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Overdiagnosis of amoebiasis in the absence of *Entamoeba histolytica* among patients presenting with diarrhoea in Wonji and Akaki, Ethiopia

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Abstract

To confirm the high reported incidence of intestinal amoebiasis among study participants at 2 cohort sites in Ethiopia where an HIV/AIDS study is taking place, stool samples of 232 patients with complaints of diarrhoea were examined for the presence of *Entamoeba histolytica* and *E. dispar* DNA between April and December 2001. By microscopy, 91 (39%) of the study participants were reported to harbour *Entamoeba* trophozoites and/or four-nucleated cysts. Using specific *E. histolytica* and *E. dispar* DNA amplification and detection, none of the study participants were found to be infected with *E. histolytica* and only 21 (9%) with *E. dispar*. The consequences of the overdiagnosis of *E. histolytica* are briefly discussed.

Keywords: amoebiasis, *Entamoeba histolytica*, *Entamoeba dispar*, diagnosis, polymerase chain reaction, Ethiopia

Introduction

Intestinal amoebiasis is one of the most commonly reported infections in Ethiopia. In a survey of 50 communities covering the central plateau of Ethiopia, *Entamoeba histolytica* was reported in 94% of the communities, with prevalence rates ranging from 3% to 55% (Kloos & Tesfa Yohanes, 1993). In a recent study on the epidemiology of infections with intestinal parasites and HIV in Ethiopia, *E. histolytica/E. dispar* was the most common parasite, with a prevalence of 25% (Fontanet *et al.*, 2000).

In Ethiopia, as in most other developing countries, diagnosis of intestinal amoebiasis is based entirely on microscopical examination of fresh stool specimens. No attempt is made to differentiate cysts and trophozoites of morphologically identical *E. histolytica* and *E. dispar*. Trophozoites and cysts are readily referred to as *E. histolytica* and widespread use of antiamoebic drugs is the result. Recent knowledge, however, indicates that, worldwide, the majority of the trophozoites and cysts reported as *E. histolytica sensu lato* are in fact *E. dispar* (Diamond & Clark, 1993). This is likely to be the case in Ethiopia as well. Very little information is available on the presence of the 2 species in Africa, more so in Ethiopia. A small study carried out in the Wonji area of central Ethiopia on 29 cultured amoebic isolates from 123 study participants revealed 27 isolates with *E. dispar* zymodemes and only 2 isolates with an *E. histolytica* zymodeme (Gatti *et al.*, 1998). A WHO/PAHO/UNESCO (1997) report recommended that *E. histolytica* should be specifically identified and, if present, treated, and if only *E. dispar* is identified treatment is unnecessary.

In this paper, we report on a survey of the presence of amoebic infection among participants of a cohort study on the natural history of HIV/AIDS in Ethiopia where baseline information is already available (Fontanet & Woldemichael, 1999). At the 2 cohort sites excretion of trophozoites and four-nucleated cysts resembling *E. histolytica/E. dispar* is reported regularly and antiprotozoal treatment is given accordingly. We prospectively collected clinical and socio-demographic data and fresh stool specimens for microscopical examination from patients presenting with diarrhoea. To confirm the microscopical diagnosis of these amoebic infections, we used *E. histolytica*- and *E. dispar*-specific DNA amplification and detection as reported earlier (Verweij *et al.*, 2000).

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Materials and Methods

Study areas and patients

The study was carried out between April and December 2001 at 2 sites in Ethiopia where a large study on HIV/AIDS is taking place (Fontanet & Woldemichael, 1999). Wonji Sugar Estate is a semi-rural site 110 km from Addis and Akaki Fibre Factory is a suburban area 25 km from Addis. Patients enrolled in the current study were either participants in the HIV/AIDS study or their family members. From the patients who were participants in the HIV/AIDS study 30 of 144 (21%) tested positive for HIV; family members were not tested. Entry criterion was the patients' complaint of diarrhoea, for which he or she presented to the cohort physician. Each patient submitted a single stool specimen for parasitological examination. For each patient a standard questionnaire containing information on symptoms and treatment was provided which the examining physician completed.

Stool processing and analysis

Microscopy. A single stool specimen was collected from all participants. Each sample was macroscopically inspected for its consistency and the presence of blood or mucus by a trained technician. Direct saline and iodine preparations were made and routine microscopical examination for any moving protozoan trophozoites and for cysts was performed. No concentration method was used.

DNA isolation and amplification. Part of the fresh stool sample was apportioned in an Eppendorf tube for DNA isolation and immediately transported in an ice-box to the main laboratory of the Ethio-Netherlands AIDS Research Project, Addis Ababa, Ethiopa, and stored frozen. For DNA isolation, a faeces suspension was made in 200 µL phosphate-buffered saline containing 2% polyvinylpolypyrolidone (Sigma, St Louis, MO, USA) and heated for 10 min at 100 °C. After sodium dodecyl sulfate–proteinase K treatment (2 h at 55 °C), DNA was isolated with QIAamp Tissue Kit spin columns (QIAGEN, Hilden, Germany) (Verweij *et al.*, 2001). DNA amplification and colourimetric detection of the product was carried out as described previously (Aguirre *et al.*, 1995; Verweij *et al.*, 2000). Briefly, polymerase chain reaction (PCR) was performed twice on each DNA sample, one with *E. histolytica*-specific primers and the other with *E. dispar*-specific primers. One primer of each primer pair was labelled with digoxigenin. The PCR product was hybridized with 5 pmol of a 5′-biotin-conjugated specific probe for *E. histolytica* and *E. dispar*, respectively. The hybridized PCR product was captured in a streptavidin-

coated microtitre plate and detected through subsequent incubation with antidigoxigenin alkaline phosphatase-labelled antibody and *p*-nitrophenyl phosphate. Absorbance was recorded at 405 nM after incubation for 1 h at room temperature. In every amplification run positive controls for *E. histolytica* and *E. dispar* and non-template (water) controls were included. Thirty microscopically-positive samples in which no specific DNA was amplified were checked for inhibition by spiking these samples with *E. histolytica* DNA and subsequent specific PCR amplification.

Results

Of the 232 patients included in the study, 154 (66%) were males and 78 (34%) were females, aged from 1 to 68 years (median = 30 years); 167 (72%) were adults (18–68 years). Overall, 38/232 (16%) of the study participants reported having received antiprotozoal treatment, mainly metronidazole or tinidazole, during the 2 months before the stool sample was submitted. In patients who reported having taken antiprotozoal treatment the prevalence of *E. histolytica/E. dispar* identified by microscopy was not different from those who had not received treatment (data not shown).

Duration of diarrhoea, and symptoms as reported by the individuals, are shown in Table 1. Less than 1 week duration of diarrhoea was most commonly reported (75%) while diarrhoea of 4 weeks or more was rare (4%). Abdominal pain, tenesmus, mucous, and/or bloody diarrhoea and flatulence were the most common complaints (66–84%). In contrast, fever, weight loss, and constipation were less common (5–30%). The duration of diarrhoea was not significantly associated with the symptoms ($P > 0.05$).

Macroscopical stool inspection showed that 136/232 (59%) were 'soft stools' while 57/232 (24%) were 'liquid stools', the rest 39/232(17%) were 'formed stools'. By microscopy, 155 (67%) of the study participants appeared to be infected with 1 or more intestinal parasites. By far the most common protozoan infection reported was *E. histolytica/E. dispar*: 91 (39%) of the study participants were thought to harbour trophozoites and/or four-nucleated cysts measuring 10–15 μm. After diagnosis of 'amoebiasis', specific treatment with an antiprotozoal drug was provided.

Upon confirmatory diagnostic analysis, using PCR–solution hybridization enzyme-linked immunoassay (PCR–SHELA), *E. dispar* was identified in only 21 of 232 cases (Table 2), while no *E. histolytica* DNA was detected. In 12 of the 21 cases in which *E. dispar* DNA was detected *Entamoeba* trophozoites and/or four-nucleated cysts were found by microscopy (Table 2). Thirty microscopically-positive samples which did not produce an amplicon in either 1 of the 2 PCRs (i.e. were 'negative'), were tested for PCR inhibition. After spiking with *E. histolytica* DNA all samples produced a specific product.

Table 1. Clinical history of patients presenting with diarrhoea to their physician in Ethiopia, April–December 2001

	No. of cases (%) ($n = 232$)
Duration (weeks)	
< 1	173 (75)
1–4	50 (21)
> 4	9 (4)
Symptoms	
Abdominal pain	196 (84)
Tenesmus	191 (82)
Mucous/bloody diarrhoea	186 (80)
Flatulence	154 (66)
Fever	70 (30)
Watery diarrhoea	44 (19)
Weight loss	31 (13)
Constipation	11 (5)

Table 2. Results of microscopical examination in comparison with polymerase chain reaction–solution hybridization enzyme-linked immunoassay (PCR–SHELA) on stool samples of patients presenting with diarrhoea to their physician in Ethiopia, April–December 2001

	PCR–SHELA[a]		
	Positive	Negative	Total
Microscopy			
Positive	12	79	91
Negative	9	132	141
Total	21	211	232

[a]Specific for *Entamoeba dispar*.

Discussion

In the present study the prevalence of amoebic infection among patients presenting with diarrhoea based on microscopy in the cohort sites was reported to be 39%. This prevalence is in the range of infection rates described by Kloos & Tesfa Yohanes (1993). The relevance of such data depends on the reliability of the microscopical differentiation of cysts and trophozoites of the various intestinal protozoa. However, this is hampered by problems in quality control for microscopical findings and moreover by the impossibility of microscopically differentiating *E. histolytica* from *E. dispar*. In view of the high prevalence of the non-pathogenic *E. dispar* in many parts of the world (Clark, 1998; Jackson, 1998), such differentiation is considered obligatory.

The difference between a mere 21 *E. dispar* cases detected by PCR–SHELA and 91 microscopically detected cases of *E. histolytica/E. dispar* (Table 2) could be explained by either a lack of sensitivity in the PCR–SHELA or by overdiagnosis in microscopy or both. Inhibition could result in false negative results in PCR, however, inhibition is rarely seen in the Leiden laboratory with the DNA isolation method used (Verweij *et al.*, 2001) and in the spiked, supposedly microscopically-positive samples there was no evidence of inhibition. Another reason for false negative PCR results could be the unexpected absence of the specific target in these particular cases. However, 15 microscopically-positive samples tested using *E. histolytica*- and *E. dispar*-specific PCRs based on the small subunit rRNA gene (Clark & Diamond, 1991) gave no results in any of the PCRs (data not shown).

The data suggest a gross overdiagnosis of *E. histolytica* by microscopy, which is the most likely explanation. First, Table 1 shows that most patients complained of diarrhoea rather than chronic diarrhoea, which is characteristic for amoebiasis. It is also noteworthy that the prevalence of *E. histolytica/E. dispar* detected by microscopy was the same in patients who had recently received treatment and those who had not. More importantly, in more than 70% of cases the diagnosis was based on the finding of trophozoites but details of the structure of the nuclei were not really taken into account. Clearly, the criteria for calling structures trophozoites or cysts of *E. histolytica* are too liberal in diagnostic settings in the field, like those in Wonji and Akaki.

Although attempts were made to implement a system of microscopical quality control, the abundance of trophozoites and cysts of commensal protozoa and the

impossibility of differentiating *E. dispar* from *E. histolytica* in combination with the operational problems of such a time consuming system, resulted in the decision to confirm diagnosis with specific PCRs instead.

On the basis of local microscopical diagnosis, 39% of the study participants examined were treated with tinidazole. The WHO/PAHO/UNESCO guidelines (1997) consider treatment unnecessary in *E. dispar* infections and only justified in cases of *E. histolytica s.str.* Clearly massive overtreatment has been the result of the use of a routinely used diagnostic system that aims to find an answer to the large numbers of people complaining of (bloody) diarrhoea.

In conclusion, the earlier study of Gatti *et al.* (1998) indicated that infections with *E. histolytica* are rare in this area and the present study indicated they may be absent, but people not infected by *E. histolytica* are consuming large amounts of drugs. Second, the commonly reported complaints of (bloody) diarrhoea in this area require alternative explanation. Third, the actual diagnostic system is unsatisfactory; therefore, training in microscopy needs improvement and alternative, reliable and affordable diagnostic systems need to be developed.

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Book Review

Textbook-Atlas of Intestinal Infections in AIDS. Daniele Dionisio (editor). Milan: Springer-Verlag Italia, 2003. xvi + 506 pp. Price €129.00. ISBN 88-470-0174-9.

Intestinal involvement is common in patients with AIDS worldwide. While a dramatic decrease in AIDS-related mortality and morbidity has been observed in developed countries in recent years due to the availability of antiretroviral therapy, gut infections remain a major problem in Africa, Asia, and South America. *Textbook-Atlas of Intestinal Infections in AIDS* is a unique book which provides excellent reviews on various gastrointestinal infections found in patients with AIDS. The 31 chapters written by authors from several continents have resulted in one of the most authoritative books on the subject. The work covers the effects of HIV on gut function; aetiology, microbiological and clinical manifestations of opportunistic gut infections; and epidemiological, pathological, histological, and electron microscopic features in great detail. The book is richly illustrated throughout by extraordinary, clear photomicrographs. *Textbook-Atlas of Intestinal Infections in AIDS* will no doubt be an invaluable resource to all medical personnel/institutions worldwide. The publishers must think about producing a low-cost edition of the book for it to be affordable for health personnel in developing countries.

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