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Molecular characterization of *Giardia intestinalis* assemblage E from goat kids in Bangladesh

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PEER REVIEW

Peer reviewer

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Comments

This is a good study in which the authors characterized the protozoan parasite *G. intestinalis* in goats. The results suggested that the zoonotic transmission, from goat kids, in Bangladesh is low. The results are interesting, especially when compared to previous studies in the field.

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ABSTRACT

Objective: To molecularly characterize *Giardia* in goat kids to elucidate the possible zoonotic hazards in Bangladesh and find out the role of *Giardia* protozoan parasite as a source of human infection.

Methods: Fecal samples of 100 goat kids were genotyped by nested PCR amplification of β-giardin gene fragment followed by sequencing and analysis.

Results: The total prevalence of *Giardia* in goat kids was 3% (3/100) and the infection is more widespread in younger (P = 0.36), Black Bengal breed (P = 0.81) and female goat kids (P = 0.58). Further analysis of *β-giardin* gene locus has shown that the gene clustered in assemblage E rather than assemblages A and B.

Conclusions: The present study suggests the low zoonotic transmission frequency from the goat kids and and giardiasis has least epidemiological significance to humans. Further study on this field is prerequisite in terms of broad geographical areas, age groups, sex and evaluation of zoonotic significance along with genetic diversity in other host species as well.

KEYWORDS

Genotype, *Giardia*, Giardiasis, Nested PCR, Phylogenetic tree, Sequencing

1. Introduction

Giardia intestinalis (*G. intestinalis*) is the most common pathogenic intestinal parasite of humans and mammals worldwide, and is a frequent cause of endemic and epidemic diarrhea, called giardiasis[1]. It becomes difficult to identify giardiasis with signs, symptoms and sensitive molecular characterization tools. Many symptoms are associated with the disease, *e.g.* significant malabsorption, diarrhea, consistent weight loss, failing to grow vigorously[2]. Immune-suppressed individuals allow the parasite to cause and spread infections more easily and often become most vulnerable hosts of parasites[3], even those with limited pathogenicity. Zoonotic transmission of *Giardia* sp. may occur from infected animals to humans through many ways like contamination of aquatic flora and fauna, environment and so on, and directly enter into human food chains[4]. Giardiasis caused by *G. intestinalis*

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is considered as a zoonotic disease, since it contaminates and infects zoonotic living systems.

G. intestinalis is currently classified into eight assemblages (A to H) on the basis of molecular characterization[5]. Generally assemblage E called hoofed assemblage is considered as the predominant gynotype of *G. intestinalis* in calves[6-8], sheep and goats[9-11], but assemblage A and B have also been observed in two ruminant species: sheep and goats[7,8,11,12]. Currently, there is considerable evidence on the epidemiology of *Giardia* infection in ruminant[9,13], especially goats in some countries[10-12], but more studies are necessary to disclose their prevalence in zoonotic transmission to human.

In Asia, Bangladesh has the largest population of goat and has 20.75 million goats at present[14]. The prevalence of *Giardia* infection in human was studied previously in Bangladesh[15]. However, no study has been put forth to evaluate involvement of goats in the *G. intestinalis* epidemiology and transmission mechanism for humans. Many risk factors are thought to be associated with *G. intestinalis* transmission in goats, *e.g.* age, housing of animals without separation of age groups, humidity, type and quality of food, hygienic condition and construction of installations inadequate for the type of animal production[16]. Therefore, the present study has focused on molecular characterization of *Giardia* in goat kids to elucidate their possible zoonotic hazards in this region, according to different age, breeds (Black Bengal and Jamunapari) and sex. In addition, we have compared the evolutionary relationship of *Giardia* infection with other *Giardia* infection not only in goats but also in other animals in different countries. It will help to understand actual epidemiological picture in terms of zoonotic potentials of *Giardia* infection in humans in different countries.

2. Materials and methods

2.1. Collection of fecal samples

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

2.2. DNA extraction

Genomic DNA was extracted from fecal samples, using the QIAamp stool mini kit (Qiagen) according to manufacturer's instructions and stored at -20 ℃. Extracted DNA concentration was measured by Thermo scientific NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

2.3. Nested PCR analysis

Nested PCR analysis was performed to identify *β-giardin* gene locus in *G. intestinalis*. Forward and reverse primers namely G7 (5'-AAGCCCGACGACCTCACCCGCAGTGC-3') and G759 (5'-GAGGCCGCCCTGGATCTTCGAGACGAC-3') have been used in primary PCR reaction to amplify about 753 bp target DNA fragments[17]. In addition, secondary PCR reaction was performed by using forward primer G7n (5'- GAACGAGATCGAGGTCCG -3') and reverse primer G759n (5'- CTCGACGAGCTTCGTGTT -3') in order to amplify about 511 bp target DNA fragments[18]. All the handling of reaction mixtures and cycling conditions were same as that of previously published standard protocols[18]. In each experiment, positive control was carried out as standard *Giardia* 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.

2.4. 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 the sequences have been submitted in GenBank NCBI database[19].

The final nucleotide sequence data reported in this paper are available in the GenBank under the accession numbers JX122074, JX122075, and JX122076.

2.5. Determination of specific genotypes and sub-genotypes

Specific assemblage of *Giardia* positive isolates was determined by comparing the sequences using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST). The variations of nucleotide position at different points were revealed using ClastalW2 multiple sequence alignment program (www.ebi.ac.uk/Tools/msa/clustalw2/) to classify genotype into subgroups.

2.6. Construction of phylogenetic tree

To compare nucleotide variability and genetic relationship of *β-giardin* gene sequences of goat kids in Bangladesh (Genbank: JX122074, JX122075 and JX12076) with that of other infected

animals in different countries, a phylogenetic tree was constructed. During this study top 23 BLAST hits with significant E-value (0.00) that corresponds to the sequence of *β-giardin* gene (including our submitted sequences JX122074, JX122075 and JX12076) were retrieved. Regenerative sequences of hits were eliminated in order to get non-redundant *β-giardin* gene sequences. The sequences were retrieved from GenBank database (http://www.ncbi.nlm.nih.gov/genbank/)[19], and were initially aligned under default conditions by using MUSCLE program (http://www.ebi.ac.uk/Tools/msa/muscle/)[20]. Furthermore, identical conserved regions of scored sequences 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[21]. The poorly aligned sequence and the internal gaps residue were taken off from the alignments to make a precise evolutionary tree by using the Jalview program[22]. Finally, the phylogenetic tree of *β-giardin* gene sequences was constructed by using ClustalW2-Phylogeny program (http://www.ebi.ac.uk/Tools/msa/clustalw2/)[23].

2.7. Data management and statistical analysis

The animal data (age, sex and breed) was imported, stored and coded accordingly using Microsoft Excel 2007 to STATA/IC-11.0 (Stata Corporation College Station) for analysis. Descriptive statistics was expressed as proportion with 95% confidence interval. Result was expressed in percentage with *P* value for *Chi*-square test. Significance was determined when $P < 0.05$.

3. Results

The *β-giardin* gene was amplified from individual genomic DNA samples by nested PCR assay during this study. The PCR analysis of 100 samples indicated that only 3% ($n = 100$) have given positive bands; the total prevalence of *Giardia* in goat kids was 3% (3 out of 100 cases) and the infection is more widespread in younger ($P = 0.36$), Black Bengal breed ($P = 0.81$) and female goat kids ($P = 0.58$) (Table 1). 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 511 bp in secondary PCR, respectively (Figure 1).

Table 1

Status of three goats having *β-giardin* gene of *Giardia* spp. in the present study.

Variables		Prevalence (*n*)	Pearson *Chi*-square value	*P* value
Breed	BB (*n*=80)	2.50 (2)	0.0573	0.811
	JP (*n*=20)	5.00 (1)		
Age (months)	1 (*n*=69)	1.45 (1)	2.0240	0.363
	2 (*n*=19)	10.52 (2)		
	3 (*n*=12)	0.00		
Sex	Male (*n*=28)	3.57 (1)	0.3038	0.582
	Female (*n*=72)	2.78 (2)		

BB: Black Bengal; JP: Jamunapari. Data was significant when $P < 0.05$.

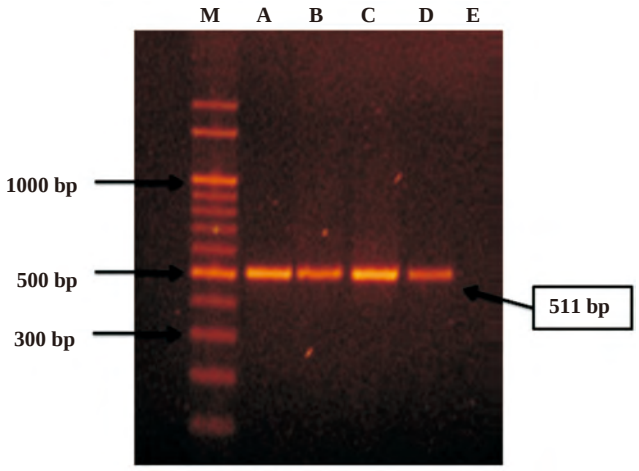

Figure 1. Electrophoretic (1.5% agarose) separation of *β-giardin* gene. Lane M: Ladder (marker); Lane A: Positive control; Lane B: JX122074; Lane C: JX122075; Lane D: JX122076; Lane E: Negative control.

The secondary PCR product of 3 representative strains were sequenced and deposited in GenBank database under following accession numbers Genbank: JX122074 (isolate N76), JX122075 (isolate N90) and JX122076 (isolate N94), respectively. The three sequences belongs to assemblage E, as confirmed by nucleotide BLAST available at NCBI databases. Furthermore, we have classified this single assemblage E into two sub-genotypes (E1 and E2) on the basis of similarities and identities of *β-giardin* gene sequences by using multiple sequence alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2). Two sequences (Genbank: JX122074, JX122075) were similar except one sequence (Genbank: JX122076) showing genetic polymorphism with one to four base variations at 14 nucleotide sites at positions 127, 131, 133, 135, 151, 164, 169, 172, 173, 198, 199, 201, 223 and 300 (data not shown). JX122074 and JX122075 have shown subtype E1 and have 100% similarity with conserved identical nucleotides of the positions, whereas JX122076 has been considered as subtype E2, and shown 97% similarity with other standard sequences of infected animals from different countries available with following Genbank: EU189375, EU189366, EU189361, DQ116625, DQ116620, DQ116614, DQ116608 and DQ1166O7. The sequence Genbank: p0-JX122076 has shown variations at 14 nucleotide sites due to its genetic mutations and diversity during evolution.

Figure 2 shows dendrogram tree, depicted an intragenotypic variation of *β-giardin* gene sequences Genbank: JX122074, JX122075 and JX122076. The degree of variability was noticed among the goat isolates in *β-giardin* gene sequences as they might have different orthologous origin, respectively. Isolate N76 (Genbank: JX122074) from goat is closely related with Genbank: EU189366 and Genbank: GQ337971 from goat and sheep, respectively. And isolate N90 (Genbank: JX122075) was genetically related closely with Genbank: DQ116604 and Genbank: EU189370 from sheep and goat, respectively. But, isolate N94 (Genbank: JX122076) has got special attention, as the sequence distantly related

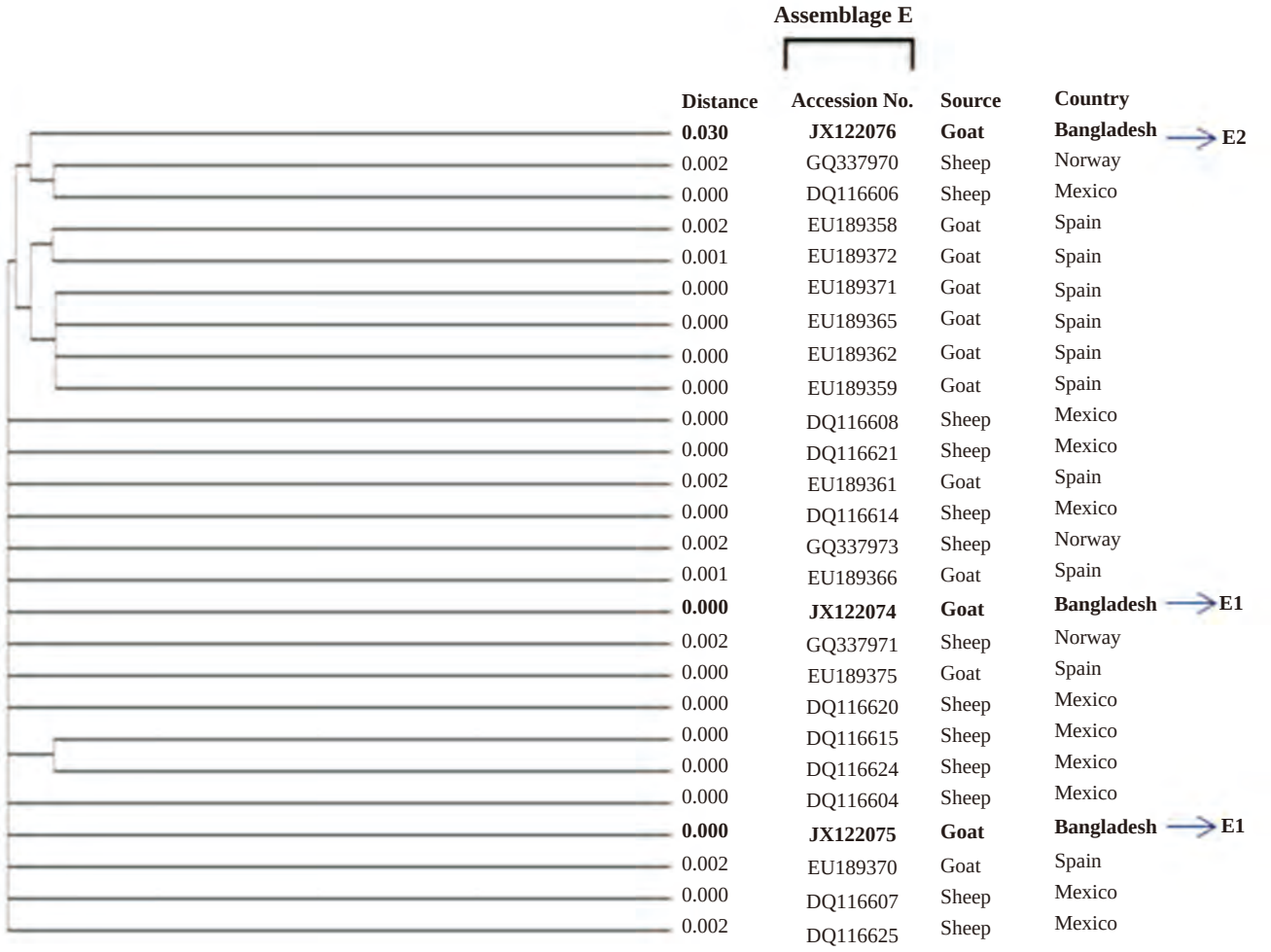

Figure 2. The genetic relationship of *G. intestinalis* inferred from *β-giardin* gene sequences following analysis using neighbor-joining method of ClustalW2-Phylogeny.

with other sequences. All sequences might be descended from this isolate N94 (Genbank: JX122076) as it has most genetic distance 0.030 among all isolates. However, pair wise genetic distances among all isolates were ranged from 0.000 to 0.030 with an average mean value 0.001, which indicates the proximity of all isolates during evolution.

4. Discussion

Samples used during this study were received from goat kids reared in Chittagong metropolitan areas. Molecular analysis revealed that the prevalence of *Giardia* was only 3% in goat kids and represent sporadic cases occurring in this part of the country. However in goat, giardiasis has been reported to occur in different parts of the world and prevalence rate was also different ranging between 6.8% and 89.2%. For example, the occurrence of giardiasis through PCR analysis was 6.8% in Malaysia[11], 89.2% in Spain[24], 25.5% in Belgium[25], 42.2% in Canary Islands, Spain[10], 12% in Maryland, USA[9]; and through microscopic observation and centrifuge flotation technique, the prevalence was 19.8% in Spain[12] and 14.3% in Brazil[26], respectively. The reasons of different occurrence rate of giardiasis such as 19.8%[12], 42.2%[10] and 89.2%[24] in Spain were the use of various techniques such as microscope[12], PCR[10] and nested PCR[24] assay, the high level environmental contamination and the stronger susceptibility of young animals than healthy adult goats. It can be proposed that the comparative high rate of infection in these countries could be due to high level of environmental contamination which is usually very different in Bangladesh, since we observed the rate only being 3%. Most small ruminants like sheep and goat in Bangladesh have little access to pasture and usually they are reared intensively or semi-intensively that it might be less possibility for the young animals to be infected by the contaminated cysts.

Age is always an important issue in any parasitic disease. Although we have assessed only young goat kids, it was interesting to see whether there is any correlation between age of goat kids and the prevalence of giardiasis. During this study, among three positive isolates, two goat kids were 2 months old and the remaining one was of 1 month of age. However, the age difference was not statistically significant in the present analysis, which might have resulted from the small sample size that have decreased the power of the statistical

test to find out a significant result if exist. Other studies observed age differences and suggested that maybe young infants have low immune complements and antibodies[10,12]. In case of cattle, it was also observed that infection of *Giardia* was highly prevalent in younger calves than in adult cattle[27]. Therefore, further study with big sample size including different age groups can be useful in future attempts to highlight the role of age in giardiasis.

To determine the relationship between goat's breed and *Giardia* infection, we have collected samples from two different local goat breeds, namely, Jamunapari and Black Bengal goat kids. We have found that Jamunapari are less vulnerable to giardiasis compared to the Black Bengal goat kids; however the association was not statistically significant. A further large scale sampling can be more informative to establish this hypothesis. Moreover, this can initiate new thoughts on breed-associated immunologic factors and future research can answer if there is any such probability or not.

Prevalence of giardiasis might occur with sex-specific preference in goat kids. We have found two female and one male infected goat kids who have shown signs and symptoms of giardiasis while association was not found statistically significant. One would assume that there is significance of sex-specific variation in illness among different groups of goat kids. Though prevalence of giardiasis regarding male and female goats are rarely studied, in case of human it was found that infection rate of *Giardia* in male and female are 1.5% and 2.18%, respectively[28]. Further study can highlight if there is any factors significantly responsible for this sex-specific vulnerability of animals as well as human suffering from giardiasis as well.

Although in Bangladesh, assemblages A and B have been associated with human infections[15], assemblage E could indirectly contribute to stimulate the zoonotic epidemiology. Assemblage E frequently found in many animal species was observed in different countries and published elsewhere[6-12], whereas recent study has suggested assemblages A and B to be present in ruminants[11]. This indicates the susceptibility of infection of assemblage E in human or vice versa. However, the assemblage E is not considered to be a strong zoonotic assemblage and therefore, giardiasis in goat could be of low epidemiological significance for possible zoonotic transmission in absence of others. The rate of infection in other small ruminants like sheep and lambs could be useful to understand the distribution of different genotypes and assemblages in any specific area. This will ultimately help to assess environmental contamination level and associated risks of *Giardia* in a particular community.

Several studies have described the intra-genotypic diversity of assemblage E in goats[10,25]. We have classified assemblage E into two subtypes (E1 and E2) on the basis of nucleotide polymorphism sites present. E1 subtype includes two isolates N76 (Genbank: JX122074) and N90 (Genbank: JX122075) and E2 has represents N94 (Genbank: JX122076). Moreover, the transmission dynamics and public health significance of the subtypes E2 need to be studied extensively for a better understanding of their unique genetic makeup. We have also analyzed the genetic diversity and/or variability of isolates N76 (Genbank: JX122074), N90 (Genbank: JX122075) and isolate N94 (Genbank: JX122076). It presents the evolutionary closed relationship with other orthologus genes from goats and sheep in Spain, Mexico and Norway. But the rate of polymorphism was not too high in terms of lower genetic distances observed. Gene flow might occur during evolution of *Giardia* and might contribute different haplotypes in animals from different countries. Therefore, the variable sequence of *β-giardin* gene in same assemblage of *Giardia* spp. 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.

It was observed that younger, Black Bengal breed and female goat kids are more vulnerable than older, Jamunapari breed and male goat kids respectively. Occurrence of assemblage E during this study suggests that zoonotic transmission of *Giardia* spp. from goat kids could be of low epidemiological significance in absence of assemblages A and B. 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 assemblages of *G. intestinalis* in animals from different countries.

Conflict of interest statement

The authors declared that they have no competing interests.

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Comments

Background

Giardiasis is a major cause of diarrheal disease in humans and animals worldwide. The zoonotic potential of its causal agent, *G. intestinalis*, has been implied for more than two decades. However, there is still a gap in the molecular characterization of *G. intestinalis* present in hosts capable of harboring zoonotic forms of the parasite.

Research frontiers

The authors performed studies in order to characterize the protozoan parasite *G. intestinalis* in goat kids in Bangladesh. They found that the prevalence of *Giardia* in goat kids was 3% and that the gene locus analyzed clustered in assemblage E rather than A and B.

Related reports

The data shows a lower number of *G. intestinalis* prevalence in this study compared to others performed in goats (Zhang *et al*., 2000; Tzanidakis *et al*., 2014; Jafari *et al*., 2014). This contrast is probably, as the authors stated in the discussion, due to the differences in the levels of environmental contamination among geographical regions. Also, it would be interesting to analyze other genes, such as triose-phosphate isomerase (Geurden *et al*., 2008).

Innovations & breakthroughs

Data regarding *Giardia* prevalence in animals in Bangladesh is scarce. Despite this study displays a low prevalence of *G. intestinalis*

in goat kids in Bangladesh, it is important to gain insight into zoonotic epidemiology and diversity of *Giardia* in the region.

Applications

It may be important to molecularly characterize *G. intestinalis* in animals in the region. The results of the present study suggest that the zoonotic transmission from goat kids is low and therefore the epidemiological significance to humans is least. However, it is important to broaden the study including other geographical areas, age groups, sex and also other host species in the region.

Peer review

This is a good study in which the authors characterized the protozoan parasite *G. intestinalis* in goats. The results suggested that the zoonotic transmission, from goat kids, in Bangladesh is low. The results are interesting, especially when compared to previous studies in the field.

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