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Diagnosis and Genotyping Detection of *Entamoeba Spp*. in Human and Some Animals

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Abstract: *The purpose of this study was diagnosed of genotypes of Entamoeba spp. By using the PCR method and for identify some of the species that infect humans and animals using the Nested -PCR , registered as infection of Cows and Sheep with (E.histolytica; E.dispar; E. bovis) and infection human with (E.histolytica; E.dispar) only.*

RT-PCR Method used to amplification of SREHP gene using SYBR GreenI dye and melting curve analysis was done , total (46) specimens which were positive by microscopic and PCR examination to E.histolytica were selected and the experiment for the period from the beginning October 2014 until the end of March 2015, shows the presence of (5) genotypes track melting temperatures (84; 83; 82; 81 ; 79) , genotype (II) was the most common compare with genotype (V) which minimal, Devoid sheep samples from the presence of genotypes (I; III; IV), while (genotype III) presence with the highest proportion in Cows, which refers to the high virulence of genotypes (II ,III) and lack of ability genotypes (genotype I; III; IV) on the infected sheep.

1. INTRODUCTION

In the first quarter of the twentieth century identified several species which date back to genus *Entamoeba* spp. [1], which spread in a different regions of the world in developing and developed countries as it includes broad spectrum of species, which is being as parasitic controversial among scientists [2] addition to a lot of species that infect more than one host, some of which are shared between more than one host [3].

The molecular tests are the best method to diagnosis parasites of this Genus and distinguish them from each other or distinguish individuals of same Species that infect different hosts , as well as the modern molecular tests of *E. histolytica* showed that there were wide hereditary differences between Human and other animals [4] recognize the fact that *E. histolytica* and *E. dispar* are similar but are not identical species was effect in the correct diagnosis of infection and Search of Amoebiasis [5].

Use tools that enable to discrimination of *E. histolytica* strains important to giving answers to many questions being asked about the virulent of parasite or its attraction to a particular organ of the host's body and to differentiate between genetic models in infections accompanied with clinical symptoms or a symptoms[6].

Use of gene SREHP promising and important method to patterning the parasite strains based on DNA as the large number of variations in the occurrences of the genes Chitinase and SREHP into the genome of Amoeba used as an indicator of the link between the genotype of the parasite and the disease caused [7].

The aims of study were to diagnosis and detection the genotype of *Entamoeba* spp. In different hosts.

2. MATERIALS AND METHODS

The current study was conducted in the laboratories of Parasites and Microbiology department at Veterinary Medicine College at Al- Qadisiya University/ Iraq where collected (94) specimens as (46) from human , (24) from cows and(24) from sheep from different areas of the Al- Qadisiya province which suffer from diarrhea .

Check all the samples by microscope and were positive for examination to Amoebiasis then was confirmed parasite presence using the PCR, identified existing of species in these samples by Nested PCR, amplified the Serine-rich *E. histolytica* protein by RT-PCR using SYBR GreenI dye and Melting curve analysis to identify genotypes exist.

2.1. Stool DNA extraction

DNA of specimens was extracted by using (Genomic DNA Stool extraction kit) which processed by the Korean Bioneer company, according to the manufacturer's instructions.

2.2. Primers

Primer		Sequence	Amplicon	Sources
E gene	**E1**	**TTTGTATTAGTACAAA**	**900 bp**	**(Haque *et al*., 1998)**
	E2	**GTA[A/G]TATTGATATACT**		
***E.histolytica* gene**	**EH1**	**AATGGCCCATTCATTCAATG**	**550 bp**	**(Yee Ling Lau *et al* ., 2013)**
	EH2	**TTTAGAAACAATGCTTCTCT**		
SREHP	**F**	**GCTAGTCCTGAAAAGCTTGAAGAAGCTG**	**549 bp**	**(Rahman *et al* ., 2006)**
	R	**GGACTTGATGCAGCATCAAGGT**		
nSREHP	**F**	**TATTATTATCGTTATCTGAACTACTTCCTG**	**450 bp**	
	R	**TGAAGATAATGAAGATGATGAAGATG**		
SREDP	**F**	**GAGGATCCATGTTCGCATTTTTATTGT**	**729 bp**	**NCBI**
	R	**GAGGATCCTTAGAAGACAATTGCCA**		
E.bovis	**F**	**AAACTGCGGACGGCTCATTA**	**174bp**	
	R	**CGCGGCATCCTTTTTCACAA**		

2.3. PCR and Nested - PCR method

PCR assay was performed using primers of specific genes (18S rRNA) which responsible for diagnosis of *Entamoeba spp.* From human and animal feces, according to [8].

2.4. Real-Time PCR

Real-Time PCR technique conducted by using primers of specific gene (SREHP) which responsible for distinguish of *E. histolytica* by the way of [9] as following :

2.4.1. Real-Time PCR master Mix

Prepared a mixture Real-Time PCR reaction using apparatus AccuPower® 2X Green Star TM qPCR Master Mix which processed by Korean Bioneer company, according to the company's instructions, as in the table following:

PCR master mix	Volume
2X Green Star master mix	25 µL
DNA template	5µL
Forward primer10pmol	2.5 µL
Reverse primer10pmol	2.5 µL
DEPC water	15µL
Total	50µL

Put the components of Real-Time PCR reaction in a special white dark tubes size 0.2ml, then transfer all tubes to vortex centrifuge speeds of 3000rpm for three minutes and then placed in a Real-Time PCR device.

2.4.2. *Real-Time PCR Thermo cycler conditions*

Application of thermal cycles to check the Real-Time PCR and by relying on several instructions AccuPower® 2X Green Star TM qPCR Master Mix as well as through account the degree Tm of primers using the device Miniopticon Real-Time PCR system BioRad. USA as following table:

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	3 min	1
Denaturation	95 °C	10 sec	45
Annealing\Extension Detection(scan)	58 °C	30 sec	
Melting	65-95°C	0.5 sec	1

2.5. Melting curve analysis

After completion of 40 cycles of PCR , the PCR products were melted by raising the temperature from 40Cº to 95Cº at a rate of one degree every minute, where iCycler iQ program displays collected data curve shows the included increasing temperature up to the Peak melting curve which represents the point of variations in DNA multiplier [10].

2.6. Statistical Analysis

The data were analyzed statistically to get a percentage and the value of Chi square.

3. RESULTS AND DISCUSSION

The study recorded overall infection (65.9%) in *Entamoeba* spp. which showed 62 sample as a result of a positive examination by PCR from 94 samples were positive by microscopic examination divided by (73.9%, 58.3%, 58.3%) in samples of human , Cows and sheep respectively fig. (1) .

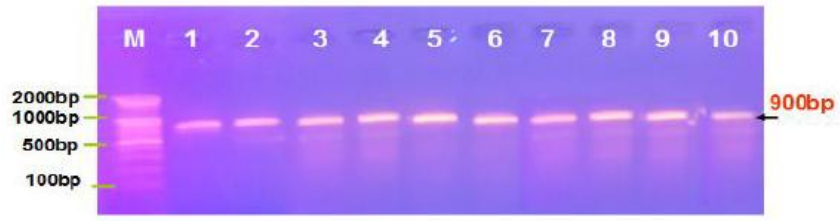

Figure1. *Agarose gel electrophoresis image that show the PCR product analysis of small subunit rRNA gene in Entamoeba spp. positive stool samples. Where M: marker (2000-100bp), lane (1-10) Entamoeba spp. positive stool samples at (900bp) PCR product.*

3.1. Infection Rate by Nested-PCR

From 62 samples were positive by PCR examined by Nested-PCR this study recorded total proportion of *E.histolytica* infection estimated (77.4%) fig. (2) and *E. dispar* infection (32.3%) fig. (3) *E.bovis* (9.6%) Fig.(4) from all samples of human ,cows and sheep (Table 1).

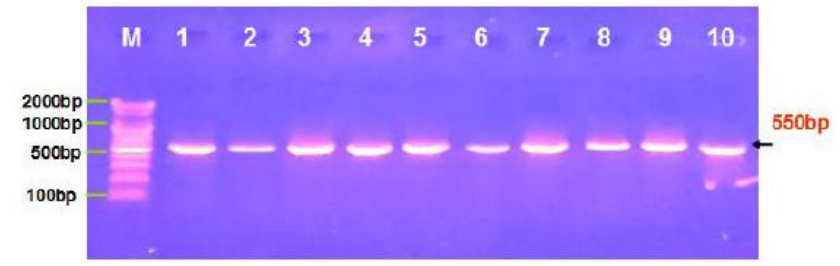

Figure2. *Agarose gel electrophoresis image that show the Nested PCR product analysis of small subunit rRNA gene in Entamoeba histolytica positive stool samples. Where M: marker (2000-100bp), lane (1-10) Entamoeba histolytica positive stool samples at (550bp) nPCR product.*

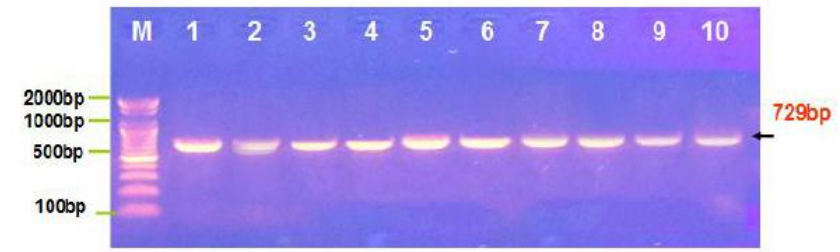

Figure3. *Agarose gel electrophoresis image that show the PCR product analysis of small subunit rRNA gene in Entamoeba dispar positive stool samples. Where M: marker (2000-100bp), lane (1-10) Entamoeba dispar positive stool samples at (729bp) PCR product.*

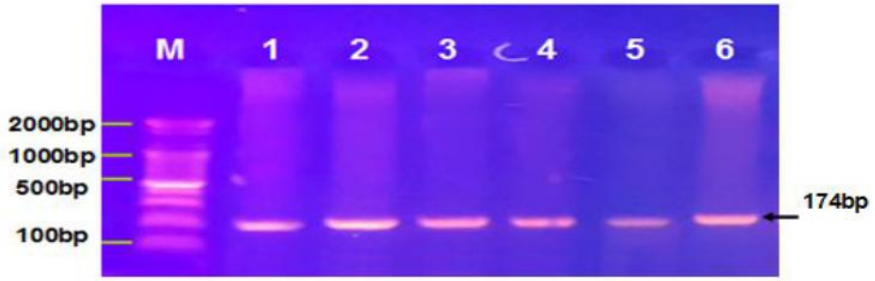

Figure4. *Agarose gel electrophoresis image that show the PCR product analysis of small subunit rRNA gene in Entamoeba bovis positive stool samples. Where M: marker (2000-100bp), lane (1-6) Entamoeba bovis positive stool samples at (174bp) PCR product.*

Table1. *total infection By Nested PCR*

E.bovis*		***E.dispar***		***E.histolytica****		**Examined**	
Negative	**Positive**	**negative**	**positive**	**negative**	**positive**		
34	0	22	12	8	26	NO	Human No:34
100	0	64.7	35.3	23.5	76.5	Percentage %	
28.5	4	11	3	2	12	NO	Cows NO:14
71.4	10	78.6	21.4	14.3	85.7	Percentage %	
14.2	2	9	5	4	10	NO	Sheep No:14
85.7	12	64.3	35.7	28.6	71.4	Percentage %	
56	6	42	20	14	48	NO	Total
90.3	9.6	67.7	32.3	22.6	77.4	Percentage %	

The study did not show a significant effect of the host in determining the incidence while type of parasite showed a significant effect at $P \geq 0.05$ as well as the significant difference in infection rates between *E.histolytica* and *E.bovis* only at $P \geq 0.05$.

3.2. The Proportion of Infection in Human Feces Samples

The present study recorded a total infection rate (73.9%) it was found that 34 of 46 samples were positive by microscopic examination showed a positive result of PCR molecular examination found no significant difference in the incidence between microscopic examination and molecular at significant level $P \geq 0.05$.

The current study found that the proportion of total infection of *E.histolythca* (76.5%) , *E.dispar* (35.3%) and empty human feces samples from *E.bovis* This explains allocate these parasites to host It is known that the human not consider host to the parasite *E.bovis*.

The results of this study were close to the results recorded by [11] When recorded the percentage of infection 62.96% in Iran , [12] recorded infection ratio 67.7% In Malaysia as it was 3.2% a single with *E.histolytica* and 13.4% of them *E.dispar*, All of these studies used PCR testing traditional way , either [13] find The number of positive samples was examined RT- PCR 124 from 205 sample checked.

The study recorded the results were higher than [14] when recorded rate reached 33.3% in Al-Qadisiya province in Iraq, as well as recorded [15] ratio reached to 7.9% of which was 69.2% of type *E.histolytica* and 10.3% *E.dispar* between patients of Qasr al-Aini hospital in Egypt, [16] in Uganda recorded infection rate of 36.7% was 7.31% *E.histolytica* and 21.6% for other species , [17] recorded the proportion amounted to 14% of the samples positive microscopic examination among children in the West Bank in Palestine .

It attributed the difference in infection rates of these parasites that these parasites irregularly spread in most countries of the world, to the different climatic conditions, customs and traditions.

3.3. Proportion of Infection in Animal Feces Samples

The present study recorded overall infection (58.3%) in 14 of 24 fecal samples of cows positive microscopic examination and the same number of samples in sheep when study recorded proportion of infection reached to (58.3%), as were 14 of the samples are positive, and the study has recorded the presence of significant difference in the incidence of molecular and microscopic examination only in cows at significant level $P \geq 0.05$.

The study recorded the presence *E.histolytica* by Nested-PCR (85.7%; 71.4%) and *E.dispar* (21.45%; 35.7%) in Cows and sheep samples respectively, indicating the possibility of transmission of these parasites between human and animal [18] but remains occurrence infection of amoeba in these animals or their work as Potential reservoir and thus its role in the transfer of infection is unclear [19] as it is known transmission of these parasites through food and water polluters where is the habits of health, culture and the use of contaminated water for irrigating crops and watering animals or the use of human and animal waste in agriculture factors help spread and transmission of the parasite between different families.

The presence of *E. bovis* in cows samples and sheep by (28.5%; 14.2%) this study proved the presence of the parasite in the normal host (Cows) and in other unusual hosts (Sheep), as previous studies have confirmed the possibility of the presence and the isolation of the parasite from other ruminants is cows such as deer and sheep [20] The reason is due to the use of contaminated water in the watering animals or animals use the same areas for grazing which facilitates the transmission of parasites themselves.

The result of the study relative to the record of [21] In the National Park in Ivory Coast, where he scored the injury ratio stood at 53.27% at study intestinal parasites which spread at the zoo also [22] recorded 57.17% infection rate in horses and donkeys in Diwaniya - Iraq.

Other studies have recorded infection rates less than the current study when,[23] recorded ratio between animals of public park in the United Kingdom reached to 28.9% , was 24.6% of which for the *E. histolytica* and 2.9% for *E. dispar*), [24] recorded Infection rate higher than the current study, when he scored infect in 45 of 64 sample which positive in microscopic examination of animal feces samples in Rome garden and infection by 8% *E. dispar* and 9% *E. histolytica*, [19] recorder 71.5% infection rate.

Can be attributed to the difference in the ratios to a lack of attention to health and culture among ranches or commitment them to health conditions in the establishment of corrals animal breeding and lack of knowledge that there is a shared or transmitted by animals or use of water contaminated feed diseases and to the difference in geographical nature and the climate of the region.

3.4. Melting Curve Analysis for Genotyping Detection

SREHP genes were amplified in (46 of 48) sample of parasite *E. histolytica*, which were positive by microscopic and molecular PCR by Real-Time PCR and using SYBR Green I form (5) while two samples only failure , the results of the melting curve analysis of the amplification products showed the presence of (5) different melting temperatures (84.83, 82.81, 79) C° for SREHP gene for *E. histolytica* form (6).

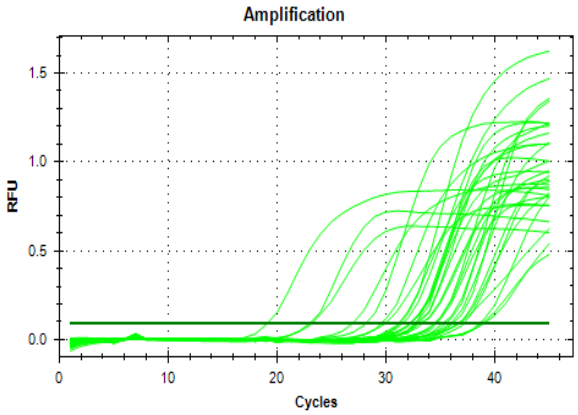

Figure5. *Real-Time PCR amplification plots that show amplification plots of nested SREHP gene of Entamoeba histolytica.*

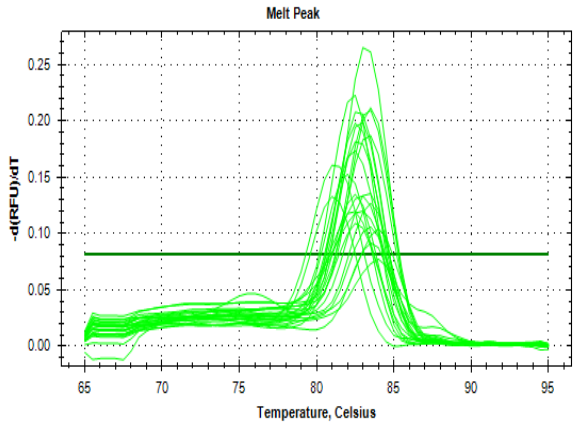

Figure7. *Display Real-Time PCR Melt peak that shows the melting point for nested SREHP gene of Entamoeba histolytica that detect the genotyping Entamoeba histolytica are Tm 84°C: Genotype-I, Tm 83°C: Genotype-II, Tm 82°C: Genotype-III, Tm 81°C: Genotype-IV and Tm 80-79°C: Genotype-V.*

SYBR green dye used in many scientific research in the diagnosis of parasites *Entamoeba* spp. [25]; [26] ,*Cryptosporidiumparvum* [27] and *Microsporidia* sp. [28].

The study showed presence of (5) curves represent (5) genotypes of *E. histolytica*, which indicates the wide difference between genotypes of this parasite depending on the different melting degrees of amplification products where these grades ranged between 79-84Cº as melting curve analysis based on the principle of quantitative GC content and the length and sequence of the target or piece genetic amplified [29] gene as the double which contains 0% of GC melts at a lower rate by four percentage points of the dual with a 100% content and the extent of a melting heat products amplification process up to 50 C º this tide widespread helped to disperse most of the products the process of amplification [30] in addition to the analysis of melting curve does not need to electric deportation but depends on the ratio between the GC / AT, but more than that, the equal in the amount of content of GC products differ in the distribution of these rules differ in the melting curve (9).

3.5. Genotypes of the Parasite E. Histolytica

Genotype result was (5) genotypes of *E. histolytica* distributed as follows (genotype: I; II; III; IV; V) which correspond to melting temperatures (84, 83.82, 81.79)Cº respectively, where the proportion of the presence of genotypes (10.86% ; 50% ; 18.75% ; 10.86% ; 8.33%) respectively table (2).

These findings are agreed with [9], In a study conducted in the United Arab Emirates Recorded [31]. Presence of 12 different genotypes of *E. histolytica* and Gene SREHP also recorded [32] Presence of 12 genotypes in Turkey, in the study [25] recorded 10 genetic models for the same parasite using the same gene [33] Using DNA microarray method in profiling parasite *E. histolytica* and *E. dispar* recorded four genotypes for *E. histolytica* and two for *E. dispar*.

Seen (Genotype II) the highest percentage (50%) reflects the high potential of this genotype to spread and transmission between different hosts especially with the presence in each host that examined contrary genotype (Genotype V) that the presence of the least ratio (8.33%) among all hosts.

3.6. Genotypes of E.histolytica in samples of human and animal feces

Table (4) showing (genotype II) as the highest percentage in samples of Human and Animal feces together (50%) as the proportion of the presence of record was (50%; 25%; 87.5%) in samples of Human, Cows and Sheep respectively.

Record (genotype III) in a higher proportion of presence in samples of Cows feces (50%),which the highest presence of a genotypes in samples of Cows feces table (3).

Five genotypes were present in samples of Human and Cows feces but deserted Sheep feces samples from genotypes (I; III; IV) Table (3).

Record genotype (V) ratio of less presence in samples of Human and Animals feces together as Seen by (8.69%) reaching its presence in Human , Cows and sheep samples feces as (7.7%; 8.3%; 12.5%) respectively table (3).

Seen genotype (II) a high proportion in all hosts refers to the high virulence of this genotype, while the presence of genotype (III) at the highest rate in Cows reflects its ability to infect this host and high specialization.
Free sheep feces samples from genotypes (I; III; IV), which may indicate these genotypes can't infect host which recorded significant difference between genotypes ratios in $P \geq 0.05$.

Table2. *different melting temperature of E.histolytica*

Percentage	No:	Genotypes	Tm C˚	Total
10.86	5	Genotype I	84	46
50	23	Genotype II	83	
19.5	9	Genotype III	82	
10.86	5	Genotype IV	81	
8.69	4	Genotype V	79	

Table3. *Compare genotypes between Human and Animals*

Sheep No: 8		**Cows No:** 12		**Human No:** 26		**Genotypes**
Perc.%	**NO:**	**Perc.%**	**NO:**	**Perc.%**	**No:**	
0	0	8.3	1	15.4	4	Genotype I
87.5	7	25	3	50	13	Genotype II
0	0	50	6	11.5	3	Genotype III
0	0	8.3	1	15.4	4	Genotype IV
12.5	1	8.3	1	7.7	2	Genotype V

4. CONCLUSIONS

Method of genotyping using SREHP gene and Melting curve analysis easy way and give guaranteed results, presence of (5) genotypes for SREHP in humans and animals and the possibility of animal infect with *E.histolytica* and *E.dispar* .Infect Cows and sheep with *E.bovis* and not infect human, which to the first study in Iraq . We recommend to using more methods of molecular evolution and the use of more than one location Jenny to draw an evolutionary tree minutes to this genus.

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