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Isolation and molecular identification of *Cryptosporidium* from human stool

Mohammad Sohel Rana, Farjana Boby, Md. Shahiduzzaman*

Department of Parasitology, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

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***Corresponding Author**

M Shahiduzzman
szaman@bau.edu.bd

ABSTRACT

Cryptosporidium is an intracellular zoonotic protozoan parasites that infect the gastrointestinal tract of humans and causes a diarrheal disease, cryptosporidiosis. Stool act as a great reservoir for contamination with water and oro-faecal is the most potential medium of transmission. The study was aimed to isolate and molecular identification of *Cryptosporidium* from stool samples of patients at Shurjo kanto Hospital, Mymensingh, Bangladesh. Samples were collected from children (under 6 years of age) and adult patients (above 20 years of age) having history of diarrhoea. *Cryptosporidium* were detected by the conventional Ziehl- Neelsen staining method and PCR. By microscopy *Cryptosporidium* were primarily detected positive in 10 from 30 samples, of 4(10) in children under 6 years of age and 6(20) in adults patients above 20 years of age, which were further confirmed by PCR. The overall prevalence of *Cryptosporidium* in 33% of all patients, of which 40% of children were at the age of less than 6 years and 30% were at the age of beyond 20 years old patients admitted in Shurjo kanto Hospital, Mymensingh, Bangladesh. Altogether, the study revealed that age has significant effects on the occurrences of the *Cryptosporidium* infection that causes diarrhea. However further studies are needed to investigate the presence of *Cryptosporidium* in more patients throughout the year in order to better understanding about the status of *Cryptosporidium* infection in human.

INTRODUCTION

Cryptosporidium is an opportunistic protozoan parasite of humans and animals worldwide and causes diarrheal disease that is typically self-limiting in immune-competent hosts but often life threatening to immune-compromised individuals (Del Coco et al., 2016). The genus *Cryptosporidium* consists of different species and genotypes which infect a wide range of hosts, including humans. The parasite is ubiquitous and lack of differentiation between the species and strains has made it difficult to track down sources of human and animal infections (Joachim, 2004).

Widespread outbreaks of disease initiated a public and animal health problem both in developed and developing countries (Fayer et al., 2008). A recent epidemiological study investigating the cause and effect of diarrhoea in over 22,000 children (under 5 years of age), residing in four African and three Asian study sites, identified cryptosporidiosis as the second most common pathogen responsible for severe diarrhoea and was also associated with death in young children (12–23 months of age) (Kotloff et al., 2013). Globally, cryptosporidiosis is estimated to be responsible for 30–50% of the deaths in children under 5 years of age and is considered the second greatest cause of diarrhoea and death in children after rotavirus (Ochoa et al., 2004; Snelling et al., 2007; Striepen et al., 2013).

In parts of Asia and Africa as many as 31.5% of all children under 2 years of age are infected with the parasite, posing a significant health risk (Checkley et al., 2014). Cryptosporidiosis is prevalent worldwide (Dalle et al., 2003; Leoni et al., 2007; Lake et al., 2008; Zintl et al., 2008). The disease was also found prevalent among the animal population in Bangladesh about three decades ago, (Rahman et al., 1985).

Cryptosporidium may be found in soil, food, water, or surfaces that have been contaminated with the feces from infected humans or animals (Xiao et al., 2004). The disease cryptosporidiosis is spread from person to person after the parasites are shed into the environment; they may be found in soil, food, water, or on surfaces that have been contaminated with feces from infected humans or

animals (Xiao et al., 2000; Medema et al., 2004). Due to improper and poor hygienic management *Cryptosporidium* oocysts from animal waste in barnyards, manure pits and field application can contaminate ground, feed stuff and water. In rural and semi-urban areas animals are washed in pond, lake, or river water where people usually take their bath, wash their cloths, and take the water to their house for drinking and household uses that contaminate with *Cryptosporidium* oocysts (Medema et al., 2004).

Bangladesh is a developing country and diarrhoea is one of the major problems of human at different ages in Bangladesh which may be caused by *Cryptosporidium* due to poor water purification, supply, and waste management. *Cryptosporidium* species are reported to be a significant cause of diarrhoeal illness of young children especially less than 5 years of age in Bangladesh (Rahman et al., 1990; Bhattacharya et al., 1997, Albert et al., 1999). About a decade ago, infection with *Cryptosporidium* species was reported in 1.4 - 8.4% diarrhoeal patients (Haque et al., 2003) from International Centre for Diarrheal Disease Research, Bangladesh in Dhaka, (ICDDR'B). However, clinical cases in most of places in Bangladesh are treated as remained undiagnosed diarrhoeal patients.The present study was conducted to isolate and identify *Cryptosporidium* oocysts from diarrhoeal patients at S. K. Hospital, Mymensingh, Bangladesh. Therefore, the present research work was done with the aims to isolate and identify *Cryptosporidium oocysts* from diarrhoeal patients at S. K. Hospital, Mymensingh, Bangladesh.

MATERIALS AND METHODS

Sample collection and processing

A total of 30 diarrhoeal stool samples were collected from Shurjo Kanto (S. K) Hospital, Mymensingh, Bangladesh. Of them 20 were collected from adult patients (above 20 years of age) and 10 were collected from children (under 6 years of age). The samples were brought to the laboratory of the Department of Parasitology, Bangladesh Agricultural University, Mymensingh and preserved at 4˚ C until use. Samples were identified as positive by observing the presence of oocysts of *Cryptosporidium* at least 2-3 in foci on at least 3 slides. The samples were then washed 2 times with distilled water and oocysts were concentrated by flotation techniques using saturated salts solution. The presence of oocysts were initially examined under following stained with modified Ziehl-Neelsen technique (Henriksen and Pohlenz, 1981) and examined under microscope. The positive samples were stored at -20˚ C until use for DNA extraction.

DNA extraction and PCR

The samples were subjected to DNA extaction by PureLink® Genomic DNA Kits. A primary PCR was performed by targeting the SSU rRNA gene of *Cryptosporidium* to confirm the detection of *Cryptosporidium* from stool sample according to the protocol described by (Xiao et al., 1999) with some modification. A PCR product of 1325 bp from target gene was amplified by forward primer (5´-TTCTAGAGCTAATACATGCG-3´) and forward primer (5´-CCCATTTCCTTCGA AACAGGA-3´) (IDT, USA). PCR reaction was performed in a total 50 µl reaction volume containing PCR Master mix (Promega®, USA), 200 nM of each forward and reverse primer and 2 µl DNA template. A total of 35 cycles were carried out, each consisting of 94˚ C for 45 seconds, 54˚ C for 45 seconds and 72˚ C for 1 minute with an initial hot start at 94˚ C for 3 minutes and a final extension at 72˚ C for 7 minutes. The PCR product was visualized in the 1.5% agarose gel by addition of ethidium bromide and examined against UV light using an image documentation system.

RESULTS AND DISCUSSION

Detection of *Cryptosporidium* by microscopy

The samples were stained by Ziehl Neelsen stain. Oval or round shaped pink color *Cryptosporidum* oocysts were observed under microscope with blue background (Figure 1, 100X). *Cryptosporidium* was found in 33% samples from Shurjo Kanto (S. K) Hospital, Mymensingh, Bangladesh.

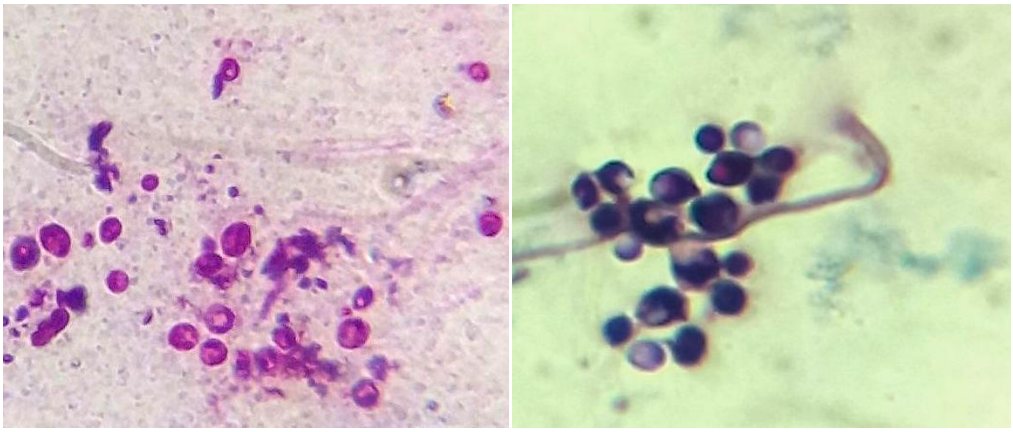

Figure 1
Oocyst under microscope 100X (oocyst of *Cryptosporidium*)

Table 1
Isolation of *Cryptosporidium* by Ziehl - Neelsen stain.

Age	Number of sample examined	Number of positive sample
Children (under 6 years)	10	4 (%)
Adults (above 20 years)	20	6 (%)
Total	30	10 (%)

Table 2
Occurrence of *Cryptosporidium* (Microscopy).

Age	Total number of sample	Nmber of positive sample	Occurrence
Children (under 6 years)	10	4	40%
Adults (above 20 years)	20	6	30%
Total	30	10	33%

The study revealed that age had significant effects on the occurrences of the *Cryptosporidium* infection that causes diarrhoea at Shurjo Kanto (S. K.) Hospital, Mymensingh, Bangladesh. Among the tested patients, 40% children under 6 years of age and 30% were infected with the *Cryptosporidium* at the age of above 20 years. The overall prevalence of *Cryptosporidium* at Shurjo Kanto (S. K.) Hospital, Mymensingh, Bangladesh is 33% due to the oro-faecal mode of transmission.

***Cryptosporidum* by PCR**

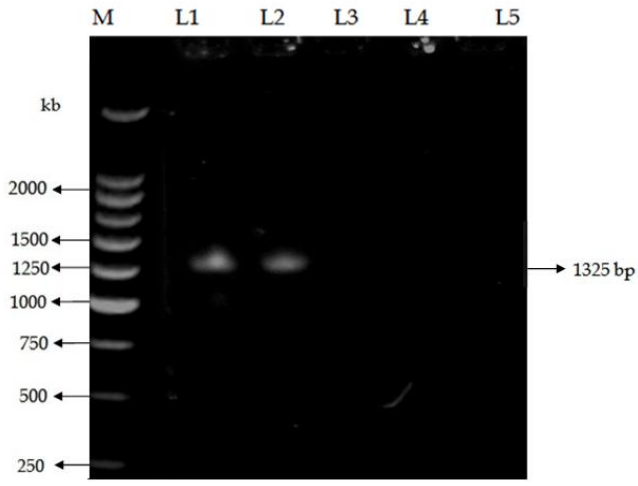

Figure 2
Confirmation of *Cryptosporidium* by PCR; Target sequence of *Cryptosporidium* is 1325 bp (lane 2). M (Ladder 1kbp), lane 1 (Positive control, DNA from Animal), lane 2 (DNA from human), lane 3 (DNA from human), lane 4 (DNA from human), lane 5 (DNA from human).

A polymerase chain reaction was used to amplify the *Cryptosporidium* DNA target. The gel image obtained from PCR products of different concentration of sample DNA demonstrated that 10 nM concentration of DNA is necessary to get clear band for detection of *Cryptosporidium*.

Only the positive samples of each category and all negative samples of each category were combined in separate tube and subjected to DNA extraction. The DNA was subjected to PCR reaction to amplify target DNA of *Cryptosporidium* (1325bp). The gel image of resolved DNA in agarose demonstrated that DNA from human samples of Shurjo Kanto Hospital (Figure 2, Lane 2) showed faint band that confirm the presence of *Cryptosporidium* infection of patients admitted in Shurjo Kanto (S. K.) Hospital Mymensingh, Bangladesh.

The overall prevalence of *Cryptosporidium* in diarrheal stool samples was 33% in this study at Shurjo Kanto (S. K.) Hospital, Mymensingh, Bangladesh. The highest prevalence (40%) was recorded in diarrheal stool of children less than 6 years. The result of our study is in conformity with the results of Khan et al. (2004) who reported 44.96% *Cryptosporidium* infection in children in Bangladesh. The findings of the study is higher than the study of Mona et al., (2013) who found *Cryptosporidium* in 22.4% diarrheal stool sample by microscopy in children. In another study of Khan et al., (2004) infections were found in 7 (25.9%) of 27 people examined where the existence of *Cryptosporidium* oocysts was confirmed by using modified Ziehl-Neelsen stain. Lower prevalence (1.7%) was also recorded by Shoaib et al. (2003). However, higher infection rate in children of this study might be due to rearing of the children especially in poor communities in unhygienic condition.

CONCLUSIONS

Cryptosporidium is prevalent among the dirrhoeal patients of Shurjo Kanto (S. K.) Hospital, Mymensingh, Bangladesh. The results confirm the presence of *Cryptosporidium* 40% in children and 30% in adults in Shurjo Kanto Hospital, Mymensingh, Bangladesh. However further studies are needed to investigate the presence of *Cryptosporidium* in more patients throughout the year in order to better understanding the status of *Cryptosporidium* infection in human.

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