

# Development and evaluation of molecular tools for detecting and differentiating intestinal amoebae in healthy individuals

## Research Article

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### Abstract

Amoebae are single-celled parasites frequently colonizing human gut. However, few molecular tools are available for accurate identification. Here, we evaluated a panel of polymerase chain reactions (PCRs) targeting *Entamoeba histolytica*, *Entamoeba dispar*, *Entamoeba coli*, *Entamoeba hartmanni*, *Entamoeba polecki*, *Endolimax nana* and *Iodamoeba bütschlii*. Thirty-six faecal samples (18 containing at least one amoeba species by microscopy and 18 microscopy negative for amoebae) were tested. Real-time PCRs were used for detection and differentiation of *E. histolytica* and *E. dispar*. Conventional PCR with Sanger sequencing were applied for detection and differentiation of *E. coli*, *E. hartmanni*, *E. polecki*, *E. nana* and *I. bütschlii*. All microscopy results were confirmed by DNA-based methods. However, more samples were positive for single and mixed amoebic species by DNA-based assays than by microscopy (22 vs 18 and 7 vs 1, respectively). DNA sequencing allowed identification of *E. coli* subtypes (ST1 and ST2), showed low intra-specific variation within *E. hartmanni*, identified two phylogenetically distinct groups within *E. nana*, and identified *Iodamoeba* at the ribosomal lineage level. Taking into account the high intra-genetic diversity within some of the species at the small subunit (SSU) rRNA gene level, amplification of SSU rRNA genes with subsequent sequencing represents a useful method for detecting, differentiating and subtyping intestinal amoebae.

## Introduction

Amoebozoa, including species of *Entamoeba* (mainly *Entamoeba coli*, *Entamoeba histolytica*, *Entamoeba dispar* and *Entamoeba hartmanni*), *Endolimax nana* and *Iodamoeba bütschlii* are among the most prevalent single-celled parasites colonizing the gut of Tunisian healthy individuals (Siala *et al.*, 2015). Unlike *E. histolytica*, none of these protists are considered pathogens, but rather thought of as commensals and not taken into consideration in the clinical-diagnostic setting (Fotedar *et al.*, 2007). Nevertheless, recent data indicate remarkable genetic diversity among these genera (Stensvold *et al.*, 2011b, 2012; Poulsen and Stensvold, 2016), the clinical and public health importance of which remains to be explored. Moreover, recent studies on gut microbiota have increased the knowledge of the influence of gut microbes on human health and disease, highlighting several associations between single-celled parasites and bacteria as potential indicators of clinical phenotypes (Berrilli *et al.*, 2012; Andersen *et al.*, 2013; Morton *et al.*, 2015; Andersen and Stensvold, 2016; Iebba *et al.*, 2016; O'Brien Andersen *et al.*, 2016; Beghini *et al.*, 2017; Stensvold and van der Giezen, 2018). Hence, studies of commensal amoeba colonizing the human gut of both healthy and diseased individuals have gained novel significance.

While microscopy is still the cornerstone of diagnostic testing for intestinal protozoa, particularly amoeba of the digestive system, this approach is labour-intensive and requires a high level of skill for accurate identification (McHardy *et al.*, 2014). Its diagnostic yield is also significantly impacted by the number of stool specimens collected and submitted for testing (Bouratbine *et al.*, 2000). In fact, many intestinal protozoa are irregularly shed, and at least three sequential stool specimens are necessary for reliable detection of intestinal parasites in routine laboratory examinations (Nazer *et al.*, 1993). In contrast, the enhanced sensitivity of molecular detection methods may require only one specimen for testing to achieve sensitivity equal to, if not greater than, microscopy (Bruijnesteijn van Coppenraet *et al.*, 2009). Furthermore, polymerase chain reaction (PCR)-based methods also enable species differentiation in case of protozoa with similar morphological features such as the pathogenic *E. histolytica* and the commensal *E. dispar* and *Entamoeba moshkovskii* (Ben Ayed *et al.*, 2008a, 2008b).

Over the past 20 years, there has been a remarkable development in molecular biology based diagnostic procedures to detect *E. histolytica*, to the point where, today, such DNA-based methods are the preferred approach (Tanyuksel and Petri, 2003). In this context, Department of

Parasitology, Pasteur Institute of Tunis has offered since 2009 two standardized real-time quantitative PCRs (qPCRs) for detection and identification of *E. histolytica* and *E. dispar*. These are routinely used for accurate identification of *E. histolytica* and *E. dispar* in food handlers with amoeba infection initially diagnosed by microscopy and patients with symptoms suggestive of intestinal amoebiasis (Bouratbine et al., 2003; Ben Ayed et al., 2008b). These two PCRs were designed and evaluated on a series of clinical stool samples analysed by microscopy and corresponding conventional PCR (data not shown). In contrast, data on the molecular identification of *E. hartmanni*, *E. coli*, *E. polecki*, *E. nana* and *I. bütschlii* has so far been very limited (Clark and Diamond, 1997; Silberman et al., 1999; Santos et al., 2010; Stensvold et al., 2011a, 2011b, 2012, 2018; Gomes Tdos et al., 2014; Poulsen and Stensvold, 2016).

The aim of this study was to develop and evaluate a panel of specific PCRs targeting 'non-pathogenic' amoebic species of epidemiological importance in Tunisia, namely *E. hartmanni*, *E. coli*, *E. nana* and *Iodamoeba* RL1 and *Iodamoeba* RL2, which altogether represent about 50% of intestinal single-celled parasites colonizing Tunisian healthy individuals (Siala et al., 2015). Although, the presence of *E. polecki* cysts have never been reported in Tunisia, their morphological features which may be confused with those of other *Entamoeba* species have justified their research by a recently available molecular tool enabling the identification of all four subtypes (ST1–ST4) of *E. polecki* (Stensvold et al., 2018).

## Materials and methods

### Faecal specimens

Thirty-six faecal samples for which the presence of parasites had been documented by microscopy were used for the study. Eighteen samples contained at least one amoeba species whereas the 18 other specimens were amoeba-negative (Table 1). All specimens were provided by the Laboratory of Parasitology and Mycology, Pasteur Institute of Tunis, and reflected anonymous samples and were received in the setting of routine diagnosis for stool parasite testing and screened using the conventional 'ova and parasites' examination. Faecal specimens used in this study had been preserved in S.T.A.R. buffer (Roche Diagnostics, Indianapolis, IN, USA) at  $-20^{\circ}\text{C}$  prior to DNA extraction.

### DNA extraction

Total genomic DNA was extracted from the stool samples using the QIAamp DNA Stool MiniKit (Qiagen, Inc., Valencia, CA, USA) with bead beating prior to DNA extraction. Briefly, 250  $\mu\text{L}$  of stool specimen was added to 1.4 mL of the stool lysis buffer (ASL) provided by the Qiagen kit, the suspension was mixed with Glass beads (500 mg; diameter, 0.5 mm; Sigma), centrifuged at 700 g for 3 min and incubated for 5 min at  $70^{\circ}\text{C}$  prior to DNA extraction according to the manufacturer's recommendations. All DNA samples were stored at  $-20^{\circ}\text{C}$  until use.

### Real-time qPCRs identifying *E. histolytica* and *E. dispar*

The 36 DNA extracts were subjected to PCR-based screening for *E. histolytica* and *E. dispar*. Two in-house real-time qPCR assays routinely used in the Laboratory of Parasitology and Mycology at the Pasteur Institute of Tunis for *E. histolytica* and *E. dispar* identification were applied in this study. These two qPCRs are based on the amplification of the small subunit (SSU) rRNA genes of *E. histolytica* and *E. dispar* and use two species-specific forward primers Eh 196F: AAATGGCCAATTCATTCAATGA and Ed 185F: GTATTAGTACAAAGTGGCCAATTTATGT and the same reverse primer Ehd294R: CATTGGTTACTTGTAAACACTGGTG and

6-FAM/MGB-labelled probe (FAM-AGGATGCCACGACAA-NF-Q) that are complementary to a region of homology between the two species. Real-time qPCRs were performed in a final volume of 20  $\mu\text{L}$  using a TaqMan universal master mixture (Applied Biosystems, USA) containing 300 nM of each primer, 200 nM of probe and 5  $\mu\text{L}$  of DNA extract. The DNA was amplified in an Applied Biosystems<sup>®</sup> PCR machine (Applied Biosystems, Foster City, CA) using 40 cycles at 95 and  $60^{\circ}\text{C}$ .

### Conventional PCRs identifying *E. coli*, *E. hartmanni*, *E. polecki*, *E. nana* and *I. bütschlii*

The 36 DNA extracts were subjected to PCR-based screening for *E. coli*, *E. hartmanni*, *E. polecki*, *E. nana* and *Iodamoeba* RL1 and *Iodamoeba* RL2. Published primers targeting *E. coli* and *E. polecki* 18S SSU rRNA genes were used for *E. coli* and *E. polecki* species identifications (Stensvold et al., 2011b, 2018) (Table S1). Moreover, four sets of primers were designed for species identification of *E. hartmanni*, *E. nana*, *Iodamoeba* RL1 and *Iodamoeba* RL2, respectively. Primers were designed using Primer/Blast software (<http://ncbi.nlm.nih.gov/tools/primer-blast>) based on SSU rRNA gene sequences available in GenBank: *E. hartmanni* AF149907 (1960 bp), *E. nana* AF149916 (2589 bp), *Iodamoeba* RL1 JN635741.1 (2376 bp) and *Iodamoeba* RL2 JN635740.1 (2187 bp). Selected primers (Table S1) were checked for sequence specificity using Nucleotide Basic Local Alignment Search Tool (BLASTN) (<https://blast.ncbi.nlm.nih.gov>). DNA amplification was performed in a total volume of 25  $\mu\text{L}$  with 1 $\times$  reaction buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl), 2.5 mM MgCl<sub>2</sub>, 200 mM (each) deoxynucleotide triphosphate, 15 pM (of each) primer, 0.25 U of Taq polymerase (ATGC, Noisy le Grand, France) and 5  $\mu\text{L}$  of template DNA or water (negative control). For *E. polecki* PCR, *E. polecki* ST4 (UNE25) DNA kindly provided by the Public Health Agency of Sweden was used as positive control. Thermal cycle reactions were set to (i) an initial denaturing step at  $95^{\circ}\text{C}$  for 7 min followed by (ii) 40 cycles of a denaturing step at  $95^{\circ}\text{C}$  for 30 s, an annealing step at ( $55^{\circ}\text{C}$  for 30 s for *E. polecki*,  $58^{\circ}\text{C}$  for 30 s for *E. nana* and *E. hartmanni*,  $60^{\circ}\text{C}$  for 30 s for *Iodamoeba* RL1 and RL2 and  $62^{\circ}\text{C}$  for 30 s for *E. coli*) and an extension step at  $72^{\circ}\text{C}$  for 30 s and (iii) a final extension step at  $72^{\circ}\text{C}$  for 7 min. Tubes were kept at  $4^{\circ}\text{C}$  until removal from the thermocycler (T100<sup>™</sup> Thermal Cycler, Bio-Rad, California, USA). PCR products were separated by electrophoresis on a 2% agarose gel in Tris/acetate/EDTA (pH 8.0) buffer, stained with Midori Green Advance DNA Stain (Nippon Genetics Europe GmbH, Germany) and visualized on an UV trans-illuminator. PCR products were purified using a NucleoSpin<sup>®</sup> extract II kit (Macherey-Nagel GmbH & Co. KG, Germany) and cycle-sequenced using an ABI Prism<sup>®</sup> Big Dye<sup>™</sup> Terminator, Cycle Sequencing Ready Reaction Kit and ABI 3130 sequencing system (ABI, PE Applied Biosystems), with the same primers as used for PCR. DNA sequences from both strands were edited and assembled into one contig using the Staden software package (<http://staden.sourceforge.net/>). MEGA version 7 software ([www.megasoftware.net](http://www.megasoftware.net)) was used to perform multiple sequence alignment (ClustalW option) and for phylogenetic analysis. For each species, relationships between specimens and reference isolates were inferred based on genetic distances using the neighbour joining (NJ) method with the Kimura 2 parameters model. Statistical support for tree distances was evaluated using bootstrapping (1000 replicates).

### Accession numbers

Nucleotides sequences were deposited in the GenBank database (<http://www.ncbi.nlm.nih.gov/>) under accession numbers MG925061–MG925076.

**Table 1.** Comparison of microscopy and PCR results on the 36 samples included in the study

Sample ID	Microscopy	Species specific PCR					
		<i>Entamoeba coli</i>	<i>Entamoeba hartmanni</i>	<i>Endolimax nana</i>	<i>Iodamoeba bütschlii</i>	<i>Entamoeba histolytica</i>	<i>Entamoeba dispar</i>
S1-S4-S5	<i>E. coli</i>	+	–	–	–	–	–
S2		+	–	–	–	–	+
S3		+	+	–	–	–	–
S6-S7-S8	<i>E. hartmanni</i>	–	+	–	–	–	–
S9-S10-S11 S13-S14	<i>E. nana</i>	–	–	+	–	–	–
S12		–	+ <sup>a</sup>	+	–	–	–
S15	<i>I. bütschlii</i>	–	–	+	+	–	+
S16	<i>E. histolytica/dispar</i>	–	–	–	–	+	–
S17		–	–	–	–	–	+
S18	<i>E. coli</i> + <i>Endolimax</i>	+	+	+	–	–	–
S19	<i>Blastocystis</i> spp.	–	–	+	–	–	–
S20		–	+	+ <sup>a</sup>	–	–	–
S21-22-24		–	–	–	–	–	–
S23		–	+ <sup>a</sup>	–	–	–	–
S25-S26	<i>Giardia intestinalis</i>	–	–	–	–	–	–
S27		+	–	+	–	–	–
S28-S29-S30	<i>Dientamoeba fragilis</i>	–	–	–	–	–	–
S31-S32-S33- S34-S35-S36	Negative	–	–	–	–	–	–
Total		7	8	11	1	1	3

<sup>a</sup>No nucleotide sequences available.

## Results

### Overall results

The 18 specimens amoeba-positive by microscopy were identified at the species/ribosomal lineage level by PCR-based methods. Molecular species identification was 100% concordant with microscopic results (Table 1). Moreover, the PCR assays identified amoebae in several microscopy negative samples and revealed more samples with a mixture of amoeba species than microscopy (22 vs 18 and 7 vs 1, respectively) (Table 1).

Moreover, species specific PCR-based methods enabled the species differentiation between *E. dispar* and *E. histolytica* (Table 1) and confirmed the absence of *E. polecki* in all study specimens.

### *Entamoeba coli*-specific PCR

*Entamoeba coli*-specific PCR was positive for seven samples. Six of these samples had been observed to contain cysts of *E. coli*, and in the remaining sample, only cysts of *Giardia* had been observed (Table 1). The *E. coli* in the seven specimens reflected four different SSU rDNA sequences (GenBank acc. nos., MG925061–MG925064). Phylogenetic analyses of these DNA sequences using *E. histolytica/dispar/moshkovskii* as out-group positioned the seven specimens in the same cluster as sequences of *E. coli* collected by other authors (Fig. 1A). Furthermore, DNA sequencing allowed the identification of *E. coli* subtypes and classified samples as ST1 ( $n = 5$ ) and ST2 ( $n = 2$ ) (Fig. 1A).

### *Entamoeba hartmanni*-specific PCR

*Entamoeba hartmanni*-specific PCR was positive for eight samples (Fig. 2A). Three of these samples were microscopy positive for

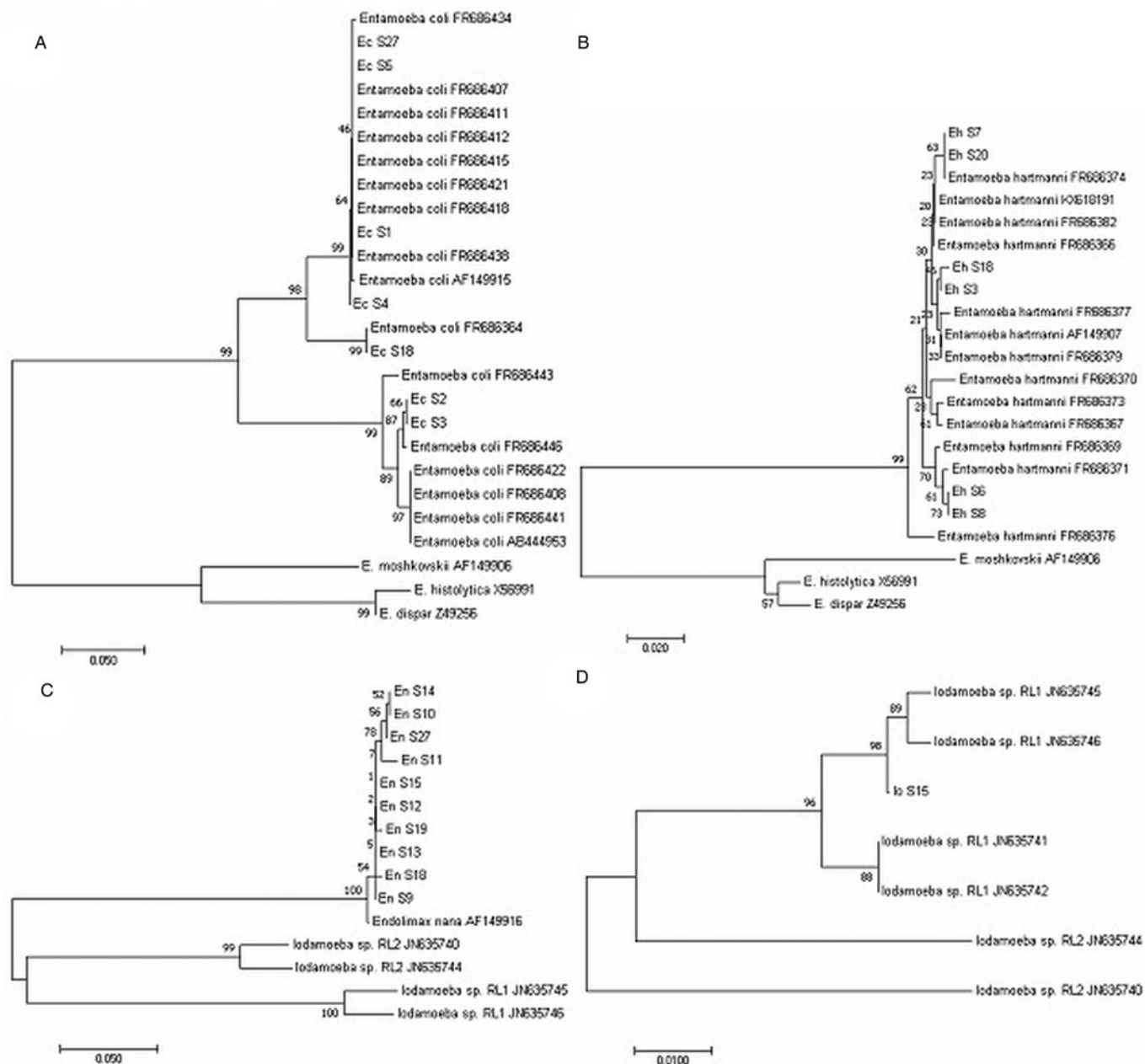
*E. hartmanni* cysts, three for cysts of *E. nana* and *E. coli* and two for *Blastocystis* only (Table 1). DNA sequencing was possible only for six samples out eight (Table 1) that corresponded to four different sequences identified by sequence alignment and visual inspection of the sequence traces and deposited in the GenBank under accession numbers MG925065–MG925068. Phylogenetic analyses of rDNA sequences with *E. histolytica/dispar/moshkovskii* as out-group positioned the six specimens in the same cluster as sequences from *E. hartmanni* collected by other authors (Fig. 1B).

### *Endolimax nana*-specific PCR

*Endolimax nana*-specific PCR was positive for 11 samples (Fig. 2B). Seven samples were positive for *Endolimax* cysts by microscopy, one for cysts of *I. bütschlii*, and three were known to contain other protists, namely *Blastocystis* and *Giardia* (Table 1). DNA sequencing was possible only for ten samples out of 11 (Table 1) that corresponded to six different sequences (GenBank acc. nos., MG925069–MG925075). Phylogenetic analyses of the DNA sequences using *Iodamoeba* as out-group positioned the ten specimens in the same cluster as the single *E. nana* reference sequence AF149916 available in the GenBank (Fig. 1C). Phylogenetic tree also indicated that *Endolimax* from this study may constitute a phylogenetically distinct group from the *Endolimax* reported in the GenBank. However, because the low bootstrap values, genetic variation in the SSU rRNA gene among *Endolimax* could not be considered (Fig. 1C).

### *Iodamoeba* specific PCRs

For *Iodamoeba*, two PCRs were applied in order to identify the two lineages RL1 and RL2. RL1 specific PCR was positive for



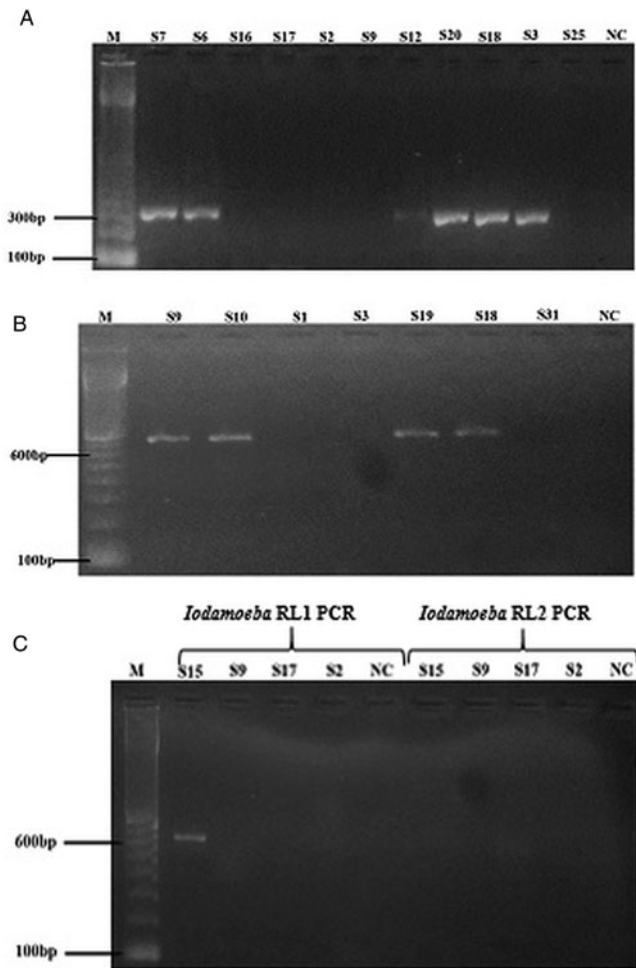
**Fig. 1.** Phylogenetic trees showing intra-specific variations in intestinal amoeba species. The consensus tree was based on the distance NJ using 1000 replicates samples. (A) *Entamoeba coli* across the 500 bp sequence, (B) *Entamoeba hartmanni* across the 300 bp sequence. Sequences from *Entamoeba histolytica*, *Entamoeba dispar* and *Entamoeba moshkovskii* were used as outgroup for both species. (C) *Endolimax nana* across the size between 400 and 700 bp sequence. Sequences from *Iodamoeba* RL1 and RL2 were used as outgroup. (D) *Iodamoeba* across the 460 bp sequence. Sequence from *E. nana* was used as outgroup.

one sample (Fig. 2C) that contained *Iodamoeba* cysts while RL2 specific PCR was negative for all samples (Table 1). Nucleotide sequence alignment was deposited in the GenBank under accession number MG925076. Phylogenetic analyses of rDNA sequences with *Endolimax* as out-group positioned the specimens in the same cluster as sequences from *Iodamoeba* of RL1 lineage characterized by other authors (Fig. 1D).

## Discussion

Based on a series of morphological key differences, microscopy allows the differentiation between the three genera of Amoebozoa infecting the human gut. In fact, mature cysts of *Endolimax* contain four nuclei and those of *Iodamoeba* contain only one, but these nuclei differ markedly in appearance from their counterparts in *Entamoeba*. Within the genus *Entamoeba*, several morphological differences including, cyst size, number of

nuclei, appearance of the peripheral chromatin, size of the karyosome, presence/absence and appearance of chromatoid bars, presence/absence of inclusion bodies are often used for *Entamoeba* species differentiation. For instance, *E. coli* which forms large cysts containing eight nuclei can easily be distinguished from other *Entamoeba* species. However, cysts of genetically different *E. coli* strains may differ in size and smaller immature forms may be mistaken for *E. histolytica*/*E. dispar* (Ben Ayed et al., 2008b). Similarly, *E. hartmanni* exhibits morphological structures similar to the features of *E. histolytica*/*dispar* complex which could lead to misdiagnosis. However, *E. hartmanni* cysts are generally smaller than those of the *E. histolytica*/*dispar* complex (Burrows, 1959). Thus, for microscopic differentiation an appropriate morphometric analysis is generally required. In the other hand, immature cysts of *E. histolytica*/*dispar* may have only one nucleus and it may be almost impossible to differentiate such cysts from those of *E. polecki*, a uninucleated species of



**Fig. 2.** Stained agarose gel electrophoresis with products amplified by specific amoeba species SSU rDNA PCRs. (A) *Entamoeba hartmanni* PCR: S7, S6, S12, S20, S18, S3 positive samples showing a band at expected size (300 pb); S16, S17, S2, S9, S25 negative samples. (B) *E. nana* PCR: S9, S10, S19, S18 positive samples showing a band at expected size (740 pb); S1, S3, S31: negative samples. (C) *Iodamoeba* RL1 and RL2 PCRs: S15 positive sample by *Iodamoeba* RL1 PCR showing a band at expected size (590 pb); S9, S17, S2: negative samples. M: 100-bp ladder; NC: negative control.

*Entamoeba*, the cysts of which are similar in size to those of *E. histolytica* and *E. dispar* (Stensvold *et al.*, 2018). Hence, morphological diagnosis of *Entamoeba* species is still a renowned challenge in clinical microbiology laboratories and lack of data on several *Entamoeba* species such as *E. polecki*, may be related to the possibility that they are confused with other *Entamoebas* (Stensvold *et al.*, 2018). Moreover, *E. histolytica* cysts are morphologically indistinguishable from those of *E. dispar* and *E. moshkovskii*, which is why they are often reported at a complex level (Fotedar *et al.*, 2007; Ben Ayed *et al.*, 2008a, 2008b).

The target gene of many diagnostic PCRs for intestinal parasites is the SSU rRNA gene which is present in all eukaryotes, often even in high copy numbers (Verweij and Stensvold, 2014). SSU rDNA sequence data are available for the vast majority of intestinal parasites and offer good resolution in terms of detecting and differentiating species and subtypes of Amoebozoa found in the human intestinal tract. These data were exploited to design oligos used in PCRs targeting *E. histolytica*, *E. dispar*, *E. coli*, *E. hartmanni*, *E. polecki*, *E. nana* and *I. bütschlii*. Each of the developed PCR was species-/lineage-specific and at least as or even more sensitive than microscopic examination, which highlights the diagnostic and epidemiological relevance of applying DNA-based methods for detecting and differentiating amoebae in faecal samples.


Genetic diversity within the genus *Entamoeba* has been subject to substantial investigation (Clark and Diamond, 1997; Silberman *et al.*, 1999; Stensvold *et al.*, 2011b). Riboprinting supported the division into groups based on the number of nuclei present in the mature cyst (Clark and Diamond, 1997). Most phylogenetic reconstructions supported monophyly of the Entamoebidae and were largely in accord with riboprinting results. Thus, species of *Entamoeba* producing cysts with the same number of nuclei formed monophyletic groups. The most basal *Entamoeba* species are those producing cysts with eight nuclei, while the group producing four-nucleated cysts was most recently derived (Silberman *et al.*, 1999). Among *Entamoeba* species, *E. coli* exhibits the highest intragenetic diversity (more than 15% polymorphic sites across the SSU rRNA gene) (Stensvold *et al.*, 2011a). It comprises at least two major subtypes, ST1 and ST2, with no absolute host specificity (Stensvold *et al.*, 2011b). In our study, using published primers, we identified both of these two subtypes. ST1 however, was more prevalent than ST2. On the other hand, primers for detecting *E. hartmanni* were published and used by Suzuki *et al.* (2008). However, recent accumulation of information on intra-specific sequence variation of *E. hartmanni* species allowed a re-evaluation of these primers (Stensvold *et al.*, 2011b). In fact, in light of recent *E. hartmanni* sequencings, it appeared that some of the *E. hartmanni* SSU rDNA sequence variants might not have been amplifiable using those primers as sequence variation exists in both of the primer binding regions, raising questions about the prevalence data generated based on those primers (Stensvold *et al.*, 2011b). Taking into account the more recently published *E. hartmanni* sequences, we designed two specific primers for PCR-based detection. These primers turned out to be both specific and sensitive. All six sequences fell into a single clade in the same cluster as sequences from *E. hartmanni* collected by other authors. Multiple sequence alignment confirmed the low intra-specific variation within this species (Clark and Diamond, 1997; Stensvold *et al.*, 2011b).

Of the so-called non-pathogenic intestinal protozoa, *Endolimax* and *Iodamoeba* belong to the least well-described amoeba infecting human gut. Most data on these parasite protists have emerged from general surveys of intestinal parasites, and the two genera remain largely unexplored in terms of genetic diversity, host specificity and molecular epidemiology (Stensvold *et al.*, 2012; Poulsen and Stensvold, 2016). In their phylogenetic analysis, Silberman *et al.* placed *Endolimax* as a sister taxon to the *Entamoeba* assemblage (Silberman *et al.*, 1999). Sequencing the SSU rRNA gene of *Iodamoeba*, Stensvold *et al.* (2012) found that *Endolimax* and *Iodamoeba* grouped together. Recently, however, and mostly due to availability of more sequences from related organisms, Zadrožilková *et al.* were able to obtain monophyly for both *Endolimax* and *Iodamoeba* (Zadrožilková *et al.*, 2015). In 2016, Poulsen and Stensvold (2016) developed specific primers based on the single complete SSU rRNA gene sequence of *Endolimax* in the GenBank. However, these later primers have proved partially effective for diagnosing *E. nana* in genomic DNA extracted from fresh stool. In-house primers developed in this study led to successful amplification in all samples positive for *Endolimax* by microscopy. DNA sequencing grouped all human specimens together and suggested two phylogenetically distinct groups within *Endolimax*: one group from human origin and the second from non-human primate. In fact, the only available complete SSU rRNA gene sequence of *Endolimax* is from grey cheeked mangabey, *Cercocebus albigena* (National Zoological Park, Washington, DC, 1991). In all cases, designing genus-specific primers based on a single DNA sequence remains problematic and more sequence data is needed to better evaluate primer design and intra-genetic variability (Poulsen and Stensvold, 2016).

Concerning *Iodamoeba*, taking into account its remarkable degree of genetic diversity and the presence of two ribosomal lineages (RL1 and RL2), two RL-specific PCRs were designed. However, given its relative rarity, only one sample containing *Iodamoeba* cyst was available for PCR analysis. This sample was positive by the RL1-specific PCR and negative by the RL2-specific PCR. DNA sequencing confirmed its ribosomal lineage (RL1).

There can be little doubt that in the next decades, screening for intestinal parasites by PCR will become more and more common, and might replace microscopy to a large extent. Real-time PCR-based methods are nowadays increasingly used for intestinal protozoa screening including *E. histolytica* (ten Hove et al., 2009; Stensvold and Nielsen, 2012; Cimino et al., 2015; Efunshile et al., 2015; Won et al., 2016; Abu-Madi et al., 2017; Incani et al., 2017). Compared with conventional PCR, it has the advantages of increased specificity because of the role of the probe, reduced risk of contamination and the capacity of multiplexing different targets and quantification (Verweij and Stensvold, 2014). However, in the interpretation of PCR results, one should keep in mind that because of the specificity of the primers and probe, the only sequences detected will be those from which they were designed. While in the future, amplicon-based approaches coupled to next generation sequencing (NGS) may prove useful and represent a viable method for detecting and differentiating amoebae in faecal samples, a major limitation to completing our ability to detect and differentiate amoebae in stool samples is the lack of data on diversity within the Amoebozoa infecting man and other host species in publicly available databases and complete ribosomal genes are necessary for phylogeny and epidemiology (host specificity). NGS/shotgun on faecal genomic DNA will only give us fragments. Nevertheless, NGS-based technology may reveal intra-specific variation within species that to some extent might prove useful for differentiation between the various subtypes and lineages.

**Supplementary material.** The supplementary material for this article can be found at <https://doi.org/10.1017/S0031182018002196>.

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