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Genetic-based investigation of three prevalent waterborne protozoa parasites in drinking water sources in Daloa district in Côte d'Ivoire

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ABSTRACT
Objective: Waterborne gastrointestinal protozoa pathogens are frequently associated with morbidity and mortality, in children under five years but these protozoa pathogens are scarcely investigated in quality control of drinking water. The objective of this study was to investigate the presence of waterborne protozoa such as *Cryptosporidium parvum*, *Giardia intestinalis,* and *Entamoeba histolytica* in various natural water sources from Daloa city using genetic markers.
Methodology and results: A total of 34 water source samples comprising 2 from springs, 5 from hydraulic pumps, and 27 from wells were investigated. Water stored in containers was also analyzed and a survey was conducted on households' water consumption habits. Species specific genetic markers were used to detect the presence of *C. parvum*, *G.intestinalis,* and *E. histolytica.* Out of the 34 sources surveyed, 14(41.2%) were infected with at least one of the 3 protozoa investigated. Twenty one (61.76%) out of the 34 Containers analyzed were found contaminated with at least one of the 3 parasites. About 15.2% of households surveyed used well water for drinking though it was shown that 48.14% of these wells are contaminated with waterborne protozoa. The detection of protozoa pathogen in some home containers and not in natural sources where the containers were filled, suggested that contamination may originate from containers through human actions. Low-income households should be aware of the risk of drinking water from wells and the need to boil or treated drinking water to avoid diarrheal diseases related thereto.
Conclusion and applications: This study shows that the spring natural water used by people does not contain parasites unlike almost wells water that have real health risks if no treatment is performed before consumption. Practice good personal hygiene regarding water's storage containers and hand washing could reduce the risk of propagation of parasitic protozoa.
Key words: water supply, waterborne parasites, *E. histolytica, C. parvum, G. intestinalis.*

INTRODUCTION
Daloa city is supplied with potable water that is regularly monitored by the national water supply company. However an important proportion of the citizens avoid using tap water as source of drinking

water mainly because of its colour and sometime dirty background. People prefer drinking natural water stored at home in domestic containers collected from natural groundwater namely from springs, hydraulic pumps, and from wells. Some others use well water for consumption rather for economic reasons as they do not need to pay for that. Natural sources of water are rarely free of microorganisms and thus, may harbour pathogens (Jones, 2001). Recently, studies on water resources in Côte d'Ivoire reported several potential of pollution of both surface water and groundwater in the southern areas of the country (Ahoussi *et al*. 2008, 2009; Soro *et al*., 2010). Studies in the field of waterborne parasitic diseases are scarce in the hinterland such as western-central Côte d'Ivoire. In general, to measure water quality, microbiological analyses for bacteria such as *Escherichia coli*, *Clostridia perfringens* and total coliforms (OMS, 2008; Ahoussy *et al*, 2013) are done. Despite waterborne gastrointestinal parasite pathogens such as *Cryptosporidium parvum*, *Giardia intestinalis,* and *Entamoeba histolytica* are rarely investigated in terms of water quality, they are frequently associated with mortality of immune-compromised individuals (Fayer, 2004; Thompson *et al*, 2005). Furthermore, *C. parvum*, *G. intestinalis* and *E. histolytica* are three major protozoan pathogens responsible for diarrhoea in children under 5 years: *Cryptosporidium parvum* infection has been linked to chronic diarrhoea, malabsorption and poor weight gain in children (Checkley *et al*., 1997). Malabsorption in *Giardia 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 & von Allmen, 2005) and *Entamoeba histolytica* is responsible for dysentery, anaemia and could impact on infant growth (Ali *et al*, 2008). Due to the influence of waterborne pathogens on households' welfare, we aim in this study to use genetic-based markers for the detection of protozoan parasites such as *C. parvum* and *G. intestinalis* and *E. histolytica* in fact 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).

MATERIAL AND METHODS

Study sites and sampling methods: Daloa is a city located in western-central Côte d'Ivoire. It is about 141 km from the political capital Yamoussoukro and 383 km from Abidjan the economic capital. It had 261,789 inhabitants in 2012 with a density of 49,348 inhabitants per km^2. It is the third biggest city in Côte d'Ivoire after Abidjan and Bouaké (Figure 1).

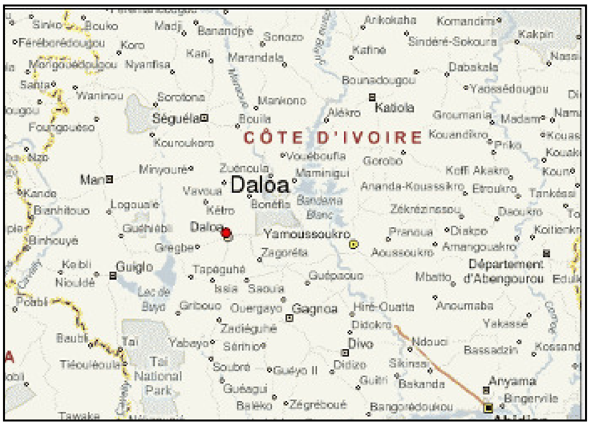


Figure 1: Localization of Daloa city in Côte d'Ivoire

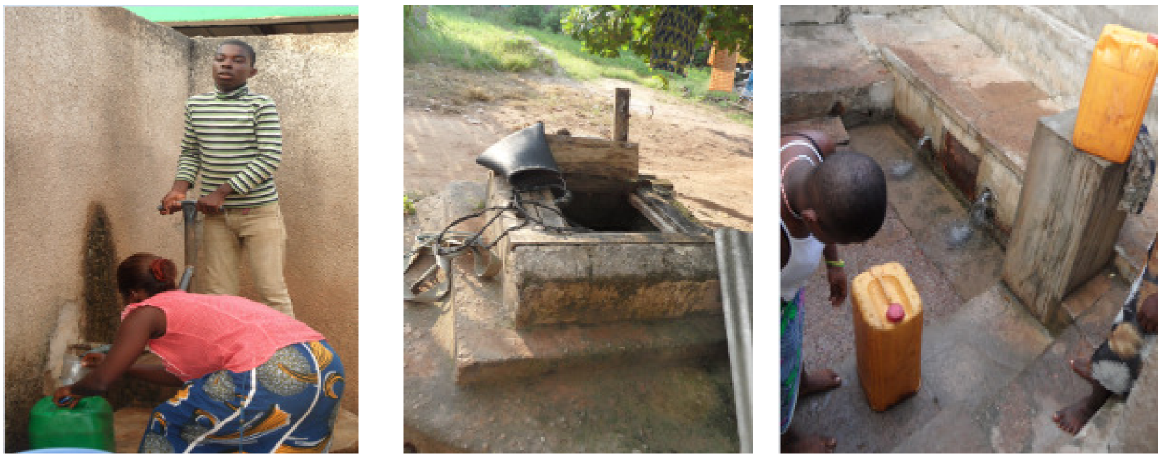
Samples collection: Water samples were collected with sterilized plastic bottles from wells, hydraulic pumps and springs distributed in the four cardinal points of the city (figure 2). In addition, specimens of natural water kept in house tanks by communities were also investigated for microorganisms. Samples were transported in a cooler with frozen ice packs and processed for analysis within 4 hours (Fotedar *et al*, 2007). A questionnaire was also given out in order to collect information on the water consumption habits of communities.

Water quality indicators: Method 1604 (U.S. EPA, 1604, 2002) on MI agar is a one-step method for the monitoring of both total coliforms and *E. coli*. Likewise, Method 1600 (U.S. EPA, 2002) on mEI agar is also one-step method, used for the detection of species belonging to Enterococcus genus. These membrane filtration (MF) based techniques do not require any additional confirmation step, mostly because they are based on the direct detection of a specific enzymatic activity.

Genetic characterization of protozoan parasite: DNA was extracted from water samples previously filtered through a 47 mm diameter, 0.45±0.02 mm pore size membrane filter using the phenol chloroform extraction method as described by Carraway (Carraway *et al*., 1996). PCR primer sequences used to amplify the genus *Giardia* and the species *G. intestinalis* are those reported by Mahbubani *et al*., 1991 and 1992 respectively. The *Cryptosporidium* 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 *BstE*II, *Hae*III digestion for RFLP analysis (Nazemalhosseini *et al*, 2011). *Entamoeba* genus was detected using genus specific PCR primers follow by nested PCR primers comprising *E. histolytica specific primers* (Khairnar and Parija, 2007).

Amplified products detection: 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 ($0.5 mgml^{-1}$) and visualized with a UV transilluminator.

Data analysis: Data were recorded using excel and analyzed with STATA 9.0 software (Stata, College Station, Texas, United States). Chi-square (x^2) tests were conducted to determine the load of each parasite species and to assess drinking consumption habits regarding social environment. Significance level was set at $p<0.05$ for all tests.



a - Hydraulic pump **b-** Well **c-** Natural Upgraded spring

Figure 2: Different types of natural water sources encountered in Daloa city

RESULTS

Drinking water sources: Out of the 34 natural sources surveyed, 14(41.2%) were infected with at least one of the 3 protozoa parasites investigated with genetic markers. *E. histolytica* was the most prevalent of the 3 pathogens studied with 29.41% but not significatively different with the prevalence of the other protozoa pathogens encounter $P>0.05$. None of the two natural springs surveyed was found infected with bacteria or protozoan parasites. Of a total of five samples from hydraulic pumps examined, only one was found infected with bacteria and protozoa (Table 1). Co-infections with combinations of *E. histolytica-C.parvum, E. histolytica-G.intestinalis* and C. parvum-*G. intestinalis* were observed respectively in 2, 3 and 1 of the total water sources investigated. Out of 27 wells investigated all around the city, 13 (48.14%) were infected with at least one pathogenic protozoa studied, that is not different from the prevalence of one of the main bacteria, **Enterococci,** used as indicator , to measure water quality $P>0.05$ (Table 1). Triple infections were observed in 3 wells (Dal P03, Dal P10 and Dal P27).

Table 1: Detailed prevalence of common microbiological indicators and 3 major protozoan pathogens found from natural water sources used as drinking consumption in Daloa

	MF method			Genetic characterization			Protozoan contamination status
Site	**E. coli**	**Total coliforms**	**Enterococci**	***E. histolytica***	***C. parvum***	***G. intestinalis***	
Dal S01	0	0	0	-	-	-	-
Dal S02	0	0	0	-	-	-	-
Dal H01	0	0	0	-	-	-	-
Dal H02	0	0	0	-	-	-	-
Dal H03	3	21	0	+	-	+	+
Dal H04	0	0	0	-	-	-	-
Dal H05	0	0	0	-	-	-	-
Dal P01	3	72	0	-	-	-	-
Dal P02	1	8	2	-	-	-	-
Dal P03	5	TNC	8	+	+	+	+
Dal P04	3	15	0	-	-	-	-
Dal P05	10	TNC	6	-	+	-	+
Dal P06	3	TNC	0	+	-	+	+
Dal P07	5	41	0	-	-	-	-
Dal P08	0	16	0	-	-	-	-
Dal P09	7	62	4	+	-	+	+
Dal P10	3	TNC	3	+	+	+	+
Dal P11	2	18	8	-	+	-	+
Dal P12	7	TNC	0	-	-	-	-
Dal P13	3	TNC	0	-	-	-	-
Dal P14	8	TNC	11	-	+	+	+
Dal P15	5	TNC	0	-	-	-	-
Dal P16	3	47	0	-	-	-	-
Dal P17	1	13	7	+	+	-	+
Dal P18	4	TNC	60	+	+	-	+
Dal P19	0	0	0	-	-	-	-
Dal P20	8	TNC	5	+	-	-	+
Dal P21	4	94	2	-	-	+	+
Dal P22	1	20	4	-	-	-	-
Dal P23	0	6	0	-	-	-	-
Dal P24	0	0	0	-	-	-	-
Dal P25	3	8	27	-	-	-	-
Dal P26	9	TNC	31	+	-	-	+
Dal P27	7	32	20	+	+	+	+

TNC: Too-numerous-to-count. According to USEPA 1604 method, plates with ≥200 total colonies and ≥100 target colonies should be considered TNC.

Domestic water tanks: We examined the prevalence of the 3 major waterborne protozoan pathogens investigated from natural water contained in domestic jugs in households. Twenty one (21) (61.76%) out of 34 samples were found contaminated with at least one of the 3 parasites investigated. *E. histolytica* was again the most prevalent with 41.2% but statistical different from the prevalence of the two others pathogens ($P<0.05$) . One of the tanks with natural spring water was found contaminated with protozoan parasite although the two springs were found free of parasite when examined. Polyparasitism was also detected in the household containers samples: 3, 4 and 2 co-infections were found with combination of *E. histolytica*-*G. intestinalis, E.*

histolytica-C. parvum and G. intestinalis-C. parvum respectively. 4 triple infections were also detected (Table 2). Protozoan parasites were detected in three of the five containers with water collected from hydraulic pumps, i.e. one from the infected hydraulic pump and two others. 17(62.96%) out of 27 jugs with water from wells were found infected with at least one protozoan parasite. All jugs containing water from the 13 wells previously contaminated were found infected.

Table 2: Detailed prevalence of 3 major waterborne protozoan pathogens assess from natural water contained in domestic jugs in Daloa.

	Genetic characterization			**Contamination status**
Site	***E. histolytica***	***G. intestinalis***	***C. parvum***	
Dal U001	-	-	-	-
Dal U002	+	-	-	+
Dal U003	+	-	+	+
Dal U004	-	-	-	-
Dal U005	+	-	+	+
Dal U006	-	-	-	-
Dal U007	+	+	-	+
Dal U008	-	-	+	+
Dal U009	-	-	-	-
Dal U010	+	+	+	+
Dal U011	-	-	-	-
Dal U012	-	+	-	+
Dal U013	+	-	+	+
Dal U014	-	-	-	-
Dal U015	-	-	-	-
Dal U016	+	-	+	+
Dal U017	+	+	+	+
Dal U018	-	+	-	+
Dal U019	+	+	+	+
Dal U020	-	-	-	-
Dal U021	-	+	+	+
Dal U022	-	-	-	-
Dal U023	-	+	+	+
Dal U024	+	+	-	+
Dal U025	+	+	-	+
Dal U026	-	+	-	+
Dal U027	+	-	-	+
Dal U028	-	-	+	+
Dal U029	-	-	-	-
Dal U030	-	-	-	-
Dal U031	-	-	-	-
Dal U032	-	-	-	-
Dal U033	+	-	-	+
Dal U034	+	+	+	+

Water consumption habits: A total of 33 households were surveyed for consumption habits and 54.54% of them used tap water for drinking which is somewhere very significance in terms of consumption habits (*P*>0.05). Natural water sources, namely spring, hydraulic pump and well represented 12.12%, 12.12% and 15.15% respectively. The remaining 6.06% households used commercial drinking water. Of the 8 households that used hydraulic pump or spring water for drinking, 15% used tap water for cooking. All households that used well water for drinking also used well water for cooking and washing dishes. Of the 54.54% who used tap water for drinking, about 17% and 30% used well water for cooking and washing dishes respectively (Table 3).

Table 3: Water consumption habits

Household	Type of water used to drink	Type of water used to cook	Type of water used to wash dishes
Dal U001	Tap	Tap	Tap
Dal U002	Hydraulic pump	Tap	Tap
Dal U003	Tap	Tap	tap
Dal U004	Tap	Tap	Tap
Dal U005	well	well	well
Dal U006	Tap	Tap	Tap
Dal U007	Spring	Spring or Tap	well
Dal U008	Commercial	Tap	Tap
Dal U009	Tap	Tap	Tap
Dal U010	Tap	Tap	Tap
Dal U011	Hydraulic pump	Hydraulic pump	Tap
Dal U012	Tap	Tap	Tap
Dal U013	well	well	well
Dal U014	Tap	Tap	Tap
Dal U015	Spring	Tap	Tap
Dal U016	well	well	well
Dal U017	Tap	well	well
Dal U018	Tap	Tap	well
Dal U019	Spring	Spring or Tap	Tap
Dal U020	Tap	Tap	Tap
Dal U021	Spring	Tap	Tap
Dal U022	well	well	well
Dal U023	Tap	Hydraulic pump or Tap	Tap
Dal U024	Tap	Tap	Tap
Dal U025	Commercial	Tap	Tap
Dal U026	Tap	Tap	Tap
Dal U027	Hydraulic pump	Hydraulic pump	Tap
Dal U028	Tap	Tap	Tap
Dal U029	Tap	Tap	Tap
Dal U030	well	well	well
Dal U031	Tap	Tap	Tap
Dal U032	Hydraulic pump	Hydraulic pump	Hydraulic pump
Dal U033	Tap	Tap	Tap

DISCUSSION

In 2004, the World Health Organization (WHO) estimated that 1.8 million people die each year because of severe diarrhoea caused by drinking contaminated water. The majority of deaths affects children under five years and occurs in developing countries (WHO, 2004). Infections transmitted by water represent then a major public health concern, particularly in developing countries (UNICEF/WHO, 2009). In the Millennium Development Goals (MDGs), the WHO plans to improve the health and development of individual. The 10th MDG target is to reduce by 2015 the number of people without access to safe drinking water and basic sanitation. For this purpose, this study is welcome to estimate the at risk proportion of the population of Daloa city regarding water consumption habits and waterborne pathogens in order to make recommendation. In the United States, which is a reference in terms of compliance with microbiological water quality, protozoa are considered as contaminants of drinking water and the Agency for Environmental Protection (EPA) said that their presence is unacceptable (U.S. EPA, 2002). Furthermore, infection studies in healthy human volunteers demonstrated a clear relationship between probability of infection and the ingested oocyst dose of a *C. parvum* strain (Dupont *et al*., 1995). At a dose of 1000 oocysts, infection probability increased to 100%. In stool surveys of patients with gastroenteritis, the reported prevalence of Cryptosporidium is 3–20% in Africa, (WHO, 2006). In developing countries, the prevalence of giardiasis in patients with diarrhea is about 20% and range from 5 to 43% (WHO, 2006). The prevalence of 41.2% of pathogenic protozoa observed in natural sources seems in accordance with what is generally found in developing country but very high compared to the 25% prevalence observed in a similar study in Brazil (Branco *et al*, 2012). Another study conducted in Egypt in the same context described a prevalence of 76% (Hanne *et al*, 2009). Thus, the prevalence of the pathogens detected in water sources can be very variable depending on the country but also on the detection techniques used. Microscopy techniques used by Branco (Branco *et al*, 2012) are recognized to underestimate the true prevalence of protozoan parasite compared to technology by molecular or genetic tools. Other methods do exist: Method 1623 is the procedure currently approved by the United States Environmental Protection Agency (U.S. EPA) for the detection of *Cryptosporidium* and *Giardia* in water and have been successfully used to recover and detect waterborne parasite in different water sources (Le Chevalier *et al*, 1995; Stinear *et al*, 1996; Lowery *et al*, 2000; Rimhanen-Finen *et al*, 2002). As a waterborne pathogen of significant concern, *Cryptosporidium* is considered a key reference pathogen in the *Guidelines for drinking-water quality (WHO,2009)*. No pathogenic parasites were detected in the springs and only one hydraulic pump was contaminated contrary to wells which had shown 48.14% prevalence with a proportion of polyparasitism. The detection of protozoa pathogen in some home containers and not in natural sources where the containers were filled, suggested that contamination may originate from containers through human actions regarding hygienic concern. This was confirmed by the fact that the water of natural upgraded springs free of pathogen have been contaminated when it is found in the container samples. This contamination of house samples suggests that the manipulation and cleanliness of the container might contribute to the microbial contamination of the samples. House tanks filling period with unwashed hand may also contribute to the presence of parasite in plastic containers due to variations in time of microbial occurrence in natural water sources (U. S. EPA, 2002). Indeed, protozoans are persistent organisms that are capable of surviving for long periods in the environment (Christon *et al*, 2007). Hand washing with soap has been shown to reduce the incidence of diarrheal disease by over 40 per cent (UNICEF/WHO, 2009). Despite complaints from people about the quality of tap water, a large proportion 54, 54% use this water for drinking. Only 3 specimens from tap water were analyzed (data not shown). No parasite was detected in the tap water but more specimens are needed to make a significant conclusion on the quality of tap water from Daloa as it is well known that this water harbour dirty background but no scientific investigation have been yet done. About 15.2% of households surveyed used well water for drinking mainly for economic reason when it was shown that 48.14% of these wells are contaminated with waterborne protozoa. This could lead to important public health concern, mainly in children under five from these communities. Increased knowledge on the characterization and epidemiology of parasites transmitted by water brought a growing interest in the importance of their detection in drinking water. *C. parvum*, *G. intestinalis* and *E. histolytica* have been identified as significant waterborne pathogens and have been found responsible for several serious outbreaks worldwide over the past two decade (Marshall *et al*, 1997). This study was the first to investigate waterborne protozoan parasite in Daloa natural water sources using genetic markers.

CONCLUSION

The high prevalence of intestinal protozoa parasite detected in natural water sources and household tanks may function as a link of transmission of different intestinal parasites due to soil and water contamination, contributing to the maintenance of parasite life cycles. Therefore, the inclusion of consistent public health interventions with measures that include the protection of springs, sanitizing wells, and primary education of the population are widely necessary, aiming the control and prevention of parasite infections.

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