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Molecular characterization of *Cryptosporidium* isolated from animal and human

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ABSTRACT

The aim of this study was molecular detection and characterization of *Cryptosporidium* isolated from animal and human stool sample. A polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) technique based on the small-subunit rRNA gene was used for the analysis of 10 positive samples (9 samples from human and 1 sample from animal). Nine human samples were collected from Shurjo Kanti (S.K.) Hospital, Mymensingh that has a diarrheal disease section and 1 animal sample was collected from Bangladesh Agricultural University campus. The samples were employed for DNA extraction using Purelink™ Genomic DNA Mini Kit (Invitrogen, USA) and then PCR was performed. Five PCR positive *Cryptosporidium* DNA were genotyped by using VspI (AseI) restriction enzyme (Lithuania, EU). *Cryptosporidium parvum,* Bovine A gene was detected in 2 samples, *Cryptosporidium parvum,* Bovine B gene was detected in 1 sample and *Cryptosporidium hominis* was detected in 2 samples. The present study confirms the *Cryptosporidium* genotype (*Cryptosporidium parvum,* Bovine A gene, *Cryptosporidium parvum,* Bovine B gene and *Cryptosporidium hominis*) in the patient admitted to Shurjo Kanti (S.K.) Hospital, Mymensingh, indicating the existence of *Cryptosporidium genotyes* in the study areas. However, further studies are needed to better understanding the transmission dynamics of these genotypes and thereafter taking necessary measure to control and/or prevent the disease.

Introduction

The protozoa under the genus *Cryptosporidium* are a zoonotic apicomplexan obligate intracellular parasites (Rossle and Latif, 2013). Cryptosporidiosis, is the term used to designate infection caused by *Cryptosporidium* spp. and is considered as one of the most common food and water borne diseases with worldwide spread. The genus *Cryptosporidium* includes a group of intracellular protozoan parasites that infect the gastrointestinal tract and other organs of mammals including human, birds, reptiles and fishes (Xiao *et al*., 1999). *Cryptosporidium* is an important cause of diarrhoeal illness and its impact is increasing both local and globally. Widespread outbreaks of disease initiated public and animal health problems both in developed and developing countries (Scott CD, 1988).

The infection is usually self-limiting in immune-competent individuals, but fatal in immuno-compromised individuals, e.g., acquired immune deficiency syndrome (AIDS) or leukaemia patients, taking immunosuppressive agents, malnourished children and elderly individuals (Mohandas *et al* 2002). Cryptosporidiosis is prevalent worldwide (Dalle *et al.,* 2003; Leoni *et al.,* 2007; Lake et al., 2008). *Cryptosporidium* is the most notorious one among more than 150 potentially waterborne pathogens (WHO, 2004), that causes gastrointestinal illness in a varitey of mammals. Water and stool is the great reservoir for contamination and potential medium of transmission of the parasite.

Cryptosporidium spp. 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 were reported in about 1.4 -8.4% of diarrhoeal patients (Haque *et al.,* 2003; Khan *et al.,* 2004) from International Centre for Diarrheal Disease Research, Bangladesh (ICDDR'B) in Dhaka. On the other hand, about three decades ago, the disease was also found prevalent among the animal population in Bangladesh (Rahman *et al.,* 1985). Clinical cases in most of places in Bangladesh are treated as undiagnosed diarrhoeal patients. Despite enormous effect on animal and human health, presently very little attention has been paid to determine the present status of the infection in Bangladesh.

Cryptosporidium oocysts, which are excreted by infected animals and humans, are commonly found in lakes, rivers and streams that serve as drinking water sources. *Cryptosporidium* oocysts are resistant to normal environmental condition and common disinfectants. Traditional epidemiological investigation only provides information on the prevalence of this parasite but does not provide any information for tracking infection sources and or transmission dynamics of cryptosporidiosis (WHO, 2004). Molecular epidemiologic studies using genotyping and sub typing tools have led to better appreciation of the public health importance of *Cryptosporidium* species/genotypes in various animals and improved understanding of infection sources in humans. Geographic, seasonal and socioeconomic differences in the distribution of *Cryptosporidium* in humans have been identified, and have been attributed to differences in sources of infection and routes of transmission.

The recent molecular tools for genotyping and sub typing analysis have greater advantages over the traditional approach for determining the infection potential of *Cryptosporidium* species in animals and humans and also for tracking the contamination sources of cryptosporidiosis. The small subunit (SSU) rRNA gene of Cryptosporidium was usually targeted to detect and differentiate *Cryptosporidium* from animal and human sources (Xiao *et al.,* 1999). The recent epidemiological data provided information on the infection potentials and transmission dynamics of *Cryptosporidium* in developed and developing countries. Bangladesh is one of the developing countries with poor water purification, supply, and waste management but *Cryptosporidium* is endemic, nonetheless, molecular epidemiological investigations are yet to be performed to determine reliable, solid and applicable information.

Genotyping is the process of determining differences in the genetic make-up (genotype) of an individual by examining the individual's DNA sequence using biological assays and comparing it to another individual's sequence or a reference sequence. It reveals the alleles an individual has inherited from their parents. Traditionally genotyping is the use of DNA sequences to define biological populations by use of molecular tools. It does not usually involve defining the genes of an individual (Follet et al., 2011).

Current methods of genotyping include restriction fragment length polymorphism identification (RFLPI) of genomic DNA, random amplified polymorphic detection (RAPD) of genomic DNA, amplified fragment length polymorphism detection (AFLPD), polymerase chain reaction (PCR), DNA sequencing, allele specific oligonucleotide (ASO) probes, and hybridization to DNA microarrays. A restriction enzyme (or restriction endonuclease) is an enzyme that cuts DNA at or near specific recognition nucleotide sequences known as restriction sites. Restriction enzymes are commonly classified into three types, which differ in their structure and whether they cut their DNA substrate at their recognition site, or if the recognition and cleavage sites are separate from one another. To cut DNA, all restriction enzymes make two incisions, once through each sugar-phosphate backbone (i.e. each strand) of the DNA double helix (Leoni et al., 2007). For genotyping of *Cryptosporidium,* VspI restriction enzyme is usually used to identify the different *Cryptosporidium* spp. in the sample.

Molecular epidemiological studies have significantly improved our knowledge on cryptosporidiosis. Recently, molecular diagnostic tools have been developed to assess the human infection potential of *Cryptosporidium* oocysts in stool and to track the sources of contamination. The study was conducted to detect *Cryptosporidium* isolated from animal and human. This study assists to confirm the types (genotypes) of *Cryptosporidium* affected both animal and human in the study area and thereafter taking necessary measure to control and prevent the disease in Bangladesh.

Materials and methods

Samples

Stool samples were collected from Shurjo Kanti (S.K.) Hospital, Mymensingh district of Bangladesh and fecal sample from cattle of Bangladesh Agricultural University, Mymensingh. The stool and fecal samples were initially processed at the collection sites and then brought to the Laboratory at the Department of Parasitology, Bangladesh Agricultural University, Mymensingh. Part of the work was done at the Laboratory of Department of Microbiology & Hygiene, and Department of Pathology, Bangladesh Agricultural University Mymensingh.

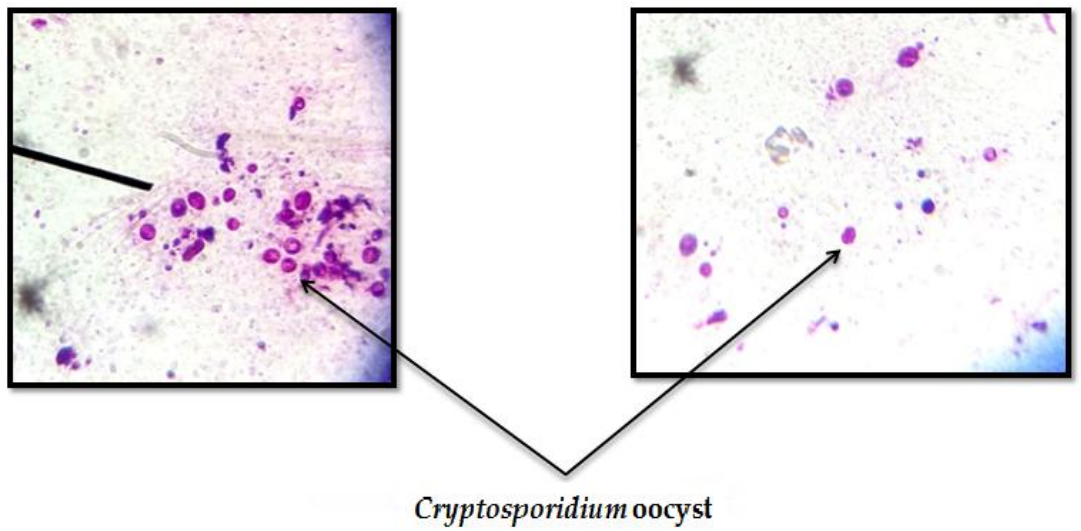

Figure 1. *Cryptosporidium* oocysts present in the stool of human (left) and cattle (right) after Ziehl-Neelsen stain (100X).

The study was conducted during the period of January 2016 to June 2016. The samples were initially screened by by Ziehl-Neelsen technique (Fig 1) and positive samples (9 samples from human and 1 sample from animal) were subjected to concentration of oocysts (Sharifuzzaman et al., 2017) and DNA extraction.

DNA extraction

Purelink™ Genomic DNA Mini Kit (Invitrogen, USA) was used to extract the DNA following manufacturer instructions.

Polymerase chain reaction (PCR)

A primary PCR was performed by targeting the Small Sub Unit (SSU) rRNA gene of *Cryptosporidium* to confirm the detection of *Cryptosporidium* from stool samples according to the protocol described by Xiao et al*.,* (1999) with some modifications. A PCR product of 1,325 bp from target gene was amplified by forward primer (5´-TTCTAGAGCTAATACATGCG-3´) and reverse primer (5´-CCCATTTCCTTCG AAACAGGA -3´), (IDT, USA). PCR reaction was performed in a total 50 µl reaction volume containing PCR Master mix (Promega, USA) 45 µl, forward primer 1 µl, reverse primer 1 µl, deionized water 1 µl, and DNA template 2 µl. A total of 35 cycles were carried out, each consisting of 94°C for 3 min in case of initial denaturation, 94°C for 45 sec in case of denaturation, 54°C for 45s in case of annealing, 72°C for 1 min in case of extension and 72°C for 7 min in case of final extension, one kb DNA ladder (Promega, USA) was used to compare the band size of target DNA.

Agarose gel electrophoresis

PCR products were analyzed in 1.5% agarose gel, stained with ethidium bromide and examined against UV light using an image documentation system.

Genotyping

For genotyping PCR products of different DNA samples were subjected to digestion by restriction enzyme. VspI (AseI) restriction enzyme (Lithuania, EU). For restriction fragment analysis, 10 µl of the PCR product was digested in a total of 25µl of reaction mixture, consisting of 1µl of VspI (AseI) restriction enzyme (Lithuania, EU), 5µl of respective restriction buffer and 9µl of distilled water at 37° C for 1 h, according to manufacturer's instructions. The digested products were fractionated on 2.0% agarose gel and visualized by ethidium bromide staining.

Results and discussion

PCR analysis showed only five *Cryptosporidium* positive samples (1325 bp, Fig. 2) out of 10 *Cryptosporidium* positive samples by Ziehl-Neelsen stained.

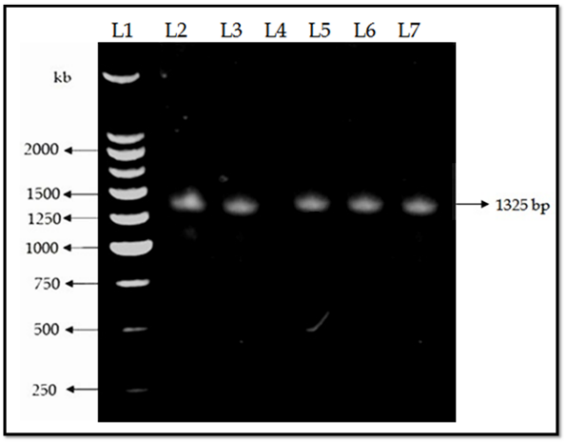

Fig. 2. *Cryptosporidium* detection by PCR. Lane 1, Molecular weight marker; Lane 2, Animal *Cryptosporidium* DNA sample; Lane3-Lane 7, Human *Cryptosporidium* DNA samples.

Digestion by VspI (AseI) restriction enzyme for genotyping

For restriction fragment analysis, PCR products of different DNA sample were subjected to digestion by VspI(AseI) restriction enzyme. The digested products were then fractionated on 2.0% agarose gel and visualized by ethidium bromide staining (Fig-3).

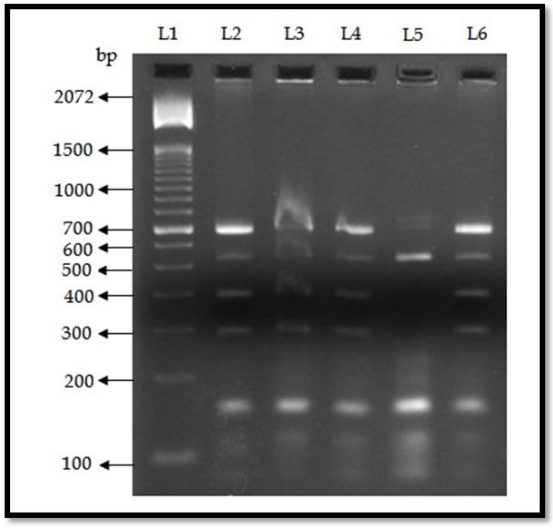

Fig 3. Genotyping by using VspI (AseI) restriction enzyme**;** Lane 1, Molecular weight marker; Lane 3, Animal *Cryptosporidium* sample; Lane 2, Lane 4, Lane 5 and Lane 6, Human *Cryptosporidium* sample.

Lane 2 and Lane 6 indicates *Cryptosporidium parvum* Bovine A gene. Lane 3 indicates *Cryptosporidium parvum* Bovine B gene. Lane 4 and Lane 5 indicate *Cryptosporidium hominis* according to RFLP profile described by Xiao *et al.,* (1999), (Fig-4).

Cryptosporidium parvum, Bovine A gene was detected in 2 samples; *Cryptosporidium parvum,*

Bovine B gene was detected in 1 samples; *Cryptosporidium hominis* (a type of *Cryptosporidium parvum*) was detected in 2 samples.

Cryptosporidium parvum, Bovine, A gene showed band of 102, 173, 303, 405, 511 and 716 bp; *Cryptosporidium parvum,* Bovine, B gene showed band size of 102, 104, 511, 628 and 716 bp; *Cryptosporidium hominis* showed band of 70, 102, 104, 407, 561 and 710 bp. In this study, Lane 2 and Lane 6 (Fig. 3) showed band size of 102, 173, 302, 403, 561 and 703 bp which is similar to *Cryptosporidium parvum,* Bovine, A gene. So it can be said that, Lane 2 and Lane 6 (Fig. 3) indicates *Cryptosporidium parvum,* Bovine A gene which source was animal origin. Lane 3 (Fig. 3) showed band size of 104, 173, 532, 642 and 723 bp which is similar to *Cryptosporidium parvum,* Bovine, B gene*,* therefore Lane 3 (Fig. 3) indicates *Cryptosporidium parvum,* Bovine, B gene which source was bovine. Lane 4 and Lane 5 (Fig. 3) showed band size of 102, 104, 412, 562, and 713 bp which is similar to *Cryptosporidium hominis*, So, it can be said that, Lane 4 and Lane 5 (Fig. 3) indicates *Cryptosporidium hominis* (a type of *Cryptosporidium parvum*) which source was human.

RFLP (in base pairs) in the SSU rRNA gene of various *Cryptosporidium* spp. and genotypes

Species	Source	PCR fragment no.	*SspI* digestion	*VspI* digestion
C. muris	Cattle, camel, hyrax	833	**385, 448**	**102, 556**
C. serpentis	Snake	831	14, 33, **370, 414**	**102**, 729, 778
C. baileyi	Chicken	826	**254, 572**	102, **104**, 620
C. felis	Cat	864	15, 33, 390, **426**	102, **104**, 182, 476
C. meleagridis	Turkey	833	11, 11, 108, **254, 449**	102, **104**, 171, 456
C. wrairi	Guinea Pig	834	11, 11, 109, **254, 449**	102, **104**, 628
Cryptosporidium sp.	Desert monitor	834	19, 33, 109, **255, 418**	102, **104**, 628
C. parvum	Human	837	11, 12, **111, 254, 449**	**70**,102,**104**,407,561,**710**
C. parvum	Monkey	835	11, 109**, 254, 461**	**70**, 102, **104**, 559
C. parvum	Bovine, A gene	834	11, 12, **108, 254, 449**	102,**173**,303,405,**511,716**
C. parvum	Bovine, B gene	831	9, **119,** 254, **449**	102, 104, **511,716**
C. parvum	Mouse	838	11, 12, **112, 254, 449**	102, **104**, 175, 457, 512
C. parvum	Dog	829	20, 33, **105, 254, 417**	94, **102**, 633
C. parvum	Ferret	837	11, 12, **111,** 254, **449**	102, **104**, 174, 457
C. parvum	Pig	838	9, 11, **365, 453**	102, **104**, 632,
C. parvum	Kangaroo, koala	837	33, **109, 254, 441**	102, **104,** 631

*Numbers in bold are the sizes of bands visible on the electrophoresis gel.

Fig. 4. RFPL (in base paris) in the SSU rRNA gene of various Cryptosporidium Spp and genotypes (Xial et al., 1999).

The present study detected the genotypes of Cryptosporidium in human and cattle in the study area by PCR-RFLP analysis. The methodologies adopted for isolation, identification of Cryptosporidium can be used for further investigation of the stool and faecal samples for better understanding the molecular characterization of *Cryptosporidium* in Bangladesh and thereafter increase understanding the transmission dynamics of Cryptosporidium in order to take necessary measures for prevention and control of this disease in human and animal in Bangladesh.

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