Molecular differentiation of Entamoeba histolytica and Entamoeba dispar from Tunisian food handlers with amoeba infection initially diagnosed by microscopy.

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MOLECULAR DIFFERENTIATION OF ENTAMOEBA HISTOLYTICA AND ENTAMOEBA DISPAR FROM TUNISIAN FOOD HANDLERS WITH AMOEBA INFECTION INITIALLY DIAGNOSED BY MICROSCOPY

BEN AYED S.*, BEN ABDALLAH R.*, MOUSLI M.**, AOUN K.*, THELLIER M.*** & BOURATBINE A.*

Summary: The purpose of the study was to obtain more reliable epidemiological data concerning Entamoeba (E.) histolytica infection in Tunisian food handlers using established molecular tools able to differentiate E. histolytica from E. dispar. From 2002 to 2005, 4,266 fresh stools specimens received in the setting of the National program of food handlers' control were analysed by optical microscopy. Twelve (2.8 %) were positive for the presence of four nuclei cysts identified as E. histolytica/E. dispar. Extraction of DNA from the 12 samples, followed by specific amplifications of E. histolytica and E. dispar SSU rDNA, showed that 11 samples (92 %) were positive for E. dispar and negative for E. histolytica. Sequencing analysis of 8 PCR products permitted to verify the results obtained with conventional PCR. The remaining sample was negative by PCR amplifying E. histolytica DNA or E. dispar DNA specifically, although it did not show any inhibition. It probably contains protozoan cysts genetically distinct from these two species but morphologically similar. Estimation of relative proportions between E. histolytica and E. dispar in cyst carriers showed that all explored individuals harboured the non pathogenic E. dispar strains. This result highlights the need of use in this population of complementary tests that allow specific diagnosis and obviate unnecessary chemotherapy.

Résumé: Différenciation moléculaire des amibes Entamoeba histolytica et Entamoeba dispar identifiées chez les manipulateurs de denrées alimentaires en Tunisie


KEY WORDS: Entamoeba histolytica, Entamoeba dispar, amibiase, épidémiologie, Tunisie.

INTRODUCTION

Medical screening protocols for Tunisian food handlers recommend stool examinations to be routinely performed. This screening is conducted to enhance the individuals’ health by treating intestinal infections as well as to protect the public health of the community. In this population at risk, reported prevalence of Entamoeba (E.) histolytica cysts was between 1 % and 3 % (Aoun et al., 1999; Bel Hadj et al., 1994; Gara et al., 1999). However, it is important to emphasize that these earlier reports have been relying upon results of microscopic examination of stool specimens that cannot differentiate the pathogenic E. histolytica from the morphologically identical species E. dispar, which occurs worldwide (Diamond & Clark, 1993). Thus in these previous studies many E. dispar infections were most probably confused with E. histolytica infections and were unnecessary treated. In fact, E. dispar is a harmless commensal protozoan and its presence in clinical specimens does not justify treatment (WHO, 1997).

It is actually admitted that misidentification of E. histolytica infection may occur if the diagnosis is based solely on stool microscopy (Tanyukel & Petri, 2003). For final confirmatory identification, biochemical techniques, immunologic assays for detection of E. histolytica antigens or molecular methods are needed (Tanyukel & Petri, 2003). Amplification of amoeba DNA fragments by PCR has proved its usefulness for
differential detection of *E. histolytica* and *E. dispar* directly from stool samples (Acuna-Soto et al., 1993; Clark & Diamond, 1992). Moreover, this PCR-based approach is suitable for molecular epidemiological studies, which have been strongly encouraged by the WHO (1997). The purpose of this study is to obtain more reliable and correct epidemiological data concerning *E. histolytica* infection in Tunisian food handlers using established molecular tools.

**MATERIAL AND METHODS**

**STOOL SAMPLES**

From 2002 to 2005, 4,266 fresh stools specimen were received for parasitic analysis in the setting of the National program of food handlers’ control. All individuals were young adults, mostly males, working in the city of Tunis. Stool analyses were prescribed in the setting of medical screening.

**PARASITIC ANALYSIS**

Microscopic examination was carried out both on direct wet preparations and on sediments obtained after formalin-ethyl acetate concentration (Golvan, 1990). To make a final diagnostic of protozoa species, a subsequent staining with Lugol’s iodine solution was done (Golvan, 1990). Every time cysts of *E. histolytica*/*E. dispar* were identified, a part of the stool was apportioned in eppendorf tubes and stored frozen for further analyses.

**DNA EXTRACTION**

DNA was extracted from samples using the QIAamp DNA Stool miniKit (Qiagen Inc, Germany) according to manufacturer’s recommendations, using approximately 200 mg of partly thawed stools for the first buffer step. Extracted DNA was used immediately for PCR assays or frozen at −20°C until analysed.

**PCR AMPLIFICATIONS**

Conventional PCR amplifications were used according to Gonin & Trudel (2003). The target for PCR was a small region (135 bp) of the small subunit ribosomal RNA gene (SSU rDNA) located on an episomal plasmid and present at approximately 200 copies per cell (Clark & Diamond, 1991). Specific amplifications of *E. histolytica* and *E. dispar* SSU rDNA were performed using two different forward primers which cover a region with six mismatches between the two species (Clark & Diamond, 1991). Forward primers were EH1 (5’-GTACAAAAATGGCCAATTCAATTACG-3’) and ED1 (5’-TACAAAGTGCCCATTTATGTAAGTA-3’) respectively (Gonin & Trudel, 2003). The same reverse primer EHD2 (5’-ACTACCAACTGATTGATAGATCAG-3’) targeted a conserved site within the two species (Gonin & Trudel, 2003). PCR amplifications were carried out in 50 µl volumes using a 50 µM concentration of each dNTP, 2 mM MgCl₂, 20 pmol of primers, 1 X HotStar Taq 10 X buffer, 5 U of HotStar Taq DNA polymerase (Roche, USA) and 5 µl of DNA. Cycling conditions were as follows: 15 min incubation at 94°C followed by 40 cycles consisting of 30 s at 94°C, 60 s at 51°C, and 40 s at 72°C, with a final 5 min elongation at 72°C. Detection of PCR products was performed conventionally on an ethidium bromide-stained 2 % agarose gel in 1 × TAE (Tris-acetate-EDTA). Two DNA samples testing positive for each species were used as positive controls. Cyst-positive samples in which no specific DNA was amplified were checked for inhibition by spiking these samples with 1 ml of positive control (Verweij et al., 2000).

**DNA SEQUENCING ANALYSIS**

At least, one DNA amplicon per samples with *E. histolytica* or *E. dispar* infection was sequenced. PCR products were purified using GenElute™ Cleanup kit (Sigma, USA). Sequencing reactions were performed directly on the amplification products using ABI PRISM 377 DNA sequencer (Applied Bio system). The sequence was aligned with deposited sequences of *E. histolytica* and *E. dispar* SSU rDNA (*E. histolytica*, GenBank accession no. X56991; *E. dispar*, GenBank accession no. Z9256) to validate these DNAs as being of *E. histolytica* or *E. dispar* origin.

**RESULTS**

Among 4,266 stool samples analysed by optical microscopy, only 12 (2.8 %) were positive for the presence of four nuclei cysts identified as *E. histolytica*/*E. dispar*. In these 12 samples, an association with cysts of *E. coli* was noted in 10 cases (83 %) (Table I).

Extraction of DNA from the 12 samples followed by PCR showed that 11 samples (92 %) were positive for *E. dispar* (as demonstrated by the amplification of the species-specific fragment of 135 pb) and negative for *E. histolytica* (Fig. 1). The remaining sample was negative by both discriminating PCR (Table I). This latter DNA sample was spiked with *E. histolytica* and *E. dispar* positive controls respectively to check for inhibition. Visualisation of specific amplifications has indicated the absence of PCR inhibitors. Moreover, detection of *E. moshkovskii* by nested PCR, as described by Ali et al., has remained negative (Ali et al., 2003). Sequencing analysis of 8 PCR products verified the results obtained with conventional PCR (Table I). Signi-
Significant alignments were obtained for all samples with *E. dispar* gene for ribosomal small subunit (EMBL accession Z49256). Very few nucleotide differences have been shown around position 190 where considerable number of nucleotide polymorphism with *E. histolytica* is reported (Clark & Diamond, 1991) (Fig. 2A). Six samples have shown a punctual insertion in position 204, whereas sequences were identical to the reference strain in one case and presented an insertion in position 208 in another case (Fig. 2B).

Table I. – Species identification of amoeba infection initially diagnosed by microscopy in food handlers.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Stool aspect*</th>
<th><em>E.b/E.d</em>*</th>
<th>Other parasites***</th>
<th>PCR (<em>E.d</em>)</th>
<th>PCR (<em>E.b</em>)</th>
<th>Sequencing</th>
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* F: formed; L: liquid; ** *E.b/E.d*: Entamoeba histolytica/Entamoeba dispar; *** *E.c*: Entamoeba coli; *D.f*: Dientamoeba fragilis; *E.n*: Endolimax nana; *P.b*: Pseudolimax butschlii.

Fig. 1. – Sample amplifications of stool specimens.
A - *E. histolytica* amplification. M: DNA molecular weight marker XIII, 100 bp ladder (Roche); lane 1, positive control; lanes 2-9, negative samples; lane 10, negative control.
B - *E. dispar* amplification. M: DNA molecular weight marker XIII, 50 bp ladder (Roche). Lane 1, positive control; lanes 2-3 and 5-9, positive samples; lane 4, negative sample; lane 10, negative control.

DISCUSSION

In food handlers, diagnosis of intestinal parasitic infection is annually attempted by stool ova and protozoan examination. Identification of parasite species still relies on microscopic examination of parasite morphology. Our current study has shown that 2.8 % of the food handlers harbour *E. histolytica*/*E. dispar* cysts. The drastic decrease of the prevalence of *E. histolytica*/*E. dispar* in food handlers in comparison with previous data could be the result of the sanitary program performed within this population at risk (Aoun et al., 1999; Bel Hadj et al., 1994; Bouratbine et al., 2004; Gara et al., 1999). However, it could also reflect previous
overvaluation of prevalence of these parasite species and current more reliable laboratory results. In fact, microscopic identification of species in fresh faecal preparations remains difficult and several factors affect adversely the results of microscopy (Tanyuksel & Petri, 2003). Actually, it’s well established that differentiation of *E. histolytica* and *E. dispar* in stool samples is not possible on the basis of microscopy alone (Tanyuksel & Petri, 2003) and diagnosis of most of the previous infections as *E. histolytica* infections based on microscopic examination only can be regarded as defective and misleading. In our study the use of molecular tools allowed the differentiation between the two species in 11 out of 12 samples containing cysts of *E. histolytica*/*E. dispar*. The remaining sample was negative by PCR amplifying *E. histolytica* DNA or *E. dispar* DNA specifically, although it didn’t show any inhibition. It probably contains protozoan cysts genetically distinct from these two species but similar in appearance, as the small cysts of *E. coli* or the large cysts of *E. histolytica*. We have not found any indications of the presence of the morphologically identical amphizoic amoeba *E. moshkovskii* which occasionally infects humans (Clark & Diamond, 1991; Tanyuksel & Petri, 2003). Estimation of relative proportions between *E. histolytica* and *E. dispers* in cyst carriers showed that all explored individuals harboured the nonpathogenic *E. dispers* strains. No one was infected by the pathogenic species *E. histolytica*. These results are in concordance with the very low incidence of the disease in northern Tunisia (Bouratbine et al., 2004). They highlight the need of use in this population at risk of complementary tests that allow specific diagnosis. In fact, differentiation between *E. histolytica* and *E. dispers* is of great importance because it obviates unnecessary chemotherapy with its attendant costs, risk of side effects and danger of drug resistance and allows the clinician to focus on early identification and treatment of *E. histolytica* infection in the minority of individuals who represent the real public health problem and are at highest personal risk.

ACKNOWLEDGEMENTS

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