin.

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INTRODUCTION

Cryptosporidium spp. are intracellular parasites that primarily infect epithelial cells of the gastrointestinal tract especially intestine and biliary ducts (Tille *et al.*, 2014). Previously, the species that infects human was called *C. parvum*; however, it is now classified as two species, one was named as the human genotype, i.e. *C. hominis*, which is transmissible between humans, and the other was a bovine genotype, *C. parvum*, which is transmissible between man and vertebrates, especially cattle (Morgan-Ryan *et al.*, 2002). Human infections with *C. parvum* are commonly seen in areas with intensive animal production. That is because the relative high occurrence of zoonotic infections among cattle and sheep in these areas (Iqbal *et al.*, 2012).

Both *C. parvum* and *C. hominis* are morphologically identical; however, there is only 97% similarities between their genomes. Thus, the differentiation between the two species is primarily depends on genotyping (Tamer *et al.*, 2007). Several genes were used for this genotyping process, the most important of which are small subunit (SSU) rRNA (Spano *et al.*, 1998), thrombospondin-related adhesive protein *Cryptosporidium*-1 (TRAP-C1) and TRAP-C2 (Elwin *et al.*, 2001).

TRAP gene is present almost in all Apicomplexan protozoa, and it encodes for protein which was found to be required for sporozoite gliding motility and together with circumsporozoite protein are necessary for penetration of the host cells (Sinnis *et al.*, 1997). In *Cryptosporidium*, TRAP-C2 gene is an example

of well-characterized gene which is used for demonstration of the polymorphic nature of the parasite genome (Elwin *et al.*, 2001), and differentiated *C. parvum* from *C. hominis* (Mojarad *et al.*, 2011). Two major genotyping methods were extensively used for detection of polymorphism in this gene: restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) and sequencing. The current study aimed to determine the genotype isolates from clinical samples using nested PCR-RFLP analysis do TRAP-C2 gene.

Subjects and Methods

A total of 64 fecal samples were collected from children (< 5 years old, 37 male and 27 female) with diarrhea who have attended to Kadhumyia and Al-Karama hospital/ Baghdad during the period from June to August 2014. Direct smear was made from each sample, and stained with modified acid fast stain for identification of *Cryptosporidium*. Fecal samples which gave positive results were mixed with 2 parts of 2.5% potassium dichromate and kept at 4 C.

DNA Extraction and TRAP-C2 Gene Amplification

Each positive fecal sample was emulsified with little amount of distilled water. Oocysts were separated from the debris by floatation using saturated NaCl solution and centrifugation for 8 min at 1000 rpm (Elwin *et al.*, 2001). The upper layer containing the oocysts was withdrawn with Pasteur pipette and put in a conical tube. Approximately three times volume of phosphate buffer saline (PBS) was added. The tube then centrifuged and the supernatant was discarded. The precipitated the resuspended with 1 ml of PBS. From washed

fecal suspension, 300 µl was used for the extraction of the parasite DNA. A ready kit ((gSYNCTM DNA Mini Kit/Geneaid/ Korea) was used according to the manufacturer's manual.

Nested-PCR was used to amplify TRAP-C2 gene. The external primers set were F-5'-CATATTCCTGTCCCTTGAGTTGT-3' and R-5'-TGGACAACCCAA ATGCAGAC-3'. This set amplifies 369-bp of TRAP-C2 gene. A ready PCR master mix (Promega/USA) was used for amplification for both genes. Template DNA (4 µl) from each sample and primers (2 µl from each) were added to 0.5 ml ependorf tube containing 25µl of master mix. Nineteen µl of deionized water was added to reach a final volume of 50µl. The mixture then put in shaker and spinner for 10 cycles for better mixing. After mixing, the master mix tubes were transferred to the thermocycler (Hybaid/UK). PCR conditions were an initial denaturation step at 95 C for 4 min followed by 38 cycles of 94 C for 30 sec, 60 C for 30 sec and 72 C, and a final extension of 72 C for 10 min.

The internal primers sequences were F-5'-GGTAATTGGTCAC GA-3' and R-5'-CCAAGTTCAGGCTTA-3' which amplifies 266-bp within the amplified 369-bp. Preparation of PCR reaction was exactly the same as for external primer except that the PCR product from the previous run were used as a template. PCR conditions involved an initial denaturation at 95 C for 3 min followed by 40 cycles of 94 C for 30 sec, 44 C for 30 sec and 72 C for 1 min, and a final extension of 72 C for 10 min. Nested-PCR products were examined by gel electrophoresis. A 10-µl aliquot of PCR product was mixed with 2 µl loading dye and loaded into the wells of the prepared gel. Power supply was adjusted into 100 volts and run for 1 hour. The gel then was stained with ethidium bromide (Biobasic/Canada)(0.5 µg/ml) for 20 min and examined using U. V. transilluminator with camera. The size of amplified products were determined by comparison with a commercial 1000 bp ladder (Kappa Biosystem/USA).

Genotyping

Two restriction enzymes were used for genotyping: *Hae*III and *Bst*EII (New England Biolabs Inc./USA). A 1µg amount DNA of each nested PCR products was mixed with a 5µl 1X NEB buffer (50mM NaCl, 10mM Tris-HCl, 10mM MgCl₂, 1mM dithiothreitol, pH 7.9), and 1µl of *Hae*III or *Bst*EII (10U). The reaction was adjusted to 50µl using sterile deionized H₂O. The solution was mixed by flicking, followed by spinning in microcentrifuge at 5000 rpm for 30 sec, then incubated at 37 °C for 60 min. *Hae*III cuts when there is GGCC sequence between the second G and first C, a sequence which characterizes *C. hominis*. On the other hand *Bst*EII cuts when there is GGTNACC sequence between the two G's, a sequence which characterizes *C. parvum*. Whenever there is digestion, there will be two fragments (237 and 29 bp) from both enzymes.

The digestion products of the two enzymes were visualized using 3% agarose gel electrophoresis. DNA marker was used for comparison of the product size.

RESULTS

Out of 64 fecal samples, only 4 samples (0.625%) gave positive result for *Cryptosporidium* using modified acid fast

staining (figure 1). Extracted DNA from the four positive samples were successfully done using nested PCR, and yielded 266-bp gene fragment (figure 2).

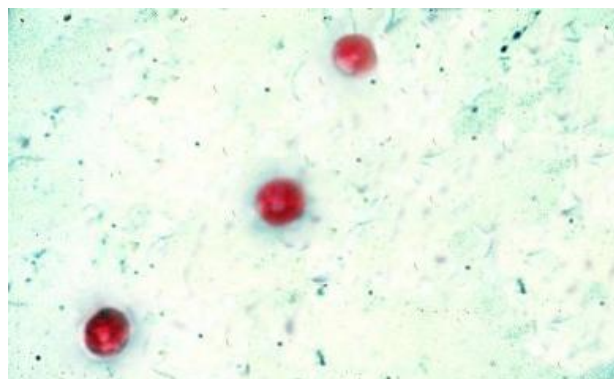


Figure 1 *Cryptosporidium* in a fecal sample using modified acid fast stain

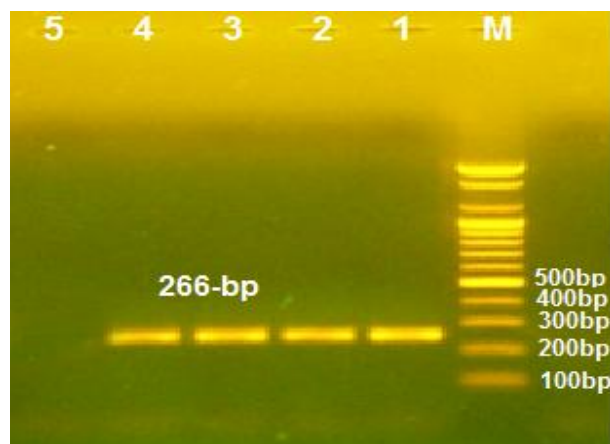


Figure 2 molecular diagnosis of *Cryptosporidium* using nested PCR for TRAP-C2 gene. M: 1000 bp DNA marker; lanes 1-4: 266- bp fragment of TRAP-C2 gene.

Genotyping with RFLP using two restriction enzymes (one specific for *C. parvum* and the other for *C. hominis*) revealed that 3 positive samples (75%) related to *C. parvum*, while only one positive sample (25%) indicated mixed infection with both *C. parvum* and *C. hominis* (figure 3).

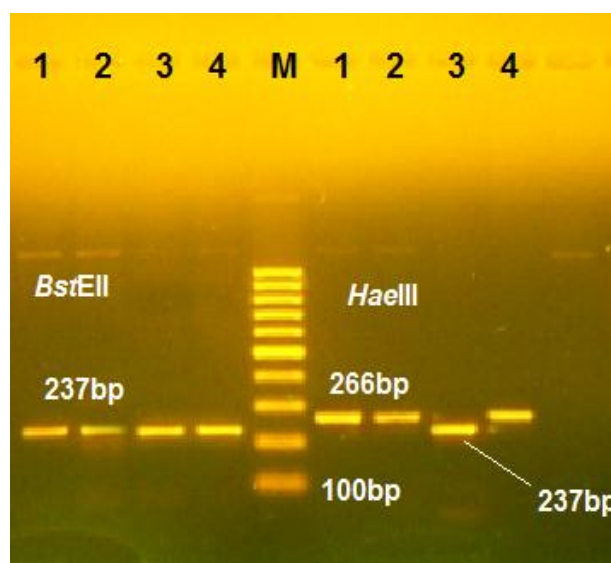


Figure 3 RFLP of nested PCR products of TRAP-C2 gene with two restriction enzymes. M: 1000 bp DNA marker; lanes 1,2,4 *C. parvum*; lane 3 mixed infection of both *C. parvum* and *C.hominis*.

DISCUSSION

Diarrhea is a major contributor to morbidity and mortality of children worldwide and is associated with more than 30% of hospitalizations and about 11% of deaths in children 5 years old (Derby *et al.*, 2014). Accurate detection of the causes of diarrhea is of paramount importance not only for effective treatment but also to adopt appropriate programs for control. In a recent study which involved 3439 children from 7 developing countries in Asia and Africa, *Cryptosporidium* was found among four most important pathogens which cause diarrhea (the other three pathogens were rotavirus, enterotoxigenic *Echerichia coli* producing heat stable toxin and *Shigella*) (Kotloff *et al.*, 2013).

As there are two important species of *Cryptosporidium* which are responsible for human infection (*C. parvum* and *C. hominis*), it is very important issue to determine the species of this protozoan in clinical cases even though the treatment could be identical. That is because such discrimination could determine the source of infection. It is well documented that *C. parvum* can transmit between human and other mammals especially bovine, while *C. hominis* is almost restricted to human (Mogan-Ryan *et al.*, 2002). The current study was not intended to investigate the prevalence of cryptosporidiosis among children; rather it was aimed to determine the species of this parasite in diarrheic children. Accordingly limited number of clinical cases were used and indicates that all the four positive cases were of bovine origin, while one of these cases was of mixed infection. This implies that in order to control this infection, it is important to control the infection in cattle or find an effective way to prevent the transmission of the infection from cattle to human. Of note, fecal samples from three cases (two for *C. parvum* and one mixed infection) were obtained from children of rural residency which involves a high opportunity to contact with cattle and subsequent getting the infection. Child of the fourth case was from urban residence; however, this does not exclude absolutely the chance for direct or indirect contact with cattle.

Many genes were used successfully for genotyping and differentiation of *Cryptosporidium* (Iqbal *et al.*, 2012; Tamer *et al.*, 2007; Mojarad *et al.*, 2011). However it seems that TRAP-C2 is suitable gene for species detection. That is because using this gene does not require for sophisticated and expensive methods such as DNA sequencing. Rather nested PCR and appropriate restriction enzyme are adequate to perform the task.

Despite the relatively small number of clinical cases used in this study, it can be concluded that most cryptosporidial-related diarrhea in children in Iraq are of bovine origin.

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