

## MOLECULAR DIFFERENTIATION OF *ENTAMOEBIA HISTOLYTICA* AND *ENTAMOEBIA DISPAR* FROM TUNISIAN FOOD HANDLERS WITH AMOEBIA INFECTION INITIALLY DIAGNOSED BY MICROSCOPY

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### 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 morphological 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 *ENTAMOEBIA HISTOLYTICA* ET *ENTAMOEBIA DISPAR* IDENTIFIÉES CHEZ LES MANIPULATEURS DE DENRÉES ALIMENTAIRES EN TUNISIE

Entre 2002 et 2005, 4266 manipulateurs de denrées alimentaires de la région de Tunis ont bénéficié d'un examen parasitologique des selles dans le cadre du contrôle systématique. Douze (2,8 %) étaient porteurs de kystes d'*Entamoeba histolytica/dispar*. L'extraction d'ADN directement à partir des échantillons de selles positives, suivie respectivement d'une amplification génique de l'ADNr d'*E. histolytica* et d'*E. dispar* et d'un séquençage des produits d'amplification a permis d'identifier *E. dispar* chez 11 porteurs de kystes. Aucun manipulateur n'était porteur de l'espèce pathogène *E. histolytica*. Ces résultats mettent en exergue la nécessité d'examen complémentaires permettant l'identification des espèces *E. histolytica* et *E. dispar* et confirment la faible prévalence de l'amibiase dans le nord de la Tunisie.

**MOTS CLÉS :** *Entamoeba histolytica*, *Entamoeba dispar*, amibiase, épidémiologie, Tunisie.

**KEY WORDS :** *Entamoeba histolytica*, *Entamoeba dispar*, amoebiasis, epidemiology, Tunisia.

## 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). Because this protozoan parasite is known as a pathogenic responsible of dysentery and liver abscess, the treatment of these individuals was systematically prescribed to interrupt parasite

transmission and to avoid the progression from infected individuals to an invasive disease (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 (Tanyuksel & Petri, 2003). For final confirmatory identification, biochemical techniques, immunologic assays for detection of *E. histolytica* antigens or molecular methods are needed (Tanyuksel & Petri, 2003). Amplification of amoeba DNA fragments by PCR has proved its usefulness for

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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  $-20^{\circ}\text{C}$  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'-GTACAAAATGGCCAATTCATTCAATG-3') and ED1 (5'-TACAAAGTGGCCAATTTATGTAAGTA-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  $\mu\text{l}$  volumes using a 50  $\mu\text{M}$  concentration of each dNTP, 2 mM  $\text{MgCl}_2$ , 20 pmol of primers, 1 X HotStar *Taq* 10 X buffer, 5 U of HotStar *Taq* DNA polymerase (Roche, USA) and 5  $\mu\text{l}$  of DNA. Cycling conditions were as follows: 15 min incubation at  $94^{\circ}\text{C}$  followed by 40 cycles consisting of 30 s at  $94^{\circ}\text{C}$ , 60 s at  $51^{\circ}\text{C}$ , and 40 s at  $72^{\circ}\text{C}$ , with a final 5 min elongation at  $72^{\circ}\text{C}$ . Detection of PCR products was performed conventionally on an ethidium bromide-stained 2 % agarose gel in  $1 \times \text{TAE}$  (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. Z49256) 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 D).

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-

Patient	Stool aspect*	<i>E.b/E.d</i> **	Other parasites***	PCR ( <i>E.d</i> )	PCR ( <i>E.b</i> )	Sequencing
1	F	Cysts	<i>E.c</i>	+	-	<i>E.d</i>
2	F	Cysts	<i>E.c</i> + <i>D.f</i>	+	-	<i>E.d</i>
3	F	Cysts	<i>D.f</i>	-	-	
4	L	Cysts	<i>E.c</i> + <i>E.n</i>	+	-	<i>E.d</i>
5	F	Cysts	<i>E.c</i>	+	-	<i>E.d</i>
6	F	Cysts	<i>E.n</i>	+	-	
7	F	Cysts	<i>E.c</i> + <i>P.b</i>	+	-	<i>E.d</i>
8	F	Cysts	<i>E.c</i>	+	-	<i>E.d</i>
9	F	Cysts	<i>E.c</i> + <i>P.b</i>	+	-	<i>E.d</i>
10	F	Cysts	<i>E.c</i>	+	-	<i>E.d</i>
11	F	Cysts	<i>E.c</i>	+	-	
12	L	Cysts	<i>E.c</i>	+	-	

\* 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*.

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

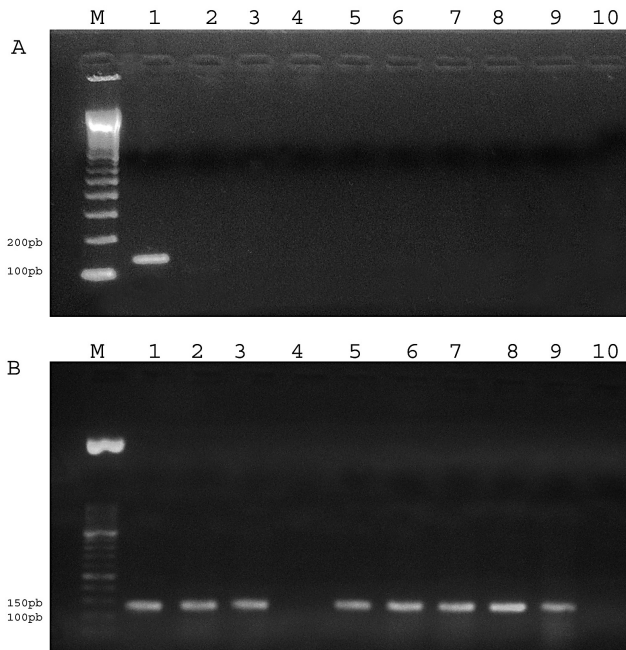


Fig. 1. – Sample amplifications of stool specimens. A - *E. histolytica* amplification. M: DNA molecular weight marker XIII, 100 bp ladder (Roche); lane 1, positive control; lanes 2-9, negative samples; lane 10, negative control. B - *E. dispar* amplification. M: DNA molecular weight marker XIII, 50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

ficant 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).



Fig. 2 – A: Sequence differences between *E. dispar* (Z 49256) and *E. histolytica* (x 56991) ribosomal RNA genes. B: Sequence differences between *E. dispar* (Z 49256) and *E. dispar* ribosomal RNA genes identified in the stool samples. \* Nucleotide sequences identified in samples 4, 7, 8, 9 and 10 are identical to those of sample 2.

## 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. bartmanni*. 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. dispar* in cyst carriers showed that all explored individuals harboured the nonpathogenic *E. dispar* 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. dispar* 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.

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