

# Real Time PCR Assay in differentiating *Entamoeba histolytica* and *Entamoeba dispar* infections in patients fecal samples attending St Camille hospital in Ouagadougou, Burkina Faso

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## ABSTRACT

Amoebiasis is a parasitic infection caused by *Entamoeba histolytica*. The diagnosis of amoebic infections, based on microscopic stool examination does not differentiate between *E. histolytica* and *E. dispar*, the commensal species because these two species are morphologically indistinguishable. The re-description of these species in 1993 and the exclusive pathogenicity recognized to *E. histolytica* have stimulated reassessment of the epidemiology of amoebiasis using molecular techniques. This study aimed to diagnose and differentiate *E. histolytica* and *E. dispar* in feces for a first time in Burkina Faso. Fecal samples from 413 patients suffering from gastrointestinal disorders, attending the Saint Camille Hospital in Ouagadougou were collected and processed by direct microscopic examination for the presence of *Entamoeba* species and other intestinal parasites. A real-time PCR was then developed and assessed for the detection and the differentiation of *E. histolytica* and *E. dispar*. Based on single fecal sample examination, the overall prevalence of *E. histolytica/E. dispar* was 22.5 % (93/413) with 84.9 % (79/93) in infected adults and 15.1 % (14/93) in children under 5 years. Fourteen (14) samples of 93 (15.05 %) *E. histolytica/E. dispar* microscopy positive samples were PCR positive. *E. dispar* was the most common, with a prevalence of 71.4 % (10/14) against 21.4 % (3/14) for *E. histolytica*. One case of mixed infection was recorded.

We showed for the first time in Burkina Faso, that real-time PCR is an excellent tool in the re-definition of the epidemiology of *E. histolytica* according to WHO recommendation and also for proper management of this tropical neglected disease.

**Keywords:** PCR, differentiation, *Entamoeba histolytica*, *dispar*, Burkina Faso.

## 1. INTRODUCTION

Amoebiasis is caused by a blood-sucking, cytotoxic and human specific protozoan parasite named *Entamoeba*

*histolytica* and is classified as a neglected tropical disease. It remains a great public health concern in

affected countries because it is responsible for nearly 100 000 deaths per year [1].

The most affected regions are Latin America, South Asia and Africa, particularly in the South and South-East of the Sahara. In Burkina Faso, studies on amebiasis between 2010 and 2012 in patients attending hospitals in Ouagadougou and Bobo-Dioulasso, have shown a prevalence between 23 % and 26 % [2-4]. A recent study in healthy school children in the Centre Ouest and Plateau Central regions of Burkina Faso showed a prevalence of 66.5 % (Erismann and *al.*, 2016).

The genus *Entamoeba* which includes many species, infects about 10 % of the world population with 10 % of cases attributed to *E. histolytica* [5]. Of these, *E. histolytica*, *E. dispar*, *E. moshkovskii*, *E. polecki*, *E. coli* and *E. hartmanni* are capable of colonizing human intestinal lumen [6,7]. *E. histolytica* and *E. dispar* are most frequently found but only *E. histolytica* is unquestionably associated with pathological effects in humans [8]. On the other hand, *E. dispar*, which is a commensal species, is not associated with invasive disease.

In the second half of the 20th century, there has been much discussion on the criteria for differentiation of these two species and their relative pathogenicity. After decade of dispute, starting with the observations of Emile Brumpt [9], the biochemical, immunological and genetic differences between *E. histolytica* and *E. dispar* previously known as pathogenic and nonpathogenic respectively have proved to be sufficient to formally set them apart as two separate species [10]. This stimulated a rapid development of new diagnostic methods for detection and differentiation of these two parasites.

The diagnosis of amoebic infections has for a long time been based on microscopic examination of feces. However deficiencies of this technique especially its non-optimal sensitivity and specificity in differentiating *Entamoeba* species have been reported [11,12]. Indeed, the cyst and trophozoite forms of *E. histolytica* and *E. dispar* are morphologically indistinguishable in optical microscopy [13].

Specific techniques such as culture of trophozoites by the method of Sargeant [14,15] and isoenzymes typing by the joint analysis of electrophoretic profile of several isoenzymes [11,16], allow differentiation between *E. histolytica* and *E. dispar*. However, these techniques are very long, expensive and not applicable in routine diagnosis. Searching of parasitic coproantigens by immunoenzymatic techniques like Enzyme-linked immunosorbent assay (ELISA) has often cross-reactivity in the distinction between these two species [17]. Serological techniques for their part have a very limited use because of seroconversion [18,19] and their inability to distinguish an old infection from a recent infection in highly endemic areas [11].

New approaches for the identification of these two species are based on molecular methods including Polymerase Chain Reaction (PCR) which is an excellent tool as recommended by WHO [1]. Real-time PCR allows a specific detection and continuous monitoring of the amplicon. Its speed, low risk of contamination of the amplicon and the absence of post-PCR analysis, greatly reduce results rendering time [11,12]. It provides greater sensitivity and is highly specific as it directly targets DNA of *E. histolytica* or *E. dispar* [20,21]

Nowadays WHO recommends a reassessment of the epidemiology of amebiasis using molecular techniques for *E. histolytica* detection, because the differentiation of highly pathogenic species *E. histolytica* from *E. dispar* is of utmost importance in the management of cases of amebiasis [1]. This differentiation between the two species allows (i) to avoid the systematic treatment of all patients whose microscopic stool examination revealed the presence of *Entamoeba* species cysts or trophozoites and (ii) to minimize the risk of resistance occurring related to misuse of antiparasitic drugs.

The aim of the present study was to develop for the first time, real-time PCR technique to identify and differentiate *E. histolytica* and *E. dispar* in feces collected from patients suffering from gastro enteritis in Ouagadougou in Burkina Faso.

## 2. MATERIALS AND METHODS

### 2.1 Stool collection

A descriptive cross-sectional study was carried out from June to October 2015 in Ouagadougou, Burkina Faso. This study involved 413 patients suffering from gastrointestinal disorders and attending Saint Camille Hospital. Pre-labeled sterile plastic containers for fecal collection were handed out to all participants. The filled containers with stool newly issued were collected on the following day and directly transported to the Medical Parasitology Laboratory of Saint Camille Hospital. The fresh fecal samples were stored at ambient temperature and processed within 2 hours post-collection by direct wet smear followed by iodine staining and examined via microscopy for the presence of *Entamoeba* species and other intestinal parasites. The optical microscope Motic BA300 (Motic Instruments, Canada) was used for analysis.

Microscopically positive samples for *Entamoeba* species cysts and trophozoites were then collected in 2.5 mL sterile cryotubes and were stored at - 20 °C and further assessed using real-time PCR technique.

### 2.2 DNA isolation from *Entamoeba* positive stool samples

Genomic DNA was extracted from microscopically positive fecal samples using Sorb-B DNA isolation kit (Sacace Biotechnologies®, Como, Italy) according to the protocol provided by the manufacturer. The purity and the final concentration of the DNA extracts were determined using the Biodrop µLITE spectrophotometer (Isogen Life Science N.V. /S.A,

Temse, Belgium). The extracted DNA was stored at -20 °C up to the amplification step.

### 2.3 Real time PCR assay

The forward primer Ehd-239F 5' ATT GTC GTG GCA TCC TAA CTC A 3' and reverse primer Ehd-88R 5' GCG GAC GGC TCA TTA TAA CA 3', generic for *E. histolytica* and *E. dispar* were used to amplify a 231 bp fragment of the 18S rRNA gene [22,23].

The differentiation of the two species was made using two TaqMan probes (Applied Biosystem, Foster City, California, USA):

-histolytica-96T: 5' TCA TTG AAT GAA TTG GCC ATT T3' specific for *E. histolytica*

-dispar-96T: 5' TTA CTT ACA TAA ATT GGC CAC TTT G3' specific for *E. dispar*.

The real-time PCR was performed in a total of 25 µL reaction volume containing 5 µL of DNA extract (4-50 µg/mL), 12 µL of TaqMan Universal PCR Master Mix, 1µL of forward primer Ehd-239F and 1µL of reverse primer Ehd-88R, 3 µL of sterile water, 3 µL of probe histolytica-96T (or dispar-96T). Detecting *E. histolytica* and *E. dispar* was made separately.

The thermocycling program set up in a 7500 FAST Real-time PCR system (Applied Biosystems, California, USA) was 95 °C for 3 min for initial denaturation, followed by 45 cycles of 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s and final extension at 60 °C for 1 min. The results were analyzed on computer using 7500 FAST software\_v2.0.6 (Applied Biosystems, California, USA).

The specificity of real-time PCR was tested using DNA extracted from 4 *E. coli* positive fecal samples, 2 *Giardia intestinalis* positive fecal samples and 5 microscopically parasites negative fecal samples. These 11 control samples underwent the same amplification protocol than DNA extracts from *E. histolytica/E. dispar* positive samples. Cross-amplification between *E. histolytica* and *E. dispar* was tested with probes specific to each species.

### 2.4 Statistical analysis

The data entry and analysis was carried out using the SPSS software (Statistical Package for the Social Sciences) version 20.0 (SPSS, Chicago, IL, USA). The chi-

square test was used for comparison and a P value was significant when <0.05.

### 2.5 Ethical considerations

This study protocol was approved by the institutional Ethics Committees of the Saint Camille Hospital of Ouagadougou and of Biomolecular Research Center Pietro Annigoni/Laboratory of Molecular Biology and Genetics of University Ouaga I Professor Joseph KIZERBO. The results of parasitological exams were transmitted immediately to the clinicians for care and appropriate treatment was given to patients.

Before sample collection, an oral briefing to describe the objectives and methodology of the study was given to the participants by the investigator. Their consent was taken either in written form (signed) or verbally followed by thumb prints (for those who were illiterate) or their parents/guardians (on behalf of their children).

## 3. RESULTS AND DISCUSSION

### 3.1 Prevalence of *E. histolytica/E. dispar* infections based on microscopy

A total of 413 patients aged from 2 months to 73 years suffering from gastrointestinal disorders were enrolled. This study population consisted mostly of 76.8 % (317/413) children aged less than 5 years with a strong representation of children aged between 12 and 24 months (35.6 %). Male patients accounted for 51.3 % of cases and sex ratio was 1.05. Sociodemographic characteristics distribution of patients is summarized in Table 1.

Among the 413 stool samples analyzed by microscopy, 93 samples showed cysts and/or trophozoites of *E. histolytica/E. dispar* with a prevalence of 22.5 % (93/413). Coinfection of *E. histolytica/E. dispar* with other intestinal parasites such as *G. intestinalis* or *T. intestinalis*, was also observed with a rate of 10.75 % (10/93).

### 3.2 Prevalence of *E. histolytica* and *E. dispar* given by real time PCR

The molecular diagnosis by Real-time PCR was performed on 93 *E. histolytica/E. dispar* microscopy positive samples for differentiating species. Fourteen (14) of them were positive by PCR (Table 2).

**Table 1:** Sociodemographic characteristics of the study population.

		N	%	p value
<b>Gender</b>	Male	212	51,3	0,5890
	Female	201	48,7	
<b>Age group</b>	2-11 months	80	19,4	0,0276
	12-24 months	147	35,6	
	25-60 months	90	21,8	
	> 60 months	96	23,2	

**Table 2:** Real-time PCR results of microscopy positive samples.

Species	N	%	p value
<i>E. histolytica</i>	3	3,23	
<i>E. dispar</i>	10	10,75	
<i>E. histolytica</i> and <i>E. dispar</i>	1	1,07	<0,0001
Negative	79	84,95	
Total	93	100	

*E. histolytica* prevalence in *Entamoeba* infections was 21.4 % (3/14) against 71.4 % (10/14) for *E. dispar* with a case of mixed infection. Two cases of *E. histolytica* infection were detected in adults against one case in a child of 42 months. A child of 36 months presented mixed infection. Adults also presented with most infections with *E. dispar* 8/10.

In the present study the overall prevalence of *E. histolytica/E. dispar* was 22.5 %. This result is lower than that found by Karou and *al.* who observed a prevalence of 30.74 % in a study conducted in 2009 on 11 728 patients aged 5 months to 72 years with gastroenteritis at the Saint Camille Hospital of Ouagadougou [2]. In another study in the same hospital with the same type of patients, Ouermi and *al.* reported a prevalence of 25.79 % [3]. The prevalence of *E. histolytica/E. dispar* was 23.4 % in a study conducted from April to August 2012 by Sangare *et al.* with 291 patients aged 0-89 years of Souro Sanou University Hospital in Bobo Dioulasso, the second town of Burkina Faso [4]. This prevalence is similar to that observed in our study in Ouagadougou.

The published data on the prevalence of *E. histolytica/E. dispar* infections in hospital setting in Burkina Faso are certainly limited but the analysis of these data allows to conclude the downward trend in the occurrence of *E. histolytica/E. dispar* infection over the years. This could be linked to the improvement of sanitary conditions in Ouagadougou.

Furthermore, the prevalence of 66.5% reported by Erismann *et al.* among 385 healthy school children, aged 8 to 14 years of the Plateau Central and Centre Ouest regions of Burkina Faso (Erismann *et al.*, 2016 in press) shows that Burkina Faso remains a country of high prevalence of *E. histolytica/E. dispar* infection.

Our results are in agreement with those of Ouattara *et al.* [24]. Indeed, in Ivory Coast, a study in Agboville among 1300 school children of primary education, the prevalence of *E. histolytica/E. dispar* was 18.8 % [24]. In 2003, a prevalence of 39.8 % was observed in a rural community at Bawku District in Northern Ghana [25]. The low sensitivity of microscopy observed in these studies reinforces the fact that single sampling would be less sensitive [26]. The removal of cysts is effected intermittently, so it is necessary to repeat examination of stool 3 times to increase the sensitivity [27]. However, all these results demonstrate that infection with *E. histolytica/E. dispar* remains endemic in West Africa and the prevalence appears to be higher in rural than in urban areas. This is explained by the low level of education of the rural population, overcrowding,

poverty, lack of hygiene and lack of sanitation are factors enhancing *E. histolytica/E. dispar* infection [28].

The implementation of adequate prevention strategy is therefore needed to reduce significantly the incidence of infection with *E. histolytica/E. dispar* and that of intestinal parasites generally. However, in order to ensure adequate treatment, it is important to detect subjects who are actually infected with *E. histolytica*, which is the species responsible for amebiasis.

In the present study, we report for the first time data on detection and differentiation of *E. histolytica* infection using real-time PCR in Burkina Faso.

The specificity of real-time PCR was 100%, no amplification of the genomic DNA of other parasites was observed. This result confirms once again the very high specificity of the real-time PCR already reported by other authors [7,22,23] using as target the 18S rRNA gene. In our study, real time PCR was positive in 14 of 93 microscopy positive samples showing a sensitivity of 15.05 %. Our results are in accordance with those found in 2003 in Ethiopia: on 91 *E. histolytica/E. dispar* microscopy positive samples, Kebede *et al.* found that 21 (23.08%) were positive only to *E. dispar* by PCR differentiation. No cases of *E. histolytica* were recorded in this study. These authors justified their result by an overestimation of positive cases by microscopy and a possible PCR inhibition [29]. Also in 2005, Lebbad *et al.* had obtained a similar result in Sweden; Among 207 microscopy positive samples, 165 cases of *E. dispar* infection were obtained against only 10 cases for *E. histolytica* after differentiation by PCR [30]. This low representation of *E. histolytica* in cases of *Entamoeba* infections had also been reported in 2003 in Ghana where a single case of infection by *E. histolytica* was registered against 72 for *E. dispar* [25]. However, these detection rate of *E. histolytica* are low compared to that found by Ngui *et al.* in a rural community in Malaysia, who shows that of 75 *E. histolytica/E. dispar* microscopy positive samples, 52 were PCR positive (69.33%) of which 39 cases for *E. histolytica* [31]. In another similar study in 2013 in Malaysia, on 65 positive samples by microscopy, Lau *et al.* found 56 (86.3%) PCR positive samples [7]. The difference with our results could be explained partly by the fact that the microscopy results would be false positive in some samples. In fact, the stool microscopy is operator-dependent. Beyond the experience and attention to detail it requires, the possibility of confusion are not negligible. It is often difficult to differentiate between a stationary trophozoite and a leukocyte, a polynuclear and a cyst, a cyst and a yeast [12,32]. On the other hand the complexity of the stool makes that false negative

reactions can be recorded during PCR due to the presence of numerous inhibitors of DNA polymerase [11,18] such as heme, bilirubin, bile salts and complex carbohydrates which are often co-extracted with the DNA of intestinal pathogens [33,34].

A negative sample for parasites by microscopy for which the patient experienced bloody diarrhea was positive for *E. histolytica* in the real-time PCR assay. This result demonstrates the importance of diagnosis by real time PCR in cases where the parasite searching is negative by microscopy and whose clinical signs are suggestive of amebiasis.

Although the sample size is not representative of the general population, our results provide an indication of the epidemiology of the two species *E. histolytica* and *E. dispar* in Burkina Faso. On 93 microscopy positive samples, finally 3 were positive for *E. histolytica* by real-time PCR against 14 for *E. dispar*. This result is consistent with the statistics of the global distribution of *E. dispar* that would be 10 times more common than *E. histolytica* [35]. The ability of real-time PCR to differentiate between *E. histolytica* and *E. dispar* can significantly reduce the number of unnecessary treatment because WHO advocates since 1997 to treat only the cases of *E. histolytica* infection [1].

We used for the first time, real-time PCR technique to detect and to differentiate *E. histolytica* and *E. dispar* in Burkina Faso. This work contributes to the WHO recommendation for a reassessment of the epidemiology of *E. histolytica* by molecular techniques.

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#### 5. Author's contribution

WAZ, SD, DO and JS conceived and designed the experiments. WAZ, TRC, TK and DI performed the experiments. WAZ, AKO, FD, OYD and STS analyzed the data. JS, SD, DO and ATY contributed reagents/materials/analysis tools. WAZ and SD wrote the paper. All the authors reviewed drafts of the paper and approved the final version of the paper.

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