

## Full Length Research Paper

# Molecular characterization of intestinal protozoan parasites from children facing diarrheal disease and associated risk factors in Yamoussoukro, Côte d'Ivoire

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Diarrheal diseases are very common in children under 5 years and may lead to a delay of physical and mental development. Despite this knowledge, data on diarrheal diseases and socioeconomic determinants are still scarce in Côte d'Ivoire. This study is then conducted with the objective to fill part of this gap and specifically assess link between infant diarrhea occurrence and some major socio-environmental factors. Stool samples were collected from children less than five suffering from diarrhea at Yamoussoukro Regional Hospital in central Côte d'Ivoire. Molecular species specific diagnosis was used to detect *Cryptosporidium parvum*, *Giardia intestinalis* and *Entamoeba histolytica*, three major protozoan parasites which cause diarrhea. Out of 306 stool samples examined, 62.75% were detected as positive at least for one of the protozoan parasite studied. Species specific prevalence was 36.93% for *C. parvum*, 20.92% for *G. intestinalis* and 22.55% for *E. histolytica*. Infection was more prevalent in children whose mothers were not educated although the difference was not statistically significant. No link was found between gender and infection while sanitation infrastructures, mother and children ages and water sources were found significantly associated with diarrhea occurrence. Awareness is then needed for women on lack of hygiene rules that could lead to diarrheal diseases burden.

**Key words:** Diarrheal diseases, children development, parasitic protozoan, molecular characterization, socio-environmental factors.

## INTRODUCTION

Diarrhea is the second leading cause of under-five deaths worldwide, after pneumonia (UNICEF/WHO, 2009). Among those who survive diarrhea, the morbidity burden may affect their development depending on the

intensity of diarrhea and pathogen in question. Malnutrition, anemia, growth restrictions, cognitive delays, irritability, increased susceptibility to other infections and acute complications are some of the consequent morbidities.

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Intestinal parasitic infections have detrimental effects on the survival, appetite, growth and physical fitness (Sharma et al., 2004), school attendance (Nematian et al., 2008) and cognitive performance of school age children (WHO, 2005). In young children, diarrhea is particularly prevalent during the first 2 years of life and caused by parasitic diseases associated with impaired cognitive performance in later childhood (Niehaus et al., 2002; Walker et al., 2012). Infection early in life is, malnutrition and diarrhea, associated with poor cognitive function at school age (Berkman et al., 2002; Ijaz and Rubino, 2012).

*Cryptosporidium parvum*, *Giardia intestinalis* and *Entamoeba histolytica* are three major protozoan pathogens responsible for diarrhea in children under 5 years (Stark et al., 2009; Stark et al., 2011). *C. parvum* infection has been linked to chronic diarrhea, malabsorption and poor weight gain in children (Checkley et al., 1997; Adjei et al., 2004; Saneian et al., 2010; Rahi et al., 2013). Malabsorption in *G. intestinalis* infected children is probably due to several factors including increased epithelial permeability, bacterial overgrowth of the small intestine, loss of intestinal brush border surface area, villus flattening and inhibition of disaccharidase activities (Müller and von Allmen, 2005). *E. histolytica* is responsible for dysentery, anemia and could impact on infant growth (Ali et al., 2008; Hegazi et al., 2013).

The prevalence of intestinal parasitic infections is one of the most accurate indicators of socioeconomic conditions of a population and may be associated with several socio-cultural and economic determinant factors. It has been demonstrated that failure of children to fulfill their developmental potential and achieve satisfactory educational levels plays an important role in the inter-generational transmission of poverty (Grantham-McGregor et al., 2007). In countries with a large proportion of such children, national development is likely to be affected and, in turn, infectious diseases will become key factors influencing public health which could impair Millennium Development Goal (MDG)4 (United Nations, 2010) regarding the under 5 mortality rate (U5MR) and infant mortality rate (IMR) (UNICEF/WHO, 2005; UNICEF/WHO, 2009).

Unlike microscopy of worm eggs, microscopic examination for the various intestinal protozoa is laborious, insensitive, and requires professional training. Sensitivity of microscopy for the different parasites does not exceed 60% even if concentration methods and skilful technical assistance are available (Hiatt et al., 1995). Nowadays a number of PCR-based assays have been developed for the identification of *G. intestinalis*, *E. histolytica* and *C. parvum* directly from human faeces. These assays have been proven to be considerably more sensitive than microscopy, highly specific and in contrast to microscopy are able to differentiate between the species and the subtype level. In addition, although a significant body of research exists on the relationship between diarrhea like-

likelihood and infrastructures, the impacts of water and sanitation infrastructures have been shown to vary by location, demanding country-specific analyses wherever diarrhea is a major cause of childhood morbidity and mortality.

Our objective is thus to investigate *G. intestinalis*, *E. histolytica* and *C. parvum* infection, based on molecular diagnosis, in children under five years suffering from diarrhea and attending Yamoussoukro CHR hospital in Central Côte d'Ivoire and examine link between infection and major socio-environmental factors. This could then contribute to appropriate policies aimed at controlling diarrheal diseases by improving mothers' hygiene practices when attending pediatric health care services.

## MATERIALS AND METHODS

### Study area and sampling

This study was conducted in the pediatric unit of regional health care services (CHR) in Yamoussoukro District. The Autonomous District of Yamoussoukro, a city whose population was estimated to be 259,373 inhabitants in 2010 with population density of 104 inhabitants per km<sup>2</sup> is the political capital of Côte d'Ivoire. It is located at 240 km north of Abidjan, the economic and administrative capital of Côte d'Ivoire. The CHR is the greatest hospital in the district.

Mothers attending pediatric health care services from March to June 2013 with their children suffering from diarrhea cases were approached and a detailed explanation of the study was given by a local nurse in local languages or in French when possible. Following the explanation, if the mother is willing she may sign or thumb-print the informed consent form which was also translated into the local languages. A community health worker went to the mother's home and took a stool sample from the child in an interval of two hours after visit to the hospital. 200 mg of each stool were aliquot in cryotube label with code from each child and stored at -20°C before DNA extraction. A socio-economic questionnaire was administered to investigate factors that could be associated with the occurrence of the diarrhea. The questions selected for analysis in this study referred to age (mother and children), shared or private toilet at home, and origin of water supply and education level of the mother. 306 stool samples were collected for analysis as well.

### Molecular characterization

#### Extraction of genomic DNA

Genomic DNA was isolated from stool specimens by a cetyltrimethylammonium bromide (CTAB) extraction method as described by Hairnar and Parija (2007). Briefly, 50 mg of stool specimen, was dispersed in 250 µl of lysis buffer (0.25% sodium dodecyl sulfate in 0.1 M EDTA, pH 8.0), and 100 µg/ml of proteinase K was added. The lysate was incubated at 55°C for 2 h. Then 75 µl of 3.5 M NaCl followed by 42 µl of 10% CTAB/0.7 M NaCl (heated to 55°C) was added. After the components were mixed, the sample was incubated at 65°C for 30 min. This was followed by extractions with equal volumes of chloroform and then phenol-chloroform-isoamyl alcohol, and the DNA was precipitated with ice cold ethanol. The dried DNA pellet was dissolved in sterile distilled water and passed over a DNA clean-up spin column. The DNA was finally eluted from the spin column in 100 µl of Tris-EDTA (TE) buffer.

**Table 1.** Sex related prevalence of protozoan species among diarrheal children.

Protozoan	Sex		X <sup>2</sup>	P. value
	Male (N=153)	Female (N=153)		
	Number (%)	Number (%)		
<i>C. parvum</i>	54 (35.3)	59(38.56)	0.35	0.56
<i>G. intestinalis</i>	28(18.3)	36(23.53)	1.27	0.26
<i>E. histolytica</i>	30(19.61)	39(25.49)	1.52	0.22
Overall infection	92 (60.13)	100 (65.36)	0.9	0.34

**Table 2.** Frequency distribution of child age groups of the study children and their mothers.

Characteristics	Frequencies			X <sup>2</sup>	P. value	
	Positive (%)	Negative	Total			
Children age groups	0-24	128(69.19)	57	185	8.53	0.01
	25-36	56(53.84)	48	104		
	>36	8(47.05)	9	17		
<b>Total</b>		<b>192(62.75)</b>	<b>114</b>	<b>306</b>		
Mothers age groups	16-20	15(53.57)	13	28	9.49	0.05
	21-25	31(50.82)	30	61		
	26-30	41(64.06)	23	64		
	31-35	79(72.48)	30	109		
	>35	26(59.09)	18	44		
<b>Total</b>		<b>192(62.75)</b>	<b>114</b>	<b>306</b>		

#### PCR amplification using species-specific oligonucleotides

The PCR method was applied to all stool sample DNA, which were stored at -20°C without preservatives using species-specific oligonucleotides. 2.5 µl of DNA solution was used in the PCR reaction. PCR primer sequences used to amplify the genus *Giardia* and the species *G. intestinalis* are those reported by Mahbubani et al. (1991, 1992). The *Cryptosporidium* isolates DNA was amplified using polymerase chain reaction amplification and restriction fragment length polymorphism (PCR-RFLP) analyses of the *TRAP-C2* gene (Thrombospondin-Related Adhesive Protein of *Cryptosporidium* 2) as described in the literature (Peng et al., 1997; Sulaiman et al., 2002). In detail, nested PCR was used to amplify a fragment (266-366 bp) of the *TRAP-C2* gene using two sets of oligonucleotide primers as described previously follow by restriction enzymes *Bst*EII, *Hae*III digestion for RFLP analysis (Nazemalhosseini et al., 2011).

*Entamoeba* genus was detected using genus specific PCR primers followed by nested multiplex PCR primers comprising *E. histolytica*, *E. dispar* specific primers (Khairnar and Parija, 2007).

#### Amplified products detection

Three microlitres of PCR-amplified DNA was detected by 2% agarose gel electrophoresis in TAE buffer (0.04 mM Tris-acetate, 0.001 mM EDTA, pH 8.0), stained in a solution of ethidium bromide (0.5 mgml<sup>-1</sup>) and visualized with a UV transilluminator.

#### Data analysis

Data recorded were analyzed using excel and STATA 9.0 software

(Stata, College Station, Texas, United States). Chi-square (x<sup>2</sup>) tests were conducted to determine the relationship between parasites infection and major socio-environmental factors. Significance level was set at p<0.05 for all tests.

## RESULTS

A total of 306 children with diarrheal diseases were included in this study out of the 405 children surveyed. One hundred and ninety two (192) of these children were infected with at least one of the three parasites detected by molecular markers, which make the overall prevalence 62.75%. Among infected children, 100 were female and 92 were male but no significant difference (p=0.34) between the two sexes was figured out (Table 1).

Children age groups were divided in three groups: birth to 24 months, 25-to 36 months and over 36 months. The group from birth to 24 months shows the highest prevalence (69.19%) and the difference was significant (p=0.01). Fewer infections (47.05%) were detected in the older children (>36 months) but this age group was less prevalent in the study (Table 2).

To see the mothers experience in caring for their children and influence on the occurrence of infection, the mothers' population was also divided in age groups with a 5-years interval between age groups. Protozoan infection was more prevalent in mothers' age group from

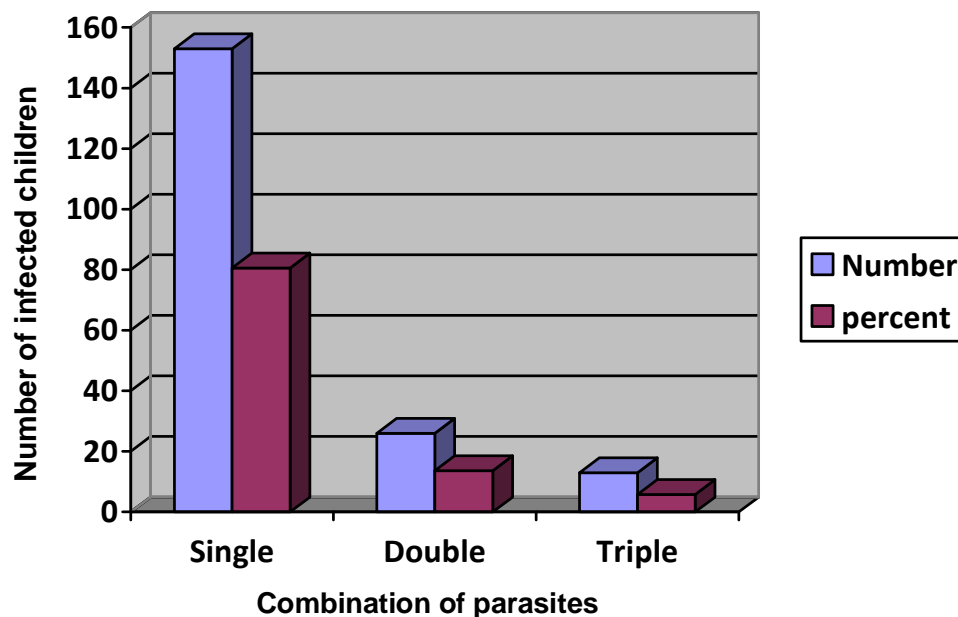


Figure 1. Single and mixed infections among children suffering from diarrheal.

Table 3. Age related prevalence of protozoan parasites among diarrheal children.

Parasites detected	Children age groups				$\chi^2$	P-value
	0-24	25-36	> 36	Total (%)		
<i>C. parvum</i>	74	33	6	113(36.93)	1.99	0.37
<i>G. intestinalis</i>	42	18	4	64(20.92)	1.27	0.53
<i>E. histolytica</i>	46	20	3	69(22.55)	1.48	0.48
Overall infection	128	56	8	192(62.75)	8.53	0.01

31 to 35 years (72.48%) and lowest prevalence (50.82%) was found in group from 21 to 25 with a significant difference ( $p=0.05$ ) between age groups (Table 2). Polyparasitism with double and triple infection was found in 26 and 13 children respectively out of the 192 positive cases but the majority was single infection (Figure 1). *C. parvum* was the most prevalent protozoan species found (36.93%) and *G. intestinalis* the less prevalent (22.55%). No significant difference was seen within each protozoan species among age groups but a significant difference was detected among children age groups ( $p=0.01$ ) when dealing with the overall infection (Table 3).

Socio-environmental factors such as mothers' education level, sanitation infrastructure used (shared or family private) and water sources were assessed (Table 4). The difference in the prevalence of protozoan infection in children was insignificant ( $p=0.06$ ) with regards to the mother education level although infection in children from not educated mother (NE) was the highest (Table 4).

Infection was less common in family with private sani-

tation as compared to community sanitation and the difference was very significant ( $p<0.0001$ ). Regarding sources of population water supply, tank and well users were more susceptible to infection than tap users with a significant difference ( $p<0.0001$ ).

## DISCUSSION

*E. histolytica*, *G. intestinalis* and *C. parvum* are known to be the most important diarrhea-causing protozoa (Stark et al., 2011) and in addition in September 2004, *Giardia* and *Cryptosporidium* were included in the "Neglected Diseases Initiative" (Savioli et al., 2006). To overcome the lack of sensitivity of the microscopic observation of intestinal protozoa (Stensvold and Nielsen, 2012), in this study, we decided to use sensitive and specific molecular markers to investigate protozoan parasites *E. histolytica*, *G. intestinalis* and *C. parvum* which are three of the most common intestinal protozoan parasites infecting humans worldwide.

**Table 4.** Major socio-environmental factors related to prevalence of protozoan parasites among diarrheal children.

Characteristic	Frequency			$\chi^2$	P. value	
	Positive (%)	Negative (%)	Total			
<b>Mothers education level*</b>	HE	31 (57.41)	23	54	6.11	0.1
	NE	65 (72.22)	25	90		
	PE	44(63.77)	25	69		
	SE	52(55.91)	41	93		
Total		192(62.,75)	114	306		
<b>Sanitation</b>	Private	61(38.61)	97	158	87.80	<0.0001
	Shared	131(88.51)	17	148		
Total		192(62.75)	114	306		
<b>Water sources</b>	Tank	51(94.44)	3	54	83.45	<0.0001
	Tap	91(84.26)	108	199		
	Well	50(94.33)	3	53		
Total		192(62.75)	114	306		

Mothers education level\*: HE (High education), NE (No education), PE (Primary education), SE (Secondary education).

A prevalence of 62.75% was observed on 306 diarrheal stools collected from children under the age of 5. The remaining 37.25% could be due to other pathogens causing diarrheal disease such as enteropathogens, bacteria (*Enterotoxigenic Escherichia coli* and other obligate pathogenic *E. coli*, *Campylobacter jejuni*, *Vibrio cholera*, *Yersinia species*, *Shigella species*, *Salmonellae*, cytotoxigenic *Clostridium difficile*), viruses (*Rotavirus*, *Noro viruses* and *Enteric adenoviruses*) and parasites such as the helminths (*Strongyloides stercoralis* and *Ascaris lumbricoides*) and protozoa (e.g. *Blastocystis hominis* and *Isospora*).

Stools of diarrhea infection are different in infant mushy stool subjected to breastfeeding which was clearly distinguished with watchful eye of physician (Messou et al., 1996). No association was found between gender and overall occurrence of parasitic infection in contrast with other studies conducted in other town in Côte d'Ivoire where authors demonstrated an association between gender and the occurrence of *G. intestinalis* and *E. histolytica* infection (Ouattara et al., 2010). Our results were nevertheless consistent with theirs in terms of a significant association between the prevalence of infection and age groups of children and their mothers. The highest prevalence of infection in children from 0 to 2 years is consistent with that reported in the literature (Guerrant et al., 1992, Walker et al., 2012). Indeed, diarrheal diseases leading to childhood malnutrition, morbidity and mortality cause 1.8 million deaths annually worldwide among children less than five years, 80% occurring in the first two years of life (Walker et al., 2012).

At species specific level, *C. parvum* species presented the highest prevalence (36.93%). *C. parvum* infection is common worldwide, and its frequency may be related to

the inadequacy of sanitation. In general, the infections are more common in developing countries and an infection rate of 33.83% is reported for children aged less than 5 years with diarrhea in Iraq (Rahi et al., 2013).

*C. parvum* is unfortunately scarcely studied in Côte d'Ivoire unlike *E. histolytica* and *G. intestinalis* (Ouattara et al., 2010; Schmidlin et al., 2013) despite its importance as a major pathogen that can cause diarrhea and even death in young children. The most recent study in Ivorian area reported prevalence of 15.0 and 14.4% of *G. intestinalis* and *E. histolytica/E. dispar*, respectively (Schmidlin et al., 2013). These rates are less than what we reported here and could be due to the fact that molecular techniques are more sensitive in addition to advantages that permit differentiation of *E. histolytica* from *E. dispar*, and also because we collected stools from children alleged as suffering from diarrheal diseases.

Literature reported that the prevalence of intestinal parasitic infections is one of the most accurate indicators of socioeconomic and environmental conditions of a population (Astal, 2004) and may be associated with several determinant factors, such as adequate sanitation, fecal pollution of water and foods, as well as host age and type of infecting parasite (Quinn, 2009). Our results confirmed that of others where sanitation and water sources influence occurrence of diarrheal diseases (Godana and Mengiste, 2013) but we found no significance influence of mother education level on pathogen transmission, contrary to some literature report (Cochrane et al., 1982; Grosse et al., 1989) despite the fact that protozoan parasites were more common in children from mother with no education level.

37.25% of children suffering from diarrhea were not in-

ected with one of the three protozoa parasite studied but could be infected with other pathogens causing diarrhea as feces were recognized as diarrheal infection stool by pediatricians. The apparent polyparasitism shows that other pathogens than those detected could co-exist in these infections.

## Conclusion

Limited published data are available from Ivorian body of research on protozoan pathogens associated with children diarrhea. This first study with specific genetic markers on diarrheal children shed light on some factors that contribute to the occurrence of protozoan diarrhea.

Although the molecular diagnosis of parasites used in this study cannot be used routinely in hospitals due to its cost, it gives the true prevalence with respect to its sensitivity and specificity and confirms microscopic misdiagnosis.

Our recommendations are consistent with research that could lead to the development of rapid diagnostic test at the hospital level. Advice on washing hands after using the toilet and the care of the drinking water for children could avoid a lot of inconvenience as a result of diarrheal diseases.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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