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Research article

Phylogenetic tree analysis of *Entamoeba* species isolated from goats

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Abstract

The present study was designed to species typing of parasite Entamoeba spp from goats by using PCR technique and phylogenetic tree analysis. The PCR technique was conducted for using specific primers were designed for 18S rRNA gene of Entamoeba spp. In this study, the sequence alignment analysis and phylogenetic tree analysis of Unweighted Pair Group method with Arithmetic were performed by using phylogenetic and molecular evolutionary analysis (MEGA 6.0 edition computer software) that analysis of 590bp for ribosomal 18S rRNA gene. Our isolates submitted to the National Center for Biotechnology Information (NCBI-GenBank) for getting accession number and then we were gotten (10) accession number for goat isolates. Entamoeba spp were detected in (10/50) (20%) of feces samples that collected from goat by PCR. Results of the phylogenetic tree analysis show that most isolates of Entamoeba spp. were closed related to NCBI-Blast Entamoeba bovis 18S ribosomal RNA gene (FN666250.1) with (80%) as an accession number (MF568371, MF568372, MF568373, MF568374, MF568375, MF568377, MF568378 and MF568380), whereas other NCBI- Blast Entamoeba spp. has been shown more related to Entamoeba histolytica isolate 18S ribosomal RNA gene (GQ423749.1) with (20%) as (MF568376 and MF568379). This study is first recording used of molecular phylogeny to Entamoeba spp. in goat at the first time in Iraq.

Keywords: *Entamoeba spp*, goat, Phylogenetic tree, 18S ribosomal gene.

Introduction

Diarrhea is a sign of disease characterized by loss of fluids from the body. The animals and human effect with it, the diarrhea occurs due to either an increased number of bowel movements, an increase in the looseness of feces; increased output of water and electrolytes out the intestine or decrease the absorption from the intestine or speed passage of feces by the intestine caused by many of factors like bacterial, viral, parasitic, nutrition...etc (1); *Entamoeba* is one from parasitic protozoa that replicate in intestines of animals and human and causes diarrhea (2). *Entamoeba* is found a live in large intestine; the genus *Entamoeba* was had many of species found in humans and animals (3, 4). *Entamoeba* is a genus consist of several species, including *Entamoeba. histolytica, E. bovis E. dispar, E. polecki, E. hartmanni, and E. Bangladeshi, E. Moshkovskii E. coli, E. chattoni, (5)(6). Entamoeba histolytica* is the parasite responsible disease called Amoebiasis (amoebic liver abscesses and amoebic dysentery) (7). *Entamoeba histolytica*is a most common protozoa parasite, which causes death due to amebic colitis and destroys of intestine wall and transmit to the liver to cause liver abscesses in hot area(8). Others such as *Entamoeba coli; Entamoeba bovis* and *Entamoeba ovis* (9). This study investigated to knowledge degree of relationship between our isolates that study in this research and compare with NCBI database after detection the isolates by PCR by using phylogenic tree analysis.

Materials and Methods

Ethical approval

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The Animal Ethical Committee of Veterinary Medicine College, University of Al-Qadisiyah, Iraq, has approved the present study under permission No: 209

Clinical samples

The study was carried out on (50) fecal samples were collected from goat from different flocks in Al-Qadisiyah governorate (Center of the city, Afak districtand and Naffar area) in a random way from october 2016 to march 2017. Fecal samples were collected from the rectum of both sex, the age of animal's ranges from 6 months to 5 years. Some of samples were diarrheic and other were normal. The fecal samples transferred in a clean, sterile plastic container, stored in coolants containers and send to the laboratory.The microscopic examination not used, The PCR method was used directly.

Feces DNA extraction:

DNA was extracted from feces samples by using kit named (Feces DNA extraction Kit, made in Bioneer. Korea). The extraction was prepared based on company directions included using feces lysis protocol method; the extracted DNA was confirmed by Nanodrop spectrophotometer apparatus, then keep at (-20ºC) at freezer for used in PCR.

Nanodrop:

The extracted DNA was estimated by Nanodrop device at 260/280nm and then kept at deep freezer until used in PCR method.

Table (1):- PCR primers 18S rRNA gene in *Entamoeba spp*:

Primer	Sequence (5 '-3 ')		Amplicon
18S rRNA gene	F	ATTGGAGGGCAAGTCTGGTG	590bp
	R	CATACTCCCCCTGAAGTCCA	

Table (2) company instructions of PCR master mix

PCR Master mix	Volume
DNA Template	5µl
18Sr RNA gene Forward primer (10 pmol)	1.5 µl
18Sr RNA gene Reverse primer (10 pmol)	1.5 µl
PCR water	12 µl
Total volume	20 µl

Table (3) PCR thermocycler conditions

PCR step	Temp.	Time	Repeat cycle
Initial Denaturation	95ºC	3min.	1
Denaturation	95ºC	30sec.	30 cycle
Annealing	60ºC	30sec.	
Extension	72ºC	1min.	
Final extension	72ºC	5min.	1
Hold	4ºC	forever	-

Primers: The PCR primers that used in this study for detection *Entamoeba spp.* based on 18S rRNA gene were designed in this study using NCBI Gene sequence database recoding *Entamoeba spp.* RL2 isolate partial 18S rRNA gene, (GenBank code:FR686362.1) and primer 3 plus design. These primers were provided by Bioneer Company, Korea as following table (1).

PCR master mix preparation:

The master mix was prepared using (Accu-Power®PCR-PreMix-Kit) master mix reagent and done depend on company instructions as following Table (2). After that, the PCR mix that revealed in the table above placed in Accu-Power PCR - PreMix that contains all other PCR components which needed to reaction such as (Taq DNA polymerase, dNTPs, 10 PCR buffer). Then, all the PCR tubes transferred into vortex centrifuge for 3 minutes, and then transferred into thermocycler my Gene, Bioneer. Korea.

PCR thermocycler conditions:

PCR product analysis:

The PCR products (590bp) were examined by electrophoresis in a 1% agarose gel using 1X TBE buffer, stained with ethidium bromide, and investigation under UV transilluminator as following table (3).

DNA sequencing method:

DNA sequencing technic has done for identification of

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Entamoeba species typing based on (18S rRNA gene) by using Phylogenetic tree analysis. The 18S rRNA gene 590bp PCR product was purified from agarose gel by using (EZ EZ-10 Spin Column DNA Gel Extraction Kit, Biobasic. Canada). The purified 18S rRNA gene PCR product samples were sent to Bioneer Company in Korea for performed the DNA sequencing using 18S rRNA forward primer by (AB DNA sequencing system). The phylogenetic analysis was performed according to on (MEGA 6.0 edition computer software).

Results

Fifty feces sample collected from the goat for detection of *Entamoeba spp*., wherever, all the isolates submit to the polymerase chain reaction test, the prevalence as a Table (4). Using specific primers designed according to the gene of 18S rRNA to using for detection of *Entamoeba spp*. protozoa at (590bp) on electrophoresis agarose gel as a Figure (1).

Table(4): Prevalence of *Entamoeba spp*

Samples	Positive Samples	Percentage (%)
Goat feces	10/50	10%

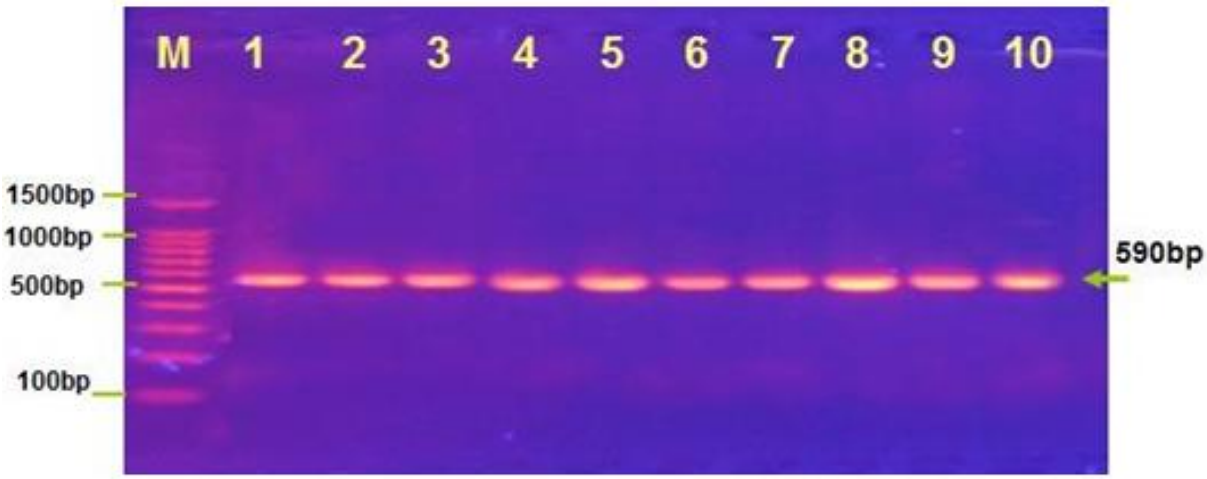

Figure (1): Agarose gel electrophoresis image that shows that PCR product analysis of *Entamoeba spp*. based on 18S rRNA gene. Where M: marker (1500-100bp), lane (1-10) positive feces samples at 590 bp PCR product.

Phylogenetic tree analysis:

The *Entamoeba spp.* (10) isolates were sent to the genetic laboratory outside of Iraq for performed (DNA sequencing), then submitted to GenBank NCBI in USA for getting accession number and then we gotten (10) accession number for goat isolates. The phylogenetic tree analysis and identity by NCBI-BLAST were show that the eight of local *Entamoeba spp*. isolates are close related to NCBI-BLAST *Entamoeba bovis* (FN666250.1) except local *Entamoeba spp.* (6) and local *Entamoeba spp*. (9) are close related to NCBI-Blast *Entamoeba histolytica* (GQ423749.1) (20%) see Figure (2), (3) and Table (5), (6) :

Table (5): Prevalence of *Entamoeba bovis* and *Entamoeba histolytica*

Isolates	**Positive results**	**Percentage (100%)**
***Entamoeba* bovis**	8/10	80%
***Entamoeba* histalytica**	2/10	20%

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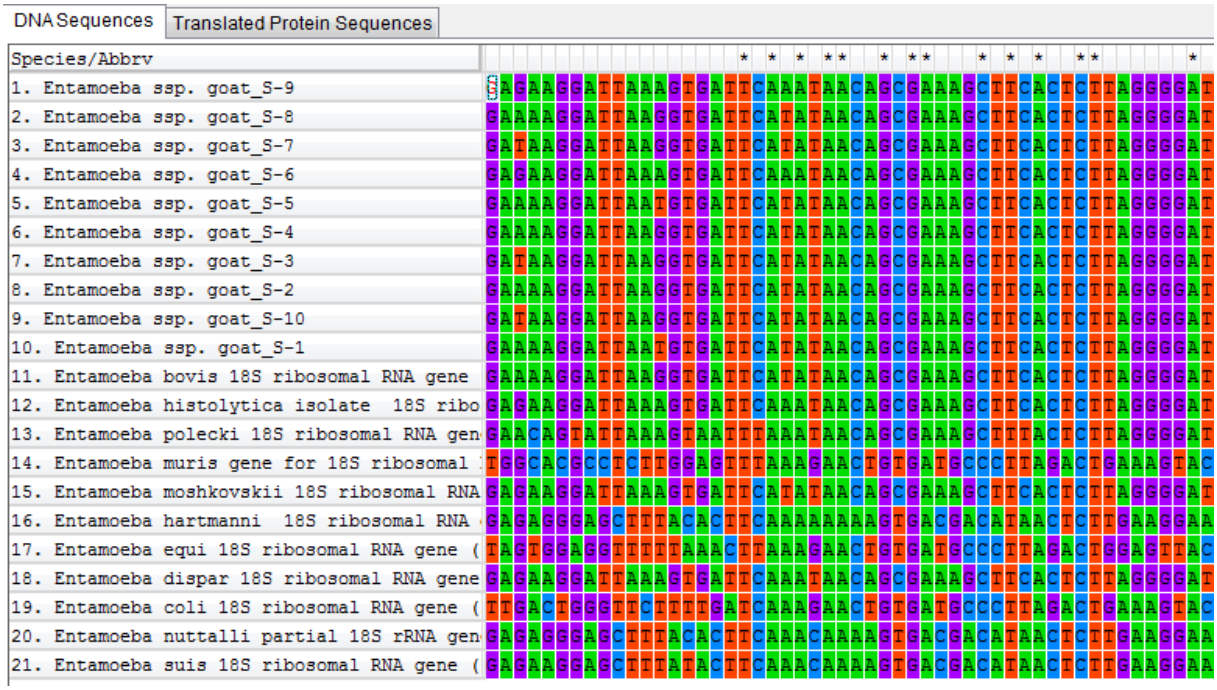
Table (6): local isolates of *Entamoeba spp*. With accession number

Isolates	Accession number
***Entamoeba spp.* (S1)**	MF568371
***Entamoeba spp.* (S2)**	MF568372
***Entamoeba spp.* (S3)**	MF568373
***Entamoeba spp.* (S4)**	MF568374
***Entamoeba spp.* (S5)**	MF568375
***Entamoeba spp.* (S6)**	MF568376
***Entamoeba spp.* (S7)**	MF568377
***Entamoeba spp.* (S8)**	MF568378
***Entamoeba spp.* (S9)**	MF568379
***Entamoeba spp.* (S10)**	MF568380



Figure(2): Multiple sequence alignment analysis of the partial 18S rRNA gene sequence in local *Entamoeba spp.* goat isolates with NCBI-Blast *Entamoeba spp*. based Clusta lW alignment analysis by using (MEGA 6.0, multiple alignment analysis tool). That show the multiple alignment analysis similarities (*) and differences in 18S rRNA gene nucleotide sequences.

Ten isolates from NCBI (GenBank):

MF568380.1 Entamoeba sp. isolate IQ-goat-10 18S ribosomal RNA gene, partial sequence
MF568379.1 Entamoeba sp. isolate IQ-goat-9 18S ribosomal RNA gene, partial sequence
MF568378.1 Entamoeba sp. isolate IQ-goat-8 18S ribosomal RNA gene, partial sequence
MF568377.1 Entamoeba sp. isolate IQ-goat-7 18S ribosomal RNA gene, partial sequence
MF568376.1 Entamoeba sp. isolate IQ-goat-6 18S ribosomal RNA gene, partial sequence
MF568375.1 Entamoeba sp. isolate IQ-goat-5 18S ribosomal RNA gene, partial sequence
MF568374.1 Entamoeba sp. isolate IQ-goat-4 18S ribosomal RNA gene, partial sequence
MF568373.1 Entamoeba sp. isolate IQ-goat-3 18S ribosomal RNA gene, partial sequence
MF568372.1 Entamoeba sp. isolate IQ-goat-2 18S ribosomal RNA gene, partial sequence
MF568371.1 Entamoeba sp. isolate IQ-goat-1 18S ribosomal RNA gene, partial sequence

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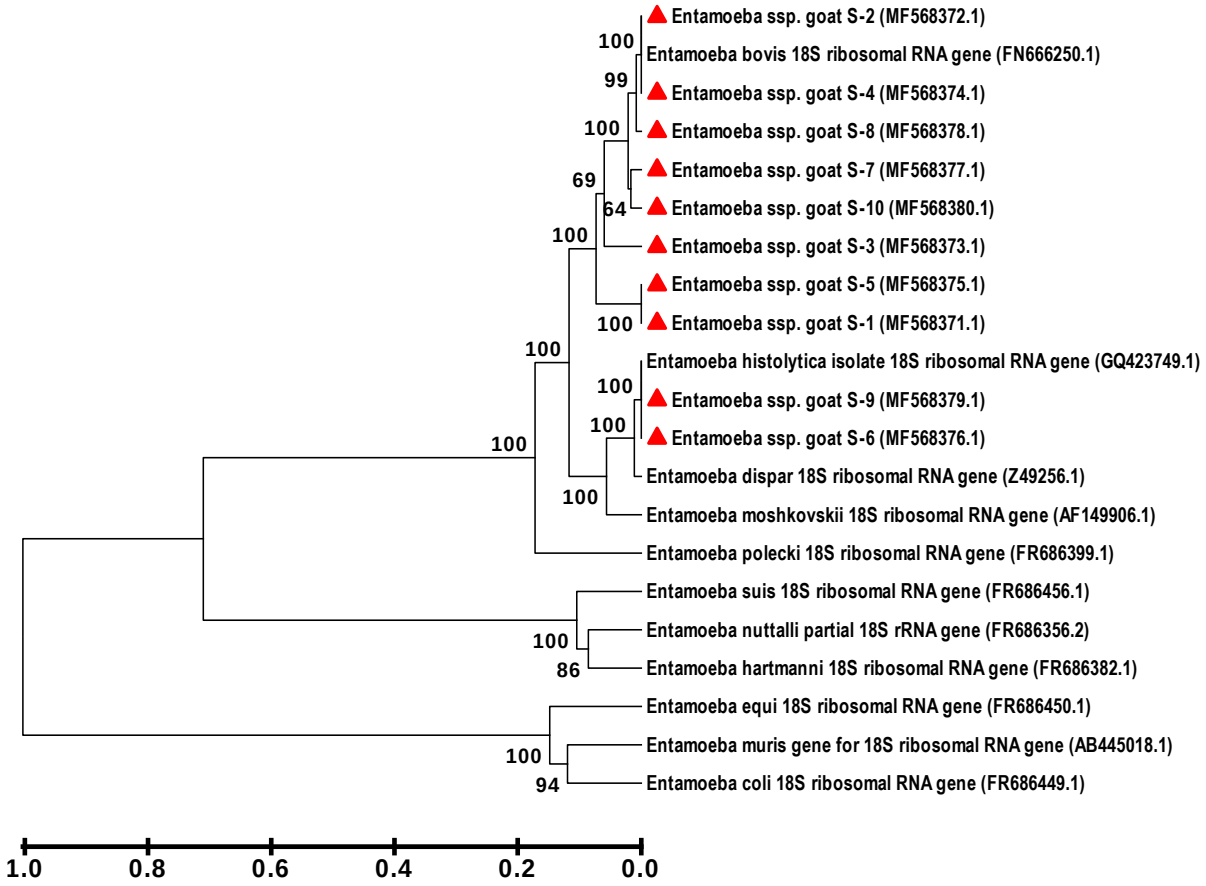

Figure (3): Phylogenetic tree analysis depends on the gene of 18S rRNA, the primer used for detection of *Entamoeba* species typing in goat isolates. The phylogenetic tree was constructed using UN weighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The local *Entamoeba spp.* (S1,S2,S3,S4,S5,S7,S8 and S10) were show close related to NCBI- Blast *Entamoeba bovis* (FN666250.1), the local *Entamoeba spp.* (S6 and S9) were shown close related to NCBI-Blast *Entamoeba histolytica* (GQ423749.1), Where, the NCBI-Blast *Entamoeba spp.* where show different out of the tree.

Discussion

There are many of researchers are studied phylogenic tree analysis of parasite of *Entamoeba spp* in the world, but our study provides new information about this parasite in Iraq for the first time, where some the researchers like (10) used 16S rRNA gene and some other like (11) used 18S rRNA gene to detection this parasite by PCR as we use. In this study, the prevalence of *Entamoeba spp* overall was 10/50 (20%) under accession number (MF568376 and MF568379), (12) in Iran and (13) in Iraq found the rate of *Entamoeba spp* was (77.4%) and (62.96%) respectively by use nested PCR, they recorded results more than our rates. While (14) in Iraq recorded rate reached to (33.3%) in Al-Qadisiyah city, that near to our results. An addition (15) in Malaysia and (16) in Egypt found results less than ours score where they found (3.2%) and (7.9%) of the parasites respectively. All of these studies used phylogenetic tree by PCR testing with conventional methods. Also (17) in Uganda confirm disease rate was (36.7%), (18) recorded (14%) in the West Bank in Palestine; that too near to our results. The percentages are contrast depend on several of causes, like climatic conditions, geographic determination, nature of the places, the degree of the contamination by the causativeagent, the parasites spread irregularly spreading in most countries (19)

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and (20). Our study has found prevalence of *Entamoeba histolytica* was (20%), (13) recorded rate (58.3%) of *Entamoeba histolytica* in cows where was higher than our rate, further more (21) found in the study presence *E. histolytica* by PCR was (85.7%) in sheep, also considered that higher than our results samples, the same with where (22) recorder (71.5%) as infection rate. (23) recorded results in center garden Rome infection by (9%) *E. histolytica*, that less than our results. In addition in human by (PCR), and for the differentiation of *E. histolytica*, (24) found the percentage was (28.7%) among HIV-positive patients this present are near to our results. There many of reports that handle about subject *Entamoeba bovis* also; our study where has found the percentage of *Entamoeba bovis* was (80%), *Entamoeba bovis* trophozoites with a percentage (80%) among buffaloes that considered same to our ratios (25). While in Turin Univ. in Italy (26) has found the percentage of *Entamoeba bovis* was (18.6%), that represent less than our results, also (27) recorded little rate of *Entamoeba bovis* in Greece was (0.5%) that less than ours. Also (13) found *Entamoeba bovis* in infection of cows and sheep were (21.5%) both of them represented less than our percentage. While (25) confirm the prevalence of *Entamoeba bovis* more than our results in (85%) in cattle.The difference in the percentage of previous prevalence caused by several factors like lack culturing health information in the owners society, health conditions that spread between the animals, type of animal breed considered important factor to infection accureance and absent of knowledge about the disease and ways of transmitted by animals or use of contaminated water. All these factors formed difference in percentage furthermore, geographical nature and the climate of the regional that considered main factors to contrast the ratios (13, 23, 28).

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