

Differentiation of *Entamoeba histolytica* and *Entamoeba dispar* by PCR: a preliminary study in Izmir, Turkey

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SUMMARY

The causative agent of amoebiasis is currently attributed to two distinct species (*E.histolytica* and *E.dispar*). The aim of this study was to differentiate these species by PCR in stool samples. Isolated genomic DNA was amplified by PCR and band products of 101 bp (*E. dispar*) were obtained. All seven stool samples were found to be *E. dispar*, not *E.histolytica*. Our results demonstrated the significance of *E.histolytica/dispar* differentiation in the diagnosis of amoebiasis. This study is preliminary to our current research project entitled "Investigation of the prevalence of amoebiasis and *Entamoeba* species in Izmir and its hinterland".

KEY WORDS: *Entamoeba histolytica*, *Entamoeba dispar*, Differentiation

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INTRODUCTION

Amoebiasis is the third-leading parasitic disease causing death after malaria and schistosomiasis. It has been estimated that approximately 480 million people, or 12% of the world's population is at risk and that the annual mortality is around 40 000 to 110 000 (Walsh, 1986). Infection is more prevalent in areas with poor sanitation, crowded communities, tropics and subtropics, rural areas and countries with low socioeconomic conditions (Markell *et al.*, 1999, Farthing *et al.*, 1996). High-risk groups include travelers, immigrants, migrant workers, immunocompromised individuals, sexually active male homosexuals, indi-

viduals in mental institutions, prisons and, children in day-care centers (Farthing *et al.*, 1996). Brumpt (1925) was the first to suggest that the differences in symptomatology and global distribution of invasive amoebiasis were due to the presence of two morphologically-identical species of amoebae, pathogen and non-pathogen. Sargeant and associates managed to distinguish pathogenic strains of *Entamoeba histolytica* (*E. histolytica*) by isoenzyme typing. *E. histolytica* was first described by Schaudinn (1903) by separating from the former *Entamoeba dispar* (*E. dispar*) determined earlier by Brumpt (1925). Recently, Diamond and Clark (1993) redescribed *E. histolytica* by its biochemical, immunological and genetic characteristics (Farthing *et al.*, 1996). Basically, diagnosis of amoebiasis relies on the microscopic examination of stool samples. *E. histolytica* and *E. dispar* are morphologically identical and could not be differentiated by staining methods. Differentiation of *E. histolytica* and *E. dispar* can only be made by isoenzyme analysis

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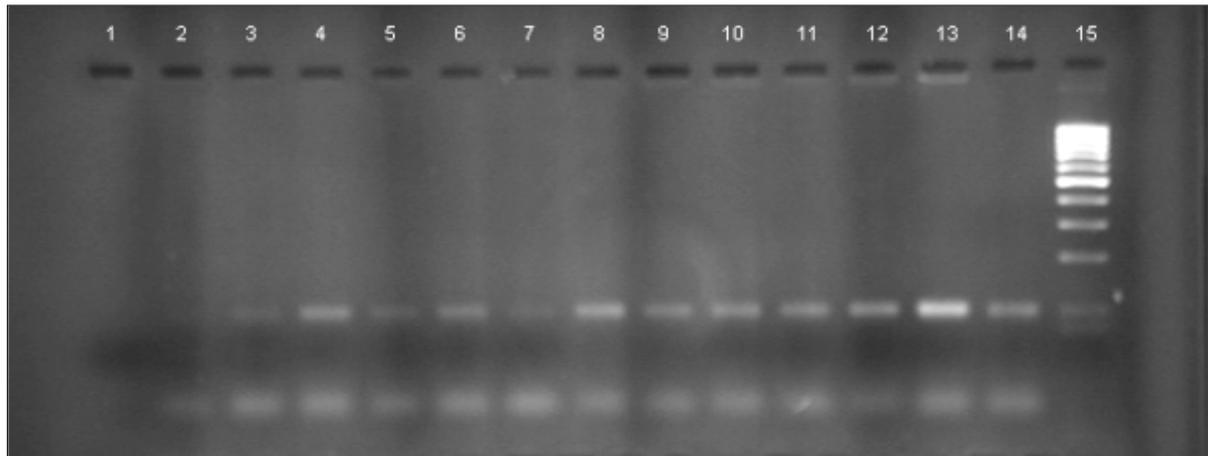


FIGURE 1 - Illustration of PCR products. Lane 1: Negative control. Lane 2: Sample 1. Lane 3: Sample 2. Lanes 4 and 5: Sample 3. Lanes 6 and 7: Samples 4. Lane 8 and 9: Sample 5. Lane 10 and 11: Sample 6. Lane 12 and 13: Sample 7. Lane 14: *E. dispar* control DNA. Lane 15: Marker (100bp).

and molecular techniques (Markell *et al.*, 1999). In this study, we aimed to differentiate *E. histolytica* and *E. dispar* by PCR (polymerase chain reaction) using the genomic DNA isolated from positive stool samples after microscopic diagnosis.

MATERIALS AND METHODS

Stool samples

Macroscopic and microscopic examination of collected stool samples: Seven stool samples, found to contain *E. histolytica* / *E. dispar* cysts and/or trophozoites by wet-mount, formalin ethyl acetate concentration and trichrome staining, as described by Garcia LS (Garcia and Bruckner 1993), were obtained from asymptomatic patients admitted to the Parasitology Laboratory of Celal Bayar University Hospital over five months. We also collected six stool samples without bacteria and other protozoa (negative samples).

Extraction of DNA from stools

DNA extraction was done with "Genomic DNA Purification Kit" (Fermentas-#K0512) and DNA was stored at -20°C until use.

PCR method

Genomic DNA segments (both extracted DNAs and controls) were amplified by PCR. Control DNAs were kindly provided by H. Tachibana.

Final concentration of the mixture contained 5 μl of 10x PCR buffer; 0.25 mM each of the four deoxynucleoside triphosphates, 1.5 mM MgCl_2 , 2 μM of each of the two primers, 2.0 U of Tth DNA polymerase (Fermentase) and 10 μl of genomic DNA as the template in a final volume of 50 μl . The reactions were amplified using the "hot-start" technique for 35 cycles in an automated PCR machine (Biometra-Uno Thermoblock). PCR was carried out using two sets of *E. histolytica* primers, p11 plus p12 and *E. dispar* p13 plus p14 (Tachibana *et al.*, 1991, Tachibana H *et al.*, 2000, Rivera *et al.*, 1996). Thirty five cycles of PCR were performed as follows: initial denaturation step at 94°C for 3 min, denaturation at 94°C for 1 min, annealing at 59°C for 1.5 min, polymerization at 72°C for 1.5 min and final polymerization step at 72°C for 8.5 min. Amplified products (10 μl) were subjected to electrophoresis in 2% agarose gels, and the presence of specific bands was visualized with UV light after ethidium bromide staining.

RESULTS

Application of PCR revealed band products of 101 bp, compatible with *E. dispar*, in all seven stool samples (Figure 1). However, there was no band (100 bp) indicating *E. histolytica* in any DNA of our study samples. No band was detected in any negative samples.

DISCUSSION

There is a wide clinical spectrum for amoebiasis: from asymptomatic infection to fulminant colitis that may cause colon perforation. Major symptoms of amoebiasis are abdominal pain, diarrhea, nausea, vomiting and flatulence. Generally, asymptomatic infections are caused by the commensal, non-pathogenic *E. dispar*, while all symptomatic infections causing dysentery and extra intestinal amoebiasis are caused by *E. histolytica*. However, asymptomatic cyst carriers could be detected for both forms of the parasite (Farthing *et al.*, 1996, Blessmann *et al.*, 2002, Tanyuksel and Petri 2003). In our study, stool samples were initially obtained from asymptomatic persons admitted to the Parasitology Laboratory for general parasitological examination.

Laboratory diagnosis of amoebiasis mainly relies on the detection of cysts and/or trophozoites in the microscopic examination of fresh or preserved stool samples by wet mount and trichrome staining. However, there are some drawbacks in the microscopic diagnosis of amoebiasis. Presence of neutrophils and macrophages in stool samples may lead to misdiagnosis and discrimination of pathogenic (*E. histolytica*) and non-pathogenic (*E. dispar*) strains is not possible in microscopic examination (Acuna-Soto *et al.*, 1993, Haque R *et al.*, 1998). Determination of pathogenic strains is currently done with molecular methods (PCR, DNA probes, riboprinting), detection of parasitic antigens in stool (Enzyme immunoassay) and isoenzyme analysis following fecal culture (Markell *et al.*, 1999, Tanyuksel and Petri 2003, Rivera *et al.*, 1998, Walderich *et al.*, 1997).

Isoenzyme analysis is an old and effective method for the differentiation of amebic species. However, it is not commonly preferred, especially for epidemiological studies due to both its crucial dependency on fecal culture as it requires high number of trophozoites for an effective analysis, and its being a labor-intensive method requiring experienced personnel and financial support (Tanyuksel and Petri 2003, Huston *et al.*, 1999).

PCR is a well-known highly sensitive and specific molecular method and is reported to be useful for epidemiological studies and determination

of pathogenic *Entamoeba* strains (Tachibana *et al.*, 1991, Rivera *et al.*, 1998, Acuna-Soto *et al.*, 1993, Huston *et al.*, 1999). The worldwide prevalence of *E. dispar* was reported to be nine times higher than *E. histolytica* (Markell *et al.*, 1999). In our preliminary study, all seven isolates were found to be *E. dispar*, indicating that the results were concordant with related studies.

Recent studies demonstrated that discrimination of pathogenic and non-pathogenic strains of amoebas in routine diagnosis would surely refrain patients from taking unnecessary treatments. In addition, it would mostly prevent misdiagnosis and help to decrease the cost of treatment (Verweij *et al.*, 2000, Zaki and Clark 2001). Our study is preliminary to a research project, with a plan to investigate the prevalence of amoebiasis and amoebic species by PCR and EIA in Izmir city and its hinterland. This is the first study of *E. histolytica* and *E. dispar* discrimination in Izmir, Turkey and will contribute to both prevalence and future treatment strategies of "real" amoebiasis in our region.

REFERENCES

- ACUNA-SOTO R. ET AL. (1993). Application of the PCR to the epidemiology of pathogenic and non-pathogenic *E. histolytica*. *Am J Trop Med Hyg.* **48**: 58-70.
- BLESSMANN J. ET AL. (2002). Real-time PCR for detection and differentiation of *E. histolytica* and *E. dispar* in fecal samples. *J Clin Microbiol.* **40** (12): 13-7.
- FARTHING M.J.G., CEVALLOS A.M., KELLY P. (1996). Intestinal Protozoa. In: Cook G.C. (Ed). "*Manson's Tropical Diseases*". 20th ed. W.B. Saunders Comp. pp.1255-1298.
- GARCIA, L.S. AND BRUCKNER D.A. (1993) Macroscopic and Microscopic Examination of Fecal Specimens. "Diagnostic Medical Parasitology" 2th edition American Society for Microbiology. 501-540.
- HAQUE R. ET AL. (1998). Comparison of PCR, isoenzyme analysis and antigen detection for diagnosis of *E. histolytica* infection. *J Clin Microbiol.* **36**: 449-452.
- HUSTON CD, ET AL. (1999) Molecular-based diagnosis *E. histolytica* infection. *Expert Reviews in Molecular Medicine.* Cambridge Uni. Press. 1-11.
- MARKELL E.K., JOHN D.T. , KROTOSKI W.A. (1999). Lumen Dwelling Protozoa. 24-89. S. Ozmat (ed.), Markell and Voge's Medical Parasitology, 8th ed. Saunders Company-Mexico. 24-89.

- RIVERA W.L., TACHIBANA H., SILVA-TAHAT M.R., UEMURA H., KANBARA H. (1996). Differentiation of *E.histolytica* and *E.dispar* DNA from cysts present in stool specimens by PCR: its field application in the Philippines. *Parasitol. Res.* **82**: 585-89.
- RIVERA W., TACHIBANA H., KANBARA H. (1998). Field study on the distribution of *E.histolytica* and *E.dispar* in northern Phillipines as detected by PCR. *Am J Trop Med Hyg.* **59**: 916-921.
- TACHIBANA H., KOBAYASHI S., NAGAKURA K., KANEDA Y, TAKEUCHI T. (2000). Asymptomatic cyst passers of *E.histolytica* but not *E.dispar* in institutions for the mentally retarded in Japan. *Parasitology International.* **49**: 31-35.
- TACHIBANA H., KOBAYASHI S., TAKEKOSHI M., IHARA S. (1991). Distinguishing pathogenic isolates of *E.histolytica* by PCR. *J Infect. Dis.* **164**: 825-6.
- TANYUKSEL M., PETRI W.A. (2003). Laboratory diagnosis of amoebiasis. *Clin Microbiol Rev.* **16** (4): 713-729.
- VERWEIJ J.J. ET AL. (2000). Differentiation of *E.histolytica* and *E.dispar* cysts using PCR on DNA isolated from faeces with spin columns. *Eur J Clin Microbiol Infect Dis.* **19**: 358-361.
- WALDERICH B., WEBER A., KNOBLOCH J. (1997). Differentiation of *E.histolytica* and *E.dispar* from German travelers and residents of endemic areas. *Am J Trop Med Hyg.* **57** (1): 70-74.
- WALSH JA. (1986) Problems in recognition and diagnosis of amoebiasis: estimation of the global magnitude of morbidity and mortality. *Rev. Infect. Dis.* **8**: 228-38.
- ZAKI M, CLARK CG. (2001), Isolation and characterization of polymorphic DNA from *E.histolytica*. *J Clin Microbiol*; **39** (3): 897-905.