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Comparison of a Stool Antigen Detection Kit and PCR for Diagnosis of *Entamoeba histolytica* and *Entamoeba dispar* Infections in Asymptomatic Cyst Passers in Iran

Shahram Solaymani-Mohammadi,[1,3] Mostafa Rezaian,[1] Zahra Babaei,[1] Azam Rajabpour,[2] Ahmad R. Meamar,[1] Ahmad A. Pourbabai,[2] and William A. Petri, Jr.[3]*

Division of Intestinal and Genital Protozoal Diseases, Department of Medical Parasitology and Mycology, School of Public Health and Institute of Public Health Research, Tehran University of Medical Sciences, 14155 Tehran 6446, Iran[1]; Department of Microbiology, School of Science, Qom Azad University, Qom, Iran[2]; and Division of Infectious Diseases and International Health, Department of Internal Medicine, University of Virginia Health System, Charlottesville, Virginia 22908-1340[3]

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The present study was conducted to compare stool antigen detection with PCR for the diagnosis of *Entamoeba* sp. infection in asymptomatic cyst passers from Iran. *Entamoeba dispar* and, in one case, *E. moshkovskii* were the *Entamoeba* spp. found in the amebic cyst passers. There was a 100% correlation between the results from the TechLab *E. histolytica* II stool antigen kit and those from nested PCR. We concluded that *E. dispar* is much more common in asymptomatic cyst passers in Iran and that antigen detection and PCR are comparable diagnostic modalities.

Entamoeba histolytica, *E. dispar*, and *E. moshkovskii* are morphologically identical but biochemically and genetically different. These parasites colonize the human gut, but only *E. histolytica* is thought to be capable of causing disease. Amebiasis, defined as asymptomatic, invasive intestinal or extraintestinal disease due to *E. histolytica* infection, is one of the most common parasitic infections worldwide, infecting about 50 million people, frequently in developing countries, resulting in 40,000 to 100,000 deaths per annum (21). Asymptomatic cyst passage is the most frequent manifestation of intestinal *Entamoeba* infection. All *E. dispar* and *E. moshkovskii* infections and 90% of *E. histolytica* infections are asymptomatic (21). The studies conducted to date that have used methods capable of differentiating between the two species suggest that, in general, *E. dispar* is much more prevalent than *E. histolytica* (9, 10, 11) and that only a small proportion of individuals specifically infected with *E. histolytica* will progress to having amebic disease (8). The necessity to identify and treat asymptomatic carriers of *E. histolytica* is emphasized by the observation that 10% of them develop invasive amebiasis in due course (6). Additionally, asymptomatic carriers are more likely to spread the disease than symptomatic persons with invasive diseases, as the latter individuals seek medical attention (12).

In nearly all of the studies of asymptomatic amebic cyst passers in which the distribution of the two species has been investigated (1, 3, 5, 11, 16, 18), *E. dispar* has been found to be much more prevalent than *E. histolytica*; however, in a few studies, including two from Mexico (14, 15) and one targeting mentally retarded individuals in Japan (20), either *E. histolytica* occult infections were more common than *E. dispar* infections or *E. histolytica* was the sole *Entamoeba* sp. found.

Several microscopy-based epidemiological studies in Iran have shown *Entamoeba* sp. infection rates of 2.2% to 30% (13, 17). The current study, therefore, was designed to address the distribution of *E. histolytica* and *E. dispar* in regions of Iran where previous microscopy-based studies showed a high prevalence of infection and to compare the commercially available TechLab *E. histolytica* II kit to PCR.

A total of 1,037 single fresh stool samples were collected from apparently healthy persons in rural and semirural settings of western (Luristan), northwestern (West Azerbaijan), and northeastern (Golestan) Iran, where the standards of hygiene are suboptimal. Stool samples were collected from persons that had given their informed consent prior to the collection according to the Iranian Ministry of Health, Treatment and Medical Training Protection Code of Human Subjects in Medical Research.

Ritchie's fecal concentration method of formalin-fixed specimens was performed on formed stool specimens, and the cysts were stained with Lugol's iodine and identified by microscopy (4). The stool samples were tested within 24 h after collection, or the samples were kept at −20°C for later analysis.

The TechLab *E. histolytica* II test (TechLab, Inc., Blacksburg, VA) was performed on stool samples containing *E. histolytica*/*E. dispar* cysts according to the manufacturer's instructions. This diagnostic kit is an antigen-based enzyme-linked immunosorbent assay and is designed to detect specifically *E. histolytica* and not the closely related nonpathogenic *E. dispar*. The well strips were measured in an automatic microtiter plate reader (Organon Teknika, Salzburg, Austria) at 450 nm. Positive results were defined as an optical density reading of ≥0.05 after subtraction of the negative control optical density.

Fecal specimen sediments (0.2 g) were used for DNA extrac-

* Corresponding author. Mailing address: Division of Infectious Diseases and International Health, P.O. Box 801340, Rm. 2115, MR4 Building, University of Virginia Health System, Charlottesville, VA 22908-1340. Phone: (434) 924-5621. Fax: (434) 924-0075. E-mail: wap3g@virginia.edu.

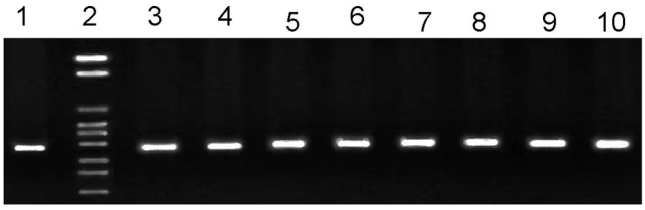

FIG. 1. Nested PCR of *E. histolytica*/*E. dispar*. A 374-bp fragment of the 30-kDa surface antigen was amplified from DNA purified from stool samples. Lanes: 1, HM1:IMSS; 2, DNA ladder; 3, AS2IR; 4 through 8, stool samples with *E. histolytica*/*E. dispar* cysts; 9 and 10, stool samples with *E. histolytica*/*E. dispar* trophozoites.

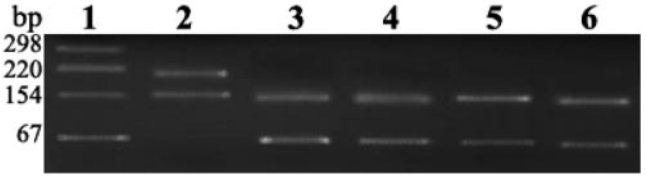

FIG. 2. Two percent agarose gel electrophoresis of the HinfI-digested PCR product. Lanes: 1, DNA ladder; 2, HM1:IMSS strain of *E. histolytica*; 3, AS2IR strain of *E. dispar*; 4 through 6, clinical samples. The 152- and 155-bp fragments overlapped and are seen as a single band.

tion by use of a QIAamp DNA stool mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Genomic DNA (5.0 μl) was subjected to PCR using primers (Alpha DNA, Quebec, Canada) P1 (5′-TAA AGC ACC AGC ATA TTG TC-3′) and P4 (5′-TTA ATT CCA TCT GGT GGT GG-3′), which were described previously (19). A second set of primers (TAG Copenhagen, Denmark), HF (5′-AAG AAA TTG ATA TTA ATG AAT ATA-3′) and HR (5′-ATC TTC CAA TTC CAT CAT CAT-3′), located within the fragment replicated by P1 and P4, making this a nested PCR, were designed, resulting in a 374-bp fragment for the axenic isolate HM1:IMSS. Amplification consisted of 35 cycles of 30 s at 94°C, 45 s at the primer-dependent annealing temperature of 54.5°C, and 1 min at 72°C, with a final extension of 7 min at 72°C. This step was followed by the nested PCR using 1 μl of the initial PCR product as the template DNA. The annealing temperature was raised from 54.5°C to 57°C for the nested PCR, while the other parameters remained unchanged. Aliquots of 15 μl of PCR products were digested with 1 μl of the restriction endonuclease HinfI (Roche Diagnostic GmbH, Mannheim, Germany) according to the supplier's instructions. Digested DNA was fragmented and visualized on 2% agarose (Merck, Darmstad, Germany).

The samples that were positive by microscopy but negative by using both the TechLab antigen detection kit and PCR for the presence of *E. histolytica* were subjected to a nested PCR with the *E. moshkovskii*-specific small-subunit (SSU) rRNA gene nested primers (Operon, Alabama) Em1 (5′-CTC TTC ACG GGG AGT GCG-3′), Em2 (5′-TCG TTA GTT TCA TTA CCT-3′), nEm1 (5′-GAA TAA GGA TGG TAT GAC-3′), and nEm2 (5′-AAG TGG AGT TAA CCA CCT-3′) to detect the presence of concurrent *E. moshkovskii* infections (2). In the initial PCR (total volume, 50 μl), 5.0 μl of stool DNA was used. The PCR included 30 cycles, each consisting of 92°C for 1 min, 55°C for 1 min and 15 s, and 72°C for 1 min, followed by a final extension of 7 min at 72°C. In the nested PCR, 1 μl of the first PCR product was used as the template DNA, the annealing temperature was increased to 62°C, and the other parameters were left unchanged. *E. moshkovskii*-specific nested SSU rRNA gene amplification product was digested with restriction endonuclease XhoI for 1 h at 37°C according to the manufacturer's instructions (Roche Diagnostic GmbH, Mannheim, Germany) to verify species identity. All PCR products were separated in 2% NuSieve 1:1 agarose gels (Invitrogen, Carlsbad, Calif.) in 1× Tris-acetate-EDTA buffer and visualized after staining with ethidium bromide (0.2 μg ml^{-1}).

Of the 1,037 stool samples examined by microscopy, 776, 158, and 103 samples were from western, northwestern, and northeastern Iran, respectively. A total of 88 persons (8.4%) of both sexes were infected by *E. histolytica*/*E. dispar* complex cysts. The combined frequencies of *E. histolytica*/*E. dispar* according to stool microscopy were 9.3% in Luristan, 5% in West Azerbaijan, and 8.2% in Golestan.

Other intestinal parasites were commonly observed in the persons examined. A total of 335 (32.3%) of the individuals examined were identified as having at least one parasite species, including *Entamoeba coli* (11.6%), *Giardia lamblia* (10.6%), *E. hartmanni* (6%), *Iodamoeba butschlii* (3.9%), *Endolimax nana* (0.9%), *Chilomastix mesnili* (0.7%), *Hymenolepis nana* (0.5%), *Dientamoeba fragilis* (0.5%), *Taenia* sp. (0.2%), and *Trichostrongylus* sp. (0.09%).

All the samples containing *E. histolytica*/*E. dispar* cysts were negative for *E. histolytica* antigen using the *E. histolytica* II kit (optical density of <0.05 after the subtraction of the negative control optical density).

An initial 540-bp fragment of genomic DNA was amplified using primers P1 and P4, followed by nested PCR using primers HR and HF to amplify an internal fragment of 374 bp (Fig. 1). The PCR products, both primary and nested, were the same size as those of the positive controls (HM1:IMSS strain of *E. histolytica* and AS2IR strain of *E. dispar*). After digestion of the nested-PCR product with the restriction enzyme HinfI, two (155-bp and 219-bp) and three (67-bp, 152-bp, and 155-bp) expected fragments were observed for *E. histolytica* and *E. dispar*, correspondingly. The 152-bp and 155-bp fragments in *E. dispar* overlapped and appeared as a single band (Fig. 2).

The restriction fragment length polymorphism analysis of the nested-PCR product of genomic DNA from all 88 *E. histolytica*/*E. dispar* cysts examined showed the *E. dispar* pattern (67-, 152-, and 155-bp fragments), while no *E. histolytica* or mixed-infection electrophoretic patterns were seen. All 88 samples with negative results by TechLab *E. histolytica* II showed an *E. dispar* electrophoretic pattern in nested PCR. According to the results of this survey, there was a 100% correlation between the *E. histolytica* II kit and nested-PCR results.

The reference strain *E. moshkovskii* Laredo (ATCC accession number 30042) gave the expected band at 258-bp with the *E. moshkovskii*-specific SSU rRNA gene nested primers, whereas samples from the control strains *E. histolytica* HM1:IMSS and *E. dispar* AS2IR were not amplified (Fig. 3). To

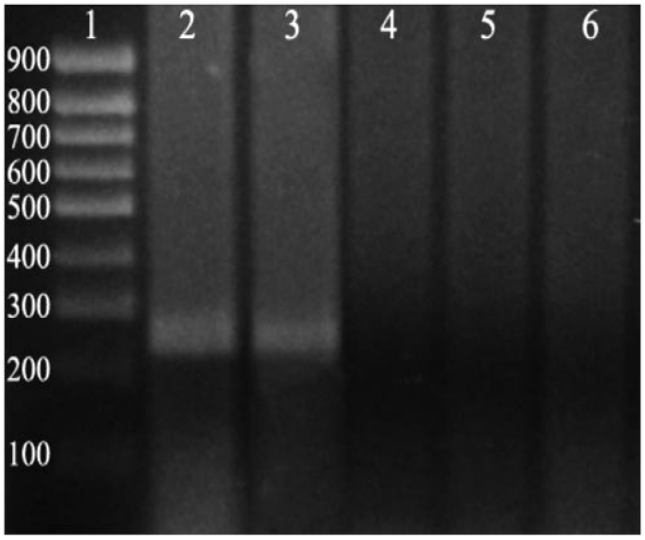

FIG. 3. SSU rRNA gene analysis of *E. moshkovskii*. Lane 1, DNA marker; lane 2, *E. moshkovskii* Laredo strain; lane 3, clinical sample; lane 4, HM1:IMSS strain of *E. histolytica*; lane 5, *E. dispar* standard strain; lane 6, negative control (water). Sizes (in base pairs) are indicated by the numbers at left.

identify species, the nested-PCR products were cut with the restriction enzyme XhoI, and two fragments (236 bp and 22 bp) were produced (data not shown). Only 1 of the 88 (1.1%) microscopy-positive stool DNA samples examined was positive for *E. moshkovskii*. The child dually infected with *E. moshkovskii* and *E. dispar* had a soft stool but no other significant gastrointestinal symptoms.

The most important findings of the present study were that (i) all Iranian asymptomatic cyst passers were infected by nonpathogenic *E. dispar*, and not pathogenic *E. histolytica*, in the regions studied; (ii) there was a 100% correlation between the results from stool antigen detection and those from the nested-PCR method; and (iii) *E. moshkovskii* was identified in one Iranian asymptomatic cyst passer.

In most regions, *E. dispar* is the commonest cause of *E. histolytica*/*E. dispar* complex infection in asymptomatic carriers. In Iran, the only previous report on the separate distributions of *E. histolytica* and *E. dispar* demonstrated that *E. dispar* was the prevailing species in Iranian amebic cyst passers (11). This was true even in the tropical areas of the south, where previous surveys showed that up to 30% of asymptomatic individuals residing in rural areas with poor sanitation were infected by *E. histolytica*/*E. dispar*. By use of an enzyme-linked immunosorbent assay-based test, it was more recently shown that just 8 of the 51 (15.6%) asymptomatic amebic cyst passers examined had anti-*E. histolytica* antibodies, supporting the hypothesis that *E. dispar* is more prevalent in asymptomatic cyst passers in this country (7).

The results of the present study showed a 100% correlation between the TechLab antigen detection kit and traditional nested-PCR results, indicating that the TechLab antigen detection kit is a specific and reliable assay for the diagnosis of *E. histolytica* and *E. dispar* infections in apparently healthy carriers compared with a sensitive nested PCR. To our knowledge, the current study is the first study to evaluate this commercially available kit for this purpose.

A high prevalence of *E. moshkovskii* infection (21.1%) has been detected in preschool children in Bangladesh, indicating that, at least in some parts of the world, it may be a true human parasite (2). In the present study, a low prevalence of infection with *E. moshkovskii* of 1.1% was recorded, showing that this putatively free-living amoeba is a rare human parasitic infectious agent in Iran. It is necessary to consider the presence of *E. moshkovskii* infections in asymptomatic cyst passers in order to avoid unnecessary antimicrobial therapy, especially when microscopy is the main route of diagnosis and when other diagnostic methods, including the TechLab kit and PCR, for *E. histolytica* and *E. dispar* infections, are negative.

This study was conducted at the Division of Molecular Biology, Department of Medical Parasitology and Mycology, Tehran University of Medical Sciences, Tehran, Iran, and the Division of Infectious Diseases and International Health, University of Virginia Health System, Charlottesville, Virginia, as part of the Ph.D. dissertation of S.S.-M. It was partially supported by a grant from Tehran University of Medical Sciences and was supported in part by NIH grant AI-43596 to W.A.P. W.A.P. reports receiving royalties from the licensing of the TechLab *E. histolyica* II kit; these are donated to the American Society of Tropical Medicine and Hygiene at no benefit to W.A.P.

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REFERENCES

1. **Abd-Alla, M. D., A. A. Wahib, and J. I. Ravdin.** 2000. Comparison of antigen-capture ELISA to stool-culture methods for the detection of asymptomatic *Entamoeba* species infection in Kafer Daoud, Egypt. Am. J. Trop. Med. Hyg. **62:**579–582.
2. **Ali, I. K., M. B. Hossain, S. Roy, P. F. Ayeh-Kumi, W. A. Petri, Jr., R. Haque, and C. G. Clark.** 2003. *Entamoeba moshkovskii* infections in children, Bangladesh. Emerg. Infect. Dis. **9:**580–584.
3. **Blessmann, J., I. K. Ali, P. A. Nu, B. T. Dinh, T. Q. Viet, A. L. Van, C. G. Clark, and E. Tannich.** 2003. Longitudinal study of intestinal *Entamoeba histolytica* infections in asymptomatic adult carriers. J. Clin. Microbiol. **41:** 4745–4750.
4. **Diamond, L. S., and C. G. Clark.** 1993. A redescription of *Entamoeba histolytica* Schaudinn, 1903 (Emended Walker, 1911) separating it from *Entamoeba dispar* Brumpt, 1925. J. Eukaryot. Microbiol. **40:**340–344.
5. **Gathiram, V., and T. F. H. G. Jackson.** 1985. Frequency distribution of *Entamoeba histolytica* zymodemes in a rural South African population. Lancet **i:**719–721.
6. **Gathiram, V., and T. F. H. G. Jackson.** 1987. A longitudinal study of asymptomatic carriers of pathogenic zymodemes of *Entamoeba histolytica*. S. Afr. Med. J. **72:**669–672.
7. **Haghighi, A., and M. Rezaeian.** 2005. Detection of serum antibody to *Entamoeba histolytica* in various population samples of amebic infection using an enzyme-linked immunosorbent assay. Parasitol. Res. **97:**209–212.
8. **Haque, R., I. M. Ali, R. B. Sack, B. M. Farr, G. Ramakrishnan, and W. A. Petri, Jr.** 2001. Amebiasis and mucosal IgA antibody against the *Entamoeba histolytica* adherence lectin in Bangladeshi children. J. Infect. Dis. **183:**1787–1793.
9. **Haque, R., A. S. Faruque, P. Hahn, D. M. Lyerly, and W. A. Petri, Jr.** 1997. *Entamoeba histolytica* and *Entamoeba dispar* infection in children in Bangladesh. J. Infect. Dis. **175:**734–736.
10. **Heckendorn, F., E. K. N′Goran, I. Felger, P. Vounatsou, A. Yapi, A. Oettli, H. P. Marti, M. Dobler, M. Trarore, K. L. Lohourignon, and C. Lengeler.** 2002. Species-specific field testing of *Entamoeba* spp. in an area of high endemicity. Trans. R. Soc. Trop. Med. Hyg. **96:**521–528.
11. **Hooshyar, H., M. Rezaian, B. Kazemi, M. Jeddi-Tehrani, and S. Solaymani-Mohammadi.** 2004. The distribution of *Entamoeba histolytica* and *Entamoeba dispar* in northern, central, and southern Iran. Parasitol. Res. **94:**96–100.
12. **Jackson, T. F. H. G.** 2000. Epidemiology of amebiasis, p. 47–63. *In* J. I. Ravdin (ed.), Amebiasis. Imperial College Press, London, United Kingdom.
13. **Nazarian, I.** 1973. Intestinal parasitic infestation in Fars province, Iran. Z. Tropenmed. Parasitol. **24:**45–50.
14. **Newton-Sanchez, O. A., K. Sturm-Ramirez, J. L. Romero-Zamora, J. I. Santos-Preciado, and J. Samuelson.** 1997. High rate of occult infection with

Entamoeba histolytica among non-dysenteric Mexican children. Arch. Med. Res. **28:**311–313.

15. **Ramos, F., P. Moran, E. Gonzalez, G. Garcia, M. Ramiro, A. Gomez, M. del Carmen, G. de Leon, E. I. Melendro, A. Valadez, and C. Ximenez.** 2005. High prevalence rate of *Entamoeba histolytica* asymptomatic infection in a rural Mexican community. Am. J. Trop. Med. Hyg. **73:**87–91.
16. **Sargeaunt, P. G., J. E. Williams, T. F. Jackson, and A. E. Simjee.** 1982. A zymodeme study of *Entamoeba histolytica* in a group of South African schoolchildren. Trans. R. Soc. Trop. Med. Hyg. **76:**401–402.
17. **Sheiban, F., and M. Rezaian.** 1981. A study on intestinal protozoa in seven villages of Bandar Abbas, southern Iran. Iran. J. Public Health **10:**45–55.
18. **Stauffer, W., M. Abd-Alla, and J. I. Ravdin.** 2006. Prevalence and incidence of *Entamoeba histolytica* infection in South Africa and Egypt. Arch. Med. Res. **37:**266–269.
19. **Tachibana, H., S. Ihara, S. Kobayashi, Y. Kaneda, T. Takeuchi, and Y. Watanabe.** 1991. Differences in genomic DNA sequences between pathogenic and nonpathogenic isolates of *Entamoeba histolytica* identified by polymerase chain reaction. J. Clin. Microbiol. **29:**2234–2239.
20. **Tachibana, H., S. Kobayashi, K. Nagakura, Y. Kaneda, and T. Takeuchi.** 2000. Asymptomatic cyst passers of *Entamoeba histolytica* but not *Entamoeba dispar* in institutions for the mentally retarded in Japan. Parasitol. Int. **49:** 31–35.
21. **World Health Organization.** 1997. Amebiasis. Wkly. Epidemiol. Rec. **72:**97–100.