Molecular Detection of *Shigella spp.* and *Entamoeba Histolytica* Causing Bacillary and Amoebic Dysentery among Children in Ramadi City

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Abstract

Background and objective:- Dysentery as any episode of diarrhea in which there is blood in loose and watery stool, there are different types of pathogens that can cause acute dysentery, including Shigella spp. and Entamoeba histolytica. The aims of this study was to isolate Shigella spp. and Entamoeba histolytica causing both bacillary and amoebic dysentery respectively, in children less than five years of age by conventional techniques. Also, molecular detection of Shigella spp. and Entamoeba histolytica was achieved.

Patients and methods:- One hundred and forty five stool specimens obtained from children were admitted Maternity and Child Teaching Hospital in Ramadi with acute diarrhea with the mean age (2.168±12.571) were enrolled in the study from January 2014 to March 2015. PCR was used to amplify specific oligonucleotide sequences with 320 base pair coding for (ial) gene for Shigella spp. and Entamoeba histolytica (16S-rRNA) gene with 439 base pair.

Results:- The stool specimens had been taken from 145 patients divided into 75(51.7%) patients yielded amoebic dysentery and others 70(48.3%) from these with bacillary dysentery. In the molecular part of this study molecular part of study, our results showed that 30 out of 75(40%) extracted DNA for E. histolytica were produced strong bands revealed positivity of PCR technique while the other 45(60%) were negative. The result of PCR technique among Shigella spp. revealed that 30 out of 70(42.86%) were positive and in contrast 40/70 (57.14%) samples were negative.

Conclusion:- The study suggested that PCR has several advantages over the conventional methods for the diagnosis of bacillary and amoebic dysentery such as safety, high sensitivity and specificity. Our results demonstrated that the value of using a combination of traditional and molecular techniques in the diagnosis of diarrheal disease in this population. This study has been providing comparable results of microscopy and PCR, none of these methods can detect all positives alone. According to our findings, microscopy is a simple analysis, but it is subjective, needs experience to evaluate and should be combined with complimentary methods such as antigen detection and PCR for identification of the species to avoid false and/ or insufficient diagnosis and treatment applications.

Key words:- Shigella spp., Entamoeba histolytica, PCR.

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Introduction:

World Health Organization (WHO) defines dysentery as any incident of diarrhea in which there is blood in loose and watery stool. Dysentery can mainly spread among people through contaminated food and water as well as poor sanitation. There are several numbers of bacteria that can cause acute dysentery, including Shigella, Salmonella, Campylobacter, and Escherichia coli[1,2]. There is an estimated 164.7 million people worldwide infected by Shigella annually, resulting in 1.1 million deaths, mostly in children under 5 years.

Shigellosis considers a major cause of morbidity and mortality among children in developing countries, and is also an important cause of morbidity in industrialized countries [3]. Shigella species are small gram negative rods, 0.3-1 µm in diameter and 1-6 µm in length, appearing singly, in pairs and in chains. Shigella species are facultative anaerobes and are non-spore formers. Unlike Salmonella, Shigella species do not possess flagella and hence are non-motile [4].

Amoebiasis causes up to 100.000 deaths annually all over the world, E. histolytica known as the main agent of intestinal amoebiasis causing amebic colitis and liver abscess, is morphologically identical with E. dispar, which is accepted as a non-pathogenic commensal parasite[5]. Amoebiasis is an infection caused by Entamoeba histolytica with or without symptoms. Replacements include entamoebiasis, amoebiosis, amoebic dysentery or bloody flux. E. dispar is a harmless commensal, which is vague from E. histolytica. The other members of the group infecting humans are E. moshkovskii, E. hartmannii, E. gingivalis, Endolimax nana and Iodamoeba butschlii [6].

Polymerase chain reaction (PCR) assays to diagnose Shigella rely mainly on the amplification of DNA fragments from invasive plasmid shared by Shigella and certain serotypes of Escherichia coli, known as enteroinvasive E. coli (EIEC). This virulence plasmid can be spontaneously lost during growth outside the host, so conventional PCR protocols use primers directed against the invasion plasmid antigen (ipaH), which is present in multiple copies in Shigella and EIEC bacterial genomes [7].

This study has been undertaken to isolate Shigella spp. and detect Entamoeba histolytica causing bacillary and amoebic dysentery respectively, in children less than five years of age in Ramadi city. Also, molecular detection of Shigella spp. using specific oligonucleotide sequences coding for (ial) gene and size 320-bp. Molecular detection of Entamoeba histolytica (16S-rRNA) gene using specific oligonucleotide sequences coding for 439-bp. Further, to determine statistical parameters regarding the role of both of conventional and molecular techniques in the diagnosis of both of Shigella spp. and Entamoeba histolytica in stool samples.

Patients and Methods:

The present study was conducted in the period from January 2014 to March 2015, considered all patients whose stool samples were sent to the laboratory in a Maternity and Child Teaching Hospital in Ramadi city, with the suspect of amoebic and bacillary dysentery (a total of 145 stool samples). Those patients were 80(55.2%) males and 65(44.8%) females. Their ages were ranging from 1 day to 5 years with the mean age (2.168±12.571).

Stool sample from each one was collected in a sterile container which were examined:

Detection of Entamoeba histolytica by microscopic examination:- Two direct smears were done, one in normal saline and the other one in lugal's iodine to screening on E. histolytica trophozoites and cysts, respectively [8].

Isolation of Shigella spp. by culture method:- The conventional culture method, which is considered to be the standard of detection of Shigella spp.[9]. Stool specimens were inoculated on MacConkey and Deoxycholate citrate agar (DCA) using sterile inoculating loop. Stools were also enriched in selenite F broth and incubated overnight at 37°C. The next day, the enriched broth was sub-cultured on MacConkey agar and DCA and incubated overnight at 37°C. Colonies morphologically resembling Shigella spp. were further evaluated with biochemical tests [4].

DNA extraction:- DNA was extracted from microscopically fecal samples using BioNeer DNA Isolation Kit (Korea) according to the manufacturer's instructions. Briefly, approximately 100 to 200 mg of stool sample was added into a 1.5ml micro centrifuge tube followed by incubation at 70°C for 10 minutes with the presence of cell lysis and disruption agent provided in the kit. Afterwards, homogenization and cell lysis procedure of the fecal sample were completed by mechanical shaking (vortexing) using vortex shaker; (Genic U.S.A. bohemia NY 11716). The extracted DNA was stored at 4°C or -20°C until required for PCR amplification.

Conventional PCR assay:-PCR assay was amplified the 16S-like ribosomal RNA was used to genetically characterize E. histolytica (439-bp) according to[10], and ial gene was used to characterize Shigella dysenteriae (320-bp) according to[11]. PCR for the detection of Entamoeba histolytica used forward primer EH-1 (5'-AAG CAT TGT TTC TAG ATC TGA G-3') and reverse primer EH-2 (5'-AAG AAG TCT AAC CGA AAT TAG-3'). The PCR, used for amplifying the 320-bp segment of associated-antigen locus (ial) gene of Shigella dysenteriae, was an upstream primer S1- (5'-AGA CTG CTA CGG GAG GCA GCA GT-3'); and a downstream primer S2- (5'-GTT GCG CTC GTT GCG GGA CTT AA-3'). Amplification of the ial and 16S-rRNA genes target was conducted in a 20 µl volume with the final mix containing 250 □M for each dNTPs, 1.5 □M MgCl**2**, 5pmol/□□l of each primer, 1U of Taq polymerase and 2.5 µl of DNA template. The sample was heated to 96 °C for 2 min, followed by 30 cycles of 92 °C for 1 min (**denaturing**), 48 °C for 1 min (**annealing**), 72 °Cfor 1 min (**extension**) and a final extension at 72 °C for 7 min, this PCR program for Entamoeba histolytica, while steps of PCR program for Shigella dysenteriae include twenty six cycles of, **denaturation** at 94 °C for 1 min, **annealing** at 43 °C for 2 min and **extension** at 72 °C for 3 min. PCR generated amplicons of 439-bp for E. histolytica and 320-bp for S. dysenteriae were subjected to electrophoresis in 2% agarose gels at 100 V for 1 hour and visualized in a UV transilluminator after staining with SYBR Safe DNA stain.

Results:

Prevalence of Entamoeba and Shigella infection via traditional examination:- a total of 145 samples were collected. About 75 out of 145(51.7%) samples were microscopically suspected for amoebic dysentery, while others 70 out of 145 (48.3%) samples were suspected for bacillary dysentery by culture method. Infection was more prevalent in males 80(55.2%) compared to females 65(44.8%), however, it was not statistically significant (data not shown). Similarly,

infants below 12 months of age were mainly affected with acute diarrhea, out of 39(27%) patients of the cases and the (13-24 months) old constituted 31(21.3%) of the cases as it is shown in the (table 1).

Higher prevalence rates were recorded in rural areas 88(60.7%), while the rest 57(39.3%) from urban area.

Table1. Distribution of the study population according to age groups

Age group (months)	Number and percentage of all tested samples	
	Number of subjects	Percentage
1-12	39	27%
13-24	31	21.3%
25-36	33	22.8%
37-48	23	15.9%
49-60	19	13%
Total	145	100%

According to result of microscopic examination for Entamoeba infection:- First group includes about sixteen samples 16/75(21.3%) were positive for E. histolytica and about 33/75(44%) for other Entamoeba species, while other 26/75(34.7%) were negative for Entamoeba spp. most samples of children that infected with amoebic dysentery ranging between (one year to three years). This result show in (table 2).

Table 2. Distribution results based on children's ages and according to result of general stool examination.

Age (months)	Positive GSE for E. histolytica	Positive GSE for E. spp.	Negative GSE for E. spp.	Total
1-12	2(12.5%)	4(12.1%)	9(34.61%)	15(20%)
13-24	5(31.25%)	10(30.3%)	3(11.53%)	18(24%)
25-36	3(18.75%)	12(36.4%)	5(19.23%)	20(26.67%)
37-48	4(25%)	4(12.1%)	4(15.4%)	12(16%)
49-60	2(12.5%)	3(9.1%)	5(19.23%)	10(13.33%)
Total	16(100%)	33(100%)	26(100%)	75(100%)

All seventy stool samples that suspected for bacillary dysentery were cultured on selective and differential media and after biochemical testes appeared only 25 out of 70 (35.7%) were positive for Shigella spp. and other 45 out of 70 (64.3%) were negative for Shigella spp. (table 3).

Table 3. Result prevalence of Shigella spp. between children depending on the age according to culture method

Age group (months)	No. Culture positive samples for Shigella spp.	No. Culture negative samples for Shigella spp.	Total
1-12	4(16%)	20(44.44%)	24(34.3%)
13-24	5(20%)	8(17.8%)	13(18.57%)
25-36	7(28%)	6(13.33%)	13(18.57%)
37-48	5(20%)	6(13.33%)	11(15.7%)
49-60	4(16%)	5(11.1%)	9(12.86%)
Total	25(100%)	45(100%)	70(100%)

Conventional PCR:-

The result of PCR for 16 positive-general stool examination samples appeared high prevalence of E. histolytica (10 out of 16), (62.5%), and among thirty-three positive-general stool examination, the study showed sixteen samples to E. histolytica, (16 out of 33)(48.5%) while only four samples from 26 (15.4%) which were negative-general stool examination were positive by PCR for E. histolytica (see figure1).

The conventional PCR demonstrated that the size of diagnostic fragments of PCR products was clearly observed for E. histolytica, the species-specific product size for E. histolytica was 439-bp for 16S-rRNA gene, as shown in the (table 4).

Table 4. Result of distribution of E. histolytica according to PCR assay

Result of G.S.E for amoebic dysentery	No. & (%)	Positive PCR (%)	Negative PCR (%)
Positive G.S.E for E. histolytica	16(21.3%)	10(33.33%)	6(13.3%)
Positive G.S.E for Entamoeba spp.	33(44%)	16(53.33%)	17(37.8%)
Negative G.S.E for Entamoeba spp.	26(34.7%)	4(13.33%)	22(48.9%)
Total	75(100%)	30(100%)	45 (100%)

In direct detection of Shigella dysenteriae by PCR based on the ial gene from fecal samples showed the high prevalence of Shigella dysenreriae in culture-negative samples and culture positive samples between the children by PCR that was twenty two (88%) samples out of 25 were positive for PCR and other 3/25 (12%) samples were negative for S. dysenteriae by PCR that may be returned for other types of Shigella spp. (figure 2).

Among the 45 samples which were negative for Shigella spp., 8 (17.8%) out of 45 were positive for S. dysenteriae. While the other samples 37/45 (82.2%) were negative for S. dysenteriae as shown in the (table 5). The results indicated that the assay could detected about 30/70 (42.86%) Shigella dysenteriae, and 30/70 (40%) Entamoeba histolytica .

Table 5. The result of PCR for Shigella dysenteriae among positive and negative culture samples.

Result of culture examination	PCR		Total
	PCR +	PCR –	
Culture + of Shigella spp.	22(88%)	3(12%)	25 (100%)
Culture – of Shigella spp.	8(17.8%)	37(82.2%)	45 (100%)
Total	30	40	70

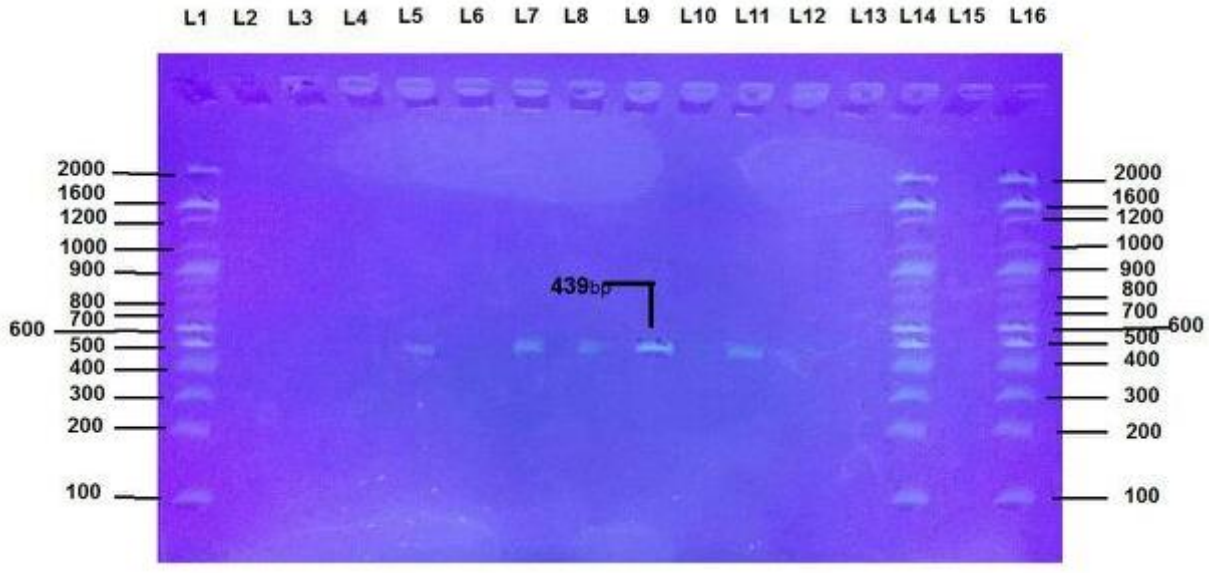

Figure 1. Amplification products of E. histolytica DNA by PCR. Amplified products of each reaction were analyzed by electrophoresis on a (2.0%) agarose gel. Lane1: 100-bp DNA ladder; lane 5,7,8,9,11: PCR amplified DNA extracted from a stool sample positive for E. histolytica, showing the 439-bp product

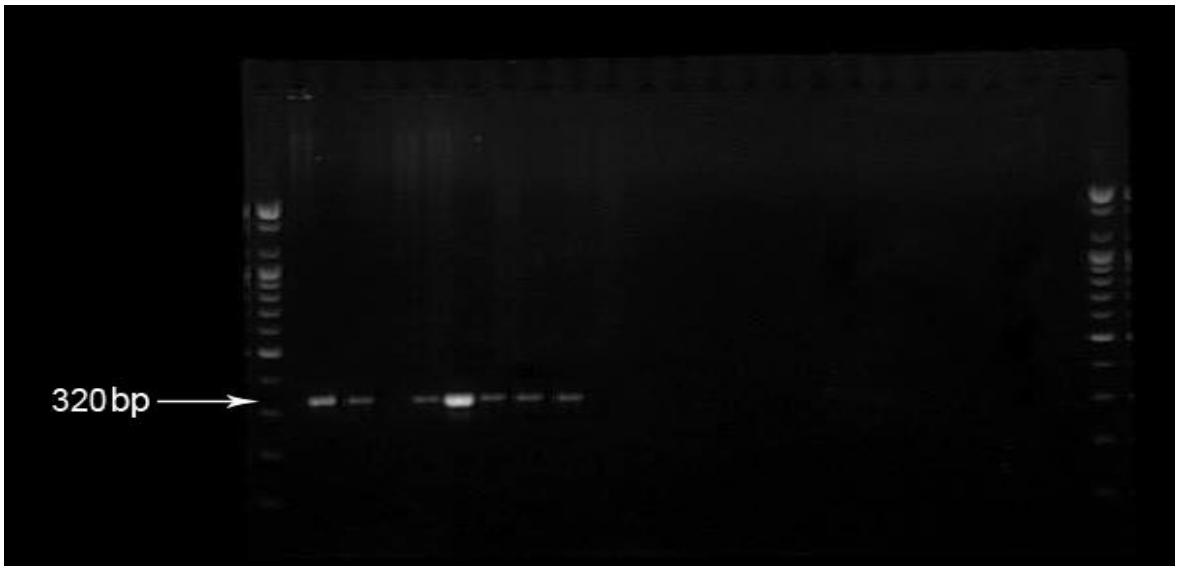

Figure 2. Amplification products of Shigella dysenteriae DNA by PCR. Amplified products of each reaction were analyzed by electrophoresis on a 2.0 % agarose gel. Lane 1: 100-bp DNA ladder; Lane 1,2,4,5,6,7,8 postive PCR amplified DNA extracted from a stool sample positive for Shigella dysenteriae, showing the 320-bp product. Note: (The photo had been taken by polaroid camera)

Discussion:

The study highlights the role of Shigella spp. and Entamoeba histolytica in the study group, this new knowledge on the etiology of diarrhea in the examined patients will help us to add plan future studies on various aspects of diarrheal diseases in this population like Rota virus. Ten percent of world's population is estimated to be infected by the parasite (4% in USA) with an estimated annual mortality of 40,000-70,000. However, 90% of those infected area symptomatic, 1% may develop invasive/ extra-intestinal amoebiasis [(12)]. Traditional diagnosis of amoebiasis has relied upon microscopic examination of fresh or fixed stool specimens [(13)].

In most laboratories around the world, and especially in developing countries like India, intestinal amoebiasis was diagnosed by demonstration of either cysts or trophozoites in stool by light microscopy [(14)].

The sensitivity of microscopy is as less as 60% and confounded with misleading results due to misidentification of macrophages as trophozoites, (polymorphonuclear and leukocytes) PMNs as cysts (particularly when lobed nuclei of PMNs break apart), and other Entamoeba species[(15)].

Shigella spp. are fragile organisms that are excreted in large numbers in the stool, but they die off quickly because stools are acidic[(16)]. Thus, routine microbiological methods used to identify Shigella spp. from stool samples are relatively inefficient, time consuming, and labor intensive, and the diagnosis often remains obscure due to the presence of low numbers of causative organisms, competition from other commensal organisms, and inappropriate sample collection.

If samples are collected after antibiotic therapy, growth of the organism may be impaired. Moreover, Dutta, et al.,[17] reported the sensitivity of the culture method to be 54% and 74%, respectively, compared to that of the conventional PCR technique.

Recent molecular diagnostic techniques based on nucleic acids, such as PCR, have shown tremendous potential for identifying Shigella spp. and have been increasingly exploited. To date, few studies have focused on the rapid diagnosis of shigellosis in underdeveloped and developing countries. However, PCR diagnostic tests have proven to be rapid and effective for the detection and identification to Shigella spp.[11]. In this study, we searched for genes unique to Shigella dysenteriae and used them to design a conventional PCR assay. Conventional PCR, which target the invasion plasmid H (ipaH) gene for detection of Shigella spp.[18].

Prevalence of amoebic and bacillary dysentery of this study according to residency was 88/145(60.7%) from rural and 57/145(39.3%) from urban, this agreement with the study of Abbass and Nazdar in Erbil,[19] which reported prevalence rate of infection was comprise (66.8) in rural and (57%) in urban area, while disagreed with other study in Erbil by Narmin and Isra,[20] which documented (25.49%) in rural and (34.69%) in urban. The findings of this study confirmed a movement of high risk of infection with Entamoeba species among the rural population as shown by other studies like[10], where prevailing poverty, poor socioeconomic condition, low standards of sanitation and hygiene and lack of education attainment may contribute to high prevalence of Entamoeba infection.

The present study reported an overall prevalence of Entamoeba histolytica as determined by microscopy about sixteen samples 16/75(21.3%) were positive for E. histolytica with high prevalence (31.25%) between age group (13-24 months) and were disagreed with the study of Basim,[21] in which the highest rate of infection was recorded in the children (1day-1year), 138/223(61.88%), and also disagreement with Al-Joudi and Ghazal in Ramadi,[22] reported 227/2810 (8.08%), but agreement with other reports demonstrating that the occurrence of diarrhea varies widely by the age of the child, and the infection was peak in children aged $\leq$ 2.0 years [23,24].

The PCR assay was performed using DNA extracted by stool extraction kit of BioNeer company directly from diarrhea stool. This assay show a novel approach for the diagnosis of diarrhea caused by Shigella dysenteriae and Entamoeba histolytica. This diagnosis efficacy of the assay was analyzed by agarose gel electrophoresis.

Microscopically positive E. histolytica and other Entamoeba spp. of our study by PCR was 30/75(40%), it is less than the values reported by Pharmongkutklao and Ramathibodi Hospitals in Bangkok, Thailand, in which the result was 27/30(90%) fecal specimens evaluated by PCR. On the other hand, Subhash, et al.,[25] were reported that the result of E. histolytica was 3/49(15.8%). The result of this study disagrees with the results of Fallah, et al.,[26] who documented that the result was 8/31(25.8%). A recent study in Malaysia reported by Ngui, et al.,[10] evaluated the level of E. histolytica was 39/52(75%). Anjana, et al.,[27] and by using the PCR assay were reported that the result of E. histolytica was 4/10(40%).

The high prevalence of Entamoeba infection may be due to the transmission and pathogenesis as well as other risk factors which favor the persistence of this infection. Given its fecal-oral route, habits related to eating, defecation, personal hygiene, cleanliness and level of education may have an impact on the prevalence rates. The findings of this study confirmed a trend of high risk of infection with Entamoeba species among the rural population, where prevailing poverty, poor socioeconomic condition, low standards of sanitation and hygiene and lack of education attainment may contribute to high prevalence of Entamoeba infection.

The failure to amplify samples could also be because samples may contain only trophozoites that could have degenerated with time or due to other pathogen like Rota virus. Several studies have confirmed that the presence of Entamoeba cysts in the fecal samples, in contrast to trophozoites, somewhat increase the chances of the PCR assay[13]. The sensitive detection of food borne pathogens in produce requires optimal cell lysis and efficient DNA purification to remove associated PCR inhibitors [28].

PCR is more sensitive and specific than conventional culture methods, Shigella dysenteriae was detected in 30/70 (42.86%)of all the stool samples when analyzed by PCR assay. By the use of PCR, we found that 8 (17.8%) out of 45 samples were positive for S. dysenteriae by PCR and not by culture, this result agreement with previous study [7,29,30]. PCR is a selective, sensitive, and specific assay that can detect a small number of cultivable as well as non-cultivable organisms. Such detection is especially important for Shigella, since they can produce disease by as few as 10 to 100 organisms. In this study, the use of PCR technique improved the rate of detecting Shigella in stool samples from Twenty two (88%) samples out of 25 were positive samples; which is higher than the conventional culture method. The false-positive reactions can result from sample contamination or carryover. To avoid false positive PCRs, must institute strict physical separation of the three main PCR steps (preparation of reaction mixture, addition of target amplification, and manipulation of PCR products).

Such inhibitors would cause false-negative PCR results with potentially serious public health consequences when a contaminated materials is not removed from circulation. our find up reagents and methods used in DNA extraction had some impact on the PCR outcome. The presence of inhibitors produced false-negative PCR results in DNA prepared from DNA extraction kits, but this effect was not seen with the boiling method, probably because the DNA and DNA inhibitors were more diluted.

The study suggested that PCR has several advantages over the conventional methods for the diagnosis of amoebic dysentery such as safety, high sensitivity and specificity. Our results highpoint the value of using a combination of traditional and molecular techniques in the diagnosis of diarrheal disease in this population. Entamoeba complex with possible differentiation has revolutionized our understanding of the epidemiology of amoebiasis and can have led to important treatment and diagnostic recommendations and to avoid unnecessary and possibly harmful chemotherapies. Molecular techniques are indeed promising tools for epidemiological studies, particularly in discriminating the pathogenic from the non-pathogenic species of the Entamoeba species. This study has been providing comparable results of microscopy and PCR, none of these methods can detect all positives alone. According to this results, microscopy is a simple analysis, but it is subjective, needs experience to evaluate and should be combined with

complimentary methods such as antigen detection and PCR for identification of the species to avoid false and/ or insufficient diagnosis and treatment applications.

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