

**MOLECULAR SURVEILLANCE OF *GIARDIA INTESTINALIS* WITH OTHER CO-
INFECTING ENTERIC PARASITES IN BARAK VALLEY, ASSAM*****Madhumita Roy^{1,2}, Joyobrato Nath^{1,3}, Shubhadeep Roychoudhury², Baby Sinha¹, Debadatta Dhar⁴**¹Department of Zoology, Gurucharan College, Silchar, Assam, India.²Department of life science and bioinformatics, Assam University, Silchar, Assam, India,³Department of Zoology, Cachar College, Silchar, Assam, India.⁴Department of microbiology, Silchar Medical college, Silchar, Assam, India.***Corresponding Author: Madhumita Roy**

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ABSTRACT

This study aims to find out the occurrences of different gastro intestinal parasites mainly *Giardia intestinalis* and its co-infection with other parasites like *Cryptosporidium sp*, *Entamoeba histolytica* and *Blastocystis* spp. in stool samples (n= 598) through microscopic analysis followed by PCR based method in a cross sectional study carried out in the Barak valley zone of Southern Assam. Of the various demographic features, prevalence was highest in the 5- 12 years age group (P= 0.0001). Our study further highlighted the rate of infection is relatively higher in case of female (P=0.3139) than that of male. In addition this study also indicated that most of the carrier of this parasite *Giardia intestinalis* does not have any diarrheal symptoms (P= 0.0095). As this study is mainly focused on the surveillance of *Giardia intestinalis*, during the investigation the occurrence of other gastro intestinal parasite was also observed but their role as a co infecting parasite is yet not clear.

KEY WORDS: Giardiasis, Demographic, Gastrointestinal, Assemblages, Epidemiology.**INTRODUCTION**

In under developed and developing countries like India disease burden and death due to intestinal protozoal infection is a great problem because of unhygienic living system and improper sanitation. *Giardia intestinalis*, the intestinal parasite with the highest prevalence followed by *Cryptosporidium parvum/ Cryptosporidium hominis*. Infection with *Entamoeba histolytica* is rare, but its high morbidity and, in particular, mortality make accurate diagnosis crucial.^[3] *Cryptosporidium spp*, *Giardia spp*. and *Blastocystis spp*. are the major diarrhea causing intestinal protozoa of human. Among those, infection with *Giardia intestinalis* is most common in the north eastern states of India, especially in the Barak valley zone of Assam as most of the slum area of this zone is situated around the Barak River causing seasonal outbreak of intestinal disease among children as well as in adults.

Giardia is a genus of intestinal flagellates that infects a wide range of vertebrate hosts. The genus currently comprises six species namely *Giardia agilis*, *Giardia ardeae*, *Giardia psittaci*, *Giardia microti*, *Giardia muris* and *Giardia intestinalis* (syn. *duodenalis*) infecting amphibians, birds, rodents and mammals.^[6] *Giardia intestinalis* is a flagellated, unicellular eukaryote that has a simple life cycle which comprises two stages: the trophozoite and cyst.

Giardia intestinalis is considered as a complex species. So far literature concern and based on genetic analysis, this protozoa has been grouped into eight assemblages (A–H). Both assemblages A and B have a wide host range and are responsible for human infections which can be transmitted zoonotically. Assemblages C–G appear to be strictly host-specific: C and D are found largely in canids, E in domestic mammals, F in cats, G in rodents and H in seals.^[9] Investigations are going on to find out the link between the infection and its severity with the different assemblages, but the results are still not satisfactory.

This study aims to find out the occurrence of *Giardia* spp. in the Barak valley region of Assam with the other co-infecting protozoan parasite such as *Entamoeba* spp., *Cryptosporidium* spp. and *Blastocystis* spp.

MATERIALS AND METHODS

Our target population comprises children without and with diarrhea those have been referred to Medicine and pediatric department of Silchar Medical college. Samples were also collected from tea garden inhabitants of Cachar, Karimganj and Hailakandi districts covering entire Barak Valley. Majority of the seasonal samples were collected through three consecutive days and considering three seasons in a year (pre monsoon, monsoon and post monsoon), so that there will be

minimum chances of error. The main objective of this study is to find out the rate of giardial infection on the mentioned population as well as the the rate of co-infection of *Giardia intestinalis* with other gastrointestinal parasites.

Study design and period

A comparative cross-sectional study based on a single fecal sample per person was conducted to figure out the true prevalence of giardiasis as well as the rate of co infection with other parasites from June 2014 to December 2015 in three districts (Cachar, Karimganj and Hailakandi) of Barak Valley zone of Assam at the levels of community, health care facilities and hospitals. A total of 598 samples were screened. All the participants' were enrolled throughout the year by systematic sampling. Samples include primary school going children and randomized human stool samples irrespective of diarrhea symptoms.

Sample preparation

In clean wide mouth capped container about 5g of fresh fecal samples were collected. The samples were collected within 2–3 h of defecation and delivered to the laboratory and divided into two aliquots. One aliquot of each of the fecal samples was used immediately for direct microscopy and the second aliquot is stored at -20°C for PCR assay. Samples from distant areas were collected in duplicate. One aliquot was preserved in 10% aqueous formalin for microscopy upon arrival in the laboratory and the rest was brought to the laboratory in unpreserved condition by maintaining temperature of approximately 4°C.^[8]

Microscopic screening

Wet preparation

To determine the presence of *Giardia* cysts or trophozoites, *Entamoeba*, and *Blastocystis*, faecal samples were concentrated by sedimentation technique were examined in Lugol-stained wet mounts. For stained preparation Trichrome stain were used for morphological confirmation.

Modified Ziehl–Neelsen staining: Presence of *Cryptosporidium*, oocysts in faecal sample were detected using the modified Ziehl–Neelsen staining technique.^[9] Briefly, it involves staining of a methanol fixed thin smear of faecal material with Kinyoun's carbolfuchsin stain for 15 minutes. Subsequently, the slide is rinsed in tap water and placed in an acid-alcohol solution to remove the stain, while acid-fast structures will resist to the acid-alcohol's destaining action. After rinsing again, the slide is placed for 5 minutes in a counter-staining product methylene blue, providing contrast between background material and acid-fast structures. The slide is rinsed once more in tap water and after that slide has been air-dried and examined under microscope using 10X eyepieces and an oil-immersion objective of 100X magnification (Olympus CX-31, Japan).

Molecular screening

DNA Extraction

DNA was extracted from cysts using Nucleo-pore Stool DNA Mini Kit (Genetics Biotech Asia Pvt. Ltd) according to the manufacturer's instruction. Briefly, 150mg stool were taken in a thrashing bead lysis tube and placed in vortex for 15 minutes after adding 750µl lysis buffer to it. The thrashing bead lysis tube then were centrifuged at 10000rpm for 1 min from which 400µl supernatant were transferred to a fast spin filter in a collection tube and were again centrifuged at 7000rpm for 1 minute. 1200µl of binding buffer were added to the filtrate in the collection tube from which 800µl were transferred to fast spin columns in a collection tube and were centrifuged at 10000 rpm for 1 minutes. This step again repeated with the remaining. Then 200µl pre wash buffer were added to the fast spin column in a new collection tube and again centrifuged at 10000rpm for another 1 minute. After that 500µl wash buffer were added to the fast spin column and centrifuged for 1 minute. It was then transferred to a 1.5ml microcentrifuge tube and 100µl elution buffer were added and waited for few minutes. The eluted DNA were transferred to a prepared post elution filter in a clean 1.5ml microcentrifuge tube and were centrifuged at 8000 rpm for 1 minute to purify the DNA. Then the PCR was performed using the following parasite specific primers.

Primers and reaction condition for PCR

Giardia intestinalis: A *gdh* gene fragment-specific primer (5'- TCAACGTCAACCGCGGCTTCCGT-3' as forward primer and 5'- GTTGTCTTGCACATCTCC-3' as reverse primer) with a PCR amplicon size of 458 bp was used. The primers were tested by standard *Giardia* DNA. All the PCR amplifications were performed in a final volume of 20µl with approximately 100ng of template DNA, 1 µM of each primer, 1X PCR buffer with 2.5 mM MgCl₂, 1X BSA, 0.2 mM dNTPs, and 1U of Taq DNA Polymerase (Thermo scientific, Waltham, USA) in the thermal cyler (Bio-Rad Laboratories, Hercules, CA). The reaction condition was 8 min at 94° C as a initial hot start step, followed by 35 cycles, of 1 min at 94° C, 90 sec at 60.5° C, 2 min at 72° C, and a final extension step 5 min at 72° C.⁽¹⁾ Distilled water used as a negative control. The PCR products were Electrophoresized on ethidium bromide-stained 1% (W/V) agarose gel.^[11]

Entamoeba histolytica : Amplification for *E. histolytica* was achieved using a nested PCR protocol targeting amplification of small ribosomal RNA gene with primer set: 5' TAAGATGCACGAGAGCGAAA- 3' and 5'GTACAAAGGGCAGGGACGTA-3' for primary PCR and 5'- AAGCATTGTTTCTAGATCTGAG -3' and (5'-AAGAGGTCTAACCGAAATTAG-3') for secondary PCR as described by Nath *et. al.*, 2015. 439 bp sized product confirmed the parasite.

Cryptosporidium sp: The primers used were those described by Xiao *et al.*, 1999 (5'-TTCTAGAGCTAATACATGCG-3' as forward primer and 5'-CCCTAATCCTTCGAAACAGGA-3' as reverse primer) giving product of amplicon size 1325 bp. The nested PCR was performed with an amplicon size of 825 bp and using 5'-GAAGGGTTGTATTATTAGATAAAG-3' as forward primer and 5'-AAGGAGTAAGGAACAACCTCCA-3' as reverse primer.

Blastocystis sp: For the amplification of *Blastocystis hominis* the primer were used (5'

TCTTGCTTCATCGGAGTC 3') as forwards primer and (3'CCTTCTCGCAGTTCTTTATC- 5') as reverse primer having the amplicon size of 462bp.^[11]

Statistical analysis

The data from the above identification procedures and the epidemiological survey were randomly matched and checked to establish consistency and validity. SPSS.14.0 was used for statistical analysis. In this study, positive cases in the inferential age group were explored for parasites (*Giardia lamblia*, *Cryptosporidium sp* and *Entamoeba histolytica*) by Multinomial Logistic Regression (MLR).

RESULT AND DISCUSSION

Table: 1 Results of microscopy and molecular parasitic detection

Name of parasites	No. of Microscopically positive samples	No. of PCR positive samples	No of samples having mixed infection
<i>Giardia sp.</i>	96	102	77
<i>Cryptosporidium sp.</i>	41	47	24
<i>Entamoeba sp.</i>	18	21	16
<i>Blastocystis sp.</i>	30	36	23

Total no of collected samples = 598

Table: 2 Statistical view of observed data.

Study group		No of sample examined	No of infected sample	% of infected	OR (CI 95%)	P value
Sex	Male	291	45	15.46	1*	0.3139
	Female	307	57	18.57	1.25 (0.81 - 1.91)	
Age group	0-5 years	133	11	8.27	1*	0.0001
	5-12years	157	43	27.39	4.18 (2.06 - 8.50)	
	12-18years	139	20	14.39	1.86 (0.86 - 4.06)	
	Adults	169	28	16.57	2.20 (1.05 - 4.61)	
Symptoms	Diarrheal	189	21	11.11	1*	0.0095
	Asymptomatic	409	81	19.80	1.98 (1.18 - 3.31)	

Socio- demographic characteristics

The total no of collected samples were 598 of which 291 samples were collected from male and 307 samples were collected from female participants. With regard to age, 133 individuals were under the age group 0-5 years old, 157 participants under the age group of 5-12years old, 139 were under the age group of 12- 18 years old and 169 participants were adult, i.e. under the age group of >18 years old. All samples were collected from the participants irrespective of their symptomology. But among the collected samples, most of the participants responded as asymptomatic. Samples were collected throughout the year.

Diagnosis of Giardiasis with other GI protozoan parasite

Of the 598 samples screened, 102(17.05%) were positive for *Giardia intestinalis*. Whereas microscopic study shows 96 samples were positive. Among these, *Giardia intestinalis* was the sole infection in 31.2% of cases and there was mixed infection with other common pathogens in the remainder. A total of 47 cases of *Cryptosporidium*

sp. were diagnosed out of the 598 samples screened (7.9%) of the total surveillance population (Table 1). Among these, *Cryptosporidium* was the sole infection in 21.4% of cases and there was mixed infection with other common pathogens in the remainder. A total of 21 cases were diagnosed with *Entamoeba histolytica* infection (3.5% of the total study population). *Entamoeba histolytica* was the sole infection in 21.5% of these cases and the remainders were mixed with other enteric parasites. A total of 36 cases were diagnosed with *Blastocystis sp.* Infection (6.02% of the total study population), where the sole infection is 13% of these cases and the remainder were mixed infection with other parasites.

Statistical Data

In our study of the total collected samples, 291 samples were collected from male individual of whom 45 samples (15.46%) were found to be positive (OR= 1*), whereas of the total collected samples 307 sample were collected from female and shows 57 samples (18.57%) were positive for *Giardia* infection (95% CI= 0.81, 1.91)

showing the P value= 0.3139. Considering the age, collected samples were categorized under four groups, i.e. 0-5 years, 5-12 years, 12-18 years and adults showing rate of infection respectively 8.27% (OR=1), 27.39% (OR= 4.18 95%CI= 2.06,8.50), 14.39% (OR= 1.86, 95%CI= 0.86,4.06) and 16.57% (OR= 2.20, 95%CI= 1.05,4.61) with the P value = 0.0001. Regarding the symptomology, of the total collected samples only 189(11.11%) samples were diarrheal (OR= 1) and the remaining *Giardia* infected participants (19.80%) claim themselves as asymptomatic (OR=1.98, 95%CI= 1.18, 3.31) with the P value=0.0095.

In spite of availability of literatures and study report, human Giardiasis is still neglected in the northeastern India were the parasitic disease burden cannot be ignored. *Giardia intestinalis* were found to be positive, can cause severe illness ranging from self-limited acute to persistent diarrhoea. Beside this malabsorption is also common.^[7, 2, 4]

In this current study, the occurrence of *Giardia intestinalis* was observed throughout the year but the prevalence of this parasite was observed a bit higher in the post monsoon and pre winter season. Whereas the other parasites also observed prevalent in a particular season, mostly during the autumn season (September to November). Seasonal prevalence may due to scarcity of water in that season which is associated with the unhygienic sanitation. However, this needs further investigation.

Among the affected individual, it has been observed that the rate of infection is relatively higher in case of children between the age group of 5-12 years age as they are vulnerable to infection while playing with contaminated water as well as taking any contaminated raw fruit or vegetable item without washing. Beside this one more cause that could be predictable is that the children belong to this age group does not have that much active immune system to fight strongly against any infection.

In other case, observation reveals the higher rate of infection in case of female rather than male which may be interpreted as mostly female are associated with household works like washing of utensils, cloths etc. with contaminated water sources. Beside this the another cause may be most of the female were being not aware about the fact of contaminated disease caused by water and they simply take river water as a source of drinking water where as the male were engaged in their profession and they take their maximum consumed water from the working place where it was not the river water.

As this study is mainly focused on the surveillance of *Giardia intestinalis*, during the investigation the occurrence of other gastro intestinal parasite was also observed but their role as a co infecting parasite is yet not clear.

CONCLUSION

The present study aims to find out the occurrence of parasitic intestinal problem among the inhabitants of the slum areas near by the river Barak of Southern Assam. All though recently few studies on this particular area reveals many cases of parasitic burdens. But the co infection of *Giardia intestinalis* with other protozoa is still unknown. According to the data derived from the current study highlights that most of the parasitic infection is symptomless, i.e. individual having protozoan parasitic infection most of them does not experience diarrhea or any other, where as in most of the cases patients having problem of abdominal cramp.

During this study, it has been observed that all the cases where *Giardia intestinalis* infection was found, there is a little morphological variation of this protozoa. However, it required a thorough investigation whether there is further genetical variation related with the morphological variation.

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