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Original Article

Entamoeba Histolytica, Giardia Lamblia and *Cryptosporidium* spp. infection in children in an urban slum area of Bangladesh

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ABSTRACT: *Entamoeba histolytica, Giardia lamblia*, and *Cryptosporidium* spp. are common diarrhea-causing parasitic protozoa. A total of 423 fecal samples from 185 children were tested for *E. histolytica, G. lamblia* and *Cryptosporidium* spp. Positivity of E. histolytica, G. lamblia and Cryptosporidium spp. were 17.5%, 50.6% and 9.2%, respectively by real-time PCR. *E. histolytica* and *G. lamblia* infection rate increases with age, while Cryptosporidium spp. infection rate decreases with age. Parasitic infection rate was lowest in the winter, gradually increased during the rainy season. Among 185 children, 25% of them showed multiple infections with 2 or 3 of these parasites. This study demonstrates the alarming parasitic infection in the children suffering from diarrhea in an urban slum area. Therefore, multiple intervention strategies should be implemented to reduce the disease burden.

KEYWORDS: *Entamoeba histolytica, Giardia lamblia, Cryptosporidium* spp and diarrhea.

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INTRODUCTION

Diarrhea is a major public health problem worldwide, especially in children. One in ten child deaths result globally from diarrheal disease before their fifth birthday, resulting in about 800 000 fatalities worldwide annually, most occurring in sub-Saharan Africa and south Asia (Liu *et al*. 2010)[1]. In South Asia, Diarrhea accounts for 26.1% of childhood deaths with a peak incidence in their early years of Life (Walker *et al.* 2012)[2]. The incidence of diarrheal diseases varies greatly with the seasons and a child's age. The rate is highest in the first two years of life and declines as a child grows older. In Bangladesh, one third of the total child death burden is due to diarrhea (Victora *et al.* 1993)[3]. Every year, a rural child suffers on average from 4.6 episodes of diarrhea, from which about 230,000 children die (Piechulek *et al*. 2003)[4].

Parasitic diseases are incriminated in causing more than 33% of global deaths of which intestinal parasitic infections are believed to take the major share (WHO 1991, WHO 1998)[5,6]. Although there could be many other causes of diarrhea, the enteric protozoa *E. histolytica, G. lamblia* and *Cryptosporidium* spp. have been recognized as important causes of diarrhea among human beings (Haque *et al.* 2003, Ortega and Adam 1997, Kosek *et al*. 2001)[7-9]. *E. histolytica* is a pathogenic parasite for which humans are the primary reservoir [10]. The clinical presentation can range from asymptomatic carriage to gastrointestinal disease and invasive disease. *E. histolytica* is morphologically identical to the nonpathogenic species *E. dispar* and *E. moshkovskii*, though genetic differences have confirmed their separation into independent species (Diamond and Clark 1993, Som *et al.* 2009)[11,12].

G. lamblia (synonyms: *G. intestinalis* and *G. duodenalis*) is the most common protozoan infection of the intestinal tract worldwide. Many countries, especially developing countries, show a high infection rate of giardiasis (Sprong *et al*.) [13]. It is believed that giardiasis is still a significant health problem. Most infected persons are children who suffer and experience growth retardation (Farthing *et al*. 1986) [14]. Cryptosporidiosis is a frequent cause of diarrheal disease in humans. Infection is acquired via the fecal-oral route, and *C. parvum* has been recognized as the cause of large waterborne and food-borne outbreaks of gastroenteritis. Patients tend to present with a self-limiting diarrhea that may last for several weeks to months. In developing countries, *Cryptosporidium* spp. infections occur mostly in children younger than 5 years of age, with a peak in children younger than 2 years of age (Tumwine *et al*. 2003, steinberg *et al*. 2004)[15, 16]. In immuno-deficient humans, especially individuals with HIV/AIDS, cryptosporidiosis can be associated with chronic, potentially life-threatening diarrhea (Cama *et al.* 2003)[17].

The aim of the present study was to determine the percentage of the three recognized enteric parasites (*E. histolytica, G. lamblia* and *Cryptosporidium* spp.) in children from an urban slum area of Bangladesh.

MATERIALS AND METHODS

Study design

The present study was carried out during January 2012 to December 2012 at the Parasitology Laboratory, Laboratory Sciences Division (LSD) of the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR, B). This study was approved by the Ethical Review Committee at the ICDDR,B. The subjects were studied living in a slum of Mirpur, in Dhaka, Bangladesh. Informed and consent was obtained from the parent or guardian, and then the child was enrolled in the study. A total of 185 children were randomly selected for the study and followed up two times per week via home visits by field research assistants. If a child had diarrhea, defined as three or more unformed stools per day, a specimen was collected and delivered from field to the laboratory within 6 hours of collection maintaining cold chain. Only one specimen was collected per episode. We considered diarrheal episodes independent if separated from another episode by 3 diarrhea-free days. The repeated diarrheal sample(s) was collected from a child who again showed diarrhea after recovery. The parasites were detected firstly by direct microscopy; we also used molecular detection techniques, ELISA and PCR, to increase the sensitivity and specificity of detection.

Microscopic screening

Immediately the samples were received, they were examined by direct microscopy. Microscopic examination was done by Olympus light microscope. In microscopic examination, liquid stool were taken into slide and examined whether it is positive or not. No concentration technique or special staining technique was applied.

Molecular Screening

Antigen-capture Enzyme Linked Immunosorbent Assay (ELISA) was performed on all the samples to detect the above-mentioned parasites using commercially available kits, i.e. GIARDIA II, E. HISTOLYTICA II and CRYPTOSPORIDIUM II (TechLab, Inc., Blacksburg, Virginia). Parasite DNA was isolated using a Stool DNA Isolation Kit (QIAGEN) according to the manufacturer's protocol, and then multiplex Real Time PCR was performed on all samples.

Primers and probes

The primers and Taqman probes for *E. histolytica* (accession no. X64142) and *G. lamblia* (accession no. M54878) were designed on small subunit ribosomal RNA gene (Roy *et al.* 2005, Verweij *et al.* 2003)[19, 20]. The primers and Taqman probes for *Cryptosporidium* spp. were designed on *Cryptosporidium* Oocyst Wall Protein (COWP; accession no. AF248743) Guy *et al.* 2003 [21]. The primers that we used for *Cryptosporidium* are capable of detecting *C. parvum*, *C. hominis*, and *C. melagiardis*. The amplified targets were 134, 62, and 151 bp for *E. histolytica*, *G. lamblia* and *Cryptosporidium* spp., respectively.

Multiplex real-time PCR assay

Amplification reactions were performed in a volume of 25 µL with Qiagen master mix (containing 100 mmol/L KCL; 40 mmol/L Tris-HCL, pH 8.4; 1.6 mmol/L deoxynucleoside triphosphate; iTaq DNA polymerase [50 units/mL], 2 mmol/L Mgcl2) and an additional 3 mmol/L MgCl2 also added; 0.4 µmol/L of each Eh-f, Eh-r primers and 0.08 µmol/L Eh-YYT probes for *E. histolytica*, 0.4 µmol/L of each Gd-80F, Gd-127R primers and 0.12 µmol/L of Gd-FT probes for *Giardia*, and 1 µmol/L of each Cp-583F, Cp-733R primers and 0.5 µmol/L of Cp-TRT probes for *Cryptosporidium* and 3 µL of the DNA sample were used in each reaction. Amplification consisted of 3 minutes at 95°C followed by 45 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C. Amplification, detection, and data analysis were performed with the i-Cycler real-time detection system (BioRad). Fluorescence was measured during the annealing step of each cycle. The ramping of the machine was 3.3°C/s in every step. Fluorescence at 530, 490, and 575 nm was measured for *E. histolytica*, *G. lamblia* and *Cryptosporidium*, respectively.

Statistical analysis

Basic demographic information, surveillance data, and clinical and laboratory findings of each diarrheal episode for which stool sample was collected were stored in data files using Fox-Pro® (Microsoft, Redmond, WA). The statistical package SPSS version 10.01 (SPSS, Inc., Chicago, IL) was used for data analysis.

RESULTS

Out of 423 fecal samples analyzed, 74 samples (17.5%) were found positive for *E. histolytica*, 214 samples (50.6%) for *G. lamblia* and 39 (9.2%) for *Cryptosporidium* spp. (Table 1). The samples that were positive either by microscopy or Antigen detection were also found positive by PCR. The real time PCR shows 100% sensitivity and specificity compared to other two methods. We have used positive controls in real time PCR for all three parasites. In addition to these, extraction controls have been used for assessing specificity of real time PCR. Among the 185 children, 55.13% (102/185) showed repeated episodes diarrheas and the repeated diarrheal samples 9, 37 and 2 cases were found re-infected with *E. histolytica*, *G. lamblia* and *Cryptosporidium* spp. Respectivel After treatment of 29 *E. histolytica* positive children (Table 3), only 17 children showed multiple diarrheas on which 9 children were found re-infected with *E. histolytica*. In case of 88 *G. lamblia* infected children, 45 showed repeated diarrheas after being introduced with antiparasitic therapy. multiple diarrheas on which 9 children were found re-infected with *E. histolytica*. In case of 88 *G. lamblia* infected children, 45 showed repeated diarrheas after being introduced with antiparasitic therapy, among them 37 were found re-infected with *G. lamblia*. Out of 19 *Cryptosporidium* spp. positive children, only 7 showed multiple diarrheas and 2 were found *Cryptosporidium* spp. positive after treatment. Examining the first diarrheal samples, 72/185 children were identified negative for all the three intestinal parasites by all the three techniques. They were left untreated with any antiparasitic therapy and found 18.77%, 50.70% & 8.45% positive for *E. histolytica*, *G. lamblia* and *Cryptosporidium* spp. respectively from their repeated samples. The highest isolation rate of *E. histolytica* and *G. lamblia* occurred in children aged 49-60 months and the highest isolation rate for *Cryptosporidium* spp. occurred in children aged 13-24 months. Low infection rate was found at the age group of 13-24 months for *E. histolytica* and *G. lamblia*. No *Cryptosporidium* spp. infection was found at the age group of 49-60 months (Table 4). Infection with *E. histolytica* (P=0.037) and/or *G. lamblia* (P=0.028) showed a steady increase with age and infection with *Cryptosporidium* spp. showed a decrease with age (P=0.459).

Table 1: Detection of *Entamoeba histolytica*, *Giardia lamblia* and *Cryptosporidium* spp. with three different techniques.

Method	No. of samples	No. of positive samples (%)		
		E. histolytica	*G. lamblia*	*Cryptosporidium* spp.
Real Time PCR	423	74 (17.5)	214 (50.6)	39 (9.2)
Antigen detection	423	14 (3.3)	113 (26.7)	20 (4.7)
Microscopy	423	1 (0.24)	14 (3.3)	ND

ND- Not Done

Table 2: Cross-classified test results obtained by the antigen detection test and a real-time PCR test.

Antigen detection test	Real time PCR	No. of samples		
		E. histolytica	*G. lamblia*	*Cryptosporidium* spp.
+	+	14	113	20
-	+	60	101	19
+	-	0	0	0
-	-	349	209	384

(+) indicates a positive and (–) indicates a negative test result.

Table 3: Results from Samples collected first time from 185 children.

Method	Number of Samples (First ds/Pre-treatment samples)	Number of positive samples (%)		
		E. histolytica	*G. lamblia*	*Cryptosporidium spp.*
Real Time PCR	185	29 (15.67)	88 (47.57)	19 (10.27)
Antigen detection	185	4 (2.16)	42 (22.7)	10 (5.4)
Microscopy	185	0 (0)	5 (2.7)	ND

ds=Diarrheal Sample

Table 4: Age wise distribution of parasites detected in 423 stool samples.

Age group (Months)	No. of samples examined	No. (%) with *E. histolytica*	No. (%) with *G. lamblia*	No. (%) with *Cryptosporidium* spp.
13-24	158	23 (14.6%)	75 (47.5%)	17 (10.8%)
25-36	166	26 (15.7%)	80 (48.2%)	15 (9%)
37-48	78	17 (21.8%)	42 (53.8%)	7 (8.9%)
49-60	21	8 (38.1%)	17 (80.9%)	0 (0%)

Seasonality: Monthly seasonal positivity for three protozoan parasitic infections gradually increased from a minimum (46%) in April to a maximum (80.1% - 88.9%) between July and November. Percentage of single infection was lowest (32.1%) in December and highest (76.3%) in November. No multiple infections were found in October but highest (23.1%) positivity was observed in July (Table 5). Peak percentages of positivity were observed in the rainy season. *E. histolytica* and *Cryptosporidium* spp. infections were observed highest in August and lowest in October. Oscillations in seasonal frequency was not dramatic in *G. lamblia* infection rather fluctuated in the remaining months of the year, and showed high positivity in September and November (Fig 1).

Multiple infections: Out of 185 children, 46 children (25%) were infected with either 2 or 3 of *Entamoeba histolytica*, *Giardia lamblia* and *Cryptosporidium* spp. Among these, 26 infections with *E. histolytica* and *Giardia lamblia*, 5 with *E. histolytica* and *Cryptosporidium* spp, 12 with *Cryptosporidium* spp and *G. lamblia* and 3 with *E. histolytica*, *G. lamblia* and *Cryptosporidium* spp. *E. histolytica*, *G. lamblia* and *Cryptosporidium* spp. is associated with diarrhoeal illness in young children in Bangladesh. In this study, the percentage of *E. histolytica* , *G. lamblia* and *Cryptosporidium* spp. was found 17.5%, 50.6% and 9.2% respectively. This prevalence is markedly higher than reported prevalence in the previous studies (Mukherjee *et al.* 2009, Haque *et al.* 2003) [22, 23]. Another study in this region demonstrated that preschool children in the urban slum are at great risk of acquiring parasitic infection, with almost half infected by five years of age (Haque *et al.* 1999) [24]. The higher infection rate the study suggests real increase in percentage but does not exclude the possibility of differences in test populations. The results of present study are significantly different from that of results of Bangladesh in the Global Enteric Multicenter Study(GEM bv Kotloff *et al*. 2013)[25]. The difference could be due to geographical location or variation in study subject, sample size as well as life style (our study subjects were living in an unhygienic conditions of slum with poor sanitation facilities). Our study showed a high risk for *E. histolytica* and *G. lamblia* infection at 49-60 month age group children, the infection rate was low at 13-24 age group and it significantly increased with age ($P<0.05$). *Cryptosporidium* spp. was more frequently detected in children less than two years of age (10.8%), and infection rate decreased with age. Similar findings were

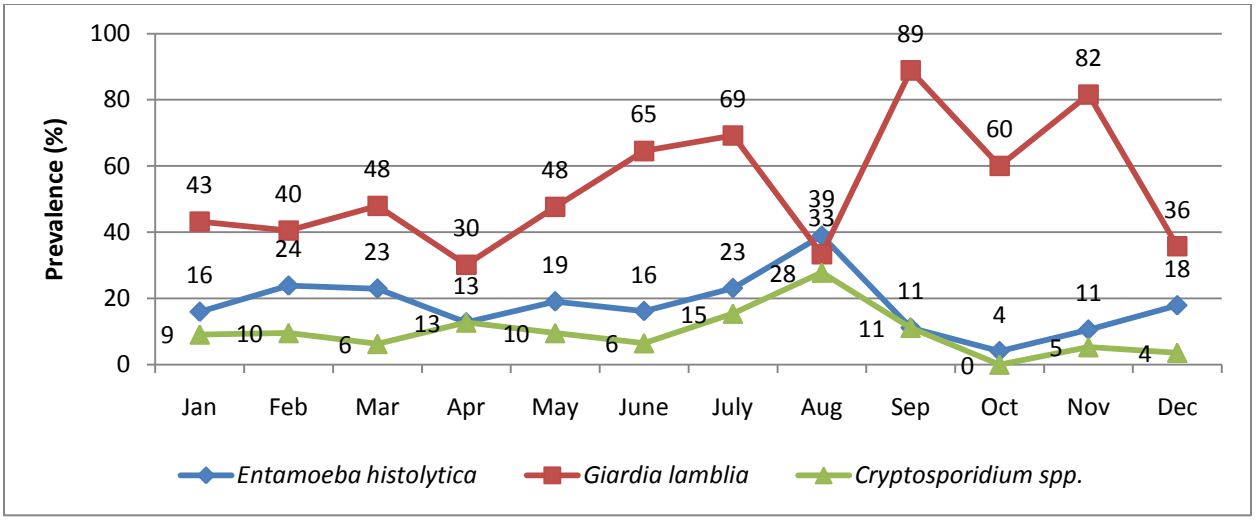

Fig 1: Seasonal prevalence of *Entamoeba histolytica*, *Giardia lamblia* and *Cryptosporidium* spp. during January 2012 to December 2012.

Table 5: Seasonal prevalence of single or multiple parasitic infections.

Months	Number of samples examined	Number (%) of samples infected		
		Single infections	Multiple infections	Total
January	44	16 (36.4)	7 (15.9)	23 (52.3)
February	42	21 (50)	5 (11.9)	26 (61.9)
March	48	21 (43.8)	8 (16.7)	29 (60.4)
April	63	24 (38.1)	5 (7.9)	29 (46)
May	42	17 (40.1)	7 (16.7)	24 (57.1)
June	31	14 (45.2)	6 (19.4)	20 (64.5)
July	26	15 (57.7)	6 (23.1)	21 (80.1)
August	18	10 (55.6)	4 (22.2)	14 (77.8)
September	18	12 (66.7)	4 (22.2)	16 (88.9)
October	25	16 (64)	0 (0)	16 (64)
November	38	29 (76.3)	4 (10.5)	33 (86.8)
December	28	9 (32.1)	4 (14.3)	13 (46.4)
Total	423	204 (48.2)	60 (14.2)	264 (62.4)

previously reported in studies from Bangladesh as well as from other countries (Haque *et al* 2003, Bhattacharya *et al.* 1997, Rahman *et al*. 1990, Shahid *et al.* 1987, Pereira *et al*. 2002, Kirkpatrick *et al.* 2002)[23, 26-30]. Positivity of *E. histolytica* was found 17.5%, which is almost similar with a study in an endemic area for amoebiasis in Turkey (Tanyuksel *et al.* 2005)[31]. We have seen a significant difference for the detection of *E. histolytica* by microscopy and antigen The detection test in comparison with real-time PCR. analytical sensitivity of our real-time PCR assay is around 0.02 parasite per reaction (Roy *et al.* 2005)[19], which is around 1 parasite in the specimen that will be extracted for DNA. The real-time PCR assay detected all of the positive specimens detected by the antigen detection test and microscopy. The real time PCR showed 100% sensitivity and 100% specificity in comparison to other methods. The specimens that were positive by real-time PCR but negative by the antigen detection test and microscopy showed higher cycle threshold (C_T) values which indicate that the low number of parasites in those samples fell below the detection limits of the antigen detection test as well as by microscopy.

Out of the 423 fecal samples analyzed, 50.6% (214/423) was detected positive for *G. lamblia* by Multiplex Real-time PCR where as the detection rate was 26.7% by antigen detection test and 3.3% by microscopy. A similar finding was found for *Cryptosporidium* spp. where 9.2 % (39/423) was detected positive by Multiplex Real-time PCR while antigen detection test detected 4.7% as positive for *Cryptosporidium* Spp. The antigen detection test showed 52.8% & 51.3% sensitivity for *G. lamblia* & *Cryptosporidium* spp. compared to Real-time PCR, respectively. However, the antigen detection test showed 100% specificity for both. This low sensitivity of antigen test was due to low number of parasites in those samples that fell below the detection limits of the antigen detection test.

From the first diarrhoeal samples of 185 children, the number of *E. histolytica* positive children was 29 (Table 3). After treatment, only 17 children showed multiple diarrheas on which 9 children were found re-infected with *E. histolytica*. The rest 8 may be due to microbial infections or other etiological agent. In case of *Giardia* infection, 37 out of 88 were found re-infected with *G. lamblia.* However, for the *Cryptosporidium* spp. infection, it was only 2 out of 19 who were found re-infected with *Cryptosporidium* spp. after treatment. Interestingly, most of the post treatment stool samples were found positive only with the real-time PCR & they showed higher cycle threshold (C_T) values compared to their first instances which indicate the low parasitic load in these samples and thus fell below the detection limits of the antigen detection test as well as by microscopy. This result also indicates a positive effectiveness of the antiparasitic therapy.

The detection limit for our Multiplex Real-time PCR was 1 trophozoite of *E. histolytica* per extraction (100 µL), 10 trophozoites of *G. lamblia* per extraction, and 100 oocysts of *Cryptosporidium* per extraction, described elsewhere (Haque *et al* 2007) [18]. Thus it clearly demonstrate that Multiplex Real time PCR is much more sensitive & specific method than the other two methods. Moreover, Microscopy cannot differentiate the *Entamoeba* species (from pathogenic *E. histolytica* to non-pathogenic *E. dispar* and others). A recent study highlighted the lack of sensitivity of Microscopy and suggested molecular methods as the diagnostic method of choice for enteric parasites (Stark *et al*. 2014)[32]. Conventional microscopy has a disadvantage due to poor sensitivity that even in experienced hands may overlook 50% of the infections Tanyuksel *et al* 2005) [31]. Numerous studies (McAuliffe *et al.* 2013, Roberts *et al.* 2011, Stark *et al.* 2008) [33-35] . have shown the benefit of molecular detection of parasites over traditional methods and, therefore, real-time PCR is now considered the diagnostic gold standard for the detection of enteric protozoa.

In the present study, 25% (46 out of 185) of the children were detected multiple infections. Interestingly, among the 46 multiple infections 41 were associated with *Giardia lamblia*, although the reason behind this is not clear. A recent study in India showed that *Giardia* infection rate play a vital role in regulation of the whole diarrhoeal disease spectrum in this endemic region (Mukherjee *et al*. 2014)[36].The result of the polyparasitism of our study is higher than the study conducted in Ethiopia where multiple parasitic infections were seen in 7.3% of the total study subjects and 17.8% among children who were infected with parasites (Beyene and Tasew 2014)[37]. The difference could be due to geographical location or variation in study subject and sample size.

E. histolytica, *G. lamblia* and *Cryptosporidium* spp. cases were evident throughout the year. Peak Positivity were observed in *G. lamblia* in the rainy season (September) which is similar to our neighboring country (Saha *et al*. 2008)[38]. *E. histolytica* infection appeared to be uniformly present throughout the years with a peak during mid monsoon seasons (July–August). Similar findings were found with other studies (Saha *et al*. 2008, and Nasiri *et al* 2009)[38, 39]. Overall, the highest parasite detection occurred during the month between July to November which corresponds to the end of the dry season and the entire monsoon season in Bangladesh. A previous study in Manila showed the number of patients with diarrhea increased with the onset of the monsoon rains and peaked during the months of maximum rainfall (Adkins *et al.* 1987) [40], Another study in Nigeria showed that the parasitic infection started in April of each year (onset of rainy season), peaked between July and August, and was the lowest at dry season between November and March (Nzeako 1992)[41;] which are similar with our present findings. Low infection rates were found during winter, cool season experience the lowest rate of detection suggesting a role for environmental factor(s) in transmission of these infection.

Intestinal parasitic infection represents a relevant clinical problem, especially in developing countries like Bangladesh, where they are responsible for morbidity and mortality in children. The positivity in the communities may be altered because of changes in social behavior and life styles during years [39]. Transmission of intestinal parasites depends on the presence of infected individuals, poor sanitation and principally, the socioeconomic and behavioral factors in the populatio (Abossie and Seid, 2014, Lin et al. 2013)[42, 43]. The present results show that the Positivity of parasitic infections in this region is still high. Therefore, multiple intervention strategies should be implemented not only for the children but also for households and the environment to reduce the disease burden.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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REFERENCES

1. Liu, L., Johnson, H.L., Cousens, S., Perin, J., Scott, S., Lawn, J.E., Rudan, I., Campbell, H., Cibulskis, R., Li, M., Mathers, C. and Black, R.E. 2010. Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. *The Lancet*. vol. 379,(9832): 2151–2161.
2. Walker, C.L.F., Aryee, M.J., Boschi-Pinto, C. and R.E. Black. 2012. Estimating diarrhea mortality among young children in low and middle income countries. *PloS One*. vol. 7, no. 1: pp. e29151.
3. Victora, C.G., Huttly, S.R., Fuchs, S.C., Barros, F.C., Garenne, M., Leroy, O., Fontaine, O., Beau, O., Fauveau, V. and Chowdhury , H.R. 1993. International differences in clinical patterns of diarrhoeal deaths: a comparison of children from Brazil, Senegal, Bangladesh, and India. *J. Diarrhoeal Dis. Res.* vol. 11 (1):25–29.

4. Piechulek, H., Al-Sabbir, A. and Mendoza-Aldana ,J. 2003. Diarrhea and ARI in rural areas of Bangladesh. *Southeast Asian J. Trop. Med. Public Health*. 34(2): 337–342.
5. WHO. 1991| Basic laboratory methods in medical parasitology ."Geneva. vol. 25, no.26,.
6. WHO. |1998. Control of tropical diseases. *Geneva World Health Organ.* vol. 201.
7. Haque, R. . Huston, C.D. Hughes, M Houpt, E. and. Petri Jr, W.A. 2003. Amebiasis. *N. Engl. J. Med.* 348 (16): 1565–1573.
8. Ortega Y.R. and. Adam R.D. 1997. *Giardia*: overview and update. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* vol. 25, no. 3, pp. 545–549; quiz 550.
9. Kosek ,M. Alcantara, C. Lima, A.A. and. Guerrant, R.L. 2001. Cryptosporidiosis: an update. *Lancet Infect. Dis.* 1 (4): 262–269,
10. Stauffer, W. Abd-Alla, M. and Ravdin, J.I. 2006. Prevalence and incidence of *Entamoeba histolytica* infection in South Africa and Egypt. *Arch. Med. Res.* 37(2): 266–269.
11. Diamond, L.S. and Clark, C.G. 1993. A redescription of *Entamoeba histolytica* Schaudinn, 1903 (Emended Walker, 1911) separating it from *Entamoeba dispar* Brumpt, 1925. *J. Eukaryot. Microbiol.* 40(3): 340–344
12. Som, I. , Azam, A., Bhattacharya, A. and Bhattacharya, S. 2009. Inter- and intra-strain variation in the 5.8S ribosomal RNA and internal transcribed spacer sequences of *Entamoeba histolytica* and comparison with *Entamoeba dispar*, *Entamoeba moshkovskii* and *Entamoeba invadens*. *Int. J. Parasitol.* 30 (6): 723–728, 13.
13. Sprong, H. Cacciò, S.M. van der Giessen, J.W.B.and ZOOPNET network and partners: Identification of zoonotic genotypes of *Giardia duodenalis*. *PLoS Negl. Trop. Dis.* 3(12): e558.
14. Farthing, M.J. Mata, L. Urrutia, J.J and Kronmal, R.A. 1986. Natural history of *Giardia* infection of infants and children in rural Guatemala and its impact on physical growth." *Am. J. Clin. Nutr.* 43(3): 395–405,
15. Tumwine, J.K., Kekitiinwa, A., Nabukeera, N. Akiyoshi, D.E., Rich, S.M., Widmer, G., Feng, X. and Tzipori, S. 2003. *Cryptosporidium parvum* in children with diarrhea in Mulago Hospital, Kampala, Uganda. *Am. J. Trop. Med. Hyg.* 68(6): 710–715,
16. Steinberg, E.B., Mendoza, C.E ., Glass, R Arana, R B., Lopez, M.B., Mejia, M., Gold, B.D., Priest, J.W., Bibb, W., Monroe, S.S., Bern, C., B.P. Bell, R.M. Hoekstra, R. Klein, E.D. Mintz, and S. Luby: 2004. Prevalence of infection with waterborne pathogens: a seroepidemiologic study in children 6-36 months old in San Juan Sacatepequez, Guatemala." *Am. J. Trop. Med. Hyg.* 70(1):83–88.
17. Cama, V.A., Bern, C., Sulaiman, I.M., Gilman, R.H., Ticona, E., Vivar, A., Kawai, V. Vargas, D., Zhou, L.,and Xiao, L. 2003. *Cryptosporidium* species and genotypes in HIV-positive patients in Lima, Peru. *J. Eukaryot. Microbiol.* 50 Suppl.: 531–533.
18. Haque, R., Roy, S., Siddique, A., Mondal, U., Rahman, S.M.M., Mondal, D., Houpt, E. and Petri Jr, W.A. 2007. Multiplex real-time PCR assay for detection of *Entamoeba histolytica, Giardia intestinalis,* and *Cryptosporidium spp*. *Am. J. Trop. Med. Hyg.* 76(4): 713–717.
19. Roy, S., Kabir, M., Mondal, D., Ali, I.K.M., Petri Jr, W.A. and Haque, R. 2005. Real-time-PCR assay for diagnosis of *Entamoeba histolytica* infection. *J. Clin. Microbiol.* 43(5): 2168–2172,
20. Verweij, J.J., Schinkel, J., Laeijendecker, D., van Rooyen ,M.A.A., van Lieshout, L. and Polderman, A.M. 2003. Real-time PCR for the detection of *Giardia lamblia. Mol. Cell. Probes*. 17(5): 223–225.
21. Guy, R.A., Payment, P., Krull, U.J. and Horgen, P.A. 2003. Real-time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage." *Appl. Environ. Microbiol.* 69(9): 5178–5185.
22. Mukherjee, A.K., Chowdhury, P., Bhattacharya, M.K., Ghosh, M., Rajendran, K. and Ganguly, S. 2009. Hospital-based surveillance of enteric parasites in Kolkata. *BMC Res. Notes*. vol. 2, pp. 110.
23. Haque, R., Mondal, D., Kirkpatrick, B.D., Akther, S., Farr, B.M. Sack, R.B. and Petri Jr, W.A. 2003. Epidemiologic and clinical characteristics of acute diarrhea with emphasis on *Entamoeba histolytica* infections in preschool children in an urban slum of Dhaka, Bangladesh." *Am. J. Trop. Med. Hyg.* 69(4): 398–405.
24. Haque, R., Ali, I.M. and Petri Jr, W.A. 1999. Prevalence and immune response to *Entamoeba histolytica* infection in preschool children in Bangladesh." *Am. J. Trop. Med. Hyg.* 60(6):1031–1034.
25. Kotloff, K.L., Nataro, J.P., Blackwelder, W.C., Nasrin, D., Farag, T.H., Panchalingam, S., Wu, Y., Sow, S.O., Sur, D., Breiman, R.F. Faruque, A.S., Zaidi, A.K., Saha, D., Alonso, P.L., Tamboura, B., Sanogo, D., Onwuchekwa, U., Manna, B., Ramamurthy, T., Kanungo, S., Ochieng, J.B., Omore, R., Oundo, J.O., Hossain, A., Das, S.K., Ahmed, S., Qureshi, S., Quadri, F., Adegbola, R.A., Antonio, M., Hossain, M.J., Akinsola, A., Mandomando, I., Nhampossa, T., Acácio, S., Biswas, K., O'Reilly, C.E., Mintz, E.D., Berkeley, L.Y., Muhsen, K., Sommerfelt, H., Robins-Browne, R.M. and Levine, M.M. 2013. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study." *Lancet*. 382 (9888): 209–222.
26. Bhattacharya, M.K., Teka, T., Faruque, A.S., and Fuchs, G.J. 1997.*Cryptosporidium* infection in children in urban Bangladesh." *J. Trop. Pediatr.* 43 (5):282–286.
27. Rahman, M., Shahid, N.S., Rahman, H., Sack, D.A., Rahman, N. and Hossain, S. 1990. Cryptosporidiosis: a cause of diarrhea in Bangladesh." *Am. J. Trop. Med. Hyg.* 42(2): 127–130.
28. Shahid, N.S., Rahman, A.S. and Sanyal. S.C. 1987. *Cryptosporidium* as a pathogen for diarrhoea in Bangladesh." *Trop. Geogr. Med.* 39(3): 265–270.
29. Pereira, M.D.G.C., Atwill, E.R., Barbosa, A.P., Silva, S.A.E. and García-Zapata, M.T.A.. 2002. Intra-familial and extra-familial risk factors associated with *Cryptosporidium parvum* infection among children hospitalized for diarrhea in Goiânia, Goiás, Brazil. *Am. J. Trop. Med. Hyg.* 66(6):787–793,
30. Kirkpatrick, B.D., Daniels, M.M., Jean, S.S., Pape, J.W., Karp, C., Littenberg, B., Fitzgerald D.W., Lederman, H.M., Nataro, J.P. and Sears, C.L. 2002.Cryptosporidiosis stimulates an inflammatory intestinal response in malnourished Haitian children." *J. Infect. Dis.* 186(1): 94–101.

31. Tanyuksel, M. Yilmaz, H., Ulukanligil, M., Araz, E., Cicek, M., Koru, O., Tas, Z. and Petri, W.A. 2005. Comparison of two methods (microscopy and enzyme-linked immunosorbent assay) for the diagnosis of amebiasis. *Exp. Parasitol.* 110(3): 322–326.
32. Stark, D., Roberts, T., Ellis, J., Marriott, T D. and Harkness, J. 2014. Evaluation of the EasyScreen™ enteric parasite detection kit for the detection of *Blastocystis spp*., *Cryptosporidium spp., Dientamoeba fragilis, Entamoeba complex,* and *Giardia intestinalis* from clinical stool samples." *Diagn. Microbiol. Infect. Dis.* 78(2):149–152.
33. McAuliffe, G.N., Anderson, T.P., Stevens, M., Adams, J., Coleman, R., Mahagamasekera, P., Young, S., Henderson, T., Hofmann, M., Jennings, L.C. and Murdoch. 2013. Systematic application of multiplex PCR enhances the detection of bacteria, parasites, and viruses in stool samples." *J. Infect.* 67(2):122–129.
34. Roberts, T., Barratt, J., Harkness, J., Ellis, J. and Stark, D. 2011.Comparison of microscopy, culture, and conventional polymerase chain reaction for detection of blastocystis sp. in clinical stool samples. *Am. J. Trop. Med. Hyg.* 84(2): 308–312.
35. Stark, D., van Hal, S., Fotedar, R., Butcher, A., Marriott, D., Ellis, J. and Harkness, J. 2008. Comparison of stool antigen detection kits to PCR for diagnosis of amebiasis. *J. Clin. Microbiol.* 46(5) 1678–1681.
36. Mukherjee, A.K., Chowdhury, P ., Rajendran, K., Nozaki, T. and Ganguly, S. 2014. Association between *Giardia duodenalis* and coinfection with other Diarrhea-Causing Pathogens in India. *BioMed Res. Int.*
37. Beyene, G. and Tasew, H. 2014. Prevalence of intestinal parasite, Shigella and Salmonella species among diarrheal children in Jimma health center, Jimma southwest Ethiopia: a cross sectional study. *Ann. Clin. Microbiol. Antimicrob.* 13(1): pp 10.
38. Saha, D.R., Rajendran, K. , Ramamurthy, T., Nandy, R.K. and Bhattacharya, 2008. Intestinal parasitism and Vibrio cholerae infection among diarrhoeal patients in Kolkata, India. *Epidemiol. Infect.* 136 (5):661–664.
39. Nasiri, V. , Esmailnia, K. Karim, G., Nasir, M. and Akhavan,O. 2009. Intestinal parasitic infections among inhabitants of Karaj City, Tehran province, Iran in 2006-2008." *Korean J. Parasitol.* 47(3): 265–268.
40. Adkins, H.J., Escamilla, J., Santiago, L.T., Rañoa, C., Echeverria, P. and Cross, J.H.1987. "Two-year survey of etiologic agents of diarrheal disease at San Lazaro Hospital, Manila, Republic of the Philippines." *J. Clin. Microbiol*. 25(7) 1143–1147.
41. Nzeako, B.C.1992. Seasonal prevalence of protozoan parasites in Nsukka, Nigeria. *J. Commun. Dis.* 24(4): 224–230.
42. Abossie, A. and Seid, M. 2014. Assessment of the prevalence of intestinal parasitosis and associated risk factors among primary school children in Chencha town, Southern Ethiopia. *BMC Public Health*. 14, pp. 166
43. Lin, A., Arnold, B.F., Afreen, S., Goto, R., Huda, T.M.N., Haque, R., Raqib, R., Unicomb, L., Ahmed, T., Colford, J.M. and Luby, S.P.2013. Household Environmental Conditions Are Associated with Enteropathy and Impaired Growth in Rural Bangladesh. Am. J. Trop. Med. Hyg. 89(1):130–137.