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Molecular characterization of *Cryptosporidium xiaoi* in goat kids in Bangladesh by nested PCR amplification of 18S rRNA gene

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PEER REVIEW

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Comments

In the current study, prevalence of *Cryptosporidium xiaoi* has been evaluated in goat kids in Bangladesh, by molecular and parasitological methods. The study describes the genotype features of this species of *Cryptosporidium* in comparison with other genotypes which have been reported from other areas of the world.
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ABSTRACT

Objective: To investigate the prevalence of *Cryptosporidium* spp. in goat kids in selected areas of Bangladesh and to elucidate the potential zoonotic hazards.

Methods: In the present study, we have used Ziehl-Neelsen staining and nested PCR approach to identify and characterize the *Cryptosporidium* sp. from diarrhoeic feces of goat kids. A total of 100 diarrhoeic feces samples were collected from Chittagong region in Southern Bangladesh. For nested PCR analysis, specific primers for amplification of 581 base pair fragments of 18S rRNA gene were used.

Results: A total of 15% and 3% samples were found positive in microscopic study and in nested PCR analysis respectively. Phylogenetic analysis of sequence data showed similarity with that of *Cryptosporidium xiaoi* recorded from sheep and goat.

Conclusions: To our knowledge, this is the first report of *Cryptosporidium xiaoi* responsible for diarrhoea in goat kids in Bangladesh. Further study can highlight their zoonotic significance along with genetic diversity in other host species inside the country.

KEYWORDS
Cryptosporidium, Nested PCR, Phylogenetic analysis, 18S rRNA

1. Introduction

Cryptosporidium has been identified as the cause of numerous outbreaks of diarrhoeal illness in human and animals including goat. While human infections are thought to be derived from animal sources such as sheep, goat and other wide range of animals, it is not yet established if there is any relationship between human and goat genotypes of *Cryptosporidium*[1,2]. In goat and goat kids, cryptosporidiosis may lead to high morbidity and mortality rate[3]. Symptoms of acute cryptosporidiosis include lack of appetite and weight loss[4]. Clinical signs are yellow diarrhoea with or without blood. Animals usually show signs of abdominal pain,

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anemia, anorexia, dehydration, tenesmus, weakness and loss of weight. Diagnosis of cryptosporidiosis is an important issue for their effective treatment and control. Alongside classical approach like microscopy, molecular tools have been developed to detect and characterize *Cryptosporidium* at the species/genotype and subtype levels. These tools have been increasingly used in characterizing the transmission of *Cryptosporidium* spp. in human and animals worldwide.

In Asia, Bangladesh has the highest population of goat and there are 20.75 million goats at present[5]. The mortality of goat and goat kids is also another major problem confronting goat rearing in Bangladesh.

The prevalence of cryptosporidiosis in sheep and goat is variable as reported in a number of previous studies. Since first report of cryptosporidial infection in goat kids appeared in Australia, several reports indicated the prevalence rate between 5% and 35%[3]. However, the zoonotic species *Cryptosporidium parvum* has been reported from goat kids in Australia, Cyprus, Zambia and Belgium[4]. Further study has also reported *Cryptosporidium hominis* and *Cryptosporidium bovis* (*C. bovis*)–like genotype in adult goat in UK and China which was later named as a new species *Cryptosporidium xiaoi* (*C. xiaoi*) based on genetic differences with other concurrent species[6-8].

Cryptosporidiosis caused by *Cryptosporidium* is a diarrheal disease outbreak worldwide and a major cause of enteric illness in man and there is significant reservoir in animals, particularly young ruminant species[9-11]. Previous reports indicated the prevalence of *Cryptosporidium* from 5% up to 77% worldwide[12-14]. In one report[4], the prevalence of cryptosporidiosis in goat kids was found as 9.5% (14 cases out of 148) in Belgium. Although a number of human cases of cryptosporidiosis have been well documented in children and adult diarrhoeal patients in Bangladesh, there is no report available about the prevalence of the *Cryptosporidium* sp. responsible for diarrhea in goat kids in the country. Until now, molecular epidemiological data concerning goat cryptosporidiosis are limited. Considering the limited data on prevalence and molecular characterization of *Cryptosporidium* especially in goat kids, the present study was undertaken to investigate the prevalence of *Cryptosporidium* spp. in goat kids in selected areas of Bangladesh and to elucidate the potential zoonotic hazards.

2. Materials and methods

2.1. Collection of fecal samples

A total of 100 fresh fecal samples were collected from goat kids of 1-6 months old from a local veterinary clinic in Chittagong, Bangladesh. All were affected with diarrhoea when admitted to the clinic. Feces were collected directly from the rectum of each goat into a plastic specimen cup followed by immediately closed and labeled properly. Feces were preserved in -20 ˚C freezer until DNA extraction was carried out.

2.2. Ziehl-Neelsen staining technique

This technique was used for the detection of oocysts of *Cryptosporidium* species in faeces as described before[15]. During this study, this classical technique was used to screen fecal samples prior to DNA extraction and PCR based diagnosis.

2.3. DNA extraction

Genomic DNA was extracted from preserved fecal samples, using the QIAamp stool mini kit (Qiagen) according to manufacturer's instructions and stored at -20 °C. The DNA concentration was measured by Thermo Scientific Nanodrop 2000® spectrophotometer (A339, USA).

2.4. Nested PCR analysis

Nested PCR analysis was performed to identify 18S rRNA gene locus in *Cryptosporidium* species. Forward and reverse primers, namely, 18 SiCF2 (GAC ATA TCA TTC AAG TTT CTG ACC) and 18 SiCR2 (CTG AAG GAG TAA GGA ACA ACC) have been used in primary PCR reaction to amplify about 763 bp target DNA fragments. In addition, secondary PCR reaction has performed by using forward primer 18 SiCF1 (CCT ATC AGC TTT AGA CGG TAG G) and reverse primer 18 SiCR1 (CTA AGA ATT TCA CCT CTG ACT G) in order to amplify about 581 bp target DNA fragments[16]. All the reaction mixtures and cycling conditions were same as previously published standard protocols[16]. In each experiment, positive control was carried out as standard *Cryptosporidium* genomic DNA along with sample run without DNA template as negative control element. A PCR thermal cycler (Applied Biosystem 2720 Thermal cycler®) was used in both primary and secondary PCR reaction cycles and the products were analyzed by 1.5% agarose gel electrophoresis.

2.5. DNA sequencing and submission

The PCR products of representative strains from each restriction group were purified with the PCR Clean-Up kit (Promega®, USA) and were sequenced on an ABI sequencer (ABI Prism, 3130, USA). After sequencing of the representative nested PCR products, the quality of the sequence was carefully assessed manually based on the corresponding electro-chromatogram and have submitted in GenBank NCBI database. The final nucleotide sequence data reported in this paper are available in the GenBank under the accession numbers JX485731-JX485732.

2.6. Construction of phylogenetic tree

To compare nucleotide variability and genetic relationship of 18S rRNA gene sequences of goat kids in Bangladesh (accession No. JX485731 and JX485732) with other infected animals in different

countries, a phylogenetic tree was constructed. During this study, top 20 BLAST derived hits of significant E-value of 18S rRNA gene sequences of JX485731 and JX485732, respectively were retrieved. Regenerative sequences of hits have eliminated in order to get non-redundant 18S rRNA gene sequences. The sequences were retrieved from GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) and were initially aligned under default conditions by using MUSCLE program (http://www.ebi.ac.uk/Tools/msa/muscle/)[17,18]. Furthermore, identical conserved regions of sequences scored in the alignment were used to generate the tree. The sequence alignment was performed under default conditions and the tree was constructed by the neighbor-joining method[19]. The poorly aligned sequence and also the internal gaps residue were taken off from the alignments to make a precise evolutionary tree by using the Jalview program[20]. Finally, the phylogenetic tree of 18S rRNA gene sequences was constructed by using MUSCLE phylogeny program (http://www.ebi.ac.uk/Tools/msa/muscle/).

3. Results

3.1. Microscopic identification

Initially after sampling the feces from diarrhoeic goat kids, classical tools like Ziehl-Neelsen stain were used to identify *Cryptosporidium* positive cases. Through microscopy, only 15 samples were found as positive out of 100 samples tested. On another note, susceptibility of goat kids were investigated based on their sex. It was recorded that out of 100 goat kids, 49 goat kids were male and remaining 51 were female goats. The data indicates that among 15 positive cases, only 6 cases were found in male while rest 9 cases of cryptosporidiosis were found in female goat.

3.2. Nested PCR amplification of 18S rRNA gene

The 18S rRNA gene was amplified from individual genomic DNA samples by nested PCR assay during this study. The PCR analysis of 100 isolates resulted only 3% (n=100) have given positive bands. Moreover, it was observed that among 3 positive isolates, 2 were female and 1 was male. This was identified by observing the band with respect to marker on 1.5% agarose gel on the basis of 753 bp in primary PCR and 581 bp in secondary PCR, respectively. Figure 1 shows the nested PCR result, where positive isolates of *Cryptosporidium* have visualized as distinct bands.

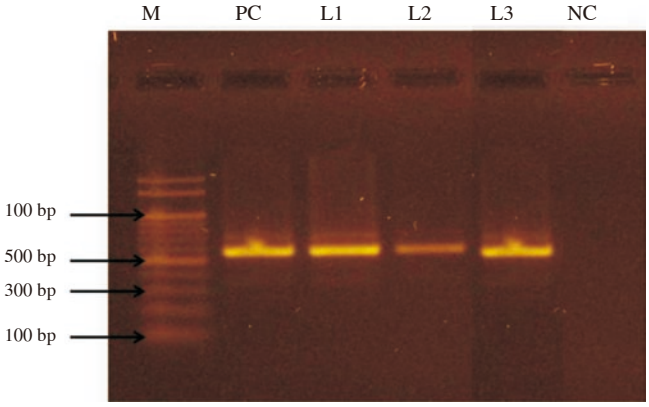

Figure 1. Electrophoretic (1.5% agarose) separation of 18S rRNA gene. Lane M: ladder (Marker); lane PC: positive control; lane L1: isolate N15; lane L2: isolate N18; lane L3: isolate N95; lane NC: negative control.

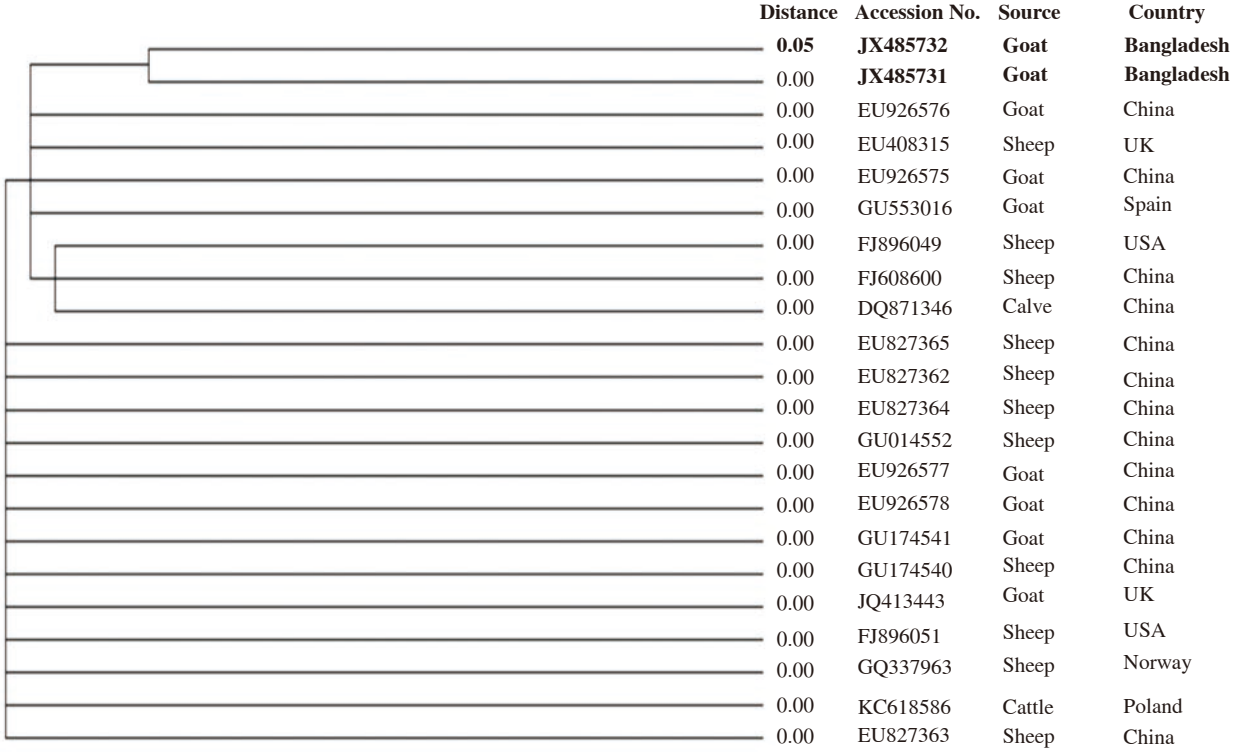

Figure 2. The genetic relationship of *Cryptosporidium xiaoi* inferred from 18S rRNA gene sequences following analysis using neighbor-joining method of ClustalW2-Phylogeny.

3.3. Sequencing of the amplicon

The PCR product was sent for DNA sequencing and the sequence data were used to conduct BLAST analysis (in the NCBI website, http://blast.ncbi.nlm.nih.gov/Blast.cgi), to characterize the *Cryptosporidium* positive isolates. The DNA sequence of three different isolates was found similar and BLAST analysis revealed that there was a 98% sequence similarity (520 nucleotides out of 531 nucleotides) with that of *C. xiaoi* which was earlier reported in Spain, Mexico and Italy. The search parameters were database: others (excluding human and mouse genome), and optimization: highly similar sequence. After BLAST searching, it was found that the highest similarity was with that of GenBank accession No. GU5530161.1. All the other hits having high *P* value shows the same species of *C. xiaoi* while a number of hits indicate similarity with *C. bovis* genotype. However, it is notable that all the hits were matched with sequence of 18S rRNA gene of *Cryptosporidium*.

3.4. Construction of phylogenetic tree

Figure 2 shows dendogram tree, depicted an intragenotypic variation of 18S rRNA gene sequences JX485731 and JX485732. The degree of variability noticed among the goat isolates in 18S rRNA gene sequences as they might have different orthologus origin, respectively. Although the genetic distance between JX485732 and JX485731 from goats was 0.05, they were closed with each other in the cluster. The cluster was genetically related with another cluster consisting of EU926576, EU408315, EU926575 and GU553016 from goat (China), sheep (UK), goat (China) and goat (Spain), respectively. It postulates the genetic polymorphism scenario of *Cryptosporidium* which not only depends on specific host but also their origin of localization. The cluster having three gene sequences of FJ896044, FJ608600 and DQ871346 was closely related with former two clusters. Finally, other remaining sequences were so diverse and descended from these three clusters. But, all sequences might be descended from this JX485732 as it has most genetic distance 0.05. However, pair wise genetic distances among all isolates were ranged from 0.000 to 0.050 with an average mean value 0.002, which indicates the proximity of all isolates during evolution. The rate of polymorphism was not too high in terms of lower genetic distances observed. Gene flow might occur during evolution of *Cryptosporidium* and might cause different haplotypes in animals from different countries. Therefore, the variable sequence of 18S rRNA genes could be a better characterizing tool to discriminate these species from each other. Such genotyping could play a vital role to elucidate the pathogenicity, epidemiology and ultimately the mechanisms of host-parasite interactions.

4. Discussion

Cryptosporidiosis in goat kids has been reported in several countries by a number of investigators. The incidence and prevalence rates vary widely according to the sample size and geographic distribution. The prevalence rate recorded during this study shows that 15% in goat kids (15 out of 100) was positive for *Cryptosporidium*. This finding was based on microscopic examination by Ziehl-Neelsen stain which is not 100% confirmatory. This is because not only cysts of *Cryptosporidium* but other organisms might interfere with the the test results. These include several acid fast bacteria including *Mycobacterium* sp. This rate of prevalence was somewhat comparatively low with other investigators such as 23% in UK[21], 24% in Romania, 40%-70% in Spain, 5.1% in lambs and 7.1% in goat kids in Greece and 24.2% in China[22-25]. Again this was comparatively higher as in Belgium where cryptosporidiosis was reported only in 9.5% diarrhoeic goat kids. However, the rate was 20% in Trinidad and Tobago and 17.6% in Iran which indicates that further study can ensure actual prevalence of cryptosporidiosis in goat kids in Bangladesh. It can be postulated that the comparative high rate of infection in different countries is due to high level of environmental contamination in Belgium and Spain which is usually very different in Bangladesh. Most small ruminants like sheep and goat in Bangladesh have little access to pasture and usually they are reared in semi-intensive method where there is little opportunity for the young animals to be infected by the contaminated oocysts. Due to lack of information in the subcontinent, we were unable to compare the occurrence of cryptosporidiosis in other small ruminants in the Indian subcontinent or Southeast Asia.

Considering the relative susceptibility, our data indicated that Jamunapari goat kids are more vulnerable to cryptosporidiosis compared to the Black Bengal goat kids. This can highlight new thoughts on breed-associated immunologic factors and further research can answer if this can increase our understanding in developing new methods of immunoprophylaxis. A large number of articles have been published on the development of DNA vaccine but still now none was completely successful. Future research should be directed to elucidate breed-specific factors that may lead to consideration of a vaccine against cryptosporidiosis.

Concerning the sex-specific vulnerability, our data showed that female goat kids were more vulnerable compared to their male counterpart. This could be associated with different hormones that may contribute to variable susceptibility of different goat kids to this infection. This observation is also important as most of the marginal farmers in Bangladesh prefer rearing female goats and reduction of morbidity and mortality due to cryptosporidiosis would be crucial to help them reducing poverty. Further research can identify the biological factors that are responsible for this sex-specific susceptibility.

When considering the age of different animals, it was revealed that goat kids of 3 months old are most vulnerable group and older goat kids are less susceptible than younger ones. This age-specific incidence could be useful in effective planning to control cryptosporidiosis in a farm or region. Further study can develop our understanding of the other associated factors such as management system, geographical and seasonal differences that may lead to better understanding of the epidemiology of cryptosporidiosis in

Bangladesh.

To our knowledge, this is the first molecular investigation of *Cryptosporidium* in goat kids in Bangladesh. The detection of *Cryptosporidium* by PCR based method is more confirmatory than traditional technique involving specific stains such as modified cold Ziehl-Neelsen stain. Although classical approach is less expensive and no highly technical instruments and facility will be required, the sensitivity of the test is compromised and they might lead to false positive result. Therefore, modern molecular tools might replace the traditional approach provided all the technical facilities are available.

During the present study, nested PCR approach was followed which is more reliable than single step PCR as it excludes the probability of getting non-specific PCR bands. A number of studies earlier reported nested PCR approach where 18S rRNA gene was amplified for molecular characterization of different assemblages. Further amplification of other *Cryptosporidium* genes such as GP-60 could be more informative in exploring the epidemiology of cryptosporidiosis in Bangladesh.

Few studies have genotyped *Cryptosporidium* from goats around the globe. It was thought that there is a *Cryptosporidium* goat genotype in goats[2]. There are minor genetic differences in the SSU rRNA gene of *C. bovis* among cattle, sheep, and goats. Data analysis during this study indicates the identified species as *C. xiaoi* which was first reported[9]. However, in all earlier reports it was described as *C. bovis* genotype and therefore after BLAST searching, we have seen a number of hits corresponding to *C. bovis*. While those are from different geographic regions like Spain, UK, China and in other hosts like sheep and lambs, we can assume that these are all the same *C. xiaoi* genotypes as the DNA sequences have significant level of sequence similarity. Further analysis in sheep and lambs in Bangladesh can ensure the existence of this species which are responsible for diarrhoea in those small ruminants.

To our knowledge, this is the first report of *C. xiaoi* in goat kids in Bangladesh. However, onle few other investigators have reported this species in several other hosts like sheep, fish, pig and kangaroo[1,26-30]. Further sequencing of *Cryptosporidium* spp. from different other animals in Bangladesh can increase our understanding whether there are other hosts available in the country who do harbor this protozoon.

One of the aims of this investigation was to assess whether the goat kids are responsible for zoonotic infections in Bangladesh. As our data showed that only *C. xiaoi* is available in the study area which is again not a zoonotic species, we can assume that goat kids are not an important source of environmental contamination of *Cryptosporidium*. However, as cattle and buffalo calves are very much susceptible to cryptosporidiosis, further analysis of feces from diarrhoeic calves may indicate whether zoonotic species are available in those animals. Another important work would be screening of municipal water sources to track any contamination from different dairy farms where oocysts are washed out through rain water which ultimately lead to human infection.

It was found that younger, Jamunapari breed and female goat kids are more vulnerable than older, Black Bengal breed and male goat kids, respectively. Occurrence of *C. xiaoi* during this study suggests that zoonotic transmission of *Cryptosporidium* from goat kids could be of low epidemiological significance. However, further study on epidemiological investigation of humans, domestic and wild animals as well as water catchment areas and drinking water sources is necessary to acquire better information about the prevalence, host affiliations and geographical distributions of the different genotypes of *Cryptosporidium* in animals from different countries.

Conflict of interest statement

The authors declared that they have no competing interests.

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Comments

Background

Cryptosporidium is a protozoan parasite of human and livestock. Different species and strains have been reported all over the world in human, cattle, sheep, goats and other animals. The authors are describing, for the first time in Bangladesh, a species of the parasite in goat kids by microscopic and molecular methods.

Research frontiers

This is the first report of *C. xiaoi* isolated and characterized from goat kids in Bangladesh.

Related reports

There are quite a few studies which described the prevalence and features of *Cryptosporidium* species in different areas of the world including Bangladesh. However, *C. xiaoi* has not been previously reported from this country and this is the main findings of this study.

Innovations and breakthroughs

Isolation and characterization of *C. xiaoi* was responsible for diarrhoea in goat kids and they are not responsible for human infection.

Applications

The study has a veterinary application since *C. xiaoi* is not a zoonotic species of *Cryptosporidium*.

Peer review

In the current study, prevalence of *C. xiaoi* has been evaluated in goat kids in Bangladesh, by molecular and parasitological methods. The study describes the genotype features of this species of *Cryptosporidium* in comparison with other genotypes which have been reported from other areas of the world.

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