The genotype of *Entamoeba histolytica* in bloody diarrhea samples of humans, cows and sheep

H.H. Naser

Zoonotic Diseases Research Unit, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Qadisiyah, Iraq
Email: hassan.naser@qu.edu.iq

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Abstract

The present study was carried out to detect the genotype of *E. histolytica* that found in human fecal specimens and animals feces with Haemorrgic diarrhea by amplifying the SREHP gene, using RT-PCR technique, Cyber green dye and by fusion curve analysis. The study also included molecular detection of amoebic parasite species using Nested-PCR technology. The study recorded presence of parasites *E. histolytica; E. dispar; E. bovis* with total infection rates 82.9, 26.8, 4.9%, respectively. The study revealed the presence of *E. histolytica* parasite in five different genotypes (I, II, III, IV, V) with rate presence 9.75, 53.65, 19.5, 9.75, 7.3%, respectively. In conclusion, there are five genotype of *E. histolytica*, in human and animals, most of these genotypes may be infect any host, *E. bovis* was recorded in sheep and cows.

***Keywords*:** *Entamoeba* spp., Real-Time PCR, SREHP, Genotyping, Melting curve analysis

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النمط الجيني للأميبا الحالة للنسج *Entamoeba histolytica* في عينات الإسهال الدموي للإنسان والأبقاروالأغنام

حسن حاجم ناصر

وحدة بحوث الأمراض الانتقالية، كلية الطب البيطري، جامعة القادسية، القادسية، العراق

الخلاصة

صممت الدراسة لتحديد النمط الجيني لطفيلي الاميبيا الحالة للنسيج *E.hitolytica* الموجودة في عينات الاسهال الدموي للإنسان والحيوان (الابقار والاغنام) باستخدام تقنية تفاعل السلسلة المتبلمرة في الوقت الحقيقي عن طريق تضخيم الجين SREHP واستعمال صبغة السايبر الخضراء. شملت الدراسة ايضا تقنية Nested-PCR لتحديد انواع طفيلي الاميبيا. سجلت الانواع *E. histolytica* و *E. Dispar* و *E. bovis* من مجموع الاصابات بنسبة ٨٢,٩ و ٢٦,٨ و ٤,٩% على التوالي. وجدت خمس انماط جينية للاميبيا الحالة للنسيج I وII و III و IV و V بنسبة ٩,٧٥ و ٥٣,٦٥ و ١٩,٥ و ٩,٧٥ و ٧,٣ على التوالي. استنتجت الدراسة هناك خمس انماط جينية للاميبيا الحالة للنسيج في الانسان والحيوان معظمها قد تصيب اي مضيف، تسجيل *E.bovis* في الابقار والاغنام.

Introduction

Many species of amoeba genus were identified in the first quarter of the twentieth century (1) and are spread in various regions of the world in the developing countries of the Indian subcontinent, Central and South America, and in the tropics of Africa (2), as well as many species that parasitize many hosts, some of which are common to more than one host (3).

The parasite has the ability to devour red blood cells by Erythrophagocytosis through the process of analyzing its plasma membrane and then digesting the base material, as

the ability of the feeding phases to digest erythrocyte may be due to the mechanism of adhesion to the surfaces of the cells and inhibit the adhesion of the parasite decreases phagocytosis and digestion of the erythrocytes during the parasite invasion of the large intestine layers (4), attributed (5) to (Hemoglobinase) and for the need of iron for the survival of the parasite, hence, the digestion of blood hemoglobin to get iron (4,5). The ability of (*E. dispar*) to cause liver injury, and some studies taken from the comparison of the genetic structure of both species (dispar and histolytica) have concluded that some sites of the gene responsible for the encoding of certain proteins of pathogenicity in the *E. histolytica* are the same in the *E. dispar* such genes as the amoebic hole Gal / GalNac-inhibitable lectin. (6,7).

In the presence of *E. histolytica* and *E. bovis* as single infection indicating that these parasites can shift from a coexistence with the host to pathogenicity (5), many studies have returned (and until recently) that have attempted to differentiate between *E. histolytica* infection and its cause, from invasions of tissues into and out of the intestine and between the infectious of *E. dispar* and other parasites, even those studies were considered unsatisfactory (8). This hypothesis is offset by recent studies that, using experimental animals, have demonstrated the events of the parasite *E. dispar* for focal ulcers and have an analytical capacity of the epithelial layer (9). performance development of a parasite which isolated from a person with no symptoms of amoebiasis in a medium containing intestinal flora, it was observed endemicity of amoebiasis taking into account the overlap between the two parasites and pathogenic bacteria or intestinal flora, which may alter the behavior of these organisms into a nurse, since some pathogenic bacteria have genes that directly or indirectly encoded molecules activated by the inflammatory response (10).

Materials and methods

Samples collection

A 34 human samples from Al-Hamza General Hospital and twenty eight animals samples were collected from different areas of Al-Hamza district, the presence of the parasite was confirmed in all samples by general stool examination via microscopic examination in the laboratories of the parasitology and microbiology branch in the College of Veterinary Medicine - Al-Qadisiyah University.

Stool DNA extraction

DNA was extracted from fecal samples using the Genomic DNA stool (AccuPrep® Stool DNA Extraction Kit) supplied by Bioneer Korean company according to the manufacturer instructions. This kit was designed to quickly and conveniently extract of DNA from genomic DNA from microorganisms in stool samples. In the presence of chaotropic salt, DNA is bound to glass fibers fixed in a column. Proteins and other contaminants are removed through washing stems, and the DNA isolated and eluted in the final elution step.

Nested PCR method

Nested PCR assay was performed detection *E. histolytica* and *E. dispar* according to (11), and *E. bovis* according to (12), the Nested PCR method was including using primary PCR primers for detection *Entamoeba* sp. Followed by Nested PCR primers that used for detection of *E. histolytica*, *E. dispar*, and *E. bovis* (Table 1).

The first round of Nested PCR master mix that include *Entamoeba* sp. Primary primers were prepared by using (AccuPower® PCR PreMix Kit, Bioneer, Korea) (Table 2).

Table 1: Nested PCR primers

Primer		Sequences (5'-3')	Product size
Entamoeba sp.	F	TTTGTATTAGTACAAA	~900bp
	R	GTAAGTATTGATATACT	
E. histolytica	F	AATGGCCCATTCATTCAATG	550bp
	R	TTTAGAAACAATGCTTCTCT	
E. dispar	F	GAGGATCCATGTTCGCATTTTTATTGT	729bp
	R	GAGGATCCTTAGAAGACAATTGCCA	
E. bovis	F	AAACTGCGGACGGCTCATTA	174bp
	R	CGCGGCATCCTTTTTCACAA	

After that, these PCR master mix components that mentioned in table above placed in standard PCR PreMix Kit that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$, stabilizer, and tracking dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. The reaction was performed in PCR thermocycler (T100 thermal cycler

BioRad. USA) by set up the following thermocycler conditions; Reactions were performed using the following protocol, initial denaturation at 95ºC for 4 min, and 35 cycles at 95ºC for 1 min, 47ºC for 1 min and 72ºC for 2 min, and a final incubation at 72ºC for 7 min.

The second round Nested PCR master mix that include *E. histolytica*, *E. dispar*, and *E. bovis* primers were prepared by using (AccuPower® PCR PreMix Kit, Bioneer, Korea) (Table 3).

Table 2: PCR master mix

First round PCR master mix	Volume
DNA template 5-50ng/µL	5µL
Entamoeba sp. Primary forward primer	1µL
Entamoeba sp. primary reverse primer	1µL
PCR water	13µL
Total volume	20µL

Table 3: Nested PCR master mix

Nested PCR master mix	Volume
First round PCR product	2µL
Second round *E. histolytica*, or *E. dispar*, and or *E. bovis* Forward primer (10pmol)	1µL
Second round *E. histolytica*, or *E. dispar*, and or *E. bovis* Reverse primer (10pmol)	1µL
PCR water	16µL
Total volume	20µL

After that, these Nested PCR master mix component that mentioned in table above placed in same PCR PreMix Kit Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes.

The reaction was performed in PCR thermocycler (T100 thermal cycler BioRad. USA) by set up the following thermocycler conditions; Reactions were performed using the following Nested PCR protocol, initial denaturation at 95ºC for 4 min, and 35 cycles at 95ºC for 1 min, (50ºC for *E. histolytica*, 60ºC for *E. dispar*, 57ºC for *E. bovis*) for 1 min and 72ºC for 1 min, and a final incubation at 72ºC for 1 min.

After that PCR products were examined by electrophoresis in a 1% agarose gel, stained with ethidium bromide, and visualized under UV Transilluminator.

Real-Time PCR method

Real-Time PCR was performed genotyping of *E. histolytica* based on allelic discrimination during melting curve of SREHP gene in positive *E. histolytica* according to (13) the method was firstly included PCR reaction for amplification of SREHP gene based on SREHP primer (Table 4).

The Real-Time PCR amplification reaction was done by using (AccuPower® Green Star™ qPCR PreMix kit, Bioneer. Korea) and the qPCR master mix were prepared for each sample according to company instruction (Table 5).

After that, these RT PCR master mix components that mentioned in table above was transferred into Green star qPCR premix standard plate tubes that contain the SYBER green dye and other PCR amplification components.

Then the plate mixed by Exispin vortex centrifuge for 3 minutes, then placed in MiniOpticon Real-Time PCR system and applied the following thermocycler conditions (Table 6).

Table 4: PCR and RT-PCR primers (SREHP) for *E. histolytica* as (12)

Primer		Sequences (5'-3')	Product size
PCR-SREHP	F	GCTAGTCCTGAAAAGCTTGAAGAAGCTG	549bp
	R	GGACTTGATGCAGCATCAAGGT	
RT PCR-SREHP	F	TATTATTATCGTTATCTGAACTACTTCCTG	450bp
	R	TGAAGATAATGAAGATGATGAAGATG	

Table 5: RT PCR master mix

RT PCR master mix	Volume
SREHP gene PCR product	2µL
RT PCR-SREHP gene forward primer	1µL
RT PCR-SREHP gene Reverse primer	1µL
PCR water	16µL
Total volume	20µL

Table 6: RT PCR master mix

qPCR step	Temperature	Time	Cycle
Initial Denaturation	95 °C	3 min	1
Denaturation	95 °C	10 sec	45
Annealing\ Extension	55 °C	30 sec	
Detection Melting	60-95°C	0.5 sec	1

Statistical analysis

The data were analyzed statistically to obtain the percentage and extract the Chi-square value (χ^2).

Results

The study recorded a total infection rate 66.1% where 41 samples showed a positive result by microscopic examination (Table 7). Percentage of infection was detected by using Nested PCR.

Table 7: Percentage of total infection using Nested PCR

Samples	Positive samples		Negative sample	
	No.	Percent%	No.	Percent%
Human	24	38.7	10	16.1
Cows	9	14.5	5	8
Sheep	8	12.9	6	9.7
Total	41	66.1	21	22.8

The study recorded the percentage of total infection with *E. histolytica* amounted to 79.1; 100; 75 (Figure 1) and *E. dispar* by 33.3; 22.2; 12.5 (Figure 2) in human, cows and sheep feces respectively, and *E. bovis* by 0; 11.1; 12.5 (Figure 3) in cattle and sheep, respectively (Table 8).

Table 8: Percentage of total infection by Nested PCR

Samples		*E. histolytica*	*E. dispar*	*E. bovis*
Human	n	19	8	0
(24)	%	79.1	33.3	0
Cows	n	9	2	1
(9)	%	100	22.2	11.1
Sheep	n	6	1	1
(8)	%	75	12.5	12.5
Total	n	34	11	2
(41)	%	82.9	26.8	4.9

The study showed the presence of the three parasites in the animals' samples, while the human samples were free from the presence of the parasite *E. bovis* (Table 8). The study showed significant effect of parasite type in the occurrence of infection with erythrocytes (F = 4.46), significant difference in the incidence between *E. histolytica* and *E. bovis* only where the animal type did not show significant effect (F = 0.848) at significant level P ≥ 0.05.

Genotypes of *E. histolytica* results

Genotyping of *E. histolytica* based on the Melting curve analysis that to detect the by Real-Time PCR assay based on amplification of SREHP gene by SYBER green dDNA binding dye which can differentiate melting temperatures (Tm) for each genotype 41. The Real-Time PCR Melting analysis results showed the presence of 5 different melting temperatures 79, 81, 82, 83, 84ºC of the SREHP gene for parasites (Figure 4). Each one melting temperature face one genotype 5 genotypes of *E. histolytica* were distributed as follows I, II, III, IV, V which corresponds to the melting temperatures 79, 81, 82, 83, 84ºC respectively, where genotypes were present 9.75, 53.65, 19.5, 9.75 and 7.3, respectively (Figure 5) (Table 9).

Table 9: Genotypes of *E. histolytica* based on melting temperature Tm

Total	Melting Tm.	Genotype	No.	%
	84	I	4	9.75
	83	II	22	53.65
41	82	III	8	19.5
	81	IV	4	9.75
	79	V	3	7.3

The relationship between the genotypes of *E. histolytica* and the presence of erythrocytes

The results showed that the presence of genotypes recorded in this study and found in fecal samples containing red blood cells was the following, where genotype II was more (22 out of 41). Genotype V was the least present in the total samples as it was found in only three samples, which was not found in cattle and sheep samples, whereas the study recorded the appearance of the five genotypes in human fecal samples (Table 10).

Table 10: Distribution of genotypes among hosts

Genotype	Human		Cows		Sheep	
	No.	%	No.	%	No.	%
I	3	7.3	1	2.4	0	0
II	13	31.7	3	7.3	6	14.6
III	3	7.3	4	9.75	1	2.4
IV	2	4.8	1	2.4	1	2.4
V	3	7.3	0	0	0	0
Total	24		9		8	

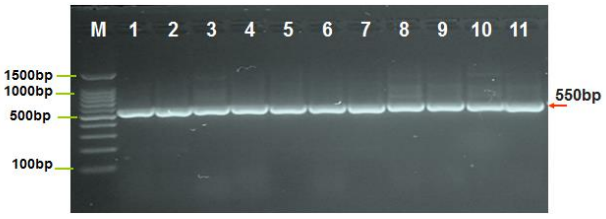

Figure 1: Electrophoresis in the agarose gel of products (550bp) PCR of rRNA for *E. histolytica.*

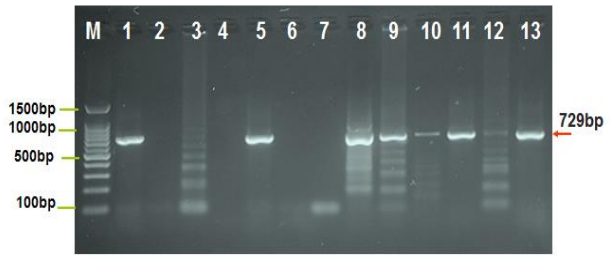

Figure 2: Electrophoresis in the agarose gel of products(729bp) PCR of rRNA for *E. dispar*.

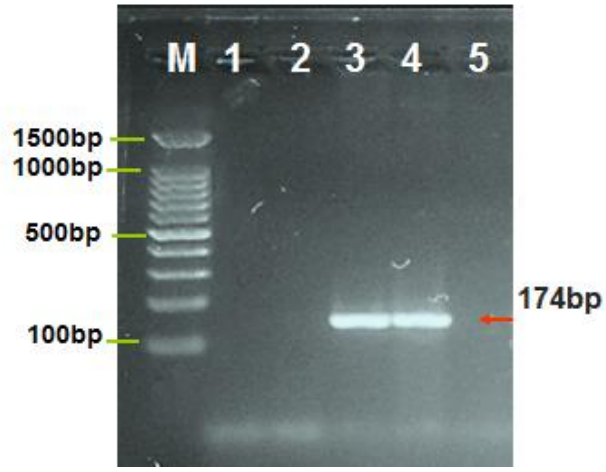

Figure 3: Electrophoresis in the agarose gel of products (174bp) PCR of rRNA for *E. bovis.*

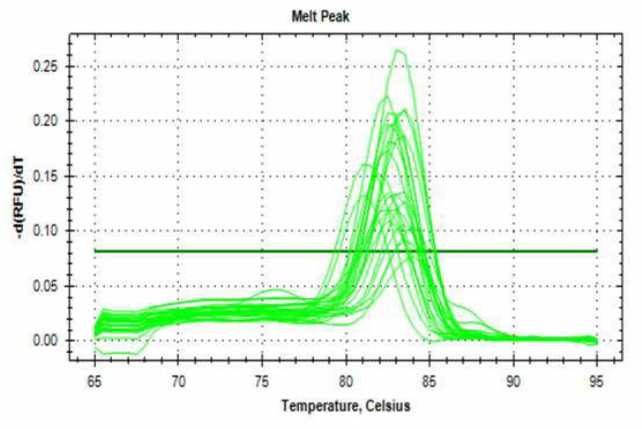

Figure 4: Different types of melting temperature peak for SREHP gene in *E. histolytica*

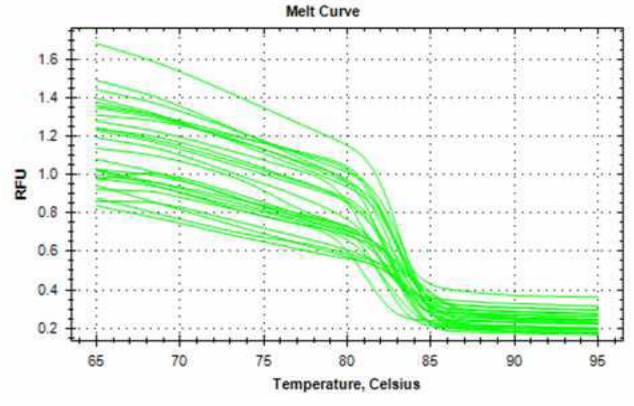

Figure 5: Melting curve for SREHP gene

Discussion

The study recorded the presence of *E. histolytica* and *E. dispar* in Humans, cows and sheep samples, indicating the possibility of transmission of these parasites between humans and animals (14) However, the incidence of infection with amoeba parasites in these animals or their functioning as potential reservoirs remains unclear. It is known that these parasites are transmitted through contaminated food and water where unhealthy habits, culture and use of contaminated water are considered. Irrigating crops, watering animals or using human and animal waste in agriculture help spread and transmission of the parasite among different hosts (15).

The presence of *E. bovis* in the host (cows) and in other hosts other than the usual (sheep), where previous studies confirmed the possibility of the presence and isolation of the parasite from other ruminants non-cattle such as deer and sheep may be due to the use of contaminated water for watering animals or the use of the same areas for grazing, which facilitates the transmission of parasites between them (16).

The results showed the high incidence of *E. histolytica*, which confirms the high ability of the parasite to analyze and devour the cells of tissues and red blood cells inside and outside the intestines (17) because they have enzymes analyzing the intestinal mucosa (8). The presence of *E. dispar* in humans may indicate a high pathogenicity of the parasite taking into account the presence of mixed infection with tissue-amoeba and the presence of a single infection of the parasite. The presence of other pathogens or auxiliary to the symptoms of infestation (15).

The presence of the parasite *E. bovis* even if only a small percentage confirms the ability of the parasite to cause infection even if the presence of another pathogen as a common infection.

The appearance of different genotypes reflecting the different melting temperatures of SREHP gene amplification products showed the large diversity in the nucleotide sequences and the content of the gene, reflecting a wide phenotypic variation of the genotype (II). Reflects the high ability of this genotype to spread and move among different hosts, especially with its presence in all hosts, Unlike Genotype V, which has the lowest ratio among all hosts (18).

The study showed the presence of four genotypes in human and cows feces in varying proportions, which the possibility of this genotype and its ability to be transmitted between humans and cows, genotype (V) which didn't appear in sheep samples, may mean that these genotypes cannot infect sheep during the period of collection time of samples.

The study showed the presence of genotype V with the lowest presence rate, which means that the ferocity of this genotype is low and its ability to infect is lower than the other genotypes. In cows as a host of the parasite.

The broad spectrum of genotypes that have emerged during the study and the appearance of some genotypes with the presence of erythrocytes in fecal samples may indicate the virulence of these genotypes and their ability to invade tissues and cause disease, but do not reveal the role of other pathogens. In the intestine, parasite virulence was observed to be proportional to ambient conditions outside the intestine, which may indicate significant variations in the parasite metabolism directly or indirectly.

Conclusion

There are five genotypes of *E. histolytica*, in human and animals, most of these genotypes may infect any host, *E. bovis* was recorded in sheep and cows.

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Conflict of interests

The Parasitology Department College of Veterinary Medicine for development and evaluation of
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