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ORIGINAL ARTICLE

Polymerase chain reaction confirmation of diagnosis of intestinal amebiasis in Puducherry

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Abstract
Background Stool microscopy, the commonly used test for diagnosis of intestinal amebiasis, is unreliable as it does not differentiate *Entamoeba histolytica*, the causative agent for amebiasis, from non-pathogenic *Entamoeba dispar* and *Entamoeba moshkovskii*.
Methods Two hundred and forty-six stool samples collected between January to February 2009, were examined microscopically for *E. histolytica*/*E. dispar*/*E. moshkovskii* complex cysts and trophozoites. Nested multiplex PCR targeting a 16 S-like rRNA gene for differential detection of all the three morphologically similar forms of *E. histolytica*, *E. moshkovskii* and *E. dispar* simultaneously was done on all microscopy positive stool samples.
Results Forty-nine stool samples were positive for *Entamoeba* spp. by microscopy. Nested multiplex PCR for *E. histolytica*, *E. moshkovskii* and *E. dispar* was positive in only 19 of these samples, and only 6 of the 19 samples were positive for *E. histolytica*.
Conclusion Stool microscopy grossly over-diagnosed intestinal amebiasis and is likely to result in unnecessary treatment with anti-protozoal drugs.

Keywords Culture · Isoenzyme analysis · Nested PCR

Introduction

Amebiasis, caused by the protozoon *Entamoeba histolytica*, is the third leading parasitic cause of death worldwide, surpassed only by malaria and schistosomiasis [1]. Worldwide, approximately 50 million cases of amebiasis occur each year with a significant number of deaths [2]. Amebiasis has been called the 10% disease because it was estimated that approximately 500 million people or 10% of the world's population were infected with *E. histolytica* [1], of whom 10% or 50 million people suffered from active amebic disease, of which again 10% (50,000 people) died each year [1]. The incidence of amebiasis is higher in developing countries and areas of high prevalence include the Indian subcontinent, southern and western Africa, the Far East, South America, and Central America [3]. Earlier estimates of *E. histolytica* infection based on stool microscopy are inaccurate because this test cannot differentiate *E. histolytica* from *E. dispar* and *E. moshkovskii,* which are morphologically identical but nonpathogenic organisms. It is not very clear as to how common infection with the pathogenic *E. histolytica* is, and how often other *Entamoeba* species are mistakenly diagnosed as *E. histolytica*. The present study was conducted to estimate the true burden of amebiasis at Puducherry using multiplex nested polymerase chain reaction.

Methods

A total of 246 stool samples were collected during January — February 2009 from patients attending our hospital with general complaints of gastrointestinal discomfort. Microscopy and culture were performed immediately on fresh unpreserved stool specimens. The samples were stored at −20^{0}C for PCR.

Microscopic examination and culture of stool

Both saline and iodine wet mounts of fresh unpreserved stool samples were examined microscopically for demonstrating *E.*

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histolytica/*E. dispar*/*E. moshkovskii* complex cysts and trophozoites using standard methods [3]. All microscopy positive stool samples were cultured for *Entamoeba* species in Locke-egg (LE) medium (NIH modification of Boeck and Drbohlav's medium) within 6 h of collection [3].

Nested multiplex-polymerase chain reaction (PCR)

The DNA was isolated from stool specimens by a cetyl trimethyl ammonium bromide (CTAB) extraction method modified as described previously [4]. The extracted DNA was passed over a DNA clean-up spin column (Bangalore Genei KT-62, Bangalore, India). Nested multiplex PCR targeting a 16 S-like rRNA gene for differential detection of all the three morphologically similar forms of *E. histolytica*, *E. moshkovskii* and *E. dispar* simultaneously in stool samples was done using indigenously designed primers described in our previous study [4]. The PCR mixture had a reaction volume of 25 μl comprising 2.5 μl of 10X PCR buffer, 1.5 μl of 25 mM MgCl2, 1.4 μl of dNTP, 0.3 μl (5 IU/μl) of *Taq* polymerase, 0.3 μM of each primer and 2.5 μl of template DNA. The PCR tubes were placed in an Eppendorf Thermal cycler (Master cycler gradient) with settings as described earlier [4]. Amplified products were separated by electrophoresis on 1.8% agarose gel and were visualized after ethidium bromide staining under UV light for bands of DNA of appropriate sizes. Positive and negative control reactions were included with each batch of samples analyzed and the final results were reported on the basis of species-specific product size for *E. histolytica; E. moshkovskii* and *E. dispar* (439, 553 and 174 base pairs respectively) (Fig. 1).

Results

A total of 246 stool samples were screened by microscopy for the presence of either cysts or trophozoites of *Entamoeba species*. Forty-nine stool samples were positive for *E. histolytica*/*E. dispar*/*E. moshkovskii* complex cysts or trophozoites. Nested multiplex PCR was performed on 49 microscopy positive stool samples and PCR was positive for *E. histolytica*/*E. dispar*/*E. moshkovskii* in 19 out of 49 stool specimens. The nested multiplex PCR detected mono-infection with *E. histolytica* in 3 (15.8%), *E. dispar* in 11 (57.9%) and *E. moshkovskii* in one (5.26%) of 19 stool samples. Mixed infections with combinations of *E. dispar* and *E. moshkovskii*, *E. dispar* and *E. histolytica*, and *E. moshkovskii* and *E. histolytica* were seen in one patient each. PCR also detected mixed infections by all the three species in one stool sample. Amebic culture was positive only in 11/49 stool samples and all culture-positive stool samples were also PCR positive for *Entamoeba* species.

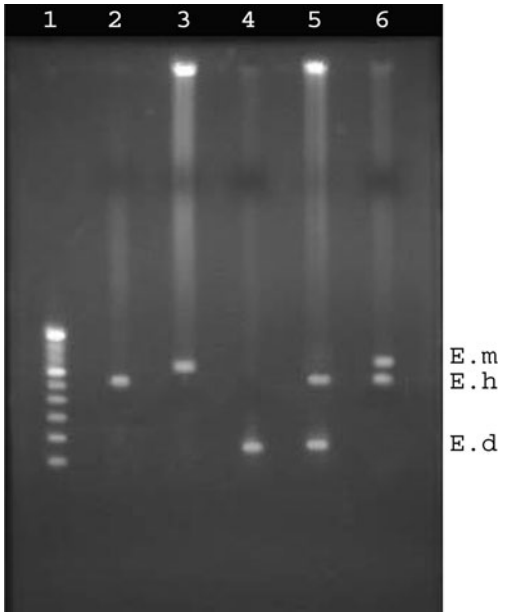

Fig. 1 Differential detection of *E. histolytica*, *E. moshkovskii* and *E. dispar* by nested multiplex PCR on stool samples. The *E. moshkovskii*, *E. histolytica* and *E. dispar* bands are 553, 439 and 174 bp, respectively. Lane-1, 100 bp DNA ladder, Lane-2, *E. histolytica* mono infection; Lane 3, *E. moshkovskii* mono infection; Lane 4, *E. dispar* mono infection; Lane 5 shows mixed infection with *E. histolytica* and *E. dispar* and Lane 6 is Showing *E. histolytica* and *E moshkovskii* mixed infection

Discussion

In most laboratories around the world, and especially in developing countries like India, intestinal amebiasis is diagnosed by demonstration of either cysts or trophozoites in stool by light microscopy [1]. *E. histolytica* is indistinguishable in its cyst and trophozoites stages from non-pathogenic *E. moshkovskii* and *E. dispar* except in cases of invasive disease when *E. histolytica* trophozoites may contain ingested red blood cells; however this finding is rarely seen [2]. This leads to over diagnosis of *E. moshkovskii* and *E. dispar* as *E. histolytica* leading to unnecessary antimicrobial treatment.

Stool culture followed by isoenzyme analysis enables the differentiation of *E. histolytica* from *E. dispar*. However, isoenzyme analysis requires one to several weeks to obtain the result and also special laboratory facilities are required; making it impractical for use in the routine diagnosis of intestinal amebiasis [2]. Several ELISA kits for antigen detection from stool are available. These ELISA tests have a sensitivity approaching that of stool culture and are rapid to perform. Antigen-based ELISA kits that are specific for *E. histolytica* use monoclonal antibodies against the Gal/GalNAc-specific lectin of *E. histolytica* (Tech Lab Kit, Blacksburg, VA) or monoclonal antibodies against serine-rich antigen of *E. histolytica* (Optimum S kit; Merlin

Diagnostika, Germany). Other ELISA kits for antigen detection include the *Entamoeba* CELISA PATH kit (Cellabs, Brookvale, Australia), and the ProSpecT EIA (Remel Inc, Sunnyvale, CA,USA) [1, 5]. In a recent study from Australia using PCR as a reference standard, the CELISA PATH Kit and Tech Lab ELISA were evaluated. The CELISA PATH kit showed 28% sensitivity and 100% specificity, however TechLab ELISA kit did not prove to be useful in detecting *E. histolytica* [6]. A recent review reported that the sensitivity of the fecal antigen test is about 100 times less than that of PCR, and it also has low specificity [5]. The only method currently available to make a confirmed diagnosis of *E. histolytica* infection is PCR; in our study only 12.3% (6/49) of microscopy-positive stool samples had *E. histolytica*. The major limitation of this study is that sample size is small as PCR was performed only on 49 microscopy positive samples.

In order to minimize undue treatment of individuals infected with other species of *Entamoeba*, efforts should be made for specific diagnosis of *E. histolytica* rather than treatment based on the microscopic examination of *Entamoeba* species in feces. In developing countries where amebiasis is endemic, microscopic diagnosis-based treatment leads to increased use of antiprotozoal agents, with a potential for resistant strains to appear [7, 8]. Thus confirmation of a microscopic diagnosis of amebiasis by PCR maybe worthwhile prior to initiation of therapy.

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Conflict of interest None declared.

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