

*Full Length Research Paper*

# Identification of Pathogenic Intestinal Parasitic Protozoa Associated with Diarrhea among Under-fives Children in Dar Es Salaam, Tanzania

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## Abstract

Diarrhea is responsible for high morbidity and mortality among children under the age of five in developing countries. Poor diet and unsafe water supply in households contributes to the prevalence of diarrhea. This study was carried out to determine intestinal parasitic protozoa causing diarrhea. Participants were children under-five years attending Municipal hospitals in Dar Es Salaam with either acute or chronic diarrhea. A total of 720 stool samples were analyzed. Parasitic protozoa were investigated by standard microscopy and PCR methods. The highest prevalence of diarrhea (29.6%) was found in the age groups of 12-23 months, followed by 24-60 months (15.6%), 6-11 months (8%) and least 0-5 months (2.4%). Microscopic method identified 41% parasitic protozoa, PCR 55.6%. However, m-PCR was more discriminative and sensitive, such that the 144 samples identified microscopically as *E. histolytica*, were differentiated as; *E. histolytica* 48 (33.3%), *E. dispar* 80 (55.6%) and *E. moshkovskii* 16 (11.1%). The most common protozoa were *Giardia lamblia* 35.6 % (256), followed by *Entamoeba histolytica* 12.2% (88), *Cryptosporidium parvum* 7.8 % (56), and Helminthes 12.7% (92). PCR methods should be advocated for differential diagnosis of parasitic causes of diarrhea in Tanzania for children.

**Key words:** Diarrhea, Parasitic protozoa, under-fives.

## INTRODUCTION

Diarrhea is the passage of watery stool for more than three times in twenty-four hours caused by consumption of contaminated food or drinks by a variety of pathogens including bacteria, fungi, viruses, protozoa or helminthes. Diarrhea can also be caused by intolerance to certain types of food or drugs and sometimes stress (Vargas *et al.*, 2004). The disease has remained to be a burden in most developing countries (Reither *et al.*, 2007; Ansari *et al.*, 2012). Furthermore, some underlying conditions that are common in our communities including malnutrition may increase the risk of contracting diarrhea diseases in developing countries.

The global deaths due to diarrheal diseases in developed countries had declined steadily due to

advanced health technologies and reliable water supply (Dean *et al.*, 2006; WHO 2013). However, despite of all advances in health technology and improved management, the situation of diarrheal infections in Africa, still remains high, it is the second leading cause of death among the five major killer diseases of children. (UNICEF, 2008; 2012; Quinn 2009; Michael *et al.*, 2010; WGO, 2012 and WHO, 2013).

The World Health Organization (WHO, 2013) and the World gastroenterology Organization (WGO) ranks diarrheal disease as the second highest cause of morbidity and mortality in children in the developing world. Knowledge and attitude, and perception of mothers and caregivers on childhood diarrhea plays a major role in the control of childhood diarrhea, as pointed out by Mwambete and Joseph (2010).

Diarrhea can be acute watery characterized by abrupt onset of diarrhea of three or more stools of loose stools per day and may last for several hours or days,

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such as cholera or acute bloody diarrhea such as dysentery and chronic or persistent diarrhea that lasts for two weeks or more (WHO, 2013).

Other studies done in Tanzania, concentrated more on bacterial and viral causes of diarrhea. However, pathogenic intestinal parasites associated with diarrhea were somehow neglected.

The diagnosis of parasitic infection for children below the age of 5 years is another enigma for Tanzania. The potentially invasive *E. histolytica* is morphologically very similar to the noninvasive *E. dispar* and *E. moshkovskii*. Thus, microscopic examination alone may not provide a definite answer about the presence of *E. histolytica* cysts and/ or trophozoites, therefore, additional methods such as antigen detection methods, or blotting methods e.g. western and southern blotting or molecular based techniques (PCR methods) are required. Otherwise, many of the infections will be missed because the sensitivity of microscopy has shown to be inadequate (Haque, 2007; Ten Hove *et al.*, 2007; Verweij *et al.*, 2004; Koffi *et al.*, 2014).

The precise data on mortality associated with diarrheal diseases caused by parasitic infections in Tanzania is not available.

Thus, the aim of this study was to investigate the incidence of pathogenic parasitic protozoa associated with acute diarrhea among children of five years of age and below and characterize them using modern laboratory methods (specifically molecular based methods) in comparison with conventional ones (ordinary microscopy and cultures).

## MATERIALS AND METHODS

The study was carried out in two Municipal hospitals in Dar Es Salaam city (Amana and Mwananyamala) and one national hospital (Muhimbili) between June 2011 and February 2013. Participants were children under the age of five who were admitted due to either acute or chronic diarrhea. A total of 720 children, (392 boys and 328 girls) were enrolled. Fresh stool samples were collected. Out of 720 samples collected; 636 were from acute diarrhea cases and 84 from chronic diarrhea cases. Samples were collected using wide mouthed sterile plastic containers by trained nurses and clinicians. Specimens were transported in Cary-Blair transport medium (Difco laboratories, USA) to Muhimbili National Hospital, central pathology.

### Laboratory investigations for parasitic agents of diarrhea

Samples were examined directly for vegetative forms of parasites and cysts by standard microscopy with iodine-stained wet-mount preparations of a formalin ether concentrate and were stained with Kinyoun's carbol-

fuchsin (Ten Hove *et al.*, 2007).

The Modified Ziehl Neelsen staining technique was also performed for the detection of *Cryptosporidium parvum*.

(i) *Parasitic protozoa DNA extraction*: DNA was extracted directly from faecal suspensions (33–50% w/v) using the semi-automated NucliSens mini-MAG instrument in combination with NucliSens Magnetic Extraction Reagents (bioMérieux, Boxtel, The Netherlands). Though, it is known that, faeces contain a lot of PCR inhibitors. However, such inhibitors were removed by optimal extraction procedures using commercial spin columns (QIAamp™ DNA stool kit) (Gonin and Trudel, 2003). The extracted DNA was then stored in a deep freezer at –20°C.

(ii) *PCR amplification and simultaneous detection of the three parasites*: The samples were analyzed by PCR without reference to the initial microscopy results. Amplification and detection of *E. histolytica*, *C. parvum* and *G. lamblia* DNA, as well as the PhHV-1 internal control DNA, were performed on all samples using a multiplex PCR. The genus *Entamoeba* was detected using genus specific PCR primers (Table 1) followed by multiplex PCR primers (Table 2) comprising of *E. histolytica*, *E. dispar* and *E. moshkovskii* specific primers as used by Verweij *et al.* (2004), Khairnar and Parija, (2007), Bruijnesteijn *et al.* (2009) and Nazemalhosseini, *et al.* (2011).

Samples that were positive for *E. histolytica* by microscopy were subjected again to multiplex PCR for differential diagnosis of *Entamoeba* species to rule out the nonpathogenic ones. *C. parvum* and *G. lamblia* were detected using their genus specific primers chosen by using Primer Express software (Applied Biosystems) on the basis of the known SSU RNA gene sequence for *G. lamblia* (GenBank accession No.M54878) that consisted of forward primer Giardia-80F, reverse primer Giardia-127R, and the *G. lamblia*-specific double-labeled probe Giardia-105T. For *C. parvum* specific primers consisted of CrF and CrR, which amplify at 138-bp fragment inside the *C. parvum*-specific 452-bp fragment. Specific DNA amplification was detected with the *C. parvum*-specific double-labeled probe (Sulaiman *et al.*, 2002; Verweij *et al.*, 2004; Bruijnesteijn *et al.* 2009; Nazemalhosseini *et al.*, 2011).

- Faecal DNA samples were considered to contain inhibitors if the PhHV-1 internal control was not detected.
- Finally the amplification products were detected following electrophoresis in agarose gel 2%.

### Ethical considerations

Ethical and research clearance "IHRDC/ IRB/ No. A29" was obtained from the Ifakara Health Institute Research and Ethical Committee. Permission to conduct the study was sought from the respective hospital authorities. An informed verbal consent was obtained from parents/

**Table 1.** Primers used in PCR assays for selected parasitic protozoa

Target organism	Oligonucleotide Sequence (5'–3')	References
<i>Giardia lamblia</i>	GAC GGC TCA GGA CAA CGG TT CA GGA CAA CGG TTG	Verweij <i>et al.</i> , 2004
<i>Cryptosporidium parvum</i>	CTT TTT ACC AAT CAC AGA ATC ATC AGA TGT GTT TGC CAA TGC ATA TGA A	Bruijnesteijn <i>et al.</i> , 2009
<i>Entamoeba histolytica</i>	ATT GTC GTG GCA TCC TAA CTC A GCG GAC GGC TCA TTA TAA CA	Verweij <i>et al.</i> , 2004
PhHV	GGG CGA ATC ACA GAT TGA ATC GCG GTT CCA AAC GTA CCA A	Ten Hove <i>et al.</i> , 2007

**Table 2.** Primer sequences used for differentiation of *Entamoeba* species in a one step differential mPCR

Primer	Primer sequence	Reference
EntaF	5'-ATG CAC GAG AGC GAA AGC AT-3';	Morgan <i>et al.</i> , 1998
EhR,	5'-GAT CTA GAA ACA ATG CTT CTC T-3'	
EdR,	5'-CAC CAC TTA CTA TCC CTA CC-3'	
EmR,	5'-TGA CCG GAG CCA GAG ACA T-3'.	

**Table 3.** Comparative analysis of parasitic protozoa diarrhea characterized by mPCR and classical microscopy

Parasites	PCR	Microscopy	p-value
<i>Giardia lamblia</i>	35.6 % (256)	16.4 % (118)	0.045
<i>Entamoeba histolytica</i>	12.2 % (88)	20 % (144)	0.043
<i>C. parvum</i>	7.8 % (56)	4.4 % (32)	0.05
<b>TOTAL</b>	<b>55.6 % (400)</b>	<b>41% (294)</b>	<b>&lt;0.05</b>

**Table 4.** Percentage occurrence of diarrhoeagenic parasitic protozoa among different age groups of the under-five (n=180) as determined by PCR

	0-5	6-11	12-23	24-60	Total
<i>Giardia lamblia</i>	2.4	6	17.2	10	<b>35.6</b>
<i>Entamoeba histolytica</i>	0	0	9.6	2.6	<b>12.2</b>
<i>Cryptosporidium parvum</i>	0	2	2.8	3.0	<b>7.8</b>

guardians of the children before enrolment into the study.

### Data analysis

Statistical analyses were performed using SPSS v.11.0.1 (SPSS Inc., Chicago, IL, USA). The p-values of <0.05 were considered to be statistically significant.

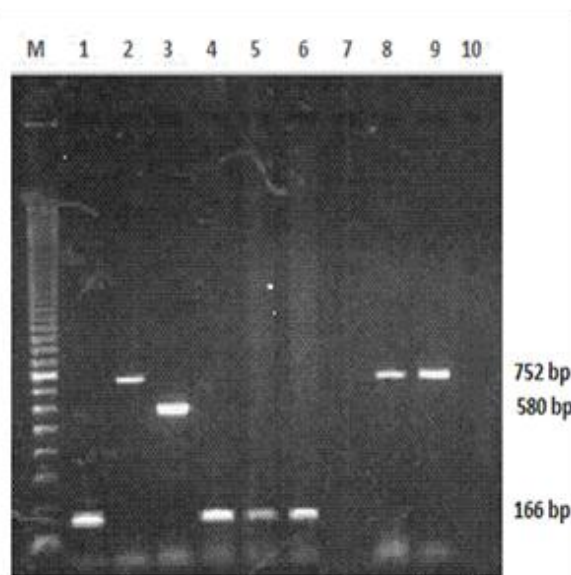
### RESULTS

Parasitic protozoa were detected and characterized microscopically and by using PCR method as summarized by table 3.

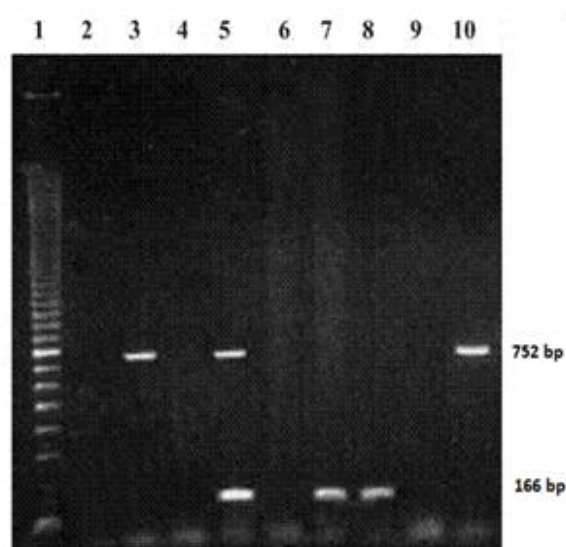
Among age groups, results showed that the age group 12-23 months was the most affected than the other two groups having the incidence of 8.6%, 4.8% and 1.4% for *Giardia lamblia*, *Entamoeba histolytica* and *Cryptosporidium parvum* respectively, (p- value < 0.05) (Table 4).

Co-infections were seen in 70 samples studied in which there were co-infection of *Giardia lamblia* and *Entamoeba histolytica* occurred in 30 samples and the remaining 40 samples were co-infections of *Giardia lamblia* and *Cryptosporidium parvum*. Figure 1 and 2.

The differential diagnostic mPCR assay successfully amplified the positive samples from *E. histolytica*, *E. dispar* and *E. moshkovskii* from the *E. histolytica* positive microscopy results, among 144 samples that were *E. histolytica* positive by classical microscopy, only 48



**Figure 1.** PCR amplification for detection and differentiation of *E. histolytica*, *E. dispar*, and *E. moshkovskii* by using primers EntaF, EhR, EdR, and EmR. **Lane M**, 100-bp ladder DNA marker; **lane 1**, *E. histolytica*; **lane 2**, *E. dispar*; **lane 3** *E. moshkovskii*; **lanes 4 to 6**, amplified products (166 bp) *E. histolytica*-positive specimens; **lane 7** negative sample; **lanes 8, 9** amplified products (752 bp) *E. dispar*-positive specimens and **lane 10** negative control.



**Figure 2.** Agarose gel electrophoresis of differential multiplex PCR product.

**Lane 1:** DNA ladder (100 bp). **Lane 2** negative control; **Lanes 3, 10** are positive for *E. dispar*. **Lanes 7, 8** are positive for *E. histolytica*. **Lane 5** is positive for both *E. histolytica* and *E. dispar* (co-infection). **Lanes 4, 6, 9** are negative samples.

**Table 5.** Differential PCR results of *E. histolytica*, *E. dispar* and *E. moshkovskii*

Type of <i>Entamoeba</i> species	Samples positive by PCR
<i>E. histolytica</i>	48/144 (33.3 %)
<i>E. dispar</i>	80/144 (55.6 %)
<i>E. moshkovskii</i>	16/144 (11.1 %)

samples (33.3 %) were positive for *E. histolytica* by differential mPCR (Table 5).

Other parasitic protozoa isolated in this study though were not in the initial objective include helminthes; hook worms (*Ankylostoma duodenale/ Necator americanus*) 6.6 % (48 samples), *Ascaris lumbricoides* 3.9 % (28 samples) and *Enterobius vermicularis* (pin worms) 2.2 % (16 samples), and these worms mostly were from children ranging from 24 months and above. Others were *Blastocystis hominis*, *Endoclimax nana* and *Isospora belli* that were seen in 6, 4 and 3 samples, respectively.

## DISCUSSION

Gastrointestinal protozoan parasites are important public health problem especially in the under-fives throughout

the world, but the development of a multiplex PCR assay which enables the simultaneous detection of all protozoan parasites discussed below was an extremely useful public health tool, and an attractive, cost-effective procedure for diagnostic laboratories and characterization of these parasitic protozoa as stated by Ten Hove *et al.* 2007; Girones *et al.* 2010 and Michael *et al.* 2010. In most cases it is likely that the real cause of diarrhea in the under-five is misdiagnosed or goes unnoticed.

Anecdotal information from doctors in Dar Es Salaam reported that, half (50%) of admissions in paediatric wards in Dar Es Salaam hospitals are suffering from diarrheal diseases of different causes.

The current study aimed at isolation and characterization of parasitic protozoa commonly associated with diarrhea in the under-fives who were admitted due to acute or chronic diarrhea in three

hospitals in the city of Dar Es Salaam, Tanzania.

The results of the study showed, the highest prevalence of diarrhea (29.6%) was found in the age groups of 12-23 months, followed by 24-60 months (15.6%), 6-11 months (8%) and least 0-5 months (2.4%). Microscopic method identified 40.8% parasitic protozoa, while PCR identified 56.6%. However, m-PCR was more discriminative and sensitive, such that the 144 samples identified microscopically as *E. histolytica*, were differentiated as; *E. histolytica* 48 (33.3%), *E. dispar* 80 (55.6%) and *E. moshkovskii* 16 (11.1%). Differential detection of *E. histolytica* and *E. dispar* in fresh stool by PCR, has helped also to detect mixed infections at a very small amount, this is in keeping with Zulhainan *et al.* (2006), Gutiérrez-Cisneros *et al.* (2010) and Ngui *et al.* (2012) that showed sensitivity at a ratio of 1:10000.

The most common protozoa isolated in this study were *Giardia lamblia* 35.6 %, which is higher compared to that found by other study in Dar Es Salaam by Moyo *et al.* (2011). Followed by *Entamoeba histolytica* (12.2%), *Cryptosporidium parvum* (7.8 %), and helminthes parasites 12.7%. this probably is due to the different methods used or due to their seasonal role in causing childhood diarrhea among children in Dar Es Salaam. Diagnosis of *G. lamblia* infections in hospitals and health centers in Tanzania is normally done by microscopic examinations of stool samples. For research purposes direct fluorescent-antibody assays and antigen detection by using enzyme-linked immune-sorbent assay are carried out.

The proportion of cases that yielded no pathogen was inversely related to age, being highest among infants 0-5 month age group and lowest among those aged 24-60 months. This could be explained by feeding habits that, very young infants are exclusively breast fed apart from older infants, and in some cases may be bottle fed or may have started being given other foods and water that may be contaminated and these are some of the common risk factors for young children to contract diarrhea diseases in majority of developing countries.

In 192 diarrheic stool samples out of 720 cases, no pathogens were detected. This could be explained by the fact that not all diarrhea cases are caused or associated with pathogens. However, it should be noted that sometimes diarrhea could be due to other non-infective factors such as intolerance to some food components and weaning diarrhea in which the intestine of the child is not well developed to digest and absorb most of the food. Poverty as measured in terms of poor hygiene and sanitation, illiteracy, lack of reliable clean water supply, lack of proper toilets at house hold levels and in schools, are always associated with parasitic diseases in most low and middle-income countries like Tanzania.

When compared with stool microscopy results, PCR results seemed to be more accurate, efficient and sensitive as it was able to determine positive samples

that were microscopically negative. Furthermore, mPCR method used in the current study was able to distinguish between *Entamoeba* species (pathogenic from non pathogenic) Table 3 and 5. This helped to solve one important drawback of microscopy which is low sensitivity as indicated by Ten Hove *et al.* (2007) that gives relatively large number of false negative results

On the contrary, other studies (Liang *et al.*, 2010) found that *E. histolytica* was more prevalent than *E. dispar*. The discrepancies between the present and other previous studies may be due to differences between studied population characteristics and/ or geographical and socioeconomic factors. It was not possible in the present study to ascertain the immune status of the children who were enrolled for the study due to some financial constraints. The non-determination of the HIV status of the participants was also a limitation as it would have proffered explanations on factors such as immune-suppression due to HIV/AIDs that may be the factor for occurrence of diarrhea. This study was also able to detect *Cryptosporidium parvum* using PCR primers which were species specific, this contradicts earlier studies that did not detect the *Cryptosporidium parvum* in Tanzanian children among under fives as a result of deficiencies in the methods employed. Another limitation of the study was the exclusion of viral pathogens to assess the level and contribution of viruses to childhood diarrhea. This could have given different results for the samples in which no pathogens were detected and even possible co-infection in cases where pathogens were detected in the current study. It was also not possible to establish a causal relationship between diarrhea and the protozoan identified in the study subjects.

Other parasites such as intestinal helminthes were detected in 36 specimens namely; hookworms, pinworms, roundworms and protozoans like *Isospora belli*, *Endoclinax nana* and *Blastocystis hominis*. Some of these have been associated with diarrhea and some are non-pathogenic but their presence indicates faecal contamination, hence, their detection is still very important as it gives a clue to water sanitation status and safety.

Urban migration contributes to the creation of informal settlements with high rates of poly-parasitism with both helminthes and protozoa which leads to higher infection rates through closer proximity of the infected to vulnerable groups (Mbae *et al.*, 2013).

This study therefore has provided molecular epidemiological data on enteric parasitic protozoans in Dar Es Salaam. The findings in the age groups studied brings up the understanding of the burden of disease in children less than 5 years of age and that the detection of protozoan parasites is important in order to improve the health conditions of these children. Hence, the need for adequate and appropriate laboratory methods such as PCR for diagnosis of diarrhea causative agents in our clinical settings is mandatory.

## CONCLUSION

Parasitic protozoa play an important role in relation to childhood diarrhea in Dar Es Salaam especially *Giardia lamblia* which was strongly associated with the disease. Among the methods used, PCR assay has shown to be a sensitive and reliable method than conventional methods in identifying parasitic protozoa from faecal samples and can assist in the rapid identification of the causative agents of diarrhea. The ability of PCR to distinguish between the pathogenic *Entamoeba histolytica* from non pathogenic species is valuable. It may facilitate epidemiological surveillance of water and edible products for human consumption to determine the risk of acute diarrheal disease at any single time in any densely populated geographic region such as some urban areas of Dar Es Salaam. And also, it may facilitate the implementation of preventive measures to decrease morbidity and mortality due to diarrhea in under-five children living in the developing world.

Classical microscopy test could not be undermined, as can be used routinely in diagnoses of intestinal parasitic protozoa in areas where molecular based methods would not be possible. In developing world such as Tanzania, there is a lot of shortcomings, e.g. electricity problems and poor water supply. Microscopy can be affordable even in rural areas of the country.

## RECOMMENDATIONS

Little attention is being given to parasitic enteric infections by health authorities and yet parasites such as *Giardia lamblia*, *Entamoeba spp.*, *Cryptosporidium spp.*, *Isoospora belli*, *Microsporidia spp.*, *Giardia intestinalis*, *Cyclospora spp.*, pose unique epidemiological constraints as they are ubiquitous in nature, treatment is still not yet available for some, and others are highly resistant to chlorination and other antiseptics used in water treatment.

This study provides a starting point for more extensive studies in the area of diarrhea in childhood and the use of effective diagnostic methods to reach for specific diagnosis and treatment.

Based on the findings of this study the following were recommended:

- Studies on causative organisms of childhood diarrhea should be carried out regularly and microbe-specific intervention strategies for the control of childhood diarrhea should not focus only on specific diagnosis of diarrheagenic bacteria but also on parasitic protozoa. However, other pathogens should not be neglected since seasonal changes may have impact on the prevalence of diarrhea due to other pathogens.
- Comprehensive studies should be carried out that will include all pathogens that are causative agents of childhood diarrhea (since this study was limited only to parasitic protozoa)

- Since gastrointestinal infections are very common in Tanzania with high associated mortality rate in infants and young children, public health prevention through fostering good hygiene and providing sanitary water and food supplies should be of the at most importance.
- Treatment should not rely on microscopic examination of stool alone. The incorporation of many new technologies into the diagnostic laboratory will provide better epidemiological data and greater understanding of the public health problems and measures to control the disease.
- The combined effort of policy makers, research scientists, clinicians and administrators will help in management and control of the diseases in Tanzania.

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