Use of PCR Amplification of tRNA Gene-Linked Short Tandem Repeats for Genotyping *Entamoeba histolytica*

Ibne Karim M. Ali,[†] Mehreen Zaki,[‡] and C. Graham Clark^{*}

Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine,
Keppel Street, London WC1E 7HT, United Kingdom

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We have developed a reliable method for PCR-based genotyping of *Entamoeba histolytica* based on variation in the numbers of short tandem repeats that are linked to tRNA genes in this species. Species-specific primer pairs were designed that differentiate *E. histolytica* from *E. dispar* as well as that reveal intraspecies PCR product length polymorphisms. The primers were tested with samples from different parts of the world, and DNA was extracted from cultured cells as well as liver abscess pus and feces by various methods. We now have the tools necessary to investigate a possible link between parasite genotype and the outcome of infection with *Entamoeba histolytica*, as well as other aspects of the organism's epidemiology.

The worldwide distribution and magnitude of infection with *Entamoeba histolytica* and *Entamoeba dispar* as separate species are not well understood, as most diagnoses are still based on microscopy, which cannot discriminate between them. According to the most cited reference (17), 10% of the world's population is infected with *E. histolytica*. However, this estimate predates the formal separation of *E. histolytica* and *E. dispar* (8) and is now being reassessed. Current data indicate that *E. dispar* is perhaps 10 times more common than *E. histolytica* worldwide, but local prevalences may vary significantly. Nevertheless, it is already clear that not all *E. histolytica* infections lead to disease in the host but that at most 1 in 10 *E. histolytica* infections progresses to the development of clinical symptoms (9). In a cohort study in Bangladesh, during a 12-month follow-up only about 3% of *E. histolytica*-infected children developed symptoms attributable to amebic dysentery (15). Similarly, during a recent 15-month longitudinal study of 43 adult asymptomatic *E. histolytica* carriers in Vietnam, none developed invasive intestinal disease, although 1 developed an amebic liver abscess (3).

What determines the outcome of an *E. histolytica* infection is still a mystery, but one possibility is that it is linked to the genotype of the parasite. To investigate this relationship, a simple, sensitive, and reliable method for strain identification is required.

A few PCR-based DNA typing methods have been reported for *E. histolytica*, making use of repetitive elements contained within both protein-coding genes and noncoding DNAs. The serine-rich *E. histolytica* protein (SREHP) gene, which encodes

an immunodominant surface antigen, has been investigated by several groups. Variations in both the numbers and the sequences of tandem repeats encoding 8- and 12-amino-acid units have been observed among different strains of *E. histolytica* (5, 10, 12, 13). A 7-amino-acid repeat-encoding region of the chitinase gene has also been used but showed comparatively little variation (10, 12, 13).

A number of polymorphic loci containing diverse, noncoding short tandem repeats (STRs) were investigated (18). All repeats were A+T rich and varied in size from 8 to 16 bp, and most of the polymorphism observed was due to variable repeat numbers. Subsequently, the development of species-specific primers for these STRs was reported, and these primers allowed the simultaneous differentiation and strain typing of *E. histolytica* and *E. dispar* (19). This is important, because in some areas of *E. histolytica* and *E. dispar* endemicity, a significant number of individuals could be coinfecting with both parasites (14, 16). These STRs were all found to be flanked by genes encoding tRNA (6a).

No single polymorphic locus can be used to detect all genotypes of *E. histolytica*, and the need to use more than one locus for strain typing has been emphasized (12, 18). To develop an optimal strain typing method, we have investigated the polymorphic potential of all tRNA-flanked STRs in *E. histolytica*, and we have designed and tested species-specific primers for those that were the most promising. We believe that the resulting method gives us the tools necessary to investigate the role of parasite genotypes in the outcome of infection with *E. histolytica* as well as to address other unanswered questions surrounding the epidemiology of this parasite.

MATERIALS AND METHODS

***E. histolytica* and *E. dispar* samples.** Eight strains of *E. histolytica* and one strain of *E. dispar* were grown axenically in LYI-S-2 medium (6) with 15% bovine serum. Information on the origins of these strains and the patients' diagnoses is provided in Table 1.

DNA samples from nine *E. histolytica* strains and nine *E. dispar* strains maintained xenically in Robinson's medium were received from the International Centre for Diarrhoeal Disease Research, Bangladesh, along with two DNA samples isolated directly from the aspirated pus of amebic liver abscess (ALA) patients and two DNA samples extracted from *E. dispar*-positive feces. Two

^{*} Corresponding author. Mailing address: Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, United Kingdom. Phone: 44-207-927-2351. Fax: 44-207-636-8739. E-mail: graham.clark@lshtm.ac.uk.

[†] Present address: Department of Microbiology, Stanford University School of Medicine, Stanford, CA 94305-5107.

[‡] Present address: School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom.

§ Supplemental material for this article may be found at <http://jcm.asm.org/>.

TABLE 1. Classification of axenic isolates

Species	Isolate	Clinical diagnosis	Suspected country of origin	Yr of isolation	ATCC ^a accession no.
<i>E. histolytica</i>	HM-1:IMSS	Amebic dysentery	Mexico	1967	30459
<i>E. histolytica</i>	200:NIH	Amebic dysentery	Isolated in the United States	1949	30458
<i>E. histolytica</i>	IULA:0593:2	Diarrhea, nausea, tenesmus, fever	Venezuela	1993	
<i>E. histolytica</i>	H-303:NIH	Amebic dysentery	Patient from Vietnam, isolated in the United States	1972	30887
<i>E. histolytica</i>	HB-301:NIH	Amebic dysentery	Myanmar (Burma)	1960	30190
<i>E. histolytica</i>	IULA:1092:1	Diarrhea, nausea, tenesmus, fever	Venezuela	1992	
<i>E. histolytica</i>	HK-9	Amebic dysentery	Korea	1951	30015
<i>E. histolytica</i>	Rahman	Asymptomatic	Sailor from India, isolated in England	1972	30886
<i>E. dispar</i>	SAW760	Asymptomatic	England	1979	50484

^a ATCC, American Type Culture Collection.

samples extracted from *E. histolytica*-positive feces in Vietnam were also obtained (courtesy of E. Tannich, Bernhard Nocht Institute, Hamburg, Germany).

Isolation of DNA. A shortened version of the cetyltrimethylammonium bromide (CTAB) DNA extraction method described previously (4) was employed for all axenic and xenic isolates, pus specimens, and the two fecal specimens from Bangladesh. Briefly, 50 μ l of culture pellet, 0.1 g of fresh or frozen stool, or 50 μ l of fresh or lyophilized ALA pus was dispersed in 250 μ l of lysis buffer (0.25% sodium dodecyl sulfate in 0.1 M EDTA, pH 8.0), and 100 μ g/ml of proteinase K was added. The lysate was incubated at 55°C for 20 min. NaCl was added to a final concentration of 0.7 M, and then CTAB was added to a concentration of 1%. After the components were mixed, the sample was incubated at 65°C for 10 min. This was followed by extractions with equal volumes of chloroform and then phenol-chloroform-isoamyl alcohol, and the DNA was precipitated with ethanol. The dried DNA pellet was dissolved in sterile distilled water and passed over a Microspin S-200 HR column (Amersham Pharmacia Biotech, Inc., United Kingdom). The Vietnamese fecal DNA samples were isolated by using the QIAamp DNA stool mini kit (QIAGEN, Hilden, Germany). One microliter of DNA, irrespective of its origin, was used as the template in each PCR. When nested PCR was employed, 1 μ l of the first amplification reaction was used as the template in the second amplification reaction.

PCR. PCR was performed with BioTaq DNA polymerase and 1.5 mM MgCl₂ (BioLine, United Kingdom). Two different groups of primers were used in this study: tRNA-specific primers, designed by using *E. histolytica* HM-1:IMSS tRNA gene sequences (GenBank accession numbers BK005648 to BK005672), which amplify both *E. histolytica* and *E. dispar* DNA (Table 2), and species-specific primers, which were designed after the comparison of homologous sequences from *E. histolytica* and *E. dispar* (Table 2) to amplify DNA from one species only. Naming of the arrays, STRs, and primers derives from the single-letter amino acid code (and anticodon, if necessary) for the relevant tRNA genes flanking the STR being amplified (6a). Consensus array unit sequences and STR organizations for *E. histolytica* HM-1:IMSS can be seen at <http://homepages.lsh.tn.ac.uk/entamoeba/units/units.htm>.

The thermal cycler settings for all PCRs were the same, except for the annealing temperatures. Standard cycling conditions consisted of 35 cycles of denaturation at 94°C for 30 s, annealing at the appropriate temperature (Table 2) for 30 s, and extension at 72°C for 30 s, followed by a final extension of 5 min at 72°C. All PCR products were separated in 1.5% agarose gels (Bio-Gene, United Kingdom) in 1× Tris-borate-EDTA buffer at 8.5 V/cm and visualized after staining with ethidium bromide. The size marker used was a 100-bp DNA ladder (Promega, United Kingdom).

Cloning and sequencing of amplified products. The amplified products chosen for sequencing were cloned by using the pGEM-T Easy Vector system (Promega) and XL1-Blue competent cells (Stratagene, The Netherlands), according to the manufacturers' instructions. Plasmid DNA was isolated by using the QIAprep Spin Mini kit (QIAGEN, United Kingdom) and was sequenced by using the ABI Prism BigDye Terminator cycle sequencing ready reaction kit, according to the manufacturer's instructions (Applied Biosystems, Inc., United Kingdom). The resulting sequences were assembled and aligned either by using the Multalin program (<http://www.prodes.toulouse.inra.fr/multalin/multalin.html>) (7) or manually by eye.

Nucleotide sequence accession numbers. The sequences have been deposited in GenBank under accession numbers AY842959 to AY842978 for *E. dispar* and AY842979 to AY843015 for *E. histolytica*.

RESULTS

Screening for STR polymorphism. In *E. histolytica*, most tRNA genes are now known to be found in long tandem arrays, with each array repeat unit containing between one and five tRNA genes which are interspersed with STRs (6a). Three arrays also contain 5S RNA genes, and one array contains an unidentified gene encoding a small RNA. We screened all the STRs present in the tRNA arrays of *E. histolytica* for polymorphism, using 46 pairs of primers (Table 2; see also Table S1 in the supplemental material) and eight axenic *E. histolytica* strains from diverse geographic locations (Table 1). A summary of the PCR product size polymorphism seen with the 46 STR primer pairs is provided in Table S2 in the supplemental material. Surprisingly, about two-thirds (28 of 46) of the STRs showed little or no variation among strains (Fig. 1A; see Table S2 in the supplemental material).

In about one-quarter (13 of 46) of the STRs, amplification produced multiple products in one or more *E. histolytica* strains (see Table S2 in the supplemental material). Some STRs showed multiple bands because there was length variation between units in the same array, but in other cases the multiple bands have other origins. For example, STRs C-K, K-S, N-K, and K-N give two or more bands in HM-1:IMSS because the corresponding tRNA gene pairs are found in two distinct arrays. Similarly, V-5 amplification gives two bands because the primers amplify sequences from both [V5] and [VME5]. A second band is sometimes produced where the product is actually more than one array unit (e.g., T-R-T-R is amplified instead of T-R). Comparing the *E. histolytica* HM-1:IMSS and *E. dispar* SAW760 results, we observed a single product at 40 STRs in these two species, of which 36 STRs are homologous.

All STRs that showed polymorphism were then investigated by using *E. histolytica* DNA isolated from nine xenic cultures and two lyophilized ALA pus samples. This allowed us to evaluate the amplification efficiency and polymorphism using DNAs and other sample types from a geographically restricted region (Bangladesh). All STRs amplified well and showed polymorphism, with S^{TGA}-D and S-Q showing the most variation (Fig. 2A). We also tested nine DNAs from xenic *E. dispar* cultures. These were successfully amplified at all STRs by using the tRNA-specific primers and also showed polymorphism in most cases.

TABLE 2. Primers used for genotyping

Primer type and name ^a	Sequence (5' to 3')	AT (°C) ^c
Selected tRNA primers ^b		
A-L5	GGATCGATACCCCTCATCTCCA	64
A-L3	CGCATCTTGCATAGCCGAG	
D-A5 (EhR1) ^d	CTGGTTAGTATCTTCGCCTGT	56
D-A3 (EhR2) ^d	GCTACACCCCATTAACAAT	
N-K5	CGAACGGCTGTTAACCGTTA	55
N-K3	TTCCTAGCTCAGTCGGTAGA	
R-R5	AGCATCAGCCTTCTAAGCTG	55
R-R3	CTTCCGACTGAGCTAACAAG	
S ^{TGA} -D5	CTCTGGATGCGTAGGTTCAA	58
S ^{TGA} -D3	GTATCTTCGCCTGTCACGTG	
S-Q5	GTGGTCTAAGGCGTGTGACT	56
S-Q3	GAGATTCTGGTTCTTAGGACCC	
<i>E. histolytica</i> -specific primers		
AL-H5	CATCTCCATTATTATCTAGATATCCT TTATTACT	57
AL-C	GGCACGAATGCTTTGATATATAA	
DA-H5 (Hsp1) ^d	GAGTTCTCTTTTTATACTTTTATA TGTT	50
DA-H3 (Hsp2) ^d	ATTAACAATAAAGAGGGAGGT	
NK2-H5	GAAGCGTCTTTTTTACTATTAGTG	59
NK2-H3	GGCGTATTTTLAGAATAGGATAAG	
RR-H5	GCGCTTTTTTATTCAATATACTCC	57
RR-H3	GGATGAAGATATCTTCACAGGG	
S ^{TGA} D-H5	AAATCCTGCCACTGTCGTAA	58
S ^{TGA} D-H3	AATCCCCGTGAAGAGTTCT	
SQ5	GTGGTCTAAGGCGTGTGACT	55
SQ-H3	GTGGGACCACTTTTTATACCTA	
<i>E. dispar</i> -specific primers		
AL-D5	CATCTCCATTATTATGTATCTATTTA TCTATTTA	60
AL-C	GGCACGAATGCTTTGATATATAA	
DA-D5 (Dsp1) ^d	TTGAAGAGTTCACTTTTTATAC TATA	50
DA-D3 (Dsp2) ^d	TAACAATAAAGGGGAGGG	
NK-D5	GCGGAAGCGTCTTTTTTACTATT ATTA	58
NK-D3	GGGCGTATTTTATAATAAGAATAG GTGG	
RR-D5	CATGAGGCGCCTTTTATCA	58
RR-D3	AGGGGATGATGATATTGAACAC ACTC	
S ^{TGA} D-D5	AAATCCTGCCACTGTCGTAC	63
S ^{TGA} D-D3	AATCCCCGTGAAGAGTTCA	
SQ-D5	AGCATAGGTGTATAGGTGTTAGG	53
SQ-D3	GGTGGGACCATATTTTATACTTC	

^a H, *E. histolytica*; D, *E. dispar*; C, common (used in both *E. histolytica* and *E. dispar*).

^b The tRNA-specific primers amplify both *E. histolytica* and *E. dispar* STRs. Only those for the six selected loci are shown. The others can be found in Table S1 in the supplemental material.

^c AT, annealing temperature used.

^d These primers were described previously (19) and were given the names inside the parentheses.

Selection of final STR panel. The selection of polymorphic STRs for the final panel was based on (i) the degree of polymorphism shown, (ii) the number of amplification products produced, (iii) the relative success of PCR amplification in different DNA sample types, and (iv) our ability to design species-specific primers. On the basis of these criteria, we selected 6 of the original 46 STRs as suitable for our purposes.

STRs C-K, E-Y, F-V, I-W, K-N, and W-I showed substantial polymorphisms; but a majority of the strains gave multiple PCR products. Because multiple bands might complicate the future genotyping of strains, we decided not to investigate these STRs further. Although STRs M-E, R-T, and S-P showed good polymorphism and produced a single product in a majority of the axenic *E. histolytica* strains, we chose not to include these STRs either. We were unable to design reliable species-specific primers for M-E, despite repeated efforts, while amplification of S-P was unreliable when DNA from xenic cultures was used, and R-T gave multiple products with fecal DNA (data not shown).

The only exception to our stated criteria is STR N-K, for which we observed two bands in all isolates except two isolates from Venezuela, which gave a single product (Fig. 1C, IULA isolates). The smaller of the two *E. histolytica* HM-1:IMSS products (designated STR N-K1) was present and was the same size in all others but was absent from the Venezuelan isolates. Analysis of the *E. histolytica* HM-1:IMSS genome data showed the presence of two N-K types that differed in the sequence of the STR and its flanking regions. The primers were therefore amplifying STRs from two distinct [NK] arrays. Only one STR is polymorphic and present in all isolates. We named this STR N-K2 and designed primers specific for this sequence, which also proved to be species specific.

Design and testing of species-specific primers. We obtained the homologous *E. dispar* SAW760 sequences for a number of the STRs in order to allow the design of species-specific primers. We also sequenced the same STRs from at least one additional strain of *E. histolytica* to ensure that the specific primer sequences were conserved within the species. Comparison of sequences between *E. histolytica* strains revealed that the PCR product size differences are mainly due to variable numbers of repeats, in agreement with earlier observations at two loci (13, 18). Although the sequences of almost all the tRNA genes are identical between *E. histolytica* and *E. dispar*, the intervening STR regions are completely different in sequence (11) (compare the sequences with GenBank accession numbers AY842959 to AY842978 with those with accession numbers AY842979 to AY843015).

In *E. histolytica*, we were able to design both 5' and 3' species-specific primers for four STRs. For the remaining two STRs, we designed one species-specific primer but had to use either the original 5' tRNA primer (S-Q) or a new but common 5' primer (A-L) because the sequence flanking the STR was too A+T rich to be suitable for primer design. In *E. dispar*, we designed both 5' and 3' species-specific primers for five STRs, which required only the new common primer for A-L, as described above. The corresponding specific primers are not always in exactly homologous positions in the two species, again because of base composition.

FIG. 1. Examples of polymorphism observed in tRNA-linked STRs of *E. histolytica*. (A) Three STRs showing little or no PCR product size polymorphism; (B) three of the selected STRs showing low to moderate polymorphisms; (C) the remaining three selected STRs showing moderate to high polymorphisms. All samples except *E. dispar* SAW760 are *E. histolytica*.

All species-specific primer pairs were tested by using two control DNAs from axenic *E. histolytica* HM-1:IMSS and *E. dispar* SAW760 cultures and DNA isolated from two xenic *E. histolytica* cultures and two xenic *E. dispar* cultures (Fig. 3). We observed specific products with all 12 pairs of primers (6 pairs for each species): *E. histolytica* species-specific primers gave products only with DNAs from *E. histolytica*, while the *E. dispar* species-specific primers gave products only with DNAs from *E. dispar* isolates. However, the *E. dispar* species-specific ST^{GA-D} primers gave faint and small (less than 100-bp) products with DNAs from the two *E. histolytica* strains from xenic cultures (Fig. 3). Because the product size for *E. dispar* obtained by using this primer pair ranges from 200 to 220 bp, we do not think that these smaller nonspecific products will interfere with species identification.

We then tested the ability of the species-specific primers to amplify *E. histolytica* DNA extracted from stool and liver abscess pus samples. For this, we used two ALA samples and two stool samples obtained from Bangladesh, which were extracted by the modified CTAB method, and two stool DNA samples from Vietnam, which had been extracted by using the QIAamp DNA stool mini kit (QIAGEN). Both the liver abscess and the stool DNA samples, regardless of the country of origin

or the method of DNA isolation, were successfully amplified at the selected STRs (Fig. 2B).

We have observed that the success rate for PCR amplification is higher for some STRs than others. In a larger xenic culture sample set, the rate of success with the S-Q species-specific primer pair was just over 80%, but all samples were positive if a nested amplification with tRNA-specific primers followed by amplification with species-specific primers was used. Nested PCR had a success rate of >99% across all STRs, irrespective of the sample origin. Therefore, we recommend the use of nested PCR when negative results are obtained with single primer pairs or when reagent cost is not a significant consideration. It is possible that the copy numbers of the STRs in the genome as well as the cell number in the sample are factors in determining the success of PCR amplification.

DISCUSSION

Tools that allow us to identify strains of *E. histolytica* are important because they may help to address some of the unanswered questions surrounding the virulence of this parasite. For example, are asymptomatic and symptomatic infections caused by

FIG. 2. STR polymorphisms obtained by using different sample types. (A) STR polymorphisms obtained by using DNA from liver abscess samples (lanes LAN-15 and LAN-39) and *E. histolytica* xenic culture DNAs (other lanes) at two of the selected STRs. The tRNA-specific primers were used (Table 2). (B) STR polymorphisms obtained by using liver abscess (LAID-19 and LAID-31) and fecal DNAs. Lanes 22027 and 29621, fecal DNAs from Bangladesh isolated by a modified CTAB method; lanes 231-1/4.00 and 484-1/4.01, fecal DNAs from Vietnam isolated by using the QIAamp DNA stool mini kit. The *E. histolytica* species-specific primers (Table 2) were used.

genetically distinct strains, and does organ tropism have a genetic component? The strain identification tools available to date have been limited. Isoenzyme analysis can detect limited diversity, but it is dependent on the establishment of the amebae in culture. A few DNA-based strain-typing methods have also been reported. SREHP gene analysis requires either restriction enzyme digestion or sequencing to detect most of the variation (5, 10, 12, 13, 21), and nested PCR is necessary to obtain reliable amplification from fecal DNA (2).

The STR-containing sequences originally studied (18) showed promise as strain identification markers (12, 13, 19–21). In the present study, we have expanded and refined this set of markers by making use of the observation that STRs are located adjacent to most tRNA genes in *E. histolytica* and *E. dispar*, a unique feature of these two species (1). The six STRs selected for use in our final panel of markers were chosen on the basis of the degree of polymorphism detected in PCR and their reliability when diverse sample types were used. The generation of *E. histolytica* and *E. dispar* species-specific primer pairs for all the STRs selected will eliminate the potential problems caused by mixed infections. The primers have been tested by using DNA samples from diverse geographic locations, and they have successfully amplified DNA isolated

directly from liver abscess pus and fecal samples, eliminating the need for culture of the amebae. The DNA can be prepared for analysis by using either a commercial kit or a modification of an existing purification method.

A considerable degree of STR length polymorphism has been observed among *E. histolytica* strains, even when the strains isolated from a restricted geographic location (12, 13, 20). The amplification products of tRNA-linked STRs obtained by using DNA prepared from cultures mirror those seen by using DNA extracted from the corresponding fecal samples (21), indicating that the establishment of a strain in culture does not lead to STR changes. We have also observed no changes in the patterns obtained at any of the STRs using DNA extracted from *E. histolytica* HM-1:IMSS or *E. dispar* SAW760 cultures at various times over the past several years (11; authors' unpublished data). Likewise, investigation of *E. histolytica* genotypes in South Africa (20) and Vietnam (3) showed that the STR patterns remain the same over the course of the same infection. The markers therefore appear to be sufficiently stable for our intended uses.

We are now using this panel of markers to investigate the distribution of parasite genotypes among a much larger number of individuals with symptomatic and asymptomatic infec-

FIG. 3. Species-specific primer testing. Each of the DNA samples was amplified by using both the *E. histolytica*-specific (H) and the *E. dispar*-specific (D) primers for two of the selected STRs, STGA-D and S-Q (Table 2). *E. histolytica* HM-1:IMSS and *E. dispar* SAW760 were used as controls. Strains 31 and 32 are known *E. histolytica* isolates, while strains 121 and 122 are known *E. dispar* isolates from Bangladesh, and the DNA was extracted from xenic cultures.

tions. It should also prove useful for the study of the patterns of transmission of this important disease and the epidemiological links between individual infections.

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