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Molecular Identification for *Entamoeba* Species by Gene Sequence in Thi_Qar Province / Iraq

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Abstract

Background: Microscopically, E.moshkovskii and E. dispar cannot be distinguished from the pathogenic species E. histolytica. A common molecular biology approach for amplifying many targets simultaneouslyis polymerase chain reaction (PCR). Methods: The use of various DNA extraction techniques in PCR assays for the identification and differentiation of the three-microscopy indistinguishable Entamoeba species in humans was investigated. The small-subunit rRNA gene's midsection was used to create a conserved forward primer, and reverse primers were created using signature sequences unique to each of thes three Entamoebaspecies. Results : The current Study used PCR for the 296 microscopically positive patients .the molecular study showed that of 144 (48.64%) were E. histolytica, 91(30.74%) were E.dispar and 52(17.56) were E.moskovski.and association of E.histolytica an E.dispar was recorded for 3.04% .and gene Sequence.

Introduction

Amoebiasis is one of the invasive extraintestinal or intestinal infections disease , affects less than 50 million individual all over the world with in 100,000 cases of death per yaer. (1). More than infections resulting from *Entamoeba histolytica*, which impairs human mortality and morbidity (2), (3). According to (2) either infection pathogenic or commensal intestinal parasites. *Entamoeba.Spp* have identical morphologies, *E. moshkovskii* and *E. dispar* are non pathogenic (4,5). amoebiasis cases, the reports from various parts worldwide,are asymptomatic according to (6)(7)(8).

Stool samples were traditionally examined under a microscope in order to identify Entamoeba species in human stool. This technique, , is unable to distinguish between commensal *E. dispar*, pathogenic *E. histolytica*, and ubiquitous *E. moshkovskiia* new species (9). In order to distinguish such amoebae, molecular techniques are required (10), (11). Polymerase chain reaction (PCR) assays were utilized in

the majority of molecular investigations to date on Entamoeba species to identify *E. dispar* and *E. histolytica,* but detection of *E. moshkovskii* was disregarded (10). In order to quickly detect and identify such three Entamoeba species, a nested multiplex PCR test has been devised (12). According to latest research, *E. moshkovskii* is the cause of gastrointestinal diseases (GIDs), and humans could be the ideal hosts for such Entamoeba (5), (13). Additionally, other investigations(14), (2) have shown a connection between clinical symptoms and E. dispar. Tissue lysis and proteolysis are a result of the protozoan parasite *E.histolytica*, which is a non-flagellated, pseudopod-forming parasite. It is regarded as the 2nd or 3rd most frequent cause of death among parasite infections and is thought to be the most aggressive protozoan disease which attacks the human bowels (15) .

Collection of stool Samples:

Area of investigation and sample gathering A significant province in southern Iraq is Thi-Qar. The location is at 31°14'N 46°19'E. Its overall area is 12,900 km2, and its population is close to 2 million. With the provinces of Basrah, Missan, Wassit, and Muthanna, it shares internal borders. The province's capital is Nasiriyah. Thi-Qar Province has a short winter and a long, hot summer with a decreasing annual rainfall rate. . Patient records at the Public Health Department of the Thi-Qar Health Office were used to collect all medical data about amoebiasis in the Thi-Qar Province for the last 12 years (from the beginning of January 2010 to the end of December 2021), including sex, date, age, and residential region. Amoebiasis patients were diagnosed by direct smears. The molecular analyses were carried out between the beginning of February 2021 and the end of October 2021 at the Mohammed Al-Mosawy and AL-Chebish Hospitals. All ages are treated in these hospitals. In order to look for trophozoites and/or cysts in the stool, 80 randomly selected stool samples from individuals with diarrhea were analyzed (general stool examination). In a -20°C refrigerator, each of the 80 samples was stored in a stool container.

General stool examination:

A: Macroscopic Examination: The stool samples were examined with naked eye to detected color, appearance, blood, mucus and smile. B. Microscopic Examination:Direct wet mount method were using for detected trophozoits and cysts by using fresh stool to examined under microscope as follow: Put on slid drop of normal saline (0.9% NaCl) on the center of the slide. Emulsified small quantity of stool in saline with an applicator stick Covered with cover slip Examined microscoply by using X10 and X40 objectives .

Molecular Diagnosis Method:

Extraction of the Genomic DNA

DNA has been obtained from 200 mg stool with the use of a commercial Kit presto^{TM} stool gDNA . in the case where the precipitates were produced in the ST-1 Buffer, this buffer is warmed in a water bath which is of a 37ºC temperature, succeeded with gently shaking until dissolution. Absolute ethanol is added (observe the label of the bottle for the volume) into the Wash Buffer and after that, mixed with the shaking for a number of seconds. The box on the bottle is checked. It has to be ensured that the bottle is

close tightly following each use for avoiding the evaporation of the ethanol. Add 800 µl of ST1 buffer to the 200 mg of stool sample in the beadbeating tube, shaking briefly and after that, Incubation at 70C° for 5min. beadbeading tubes are horizontally attached to the vortex with tape or adapter vortex at the maximal speed at room temperature for 10min (centrifuging for 2min. at 8,000xg in the room temperature) for the purpose of eliminating foam which results from the detergents which are present in the ST-1 buffer. 500µl of the supernatant is transferred into a new 1.50ml micro-centrifuge tube. The elution buffer is Pre-heated (100µl for each sample) to 60 °C for the elution of the DNA. 150µl of the ST-2 buffer has been added and vortexed for 5sec. After that, it has been incubated for 5min at 0°C - 4°C. Afterwards, it is centrifuged for 3min at 16000 xg at the temperature of the room for precipitating the PCR inhibitors and the insoluble particles. A column of inhibitor removal (i.e. a purple ring) has been put in a 2ml centrifuging tube, and 500µl of the supernatant has been transferred into the column of the inhibitor removal, after that, it has been centrifuged for 1min at 16000 xg at the room temperature and the column is then discarded. The flowing is saved in 2.0ml centrifuging tube for the binding of the DNA. An amount of 800ml of the ST-3 buffer has been put into the flow and mixed right away through shaking well for 5sec. The AGD column (i.e. the green ring) has been put in a 2.0ml collecting tube. An amount of 700µl of the sample has been added then mixed with the GD column (centrifuged 1min at 16000 xg) and the flow is discarded – through then placing GD column back into the 2.0ml tube of the collection. The rest of the sample mix is transferred into the column of the GD. (centrifuged for 1min at 16000 xg). Then flow is discarded –through then, GD column is placed back into 2.0ml tube of collection. An amount of 400µl of the ST-3 buffer has been added into the column of the GD (centrifuged for 30sec. at the room temperature, at 16000 xg). Discarding flow –through and after that, GD column is placed back into the 2ml tube of collection. After that, 600ml wash buffer is added (it has to be ensured that the absolute ethanol has been added) to GD column (centrifuged for 30sec. at 16000 xg at the room temperatures. Discarding flow- through after that placing GD column back into 2ml tube of collection. An amount of 600µl of the wash buffer has been added (i.e. the absolute ethonal) into GD column (centrifuged for 30sec. at 16000 xg). Discarding flow – through and after that, placing GD column back into 2.0ml tube of collection (centrifuging for 3min at 16000 xg at the room temperature for the purpose of drying the matrix of the column. Dry GD column is transferred into new 1.50ml microcentrifuge tube. 30µl -100µl of pre-heated was add to Elution buffer. Water or TE will be absorbed entirely. Then centrifuged for 2min at 16000 x g at the room temperature for eluting purified DNA.

The PCR amplification of targeted genes:

sequences of the primer that were utilized have been: for EntaF, 5ATG CAC GAG AGC GAA AGC AT3; for EhR, 5GAT CTA GAA ACA ATG CTT CTC T3; for EdR, 5CAC CAC TTA CTA TCC CTA CC3; and for the EmR, 5TGA CCG GAG CCA GAG ACA T3. All of the primer sequences have been compared with the sequences in the GenBank. Which has shown that forward primer (Enta-F) sequence has only been found in the Entamoeba and that the 3 reverse primer (EdR, EmR and EhR) sequences have been defined as species specific. Which is why, they're proper for the differentiation of the species. Forward primer in combination with suitable reverse primer produces 573bp PCR product with the E. histolytica DNA, 390bp PCR product with the E. dispar DNA, and 553bp product with the E. moshkovskii DNA. Reaction of the PCR amplification has been carried out in 51µl final volume in

0.1ml PCR tubes with the use of Px-2 thermal cycler (ThermoHybaid, UK). The conditions of the reaction have been optimized for thte purpose of combining forward primer (EntaF) with every one of the 3 reverse primers (EdR, EmR and EhR) in one reaction mix and under similar conditions. The mix of the reaction included 200M of deoxy-nucleoside triphosphate, 0.1M of every one of the reverse and forward primers, 6mM MgCl2, 1 Taq buffer, 0.5U of the Taq polymerase, and 10l of the extracted samples of the DNA. The amplification of every one of the species-specific DNA fragments started with initial denaturation at 94ºC for 3min, which is succeeded by 30 cycles of 94ºC for 1 min, 58ºC for 1 min, and 72ºC for 1min, with final extension at 72ºC for 7 min. Amplified products have been visualized with the etBr staining after the electrophoresis on 1.50% of the agarose gel.

DNA sequencing

All genes under study of DNA product with primer f and primer R and results were reading according to the BLAS (Basic local Alignment search tool) and available on the NCB1 national center for biotechnology information) and determine the types of genetic tree in detection strain in the Entamoeba histolytica , Entamoeba dispar and moshkovskii .and determine of virulence factor genes in Entamoeba histolytica spceies by gene sequence .

Statistical analyses

Statistical analyses proceeded in every group of this study, descriptive statistics analyzed with the use of the Analysis of variance(ANOVA) have been carried out with the use of the mean and standard errors (SE) with the LSD test for the continuous variables ($p < 00.05$) has been determined as significant, and X (P_{value}) has been considered as significant. Every analysis has been carried out with the SPSS program for Windows (v. 23. /2010).

Results

Table (1)

No.	Anbameobiasis spp.	Positive n(%)
1	E. histolytic	144(48.64)
2	E. dispar	91(30.74)
3	E. moskovski	52(17.56)
4	E. histolytic + E. dispar	9 (3.04)
Total		296(99.98)
X^2		133.757
P value		0.000*
*Significant P <0.05. PCR according to age		

Table (2)

No.	Age (years)	Number [n (%)]	Gender	Number [n (%)]	Residential area	Number [n (%)]
1	<1-10	61(42.36)	Male	74(51.38)	Urban	71(49.30)
2	11-20	31(21.52)	Female	70(48.61)	Rural	73(50.69)
3	21-30	22(15.27)				
4	31-40	20(13.88)				
5	> 40	10(6.94)				
Total	144(99.97)			144(99.97)		144(99.99)
X^2	52.736			0.111		0.028
P value	0.00*			0.73901¥		0.86711 ¥

We have performed PCR for the 296 microscopically positive patients. The molecular study showed that E. histolytica was the 1st with 48.64% of cases. E. dispar was the 2nd with 30.74% of cases. And E. moskovski the 3rd with 17.56% of cases. The association of E. hitolytica and E. dispar was recorded for 3.04% of cases

Significant P <0.05 ¥Non-significant ¥Non-significant PCR according to gender

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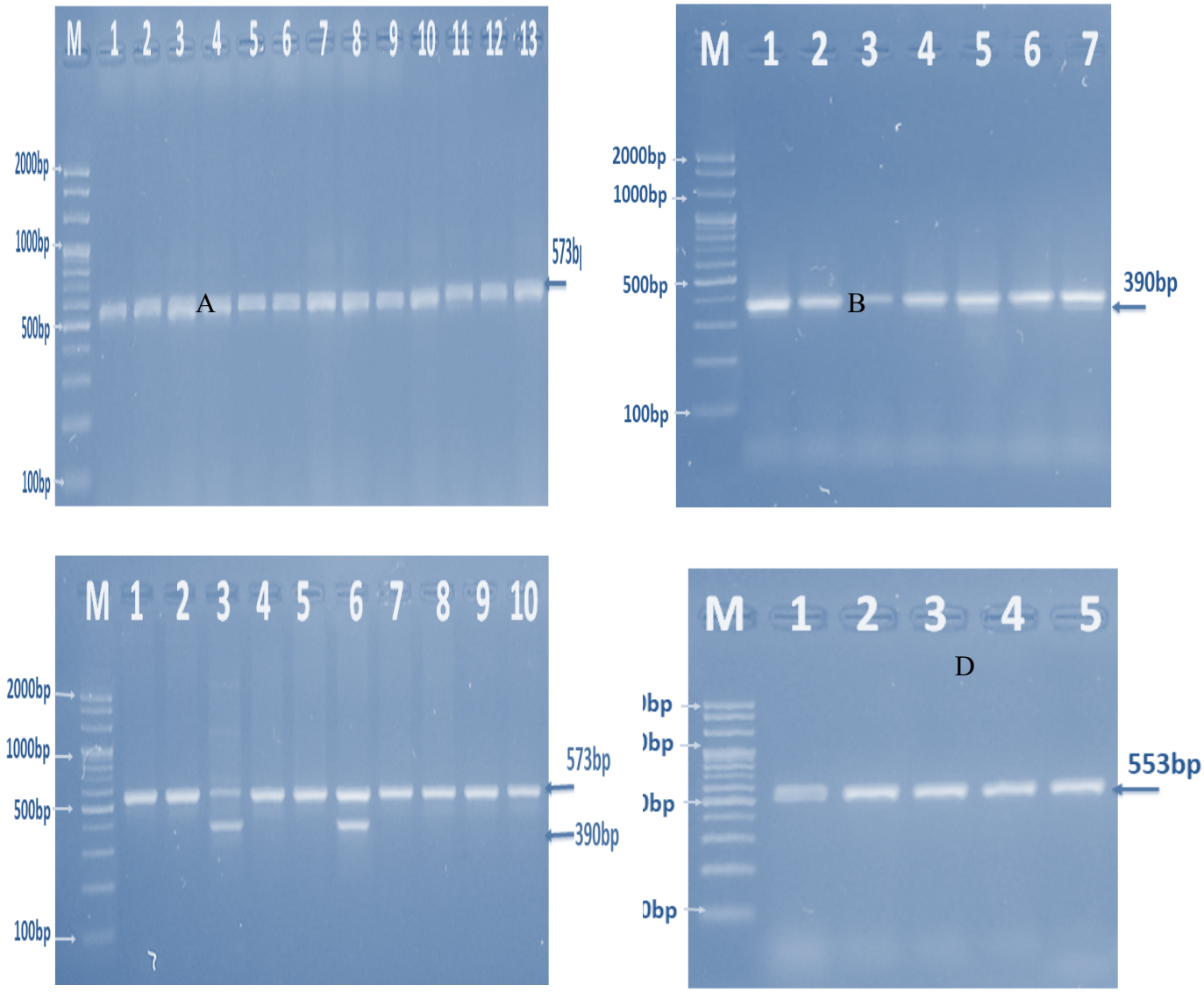

Figure (1): images of *Entamoeba species* that have been detected.(A) *E. histolytica*(B),*E.dispar* (C*), E. moshkovskii* (D) , mixed *E. histolytica* and *E.dispar* . Agarose gel electrophoresis image that showed the PCR product analysis of small subunit ribosomal RNA gene in *E. histolytica* from Human stool samples. Where , the Lane (M): DNA marker ladder (2000-100bp) and the Lane (1-13) were showed only positive PCR *E.histolytica* samples at 573bp PCR product size(A). , Only positive PCR *E. dispar* results were shown on the Lane (M): DNA marker ladder (2000-100bp) and the Lane (1-7) at 390bp PCR product size (B). Only positive PCR *E. moshkovskii* results were shown on the Lane (M): DNA marker ladder (2000-100bp) and the Lane (1-5) at 553bp PCR product size (C). At 573bp PCR product size, the Lane (M): DNA marker ladder (2000-100bp) and the Lane (1-10) demonstrated positive PCR *E. histolytica* samples. The Lane (3 and 6) were showed only positive PCR *E.dispar* samples at 390bp PCR product size(D).

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Entamoeba histolytica Spp DNA Sequence results

DNA Sequences Translated Protein Sequences

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Species/Abbrv
1 Entamoeba histolytica IQ No 1 small subunit ribosomal RNA GGTGGAGTGATTTGTCAGGTTAATTCCGGTAACGAACGAGAC
2 Entamoeba histolytica IQ No 2 small subunit ribosomal RNA GGTGGAGTGATTTGTCAGGTTAATTCCGGTAACGAACGAGAC
3 AB002794 1 15-365 Entamoeba histolytica                 GGTGGAGTGATTTGTCAGGTTAATTCCGGTAACGAACGAGAC
4 MF421529 1 15-389 Entamoeba histolytica                   GGTGGAGTGATTTGTCAGGTTAATTCCGGTAACGAACGAGAC
5 KP233840 1 1233-1607 Entamoeba histolytica isolate EH_I GGTGGAGTGATTTGTCAGGTTAATTCCGGTAACGAACGAGAC
6 OP808370 1 1231-1605 Entamoeba histolytica isolate Y48 GGTGGAGTGATTTGTCAGGTTAATTCCGGTAACGAACGAGAC
7 MK332025 1 1317-1691 Entamoeba histolytica strain A1      GGTGGAGTGATTTGTCAGGTTAATTCCGGTAACGAACGAGAC
8 OP626165 1 395-769 Entamoeba histolytica strain Y74 1     GGTGGAGTGATTTGTCAGGTTAATTCCGGTAACGAACGAGAC
9 OP537204 1 395-769 Entamoeba histolytica strain FCK773 GGTGGAGTGATTTGTCAGGTTAATTCCGGTAACGAACGAGAC
10 OP456537 1 395-769 Entamoeba histolytica strain RR967 GGTGGAGTGATTTGTCAGGTTAATTCCGGTAACGAACGAGAC

Species/Abbrv
1 Entamoeba histolytica IQ No 1 small subunit ribosomal RNA TGAAACCTATTAATTAGTTTTCTGCCTATAAGACAGAAATG
2 Entamoeba histolytica IQ No 2 small subunit ribosomal RNA TGAAACCTATTAATTAGTTTTCTGCCTATAAGACAGAAATG
3 AB002794 1 15-365 Entamoeba histolytica                 TGAAACCTATTAATTAGTTTTCTGCCTATAAGACAGAAATG
4 MF421529 1 15-389 Entamoeba histolytica                   TGAAACCTATTAATTAGTTTTCTGCCTATAAGACAGAAATG
5 KP233840 1 1233-1607 Entamoeba histolytica isolate EH_I TGAAACCTATTAATTAGTTTTCTGCCTATAAGACAGAAATG
6 OP808370 1 1231-1605 Entamoeba histolytica isolate Y48 TGAAACCTATTAATTAGTTTTCTGCCTATAAGACAGAAATG
7 MK332025 1 1317-1691 Entamoeba histolytica strain A1      TGAAACCTATTAATTAGTTTTCTGCCTATAAGACAGAAATG
8 OP626165 1 395-769 Entamoeba histolytica strain Y74 1     TGAAACCTATTAATTAGTTTTCTGCCTATAAGACAGAAATG
9 OP537204 1 395-769 Entamoeba histolytica strain FCK773 TGAAACCTATTAATTAGTTTTCTGCCTATAAGACAGAAATG
10 OP456537 1 395-769 Entamoeba histolytica strain RR967 TGAAACCTATTAATTAGTTTTCTGCCTATAAGACAGAAATG

Species/Abbrv
1 Entamoeba histolytica IQ No 1 small subunit ribosomal RNA CATTTCAATTGTCCTATTTTAATTGTAGTTATCTAATTTCG
2 Entamoeba histolytica IQ No 2 small subunit ribosomal RNA CATTTCAATTGTCCTATTTTAATTGTAGTTATCTAATTTCG
3 AB002794 1 15-365 Entamoeba histolytica                 CATTTCAATTGTCCTATTTTAATTGTAGTTATCTAATTTCG
4 MF421529 1 15-389 Entamoeba histolytica                   CATTTCAATTGTCCTATTTTAATTGTAGTTATCTAATTTCG
5 KP233840 1 1233-1607 Entamoeba histolytica isolate EH_I CATTTCAATTGTCCTATTTTAATTGTAGTTATCTAATTTCG
6 OP808370 1 1231-1605 Entamoeba histolytica isolate Y48 CATTTCAATTGTCCTATTTTAATTGTAGTTATCTAATTTCG
7 MK332025 1 1317-1691 Entamoeba histolytica strain A1      CATTTCAATTGTCCTATTTTAATTGTAGTTATCTAATTTCG
8 OP626165 1 395-769 Entamoeba histolytica strain Y74 1     CATTTCAATTGTCCTATTTTAATTGTAGTTATCTAATTTCG
9 OP537204 1 395-769 Entamoeba histolytica strain FCK773 CATTTCAATTGTCCTATTTTAATTGTAGTTATCTAATTTCG
10 OP456537 1 395-769 Entamoeba histolytica strain RR967 CATTTCAATTGTCCTATTTTAATTGTAGTTATCTAATTTCG
Species/Abbrv
1 Entamoeba histolytica IQ No 1 small subunit ribosomal RNA TTCGCAAGAACAGGTGCGTAAGTACCACTTCTTAAAGGGAC
2 Entamoeba histolytica IQ No 2 small subunit ribosomal RNA TTCGCAAGAACAGGTGCGTAAGTACCACTTCTTAAAGGGAC
3 AB002794 1 15-365 Entamoeba histolytica                 TTCGCAAGAACAGGTGCGTAAGTACCACTTCTTAAAGGGAC
4 MF421529 1 15-389 Entamoeba histolytica                   TTCGCAAGAACAGGTGCGTAAGTACCACTTCTTAAAGGGAC
5 KP233840 1 1233-1607 Entamoeba histolytica isolate EH_I TTCGCAAGAACAGGTGCGTAAGTACCACTTCTTAAAGGGAC
6 OP808370 1 1231-1605 Entamoeba histolytica isolate Y48 TTCGCAAGAACAGGTGCGTAAGTACCACTTCTTAAAGGGAC
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10 OP456537 1 395-769 Entamoeba histolytica strain RR967 TTCGCAAGAACAGGTGCGTAAGTACCACTTCTTAAAGGGAC
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Figure (2): Study of multiple sequence alignments of the short subunit ribosomal RNA gene in local Entamoeba histolytica IQ isolates and related Entamoeba histolytica isolates from NCBI-Genbank. The online ClustalW alignment program was used to create the multiple alignment analysis. This alignment study revealed substitution mutations in the small subunit ribosomal RNA gene between isolates as well as nucleotide alignment similarity as (*).

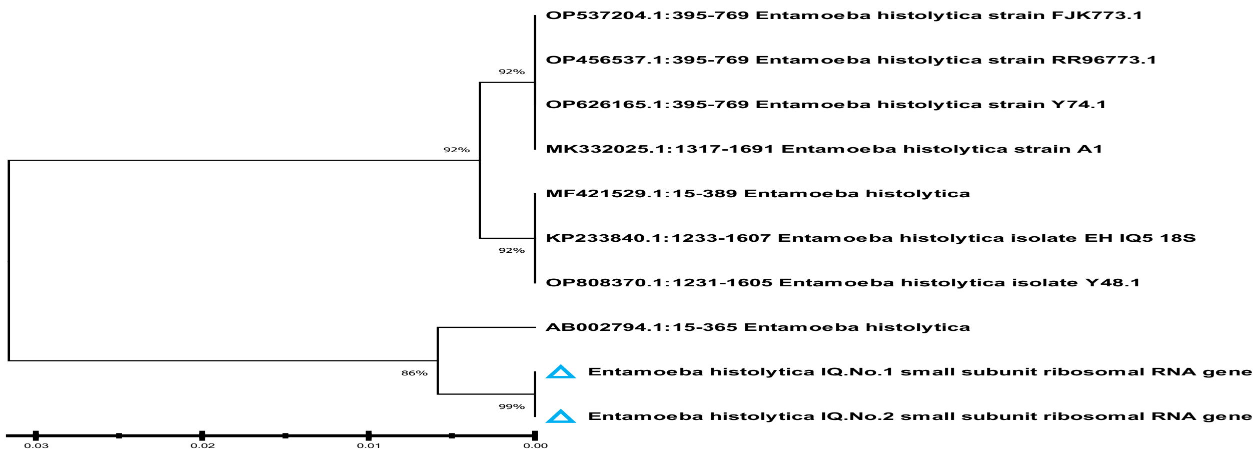

Figure (3): The genetic analysis of local *E. histolytica* IQ isolates used a phylogenetic tree based on the partial sequencing of the small subunit ribosomal RNA gene. Using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA tree) in the Mega 6.0 version, the phylogenetic tree was created. The local *E. histolytica* IQ isolates were showed closed related to NCBI-BLAST *E.histolytica* (AB002794.1) at total genetic changes (0.20-0.050%).

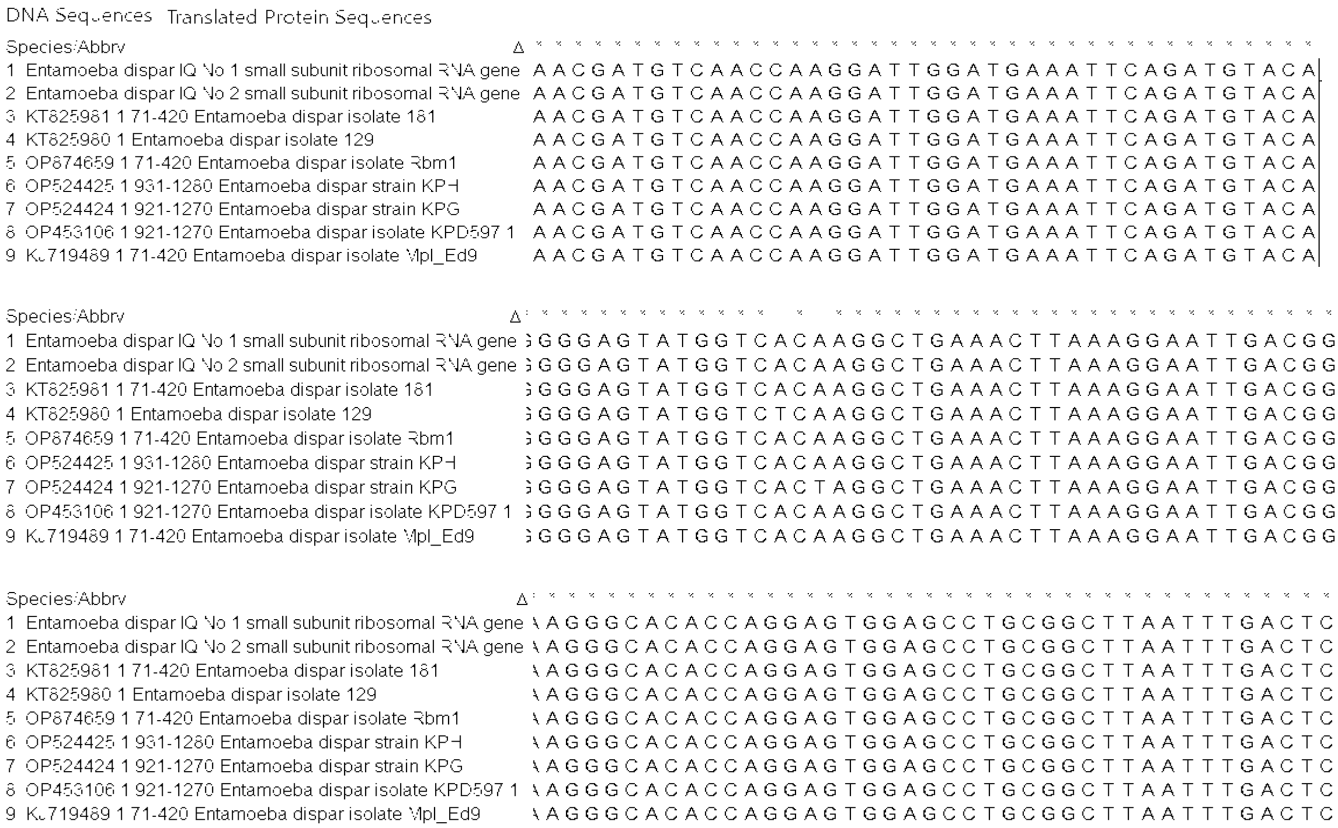

Figure (4): Analysis of multiple sequence alignments for the short subunit ribosomal RNA gene in Entamoeba dispar IQ isolates from the locale and related Entamoeba dispar isolates from NCBI-Genbank. Utilizing the online ClustalW alignment tool, the multiple alignment analysis was created. This alignment study demonstrated the nucleotide alignment similarities as (*) and substitution mutations in the small subunit ribosomal RNA gene between isolates.

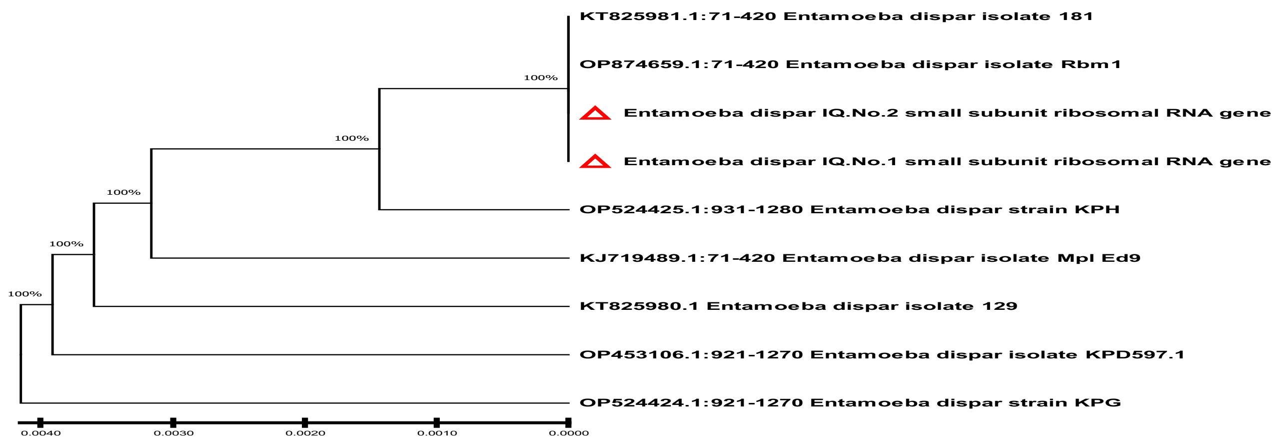

Figure (5): Local *E. dispa*r IQ isolates utilized for genetic analysis were based on a phylogenetic tree analysis of the small subunit ribosomal RNA gene partial sequence. The phylogenetic tree was created using MEGA 6.0's Unweighted Pair Group Method with Arithmetic Mean (UPGMA tree). The local *E. dispar* IQ isolates were found to be closely related to NCBI-BLAST isolate 181 (KT825981.1) of *E. dispar* with total genetic alterations of (0.0040-0.0010%).

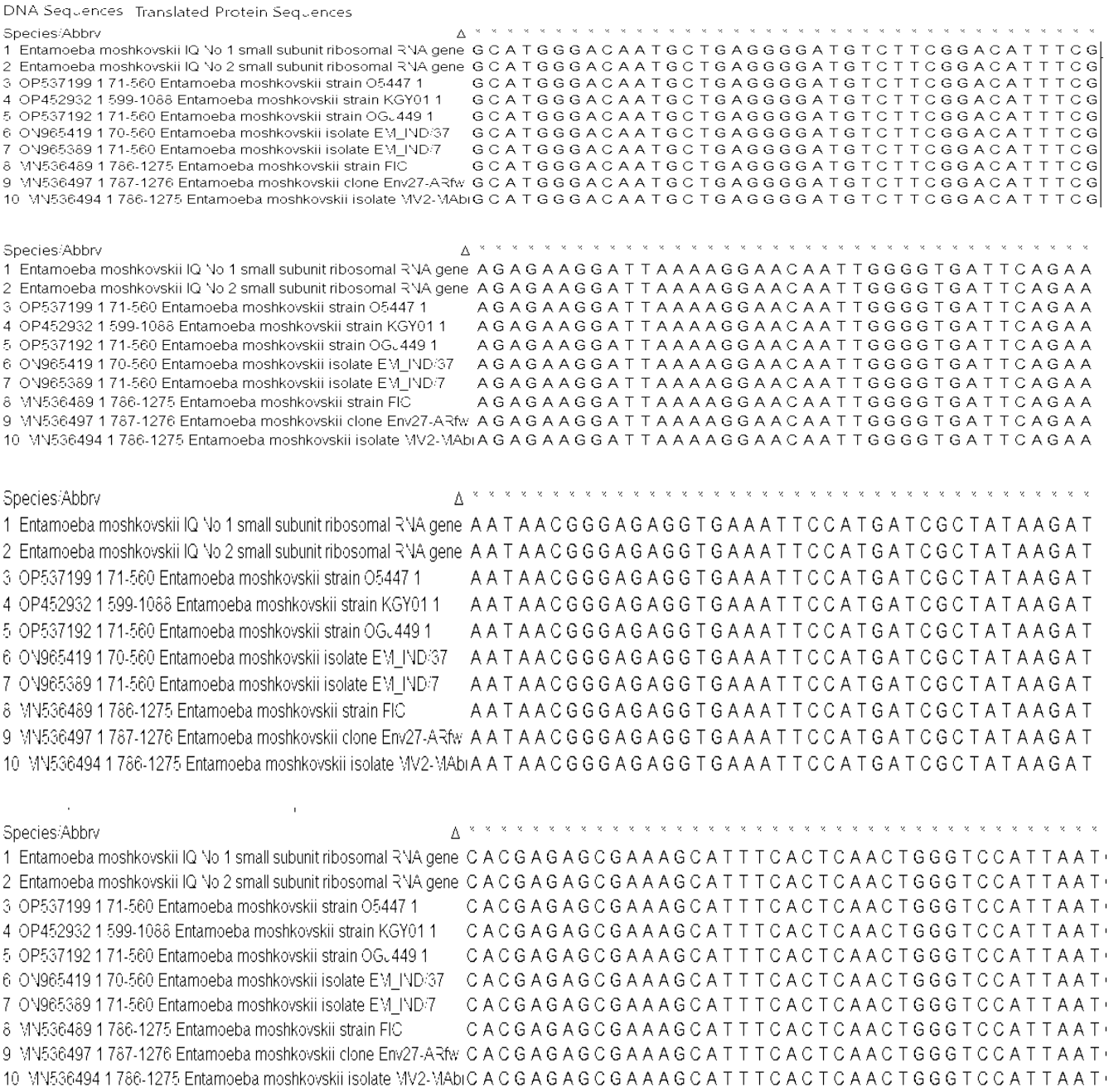

Figure (6): Local Entamoeba moshkovskii IQ isolates and related Entamoeba moshkovskii isolates were subjected to multiple sequence alignment analyses for the small subunit ribosomal RNA gene. Utilizing the online ClustalW alignment tool, the multiple alignment analysis was created. The alignment analysis demonstrated the nucleotide alignment similarity as (*) and substitution mal RNA gene between isolates. ons of (0.0040-0.0010%).

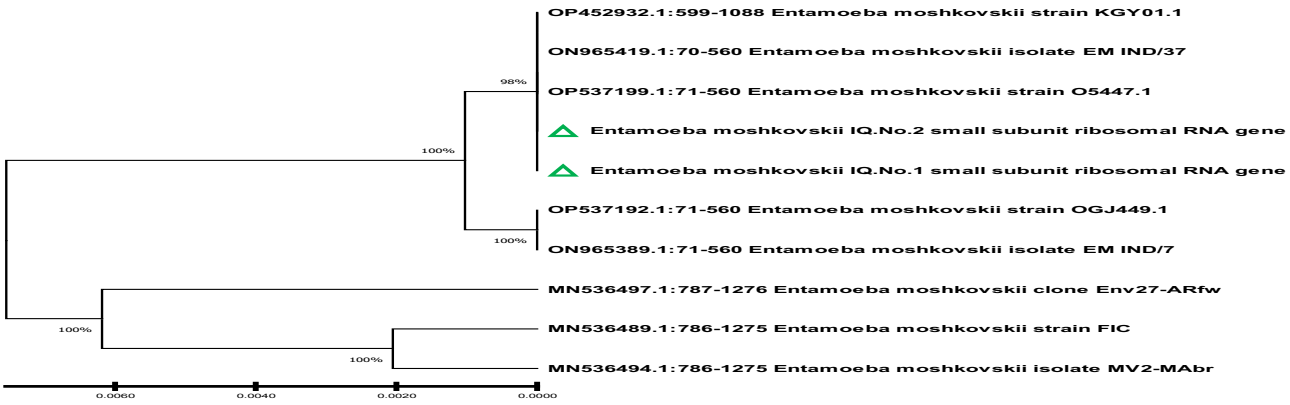

Figure (7): Local *E. moshkovskii* IQ isolates utilized for genetic study were based on a phylogenetic tree analysis of the small subunit ribosomal RNA gene partial sequence. The phylogenetic tree was created using MEGA 6.0's Unweighted Pair Group Method with Arithmetic Mean (UPGMA tree). The local *E. moshkovskii* IQ isolates were shown to be closely related to *E. moshkovskii* strain O5447.1 (OP537199.1) by NCBI-BLAST, with total genomic alterations between 0.0060 - 0.0020 %.

Discussion

One of the most prevalent human infections all over the world is amoebiasis the pathological and genetic variations, the three examined species are morphologically similar(16).in the curnt study, Entamoeba species were distinguished using molecular and microscopic techniques. The identification regarding trophozoites or cysts under the microscope serves as the basis for *Entamoeba* detection in medical labs of several nations, (17). our research showed that 48.64% of those cases were caused by *E. histolytica* ,(30.74*) E. dispar* and *E. moshkovskii* in this work has been comparable to that in the majority of earlier research done in central, northern, and southern Iran. Hooshyar H et al (18). studies from Malaysia(10),(19),(20), (21), (15), (4), (22), and (23) from the Netherlands, South Africa, Australia, and Northwest Ethiopia. Yet, research from the south-west of Iran's (24) In regions where *E. histolytica* was more common, (25), (26), and Gaza Strip (27) reported comparable findings. The majority of of E. histolytica cases could actually be E. dispar that is non-pathogenic, according to a (28). Yet, patients with no symptoms have also been shown to have *E. histolytica* infection. For instance, most positive *E. histolytica* cases have been regarded as asymptomatic in investigations from the Philippines (29) and Japan(30). Abdominal pain and persistent diarrhea have been recorded in every *E. histolytica* infection case (mixed as well as single infections), which had a prevalence of 0.36 % in our community. This observation is in line with a number of studies from South Africa (15) and Pakistan (31) that demonstrated that *E. histolytica* frequently causes clinical signs in the patients. According to(2), (32) Have also discovered that the *E. dispar* has been non-pathogenic and commensal to people. (33) (14) studies claim that Brazilian *E. dispar* strain is pathogenic and can, in vivo, result in an amoebic liver abscess. (34). According to our research, 7 of 30 patients who have E. dispar had a single infection, and in 3 cases, the GIDs that include the stomach pain were present.(35) first proposed the idea that the *E. moshkovskii* represents a nonvirulent, free-living *Entamoeba species*. Which contradicts our results, though, as one patient with *E. moshkovskii* infection experienced gastroenteritis symptoms as persistent diarrhea and abdominal pain.(10) ,(5), A (10), Khairnar K and Parija SC have connected the *E. moshkovskii* infection to GIDs in Australia, Malaysia, and India (2005). According to a Malaysian study, additional research is required to establish the connection between GIDs and *E. moshkovskii* and to discover this species' potential pathogenicity. Other potential factors, like fungal, bacterial, and viral infections, or other non-infectious illnesses that are related

to gastro-enteritis symptoms have not been examined in this work, as a result of a low number of the positive cases. Although E. dispar is known as non-pathogenic, but in the current study, indicating the probability that E. dispar might have some pathogenic appearances, or these abdominal pains may be due to other causes. These results are similar obtained by (36). Other study recorded the mixed parasite infection rate of the E .dispar and E .moshkovskii which has been (1/54 and 1.85)% recorded in Tunis, as the mixed infection record's proportion 7.40% by using PCR method. In sequencing the strain separated in current study from patients with diarrhea have different surface antigen to distinguished through DNA probe, to investigate these stain differ in their rRNA. The sequence that using give result show that the gene of pathogenic strain differ from nonpathogenic one level and suggested that the 18SrRNA probe could be of value to detection studies (37,38).

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