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A New PCR-Based Species Genotyping Differentiation Approach in Entamoeaba

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The most commonly used approach for Entamoeba species differentiation up to date is the tRNA-linked STR regions of the parasite's genome. In the present study, a new reliable, fast and easy molecular tool for species differentiation was developed. DNA was isolated from fecal samples collected from infected subjects with either Entamoeba histolytica (EH) or Entamoeba disper (ED) in Saudi Arabia. Two types of primer sets were compared in which the first targeted tRNA-linked STR regions, while the second was designed after multiple contig alignment of the two genomes using NUCmer program in aligned areas with high similarity (~90%) and difference between of ~90 bp. The selection criteria secures that designed primers should pair with both EH and ED contig sequences at homologous regions of 200-500 bp of both species except for the presence of indels that result in the recovery of amplicons of two species with different sizes. Banding patterns in the tRNA-linked STR region resulted in the occurrence of several common amplicons. We speculate that primers mismatch with regions other than the specified STR arrays of Entamoeba histolytica or Entamoeba disper with organisms other than Entamoeba existed in the fecal sample. However, the STR-based approach looked very useful in studying strain differentiation and parasite diversity. The results for the new approach complemented those of the STR-based approach, except that the latter failed to detect coinfected subjects. The new approach proved to be useful at the species level, while the tRNA-linked STR approach can still be a good choice for strain differentiation.

Keywords: Amplicon; Contig; Nucmer; pecies differentiation; STR; Strain Differentiation.

Amebiasis is a disease basically caused by the pseudopod-forming protozoan parasite Entamoeba histolytica (EH). The disease can either be asymptomatic[1] or can result in severe infection with amebic colitis (AC) and amebic liver abscess (ALA). AC is the main cause of diarrhea worldwide for children up to two years old especially those living in the rural developing countries. The disease represents the third leading cause of death, accounting for 9% of all deaths in

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children up to five years of age[2-4]. In Saudi Arabia, there are several communicable diseases affecting the human digestive system likely associated with Hajj season. EH is likely to be one of the most common causes of infectious diarrhea among Hajji individuals in addition to the diseases transferred from individuals coming from endemic areas[5]. The main problem lies in the possible transmission of new invasive strains of the parasite during this season.

Previous efforts of differentiation at the microscopic level failed to discriminate among Entamoeba species. Advances in molecular diagnostic methodologies have resulted in the recognition and separation of EH from the three other nonpathogenic species that infect humans. These other species are morphologically indistinguishable from EH. They are E. moshkovskii that may cause diarrhea[6], in addition to the nonpathogenic E. dispar (ED) and the newly described E. Bangladeshi[7-9]. This scenario changed when ED strains were lately isolated from symptomatic patients in Brazil[10]. These ED strains were able to cause AC and ALA that are occasionally indistinguishable from those produced by EH. This finding revived the possibility that ED can produce lesions in humans.

As ED was reported to be several times more common than EH worldwide[4], it is a must to detect discrete characteristics of this species as compared to EH. However, as indicated earlier, discrimination between Entamoeba histolytica (EH) and Entamoeba dispar (ED) as two separate species is still a difficult task. As a more complication, not all EH infections lead to disease in the host as only ~10% infections progresses to the development of clinical symptoms [6]. These findings indicate that the outcome of EH infection is still a mystery but we speculate it is strain-specific. In addition, evidences of coinfection with EH and ED was reported in some areas of endemicity with both parasites[11]. The later poses more burden into the characterization of either species, a phenomenon that exists in our study.

Molecular characterization based on the short tandem repeats (STRs) linked to tRNA genes of either species indicates the existence of a large number of subspecies or strains[12]. The latter type of differentiation, e.g., STRs, poses more burden in detecting a certain marker with a specific molecular weight for either species, on one hand, in addition to the possibility to get successive PCR products with similar sizes in other Entamoaba or non-Entamoeba species, on the other hand. Therefore, we found it useful to develop a reliable, fast and easy molecular tool for species differentiation, while recommend the use of STR markers only for strain differentiation. The first approach requires studying no more than one locus, while the second requires studying several loci for discrete strain differentiation[11,13].

MATERIALS AND METHODS

Sample collection and DNA isolation

Fecal samples were collected from 37 dysentery-infected subjects, either Saudi or non-Saudi, in four hospitals in Jeddah, KAU. An ethical approval (no. A00451) has been issued by the Ministry of Health, Saudi Arabia following the regulation of the General Administration for Research and Studies at the Ministry of Health (registration no. 1195437) and the National Committee for Medical and Biological Ethics (registration no. H-02-J-002). Consent forms were filled by infected subjects or their relatives at sampling time.

The fecal samples used for molecular characterization were kept fresh at 4°C and diagnosed by microscopic examination and positive samples were subjected to DNA extraction by using QIAMP mini kit specific for stool purification (QIAamp® DNA Mini kit, Qiagen GmbH, Hilden, Germany) following manufacturer's protocol. In order to remove RNA contamination, RNase A (10 mg/ml, Sigma, USA) was further used to DNA samples and incubated at 37oC for 30 min. The purity and concentration of DNA in the extracts were checked by the nanodrop (NanoDrop 2000 spectrophotometer, Thermo Scientific™, Thermo-Fisher scientific, DE, USA).

Primers used and PCR conditions

Two types of primers were used in the present study. The first type of primers (either genus- or species-specific) were originally designed by Ali *et al*[11] in the tRNA-linked STR regions to amplify those of the EH HM-1:IMSS tRNA gene sequences (GenBank accession numbers BK005648-BK005672). Genus-specific primers of tRNA-linked STR of RTCT and NK1 arrays were used to amplify both EH and ED

DNAs, while species-speci?c primers of tRNA-linked STR of RTCT were designed to amplify DNAs from one species only (Table 1 & Figure S1). Nomenclature of these strain differentiation primers derived from the single-letter amino acid code for the relevant tRNA genes ?anking the STRs being ampli?ed. Consensus array unit sequences and STR organizations are shown in the link http://homepages.lshtm.ac.uk/entamoeba/units/units.htm. Accession nos. used in designing primers of the RTCT and NK1 tRNA-linked STR arrays are BK005654.1 and BK005655.1 for EH, while HQ439972.1 and EF421344.1 for ED, respectively (Figure S1). PCR was performed using a ready-to-use master mix (BioTaq Green Master Mix, Promega) with DNA concentration of ~50 ng and conditions were 95ºC/5 min (initial denaturation), 95ºC/30 sec, 55-58ºC/45 sec and 72ºC/1 min (36 cycles), 72ºC (final extension), then reaction was held at 4ºC. Amplicons were originally run on agarose gel (1.5% in 1x TBE buffer) and successful amplicons were run using polyacrylamide gel electrophoresis (PAGE)[14] to recover amplicons with higher resolution. Either gel type was stained with ethidium bromide (0.3 ug/ml), then visually examined with UV transilluminator and photographed using a CCD camera (UVP, Cambridge, UK).

In order to design the new species-specific primers, genomes of EH (https://www.ncbi.nlm.nih.gov/genome/27) and ED (https://www.ncbi.nlm.nih.gov/genome/372) were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/genome/?term=entamoeba). Contigs of the two genomes (https://www.ncbi.nlm.nih.gov/Traces/wgs/AAFB02?display=contigs, https://www.ncbi.nlm.nih.gov/Traces/wgs/AANV02?display=contigs) were downloaded from NCBI. Multiple contig alignment of the two genomes was done using NUCmer module 3.0 (NUCleotide MUMmer, part of mummer software) to determine the position and orientation of a set of sequence contigs, and to find all of the maximal unique matches of a given length between the two input sequences, a step to increase the overall coverage of the alignment[15]. Only about 100 contigs of each species showed partial matching in DNA sequences. Commands were made to recover a delta file, which was converted to a coords file. The latter file type is accessible by Excel (xlxs). The contig pairs with high similarity (~90%) and difference between aligned area with ~90 bp (sequence similarity/difference criteria) were selected in the recovered Excel file. Different groups of primers were detected in this study for contig pairs meeting the sequence similarity/difference criteria. PCR was performed using ready master mix (BioTaq Green Master Mix, Promega) and conditions were 95ºC/5 min (initial denaturation), 95ºC/30 sec, 52ºC/45 sec and 72ºC/1 min (40 cycles), 72ºC (final extension), then reaction was held at 4ºC. Amplicons were run on agarose gel, stained with ethidium bromide (0.3 ug/ml), then visually examined and photographed.

RESULTS

A number of 37 subjects (19 males and 18 females) aged between 1 and ? 50 years with background from more than seven

Table 1. Primers generated used for strain differentiation

tRNA primers	Sequence (5‘ to 3‘)	Annealing temp. (°C)
General primers		
R-R5	AGCATCAGCCTTCTAAGCTG	55
R-R3	CTTCCGACTGAGCTAACAAG	
N-K5	CGAACGGCTGTTAACCGTTA	55
N-K3	TTCCTAGCTCAGTCGGTAGA	
EH -specific primers		
RR-H5	GCGCCTTTTTATTCAATATACTCC	57
RR-H3	GGATGAAGATATCTTCACAGGG	
ED-specific primers		
RR-D5	CATGAGGCGCCTTTTTATCA	58
RR-D3	AGGGATGATGATATTGAACACACTC	

countries participated in the study (Table 2). Saudi individuals (~66%) and those of 1-10 years old (~46%) represented the most frequent subjects of the two categories (e.g., nationality and age) in the study. Double survey of infection in tRNA-linked STR arrays indicated that all these subjects are infected with Entamoeba (Figures 1 & 2). All types of tRNA-linked STRs with Entamoeba

Fig. S1. Sequences of the RTCT (a & b) and NK1 (c & d) tRNA arrays of the EH species with accession nos. BK005654.1 (a) and BK005655.1 (c) and ED species with accession nos

general primers generated by Ali *et al*[11] indicated that only two arrays, e.g., RTCT and NK1, out of the six arrays of STRs were successful in detecting the disease in the respective subjects (Figures 1 & 2). Gradient PCRs for either array (e.g., RTCT or NK1) were done in the present study to detect the best PCR thermal conditions and avoid the occurrence of false positives. The results indicated that annealing temperature previously indicated by Ali *et al*[11] is the best.

Analysis of STR markers with Entamoeba general primers

The expected number and sizes of amplicons to be generated by both types of markers, either general or species-specific, are shown in Table S1. For the general markers, numbers of three

Table 2. Detailed sociodemographic characteristics of the infectedsubjects living in Jeddah, Saudi Arabia

Serial no.	Sex	Nationality	Age (years old)	Species	Overall numbers	
1	Male	Syrian	1-10	ED	Gender	
2		Saudi			Male	19
3					Female	18
4				EH	Age (years old)	
5		Yamani			1-10	17
6		Saudi			11-20	1
7		Yamani	21-30	ED	21-30	7
8				EH	31-40	7
9		Saudi			41-50	3
10		Pakistani			>50	2
11		Egyptian	31-40	ED	Nationality	
12		Saudi			Saudi	22
13					Egyptian	5
14		Egyptian			Yemeni	5
15				EH	Pakistani	2
16		Saudi			Other	3
17		Bangladesh	41-50	ED	Infecting species	
18		Saudi		EH	EH	22
19			>50		ED	12
20	Female	Saudi	1-10	ED	EH/ED	3
21						
22		Yamani		EH		
23						
24		Philippian				
25		Saudi				
26				ED		
27				EH		
28						
29						
30						
31			11-20	ED		
32		Pakistani	21-30	EH		
33		Saudi				
34						
35			31-40	ED		
36		Egyptian	41-50	EH		
37			>50			

Percentage of non-Saudi male subjects is 47.4% as compared to Saudi (52.6%)
Percentage of non-Saudi female subjects is 33.3% as compared to Saudi (66.7%)

and six amplicons in RTCT array of EH (e.g., 678, 686, 694 bp) and ED (e.g., 586, 686, 696, 702, 712, 728 bp), and numbers of two and one amplicons in NK1 array of EH (e.g., 527, 598 bp) and ED (e.g., 597 bp), respectively, were generated in Entamoeba sp. strains available in the NCBI (https://blast.ncbi.nlm.nih.gov/). This data scopes the light on the divergence of Entamoeba sp. strains in the linked-tRNA STR arrays. As our subjects are from different geographic backgrounds, we expected to detect several new strains in the studied subjects.

Banding patterns of either marker in the present study indicated the occurrence of several common and specific amplicons (Figures 1 & 2). We can simply explain the occurrence of specific amplicons that indicate the existence of new strains

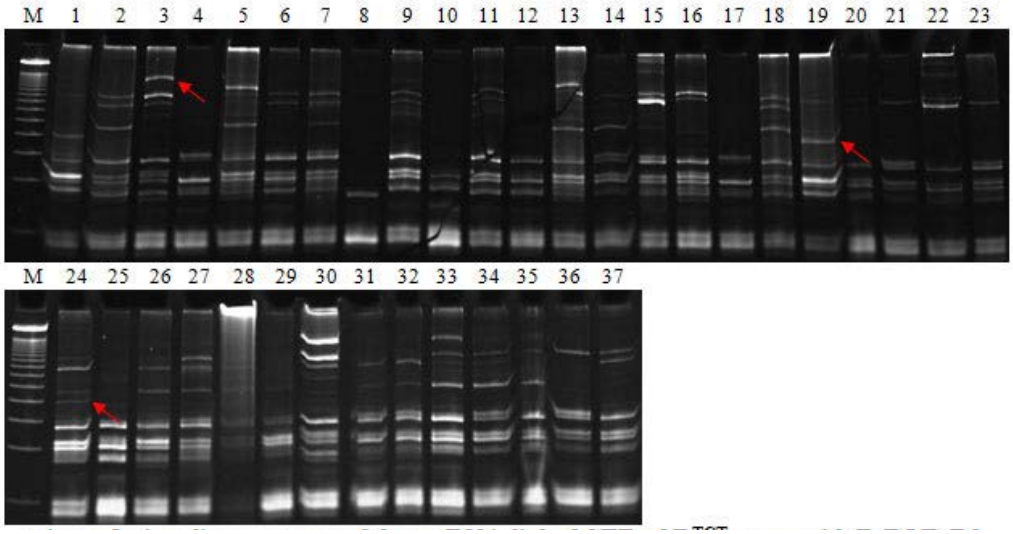

Fig. 1. Amplicons generated from tRNA-linked STR of RTCT array with R-R5/R-R3 primers for subjects infected with either Entamoeba species

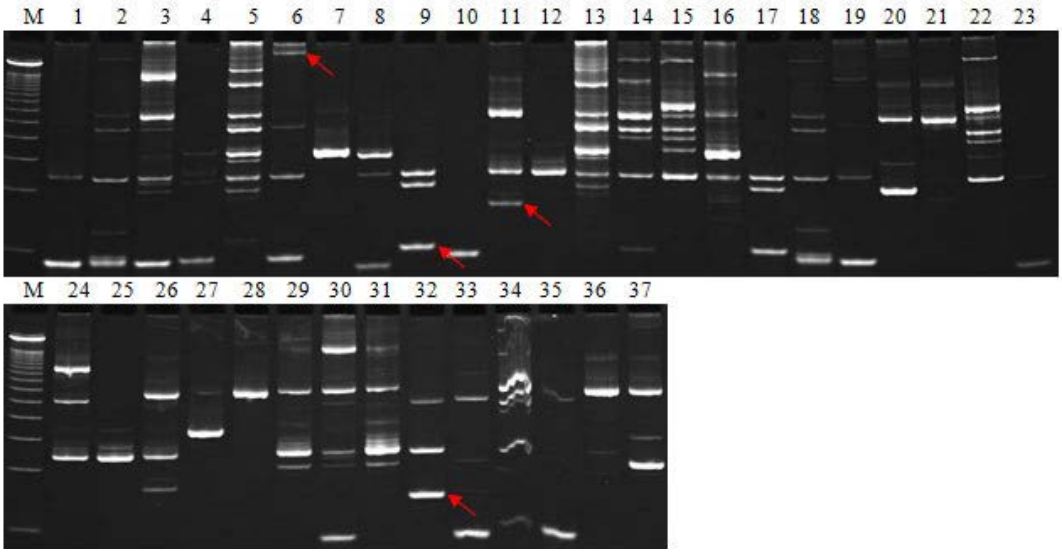

Fig. 2. Amplicons generated from tRNA-linked STR of NK1 array with N-K5/N-K3 primers for subjects infected with either Entamoeba species. Sociodemographic characteristics details of different subjects are shown in Table 2. M=100 bp ladder. Specific markers are indicated by the red arrows

Table S1. Primer pairs generated to amplify short tandem repeats (STRs) regions of the tRNA arrays either in Entamoeba genus (general primers, e.g., R-R5/R-R3, N-K5/N-K3) or in a given Entamoeba species

Primer pair R-R5/R-R3 (general) of R^{TCT} array

	Sequence (5'->3')	Length
Forward primer	AGCATCAGCCTTCTAAGCTG	20
Reverse primer	CTTCCGACTGAGCTAACAAG	20

Products on target templates: EH (678, 686, 694 bp), ED (586, 686, 696, 702, 712, 728 bp)

>AY843014.1 Entamoeba histolytica strain IULA:0593:2 array unit [R(TCT)]encoding tRNA-ArgTCT genomic sequence
product length = 678
Forward primer 1 AGCATCAGCCTTCTAAGCTG 20
Template 1 20
Reverse primer 1 CTTCCGACTGAGCTAACAAG 20
Template 678 659

>AY843012.1 Entamoeba histolytica strain Rahman array unit [R(TCT)]encoding tRNA-ArgTCT genomic sequence
product length = 678
Forward primer 1 AGCATCAGCCTTCTAAGCTG 20
Template 1 20
Reverse primer 1 CTTCCGACTGAGCTAACAAG 20
Template 678 659

>AY843011.1 Entamoeba histolytica strain MS34-199 array unit [R(TCT)]encoding tRNA-ArgTCT genomic sequence
product length = 686
Forward primer 1 AGCATCAGCCTTCTAAGCTG 20
Template 1 20
Reverse primer 1 CTTCCGACTGAGCTAACAAG 20
Template 686 667

>AY843013.1 Entamoeba histolytica strain 200:NIH array unit [R(TCT)]encoding tRNA-ArgTCT genomic sequence
product length = 694
Forward primer 1 AGCATCAGCCTTCTAAGCTG 20
Template 1 20
Reverse primer 1 CTTCCGACTGAGCTAACAAG 20
Template 694 675

>HQ439967.1 Entamoeba dispar isolate NH_9IR tmR-tmR intergenic spacer, partial sequence
product length = 586
Forward primer 1 AGCATCAGCCTTCTAAGCTG 20
Template 1 20
Reverse primer 1 CTTCCGACTGAGCTAACAAG 20
Template 586 567

>HQ439961.1 Entamoeba dispar isolate NH_3IR tmR-tmR intergenic spacer, partial sequence
product length = 586
Forward primer 1 AGCATCAGCCTTCTAAGCTG 20
Template 1 20
Reverse primer 1 CTTCCGACTGAGCTAACAAG 20
Template 586 --.................. 567

>AF525284.1 Entamoeba dispar tRNA-Arg genes, partial sequence
product length = 686
Forward primer 1 AGCATCAGCCTTCTAAGCTG 20
Template 1 20
Reverse primer 1 CTTCCGACTGAGCTAACAAG 20
Template 686 667

>HQ439972.1 Entamoeba dispar isolate NH_14IR tmR-tmR intergenic spacer, partial sequence
product length = 696
Forward primer 1 AGCATCAGCCTTCTAAGCTG 20
Template 1 20
Reverse primer 1 CTTCCGACTGAGCTAACAAG 20
Template 696 --.................. 677

>HQ439968.1 Entamoeba dispar isolate NH_10IR tmR-tmR intergenic spacer, partial sequence
product length = 702
Forward primer 1 AGCATCAGCCTTCTAAGCTG 20
Template 1 20
Reverse primer 1 CTTCCGACTGAGCTAACAAG 20
Template 702 683

>HQ439970.1 Entamoeba dispar isolate NH_12IR tmR-tmR intergenic spacer, partial sequence
product length = 712

```
Forward primer   1  AGCATCAGCCTTCTAAGCTG   20
Template         1  ....................   20
Reverse primer   1    CTTCCGACTGAGCTAACAAG  20
Template       710  --....................  693
```

>HQ439959.1 Entamoeba dispar isolate NH_1IR tmR-tmR intergenic spacer, partial sequence

```
product length = 725
Forward primer   1  AGCATCAGCCTTCTAAGCTG   20
Template         1  ....................   20
Reverse primer   1    CTTCCGACTGAGCTAACAAG  20
Template       726  --....................  709
```

Primer pair N-K5/N-K3 (general) of NK1 array

	Sequence (5'⇒3')	Length
Forward primer	CGAACGGCTGTTAACCGTTA	20
Reverse primer	TTCCTAGCTCAGTCGGTAGA	20

Products on target templates: EH (527, 598 bp), ED (597 bp)

>BK005655.1 TPA: Entamoeba histolytica tRNA-encoding array unit NK1

```
product length = 527
Forward primer   1   CGAACGGCTGTTAACCGTTA   20
Template        25   ....................   44
Reverse primer   1    TTCCTAGCTCAGTCGGTAGA  20
Template       551   ....................  532
```

>BK005656.1 TPA: Entamoeba histolytica tRNA-encoding array unit NK2

```
product length = 595
Forward primer   1   CGAACGGCTGTTAACCGTTA   20
Template        25   ....................   44
Reverse primer   1    TTCCTAGCTCAGTCGGTAGA  20
Template       622   ....................  603
```

>AY842975.1 Entamoeba dispar strain SAW 760 array unit [NK], between tRNA-AsnGTT and tRNA-LysCTT genomic sequence

```
product length = 597
Forward primer   1  CGAACGGCTGTTAACCGTTA   20
Template         1  ....................   20
Reverse primer   1  TTCCTAGCTCAGTCGGTAGA   20
Template       597  ....................578
```

>EF421344.1 Entamoeba dispar strain SAW760 tRNA array unit NK genomic sequence

```
product length = 597
Forward primer   1   CGAACGGCTGTTAACCGTTA  20
Template        25   ....................  44
Reverse primer   1   TTCCTAGCTCAGTCGGTAGA  20
Template       621   ....................602
```

Primer pair RR-H5/RR-H3 (EH-specific) of R^{TCT} array

	Sequence (5'⇒3')	Length
Forward primer	GCGCCTTTTTATTCAATATACTCC	24
Reverse primer	GGATGAAGATATCTTCACAGGG	22

Products on target templates: EH (477, 485, 557, 565, 573, 581, 589, 597, 605 bp)

>EF421386.1 Entamoeba histolytica strain LAID-02 tRNA array unit STR R-R type 12RR genomic sequence

```
product length = 477
Forward primer   1  GCGCCTTTTTATTCAATATACTCC   24
Template         1  ........................   24
Reverse primer   1    GGATGAAGATATCTTCACAGGG   22
Template       477  ........................  456
```

>EF421385.1 Entamoeba histolytica strain 1057-D811 tRNA array unit STR R-R type 11RR genomic sequence

```
product length = 477
Forward primer   1  GCGCCTTTTTATTCAATATACTCC   24
Template         1  ........................   24
Reverse primer   1    GGATGAAGATATCTTCACAGGG   22
Template       477  ........................  456
```

>EF421384.1 Entamoeba histolytica strain 3646-D89 tRNA array unit STR R-R type 10RR genomic sequence

```
product length = 455
Forward primer   1  GCGCCTTTTTATTCAATATACTCC   24
Template         1  ........................   24
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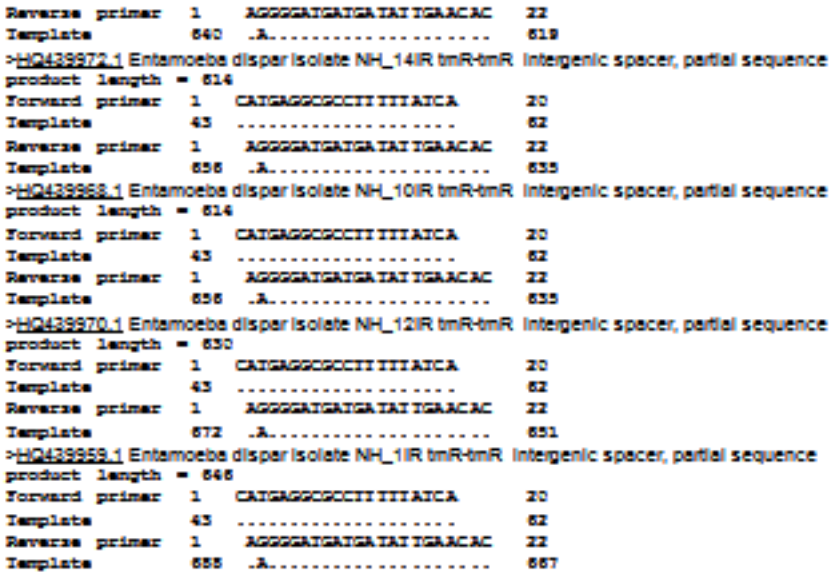
```
Reverse primer   1    GGATGAAGATATCTTCACAGGG   22
Template         455  ........................  434
>EF421383.1 Entamoeba histolytica strain LAID-04 tRNA array unit STR R-R type 9RR genomic sequence
product length = 557
Forward primer   1  GCGCCTTTTTATTCAATATACTCC   24
Template         1  ........................   24
Reverse primer   1    GGATGAAGATATCTTCACAGGG   22
Template         557  ........................  536
>EF421380.1 Entamoeba histolytica strain SA46 tRNA array unit STR R-R type 6RR genomic sequence
product length = 565
Forward primer   1  GCGCCTTTTTATTCAATATACTCC   24
Template         1  ........................   24
Reverse primer   1    GGATGAAGATATCTTCACAGGG   22
Template         565  ........................  544
>EF421377.1 Entamoeba histolytica strain YS-27 tRNA array unit STR R-R type 3RR genomic sequence
product length = 573
Forward primer   1  GCGCCTTTTTATTCAATATACTCC   24
Template         1  ........................   24
Reverse primer   1    GGATGAAGATATCTTCACAGGG   22
Template         573  ........................  552
>EF421379.1 Entamoeba histolytica strain LAID-29 tRNA array unit STR R-R type 5RR genomic sequence
product length = 551
Forward primer   1  GCGCCTTTTTATTCAATATACTCC   24
Template         1  ........................   24
Reverse primer   1    GGATGAAGATATCTTCACAGGG   22
Template         551  ........................  560
>EF421376.1 Entamoeba histolytica strain LAID-01 tRNA array unit STR R-R type 2RR genomic sequence
product length = 551
Forward primer   1  GCGCCTTTTTATTCAATATACTCC   24
Template         1  ........................   24
Reverse primer   1    GGATGAAGATATCTTCACAGGG   22
Template         551  ........................  560
>AY843014.1 Entamoeba histolytica strain IULA:0593:2 array unit [R(TCT)]encoding tRNA-ArgTCT genomic
sequence
product length = 551
Forward primer   1    GCGCCTTTTTATTCAATATACTCC  24
Template         49  ........................  72
Reverse primer   1    GGATGAAGATATCTTCACAGGG   22
Template         629  ........................  608
>AY843012.1 Entamoeba histolytica strain Rahman array unit [R(TCT)]encoding tRNA-ArgTCT genomic sequence
product length = 551
Forward primer   1    GCGCCTTTTTATTCAATATACTCC  24
Template         49  ........................  72
Reverse primer   1    GGATGAAGATATCTTCACAGGG   22
Template         629  ........................  608
>AY843011.1 Entamoeba histolytica strain M834-199 array unit [R(TCT)]encoding tRNA-ArgTCT genomic sequence
product length = 559
Forward primer   1    GCGCCTTTTTATTCAATATACTCC  24
Template         49  ........................  72
Reverse primer   1    GGATGAAGATATCTTCACAGGG   22
Template         637  ........................  616
>EF421375.1 Entamoeba histolytica strain 462 tRNA array unit STR R-R type 1RR genomic sequence
product length = 559
Forward primer   1  GCGCCTTTTTATTCAATATACTCC   24
Template         1  ........................   24
Reverse primer   1    GGATGAAGATATCTTCACAGGG   22
Template         559  ........................  568
>BK005654.1 TPA: Entamoeba histolytica tRNA-encoding array unit R^TCT
product length = 559
Forward primer   1    GCGCCTTTTTATTCAATATACTCC  24
Template         71  ........................  94
Reverse primer   1    GGATGAAGATATCTTCACAGGG   22
Template         659  ........................  638
```

>EF421378.1 Entamoeba histolytica strain 199-M834 tRNA array unit STR R-R type 4RR genomic sequence
product length = 589
Forward primer 1 GCGCCTTTTTATTCAATAIACICC 24
Template 1 24
Reverse primer 1 GGATGAAGATATCTTCACAGGG 22
Template 589 568
>EF421382.1 Entamoeba histolytica strain 3514-M843 tRNA array unit STR R-R type 8RR genomic sequence
product length = 597
Forward primer 1 GCGCCTTTTTATTCAATAIACICC 24
Template 1 24
Reverse primer 1 GGATGAAGATATCTTCACAGGG 22
Template 597 576
>AY843013.1 Entamoeba histolytica strain 200:NIH array unit [R(TCT)]encoding tRNA-ArgTCT genomic sequence
product length = 597
Forward primer 1 GCGCCTTTTTATTCAATAIACICC 24
Template 49 72
Reverse primer 1 GGATGAAGATATCTTCACAGGG 22
Template 645 624
>EF421381.1 Entamoeba histolytica strain J4 tRNA array unit STR R-R type 7RR genomic sequence
product length = 605
Forward primer 1 GCGCCTTTTTATTCAATAIACICC 24
Template 1 24
Reverse primer 1 GGATGAAGATATCTTCACAGGG 22
Template 605 584

Primer pair RR-D5/RR-D3 (ED-specific) of R^{TCT} array

	Sequence (5'->3')	Length
Forward primer	CATGAGGCGCCTTTTTATCA	20
Reverse primer	AGGGATGATGATATTGAACAC	22

Products on target templates: ED (272, 598, 614, 630, 634, 646 bp)
>KJ149296.1 Entamoeba dispar isolate PRIR1 tmR-tmR intergenic spacer, partial sequence
product length = 272
Forward primer 1 CATGAGGCGCCTTTTTATCA 20
Template 270 T..... 259
Reverse primer 1 AGGGGATGATGATATTGAACAC 22
Template 541 520
product length = 634
Forward primer 1 CATGAGGCGCCTTTTTATCA 20
Template 270 T..... 259
Reverse primer 1 AGGGGATGATGATATTGAACAC 22
Template 900 ---..... 882
>HQ439967.1 Entamoeba dispar isolate NH_9IR tmR-tmR intergenic spacer, partial sequence
product length = 495
Forward primer 1 CATGAGGCGCCTTTTTATCA 20
Template 43 62
Reverse primer 1 AGGGGATGATGATATTGAACAC 22
Template 540 .A..... 519
>HQ439961.1 Entamoeba dispar isolate NH_3IR tmR-tmR intergenic spacer, partial sequence
product length = 495
Forward primer 1 CATGAGGCGCCTTTTTATCA 20
Template 43 62
Reverse primer 1 AGGGGATGATGATATTGAACAC 22
Template 540 .A..... 519
>EF421343.1 Entamoeba dispar strain SAW760 tRNA array unit ArgTCT genomic sequence
product length = 595
Forward primer 1 CATGAGGCGCCTTTTTATCA 20
Template 65 56
Reverse primer 1 AGGGGATGATGATATTGAACAC 22
Template 662 .A..... 641
>AF525284.1 Entamoeba dispar tRNA-Arg genes, partial sequence
product length = 595
Forward primer 1 CATGAGGCGCCTTTTTATCA 20
Template 43 62



but we found it difficult to explain the occurrence of several common bands except that the two sets of primers mismatch with regions other than the two specified arrays in Entamoeba species or in organisms other than Entamoeba that surely exist in the fecal sample. Results of the RTCT array in the present study indicated the existence of three strain-specific markers in subjects 3 (>900 bp), 19 (~380 bp) and 24 (~400 bp). Amplicon sizes of these possibly new strains match none of those available in the NCBI link. The first two subjects are Saudi aged 1-10 and >50 years old, while the third is Philippian aged 1-10 years old (Figure 1). Results of the NK1 array indicated the existence of four strain-specific markers in subjects 6 (~1500 bp), 9 (~180 bp), 11 (~50 bp) and 32 (~160 bp). The first two subjects are Saudi aged 1-10 and 21-30 years old, while the third is Egyptian aged 31-40 years old and the forth is Pakistani aged 21-30 years old (Figure 2).

Analysis of STR markers with Entamoeba species-specific primers

For the species-specific markers in Table S1, numbers of nine and six amplicons in RTCT array of EH (e.g., 477, 485, 557, 565, 573, 581, 589, 597, 605 bp) and ED (e.g., 272, 598, 614, 630, 634, 646 bp), respectively, were generated in Entamoeba sp. strains available in the NCBI (https://blast.ncbi.nlm.nih.gov/). Based on the studied tRNA-linked STR of RTCT with species-specific primers (e.g., EH or ED) in the present study, 14 out of all subjects interestingly infected with symptomic ED strain(s). This number represents ~38% of the studied cases (Table 2).

Results of the RTCT array of EH-infected subjects indicated the possible existence of two strain-specific markers in subjects 18 (>180 bp) and 24 (~400 bp) (Figure 3). The latter marker was detected for the same subject (Philippian) using general primer of the same array (Figure 1), while the first marker was generated for a Saudi subject aged 31-40 years old (Figure 3). Amplicon sizes of these possibly new strains match none of the nine EH strains available in the NCBI link. On the other hand, results of RTCT array in ED-infected subjects indicated the possible existence of as high as 10 new strain-specific markers in eight subjects (Figure 4). Marker sizes are 900 bp for subject 1, 200 bp for subject 2, 1300 bp for subject 3, 380 bp for subject 11, 200 bp for subject 12, 1400 and 280 bp for subject 14, 350 bp for subject 20 and 100 and 120 bp for subject 26. Two of these subjects are Egyptian (11 and 14), while one (1) is Syrian, while the other six subjects are Saudi (Table 2). Six of these markers are for subjects aged 1-10 years old, while four markers are for subject aged 31-40

years old (Figure 4). Markers of these possibly new strains match none of those available in the NCBI link, except for the marker sized ~280 bp of the Egyptian subject 14 that matches the size of the marker of ED isolate PRIR1 (272 bp) available in the NCBI link.

It is likely to detect Entamoeba strains with specific markers in Egyptian (four markers), Syrian (one marker), Philippian (one marker), and Pakistani (one marker) individuals, but this is unlikely for Saudi individuals unless they were originally infected while they are abroad. Egyptian individuals are strong candidate of disease transmission to the Saudi habitat. Possible new strain-specific markers frequently found for subjects aged 1-10 years old across nationalities. Overall data indicate the possible occurrence of new strains in 14 out of the 37 subjects of which 10 of them are males (subjects 1, 2, 3, 6, 9, 11, 12, 14, 18, and 19), while four are females (subjects 20, 24, 26 and 32). This indicates that males are higher transmitter of the new strains than female.

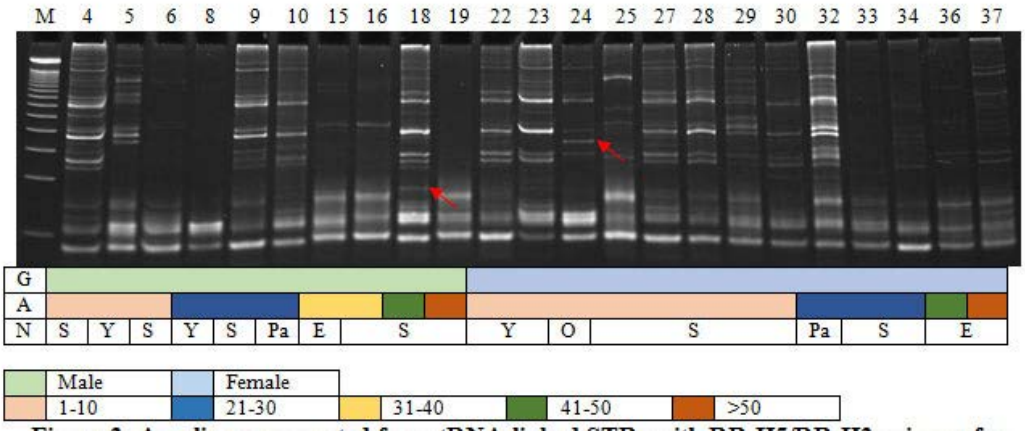


Fig. 3. Amplicons generated from tRNA-linked STRs with RR-H5/RR-H3 primers for subjects infected with EH. Sociodemographic characteristics details of different subjects are shown in Table 2. G=Gender, A=age (in years), N=Nationality, S=Saudi, E=Egyptian, Y=Yemeni, Pa=Pakistan, O=others. M=100 bp ladder. Specific markers are indicated by the red arrows

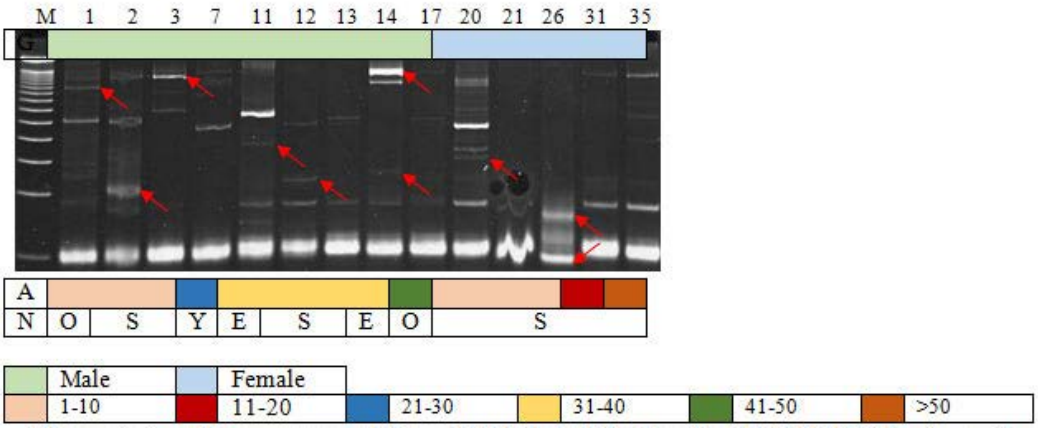


Fig. 4. Amplicons generated from tRNA-linked STRs with RR-D5/RR-D3 primers for subjects infected with ED. Sociodemographic characteristics details of different subjects are shown in Table 2. G=Gender, A=age (in years), N=Nationality, S=Saudi, E=Egyptian, Y=Yemeni, Pa=Pakistan, O=others. M=100 bp ladder. Specific markers are indicated by the red arrows

Table S3. Statistics of paired alignment of two selected contigs of the two genomes of ED (NW_001855238.1) and EH (NW_001915012.1) using Nucmer module (NUCleotide MUMmer, part of mummer software). The contig pair showed high similarity (~90%) in three areas with ~4900, 4000 and 5700 bp total size. The difference in sizes of the three areas of the two contigs are 58, 126 and 28 bp, respectively. The area with 4900 bpwith the largest difference of 126 bp, then the region within this area with the longest indel of 45 bp were selected. Sequences flanking this indel was utilized in designing primersforspecies-specific marker detection to generate 217 and 262 bp for the ED and EH contigs, respectively.

S1	E1	length R (ED)	S2	E2	length Q (EH)	difference (ED-EH)	% IDY	[LEN R]	[LEN Q]	[COV R]	[COV Q]	contig no. (ED)	contig no. (EH)
10179	15042	4863	27301	22380	-4921	58	89.41	41758	39477	11.65	12.47	NW_001855238.1	NW_001915012.1
15513	19503	3990	21944	17828	-4116	126	87.11	41758	39477	9.56	10.43	NW_001855238.1	NW_001915012.1
19774	25478	5704	17692	11960	-5732	28	91.04	41758	39477	13.66	14.52	NW_001855238.1	NW_001915012.1

[S1] start of the alignment region in the reference sequence (ED)
[E1] end of the alignment region in the reference sequence (ED)
[S2] start of the alignment region in the query sequence (EH)
[E2] end of the alignment region in the query sequence (EH)
[LEN 1] length of the alignment region in the reference sequence (ED)
[LEN 2] length of the alignment region in the query sequence (ED)
[% IDY] percent identity of the alignment (ED/EH)
[LEN R] length of the reference sequence (ED)
[LEN Q] length of the query sequence (EH)
[COV R] percent alignment coverage in the reference sequence (ED)
[COV Q] percent alignment coverage in the query sequence (EH)

Analysis of the new approach of species differentiation in Entamoeba

The new type of species-specific markers was developed to overcome the complications of the STR-based species differentiation method. In addition, we speculate that primers used for the tRNA-linked STR markers might mismatch with organisms other than Entamoeba existing in the fecal sample. However, we have followed an approach that is not devoid the same assumption. Therefore, we have developed five sets of primer pairs utilizing our new approach in order to choose the one that clearly matches with both Entamoeba species with no other amplicon's background.

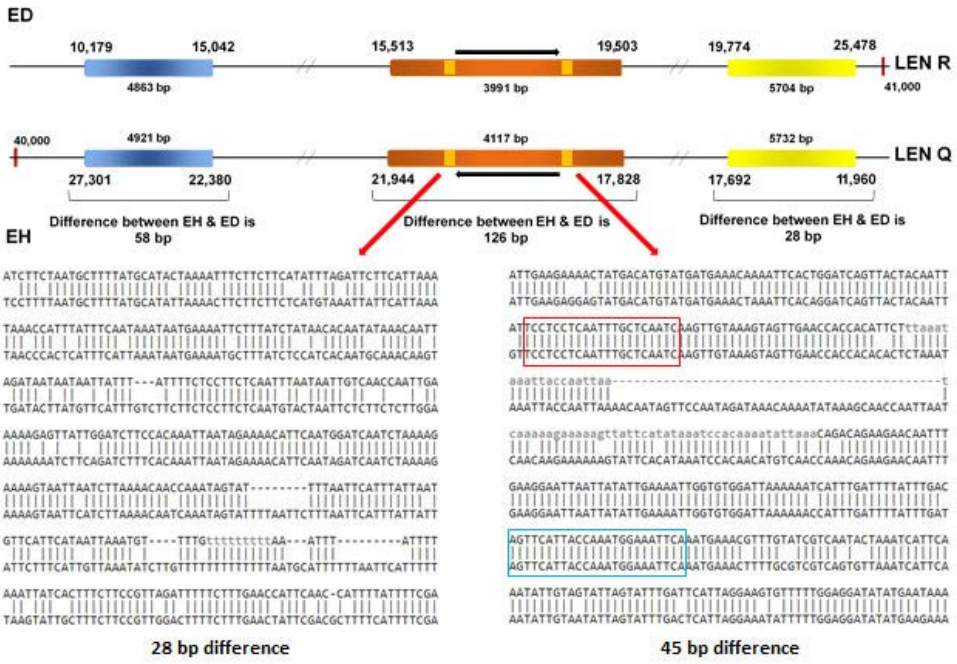


Fig. 5. A model for the approach used in selecting contigs of the ED and EH genomes for generating the new type of species-specific markers

Table 3. Contigs of EH and ED meeting the criteria of selecting contig pairs from multiple sequence alignment of contigs of the two genomes using nucmer module (part of mummer software)

Contig pair no.	Species	Contig accession no.	Contig length (bp)	Diff. (bp)	Similarity (%)
1	ED	NW_001854967.1	13230	128	91.88
	EH	NW_001914891.1	13102		
2	ED	NW_001855840.1	12450	180	91.14
	EH	NW_001915091.1	12630		
3	ED	NW_001853623.1	3840	111	89.99
	EH	NW_001915426.1	3951		
4	ED	NW_001854085.1	5132	107	90.01
	EH	NW_001915137.1	5025		
5	ED	NW_001855238.1	3991	126	87.11
	EH	NW_001915012.1	4117		

In the new approach, genomes of EH and ED were retrieved from NCBI

(https://www.ncbi.nlm.nih.gov/genome/?term=entamoeba). Multiple contig alignment of the two genomes was done using NUCmer module (part of mummer 3.0 software, http://mummer.sourceforge.net/). This module is the most user-friendly alignment script for standard DNA sequence alignment. It is used to determine the position and orientation of a set of sequence contigs in relation to a finished sequence. The program is a three-step process comprising maximal exact matching, match clustering, and alignment extension. It searches the maximal unique matches of a given length between the two input sequences. Then, individual matches are clustered into closely grouped sets and the non-exact sequence between matches is aligned via a modified Smith-Waterman algorithm.

Commands were made to recover a delta file, which is converted to a coords file. The latter is accessible by Excel (xlxs). As outputs of the analysis, [S1] is the start of the alignment region, while [E1] is the end of the alignment region in the ED contig. [S2] is the start of the alignment region, while [E2] is the end of the alignment region of EH contig. [LEN 1] is the length of the alignment region in the ED contig, while [LEN 2] is the length of the alignment region in the EH contig. [% IDY] is the percent identity of the alignment. [LEN R] is the length of the ED contig, while [LEN Q] is the length of the EH contig. [COV R] is the percent

Table 4. Primers designed based on contig pair analysis of EH and ED meeting the criteria of selecting contig pairs from multiple contig alignment of the EH and ED genomes using nucmer module (part of mummer software)

Contig pair no.	Name	Sequence	Product size (bp) ED	EH
1	P1_HD-F	AAAwCTTTCTTyrACTTCTTCTTCC	424	531
	P1_HD-R	TTTAGGTTTTTCAGTTGCCAATC		
2	P2_HD-F	CATTGACTTTCAGGAGGrAATTG	423	476
	P2_HD-R	YTGYTYTGCTTTTAAAGCATGG		
3	P3_HD-F	GCTACTTTCAGACACTTAACAAATC	491	552
	P3_HD-R	CCAAGAGAAATATGAACACATTTYC		
4	P4_HD-F	TGCCATTCAATAyCGTCTTTG	410	353
	P4_HD-R	AAATCCACAGTGATGAAATAACTTG		
5	P5_HD-F	TCCTCCTCAATTTGCTCAATC	217	262
	P5_HD-R	TGAATTTCCATTTGGTAATGAACT		

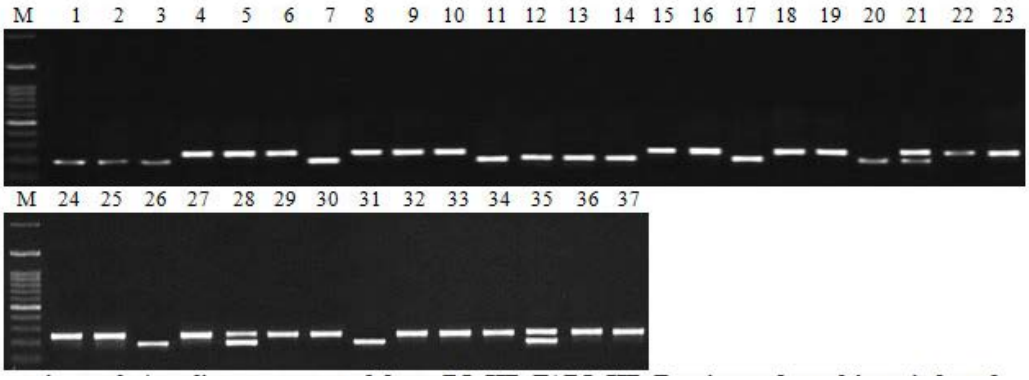


Fig. 6. Amplicons generated from P5_HD-F/ P5_HD-R primers for subjects infected with either Entamoeba species. Amplicons with 262 bp were generated from EH-infected subjects, while those with 217 bp were generated from subjects with ED. Cases of the two amplicons indicate the coinfection of respectivesubjects (e.g., 21, 28 and 35). Sociodemographic characteristics details of different subjects are shown in Tables 1 and 2. M=100 bp ladder

alignment coverage in the ED contig, while [COV Q] is the percent alignment coverage in the EH contig.

Table S2 indicates the contig pairs of ED and EH ~90 bp difference and similarity between aligned area of ~90% were selected in the recovered Excel file. The selection criteria secures that species-specific primers to be designed should pair with both EH and ED contig sequences at completely homologous regions of 200-500 bp of both species except for the presence of indels that result in the amplification of DNA fragments of the two species with difference in sequence length. Five candidate contig pairs of ED and EH were found suitable for primer design in which each primer pair can amplify different product sizes for the two species (Table 3). Primers generated from these five pairs of contigs are shown in Table 4. A model of the alignment is shown in Table S3 and described in Figure 5 of which two contigs of ED (NW_001855238.1) and EH (NW_001915012.1) showed three aligned regions with size differences of 28, 58 and 126 bp. The region with the largest difference, e.g., 126 bp, was selected for primer design. In the model case, a difference of 45 bp length was recovered between amplicons of the two species (Table S3 & Figure 5).

Figure 6 indicates the amplicons generated from P5_HD-F/ P5_HD-R primers for the 37 subjects. The figure indicates a single amplicon for each subject referring to ED (217 bp) or EH (262 bp) aligning with the results of the tRNA-linked STR approach (Figures 3 & 4), except for the three female subjects, e.g., 21, 27 and 35 where two amplicons referring to the two species were generated. The latter results indicates the coinfection with the two species in the three subjects. This conclusion was not reached using the tRNA-linked STR approach. We hypothesize that if other species-specific primers following the new approach were used, other species of Entamoaba might be found as a coinfection. The new approach has overcome the problem of primer mismatches and the occurrence of non-specific priming that could not be overcome utilizing the tRNA-linked STR approach despite our efforts to adjust the annealing temperatures. The major problem with the non-specific priming is the inability to recognize the right from the false products, thus, cannot refer a certain amplicon with a certain size to a certain species. The new approach is important in resolving this problem at the species level, while the tRNA-linked STR approach can still be a good choice for strain differentiation.

DISCUSSION

Strain differentiation of EH and ED are important in getting a better insights on the mysterious virulence of this parasite. Ali *et al*[11] indicated that symptomatic and asymptomatic infections can be caused by genetically distinct strains. The STR-based strain differentiation might be a good approach in addressing unanswered questions on virulence of the parasite. Ali *et al*[11] claimed that they were able to detect a unique feature of these two species and eliminated the potential problems caused by mixed infections or cultures. However, BLAST analysis for the first type of primers used in the present study indicates similar-sized amplicons of EH and ED. The latter conclusion indicates the possible confusion in characterizing the parasite in case of coinfection. Additionally, Feng *et al*[16] detected seven different genotypes in ED from eight samples by sequence analysis of tRNA-linked short tandem repeats. These different genotypes were found within the same family. Therefore, we suggest that it is important first to use a more accurate species-specific markers way before studying strain differentiation and parasitology. Ali *et al*[11] also claimed that they were able to distinguish clearly between the two species in fecal samples and eliminate the need for culturing the parasite using their approach. Our results indicated that the STR approach failed to clearly prove the existence of only one species in the studied samples as several amplicons were recovered for the different primers (either genus- or specific-primers) used with similar sizes in both species. Our results did not eliminate the possibility of coinfection in our samples.

There are several reports that address the high degree of STR length polymorphism among EH strains, even when the strains isolated from a restricted geographic location[13,17,18]. On top of this, our results indicated the existence of several amplicons that are common in subjects with diverse genetic makeup, therefore, we do not eliminate the possibility of having polymorphism in a given STR of the same parasite. The latter observations

makes the use of strain differentiation alone more complicated and reject the claim of detecting patterns of transmission of this important disease and the epidemiological links between individual infection via the use of STR approach. Ghosh *et al*[19] reported the occurrence of recombinational loss of a ribosomal DNA unit from the circular episome of Entamoeba histolytica HM-1:IMSS. Consequently, there is a possibility that recombinational gain can take place in circular episomes or in circular structures of tRNA as we claim. Investigations of EH genotypes in South Africa[18] and Vietnam[20] indicated similar STR patterns over the course of the same infection. These results are not controversy to ours as these patterns can still hold the possibility of having polymorphism not only among different STRs, but also within a given STR. This phenomenon is similar to the phenomenon of heteroplasmy in mitochondrial genomes. We hypothesize that if other species-specific primers following the new approach were used, other species of Entamoaba might be found as a coinfection. Finally, our results recommend the use of the new approach in species differentiation, while use tRNA-linked STR approach in strain differentiation.

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