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The use of real-time PCR to identify *Entamoeba histolytica* and *E. dispar* infections in prisoners and primary-school children in Ethiopia

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In Ethiopia, it is generally unknown what proportion of the amoebic infections commonly found, by microscopy, in humans are caused by non-invasive *Entamoeba dispar* rather than the potentially invasive *E. histolytica*. Faecal samples were therefore collected from 363 primary-school students and 409 prisoners from various regions of Ethiopia. Each of these samples was checked for *Entamoeba* infection by the microscopical examination of formol–ether concentrates. DNA was then extracted from the 213 samples (27.6%) found *Entamoeba*-positive, and run in a real-time PCR with primers, based on the SSU-rRNA gene sequences of *E. histolytica* and *E. dispar*, that allow DNA from the two species to be distinguished. Although *E. dispar* DNA was identified in 195 (91.5%) of the 213 samples checked by PCR, no *E. histolytica* DNA was detected. This finding is consistent with the conclusion of a previous, smaller investigation: that many amoebic infections in Ethiopia are incorrectly attributed to *E. histolytica* and then treated, unnecessarily, with amoebicidal drugs.

Intestinal amoebiasis caused by *Entamoeba histolytica* is thought to be responsible for many cases of dysentery in Ethiopia. The prevalence of amoebic infection in parts of Ethiopia may exceed 50%. Torrey (1965), for example, found that 55% of the Saysay pastoralists from the Blue Nile gorge who he investigated were excreting *E. histolytica*/*E. dispar*. According to the unpublished records of the national Ministry of Health, amoebic infection is one of the most common infections discovered during routine diagnosis in many hospitals and health centres in Ethiopia. In most such institutions, the detection of amoebic infection is solely based on the microscopical examination of fresh stool samples. This method is not very sensitive, however, and cannot be used to distinguish the non-pathogenic *E. dispar* from the potentially pathogenic *E. histolytica* (Anon., 1997). *Entamoeba dispar*, with cysts and small trophozoites that are morphologically identical to those of *E. histolytica*, lives in the colonic lumen of its human host, as a harmless commensal. Unfortunately, in all surveys carried out before *E. dispar* was recognized as a separate species, and in many subsequent surveys and diagnostic tests, all quadrinucleate cysts and trophozoites seen in faecal samples were recorded as

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E. histolytica. The failure to distinguish *E. dispar* from *E. histolytica* and the frequent mis-identification of many other cysts and trophozoites of genera other than *Entamoeba* as those of *E. histolytica* s.l. (Kebede *et al.*, 2003) have generally led to the prevalence of *E. histolytica* infection being grossly over-estimated.

In a survey on the Seychelles, Sargeaunt (1992) demonstrated that the risk of over-diagnosing *E. histolytica* infections could be drastically reduced by replacing routine microscopy with culture and zymodeme characterization. *Entamoeba histolytica* and *E. dispar* can now also be detected and distinguished in 'conventional' PCR that amplify any of several species-specific gene fragments (Tachibana *et al.*, 1991; Diamond and Clark, 1993; Verweij *et al.*, 2000). These conventional PCR are relatively time-consuming, however, and prone to false-positive results caused by cross-contamination. 'Real-time' PCR, in which specific amplification is detected with fluorescent-labelled probes during the reaction, offer a more rapid and sensitive alternative (Verweij *et al.*, 2004).

Infection with the true *E. histolytica* appears to be relatively uncommon in Addis Ababa, Wonji and Akaki (Gatti *et al.*, 1998; Kebede *et al.*, 2003), perhaps because of the extensive use of metronidazole to treat amoebic infections caused by this species or *E. dispar*. The aim of the present study — in which real-time PCR based on the genes coding for the small-subunit ribosomal RNA (SSU rRNA) of *E. histolytica*/*E. dispar* were employed to test faecal samples collected from various sites in Ethiopia — was to determine the percentage of *E. histolytica*/*E. dispar* infections attributable to *E. dispar*.

SUBJECTS AND METHODS

Study Population and Sample Collection

The faecal samples investigated (one/subject) came from 772 apparently healthy individuals who lived in various regions of Ethiopia. The subjects were either children attending one of three primary schools or inmates of one of three prisons (Table 1). The schools and prisons investigated were all in areas where, according to the records of local hospitals or the results of parasitological surveys based on microscopical diagnosis, there was a relatively high prevalence of intestinal infection with *E. histolytica*/*E. dispar*. Systematic random sampling was used to select the 10% of the prisoners/schoolchildren at each site who were each asked to provide a single stool specimen, which was collected in a clean and labelled container. Part of each specimen was preserved in sodium-acetate–acetic-acid–formalin (SAF), for later microscopical examination, and the rest was preserved, by mixing with absolute ethanol (approximately 2 ml/g faeces) and storage at 4°C, for DNA isolation.

Microscopy

For the microscopy, each stool subsample was concentrated by formol–ether centrifugation. Each concentrate was then checked under a light microscope for parasitic cysts, trophozoites and ova, as a wet mount.

DNA Analysis

ISOLATION

For each DNA isolation, the solids in 250 µl of a faecal suspension in absolute ethanol

TABLE 1. *Demographic characteristics of the 772 subjects from three primary schools and prisons in Ethiopia*

	No. and (%) of subjects
CHILDREN FROM:	
Sirba Abay primary school (Blue Nile gorge)	146 (40.2)
Burka Jato primary school (Nekemte)	136 (37.5)
Hamle primary school (Asella)	81 (22.3)
INMATES OF:	
Ziway prison	138 (33.7)
Shewa Robbit prison	125 (30.6)
Nekemte prison	146 (35.7)
Male subjects	587 (76.1)
Female subjects	185 (23.9)

were washed twice with phosphate-buffered saline (pH 7.2; 1 ml/wash). The final pellet was resuspended in 200 µl 2% polyvinylpolypyrolidone (Sigma) and heated to 100°C for 10 min. After SDS–proteinase-K treatment for 2 h at 55°C (Verweij *et al.*, 2001), the DNA in each sample was isolated using a spin column from a QIAamp tissue kit (Qiagen, Hilden, Germany).

AMPLIFICATION AND DETECTION

The real-time PCR described by Verweij *et al.* (2004) was used to amplify, detect and distinguish the SSU-rRNA sequences of *E. histolytica* and *E. dispar*. The forward (Ehd-239F; 5′-ATT GTC GTG GCA TCC TAA CTCA-3′) and reverse (Ehd-88R; 5′-GCG GAC GGC TCA TTA TAACA-3′) primers used amplify a 172-bp fragment inside the SSU rRNA gene. Two minor-groove-binding (MGB) TaqMan® probes, one specific for *E. histolytica* (histolytica-96T; VIC™–5′-TCA TTG AAT GAA TTG GCC ATTT-3′–non-fluorescent quencher; Applied Biosystems, Foster City, CA) and one specific for *E. dispar* (dispar-96T; FAM–5′-TTA CTT ACA TAA ATT GGC CAC TTTG-3′–non-fluorescent quencher; Applied Biosystems), were then used to distinguish the two species. Other PCR primers [PhHV-267s (5′-GGG CGA ATC ACA GAT TGA ATC-3′) and PhHV-337 (5′-GCG GTT CCA AAC GTA CCAA-3′)] and a double-labelled detection probe (PhHV-305tq; Cy5–5′-TTT TTA TGT GTC CGC CAC CAT CTG GATC-3′–Black Hole Quencher 2), all from Biolegio (Malden, The Netherlands), were used to amplify and detect the phocid herpesvirus type-1 (PhHV-1) used as the internal control (Niesters, 2002). Each set of amplification reactions was performed in a 50-µl reaction mixture containing PCR buffer (HotStart Taq master mix; Qiagen), 5 mM $MgCl_2$, 25 pmol of each *E. histolytica*-/*E. dispar*-specific primer, 15 pmol of each PhHV-1-specific primer, 5 pmol of the *E. histolytica*-specific MGB TaqMan probe, 5 pmol of the *E. dispar*-specific MGB TaqMan probe, 2.5 pmol of the PhHV-1-specific double-labelled probe and 5 µl of the DNA sample. A Bio-Rad I thermocycler (Bio-Rad, Hercules, CA) was set to give 15 min at 95°C followed by 50 cycles, each of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C.

RESULTS

The 772 subjects were from various parts of Ethiopia and either primary-school children (47%) with a median age of 12 years or prisoners (57%) with a median age of 27 years (Table 1). As almost all the prisoners were male, there were more male subjects than female. In general, the schoolchildren and prisoners were similar in terms of the prevalence of each protozoan or helminth infection observed (Table 2). *Schistosoma mansoni* was, however, particularly common

TABLE 2. *Prevalences of intestinal protozoan and helminth infections among the 772 subjects, as determined by microscopy*

Recorded parasite	No and (%) of subjects found infected
PROTOZOA	
Blastocystis hominis	233 (30.2)
Entamoeba coli	229 (29.7)
Entamoeba histolytica/*E. dispar*	213 (27.6)
Iodamoeba buetschii	106 (13.7)
Amoeba with mononucleate cyst	56 (7.3)
Giardia lamblia	45 (5.8)
Entamoeba hartmanni	34 (4.4)
Endolimax nana	29 (3.8)
Chilomastix mesnili	21 (2.7)
Isospora sp.	2 (0.3)
HELMINTHS	
Hookworm	115 (14.9)
Ascaris lumbricoides	33 (4.3)
Schistosoma mansoni	26 (3.4)
Trichuris trichiura	16 (2.1)
Enterobius vermicularis	2 (0.3)
Strongyloides stercoralis	2 (0.3)
Taenia sp.	2 (0.3)
Hymenolepis nana	1 (0.1)

among the subjects from Sirba Abay primary school (Blue Nile gorge), with a prevalence of 15.8%.

By microscopy, 71% of the subjects (71.1% of the schoolchildren and 70.7% of the prisoners; $P > 0.05$) were each found to be carrying at least one species of intestinal parasite. Infection with the *E. histolytica*/*E. dispar* species complex represented one of the most common parasitic infections, with a prevalence of 27.6%. Hookworms appeared to be the commonest of the intestinal helminths (Table 2).

Overall, 213 faecal subsamples — one of every faecal sample found positive for *E. histolytica*/*E. dispar* cysts and/or trophozoites by microscopy — were tested with the real-time PCR (Table 3). The results of the PCR indicated that 195 (91.5%) of the subsamples were positive for *E. dispar* whereas none was PCR-positive for *E. histolytica*. In the PCR, *E. dispar*-specific amplification could be detected after 19–46 cycles (median = 33) and usually before 35 cycles. Only three (2.7%) of the 111 samples found cyst-positive by microscopy but 15 (14.7%) of the 102 found cyst-negative but trophozoite-positive were PCR-negative. When every PCR-negative sample was re-tested in the PCR, each was again found PCR-negative. In all samples except one, amplification of the internal control was detected, at the expected threshold of approximately 33 cycles. The sample that did not show amplification of the control was one of the cyst-positive samples that appeared PCR-negative for *Entamoeba*.

DISCUSSION

As in the present study, most of the subjects (with diarrhoea) investigated in Ethiopia by Kebede *et al.* (2003) were found PCR-positive for *E. dispar* and none was PCR-positive for *E. histolytica*. In these earlier studies, the absence or very low prevalence of *E. histolytica* infection was attributed to the extensive use of metronidazole, for treatment of patients with cysts or trophozoites, in the study sites (Kebede *et al.*, 2003). The main aim of the present study was to see if true infection with *E. histolytica* was generally rare throughout Ethiopia. The subjects came from six sites where the prevalence of amoebic infection, as determined by microscopy, was known to be high, thanks, probably, to very poor standards of hygiene and high population densities. Children attending primary schools were investigated as they are unlikely to have received much, if any, metronidazole treatment. That the prevalence of *E. histolytica*/*E. dispar* infection recorded, by microscopy, among the schoolchildren from Sirba Abay (55%) was among the highest ever reported in Ethiopia (Kloos and Tesfa Yohannes, 1993) supports the notion that such children have received little if any effective treatment.

In the present study, specific DNA amplification did not reveal any true infections with *E. histolytica,* even though many (27.6%) of the subjects were found to be infected with *E. histolytica*/*E. dispar* by microscopy. Almost all (91.5%) of the 213 microscopically positive subjects were found PCR-positive for *E. dispar* only. Similar observations, of low prevalences

TABLE 3. *Comparison of the results of the microscopical detection of* Entamoeba histolytica/E. dispar *cysts and trophozoites with those of the real-time PCR*

	PCR result (no. of samples):			
Results of microscopy:	Positive for *E. histolytica*	Positive for *E. dispar*	Negative	Any
Cysts only	0	79	1	80
Trophozoites only	0	87	15	102
Cysts and trophozoites	0	29	2	31
Any	0	195	18	213

of infection with *E. histolytica* s.s., have been reported elsewhere in Africa, notably in Ivory Coast and Ghana (Heckendorn *et al.*, 2002; Verweij *et al.*, 2003). The epidemiological picture seen in countries such as Mexico, Bangladesh and Vietnam (Haque *et al.*, 1997; Palacio-Sanchez *et al.*, 1997; Blessmann *et al.*, 2002), where high proportions of cyst-excreters are reported to be infected with true and potentially invasive *E. histolytica*, appears to be very different from that encountered in Ethiopia.

The present results, for apparently healthy subjects, are consistent with those of Kebede *et al.* (2003), for subjects with diarrhoea. The absence or low prevalence of infection with *E. histolytica* in Ethiopia appears to be the rule rather than the exception, both in areas with frequent utilization of metronidazole as well as in those where treatment is given less often. The implications, for treatment and management, are far-reaching. All too often it appears, in routine clinical practice in Ethiopia, quadrinucleate cysts and trophozoites in faecal samples are assumed to be *E. histolytica* s.s. and the patient is given unnecessary treatment with an amoebicidal drug.

The results of the real-time PCR show better concordance with the microscopical finding of *E. histolytica*/*E. dispar* cysts than with the microscopical detection of *E. histolytica*/*E. dispar* trophozoites (Table 3). It therefore appears that, compared with the trophozoites, the cysts of *Entamoeba* are more easy to identify by microscopy, with the trophozoites of amoebae other than *E. histolytica*/*E. dispar* and even faecal leucocytes being mis-identified as *E. histolytica*/*E. dispar* trophozoites.

In the present study, of the three cyst-positive cases who were found negative in the real-time PCR, one showed inhibition (i.e. non-amplification of the internal control). When faecal samples from the other two cases were investigated further, by species-specific DNA hybridization after amplification of a part of the SSU-rRNA gene (Verweij *et al.*, 2003), they were found positive for *E. coli* and *E. hartmanni* or *E. coli*, *E. hartmanni* and an *E. polecki*-like *Entamoeba* species (unpubl. obs.). In these two cases, the small cysts of *E. coli* or the larger ones of *E. hartmanni* or the *E. polecki*-like species were probably mis-identified microscopically as the cysts of *E. histolytica*/*E. dispar*.

The outcomes of the present and recent, related studies indicate that infection with *E. histolytica* is much rarer in Ethiopia than previously thought. Intestinal amoebiasis is routinely over-diagnosed in Ethiopia, resulting in much unnecessary use of metronidazole and other amoebicidal drugs.

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