Molecular study to detection and genotyping of *Entamoeba* spp. in cattle and sheep

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Abstract

The aim of the study was to diagnose the *Entamoeba* spp. in cattle and sheep by using PCR method, for identified some of these species used the Nested-PCR, and identified genotypes of *E. histolytica* by RT-PCR and Syber Green I dye. Forty eight (48) fecal samples (24 cows and 24 sheep) positive with microscopic test were collected from the beginning of October 2014 until the end of March 2015, then examined with PCR for detection of *Entamoeba* spp., species identified by Nested –PCR, then genotypes of *E. histolytica* determined by amplification of SREHP gene using RT-PCR with SYBR Green I dye and melting curve analysis. Results were recorded infection of cows and sheep by (*E. histolytica, E. dispar, E. bovis*) in (78.5%, 28.5%, 21.5%) ratio respectively. Five genotypes (I, II, III, IV, V) of *E. histolytica* were detected. All the genotypes infect the cows but the sheep infected with two (II,V) genotypes only. In conclusion: *E. histolytica* has wide spectrum of genotypes, some of these genotypes unable to infection sheep.
Key words: *Entamoeba* bovis, RT-PCR, SREHP, genotyping, melting curve analysis.

دراسة جزيئية لتشخيص وتنميط طفيلي *Entamoeba* spp. في الأبقار و الأغنام

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الخلاصة

أجريت الدراسة الحالية للكشف عن تواجد انواع طفيلي *Entamoeba* spp. التي تصيب الأبقار والأغنام باستخدام طريقة PCR ولتمييز أنواع الطفيلي استخدمت تقنية Nested –PCR وللكشف عن التنميط الوراثي لطفيلي *Entamoeba histolytica* من خلال تضخيم جينSREHP باستخدام تقنيةRT-PCR وصبغة السيابر الخضراء وتحليل منحنى الانصهار. جُمِعت 48 عينة براز (24 من الأبقار و24 أخرى من الأغنام) موجبة بالفحص المجهري خلال الفترة من بداية شهر تشرين الأول 2014 وحتى نهاية شهر آذار 2015 ، فُحِصت العينات الموجبة بتقنية PCR للتأكد من وجود الطفيلي ، بعدها تم تمييز أنواع الطفيلي باستخدام طريقة Nested PCR ، حددت الأنماط الوراثية لطفيلي *E. histolytica* باستخدام طريقة RT-PCR. سجلت الدراسة إصابة الأبقار والأغنام بالأنواع (*E. histolytica, E. dispar, E. bovis*) حيث بلغت نسبة الإصابة الكلية 78.5% ، 28.5% ، 21.5% على التوالي. كشفت الدراسة تواجد خمسة طرز وراثية (I, II, III, IV, V) لطفيلي *E. histolytica* تواجدت جميع الطرز الوراثية في عينات الأبقار بينما خلت عينات الأغنام من الطرز الوراثية (I, III, IV). أثبتت الدراسة إمكانية إصابة الأبقار والأغنام بأنواع طفيلي الأميبا (*E. histolytica, E.dispar, E. bovis*) ، كما إن لطفيلي *E. histolytica* مدى واسع من الطرز الوراثية ، إن تواجد اثنان فقط من الطرز الوراثية في براز الأغنام يشير إلى عدم إمكانية هذه الأنماط على إصابة المضيف في حين تواجد الأنماط الخمسة في براز الأبقار قد يؤشر قدرة هذه الأنماط على إحداث الإصابة بالأبقار.
الكلمات المفتاحية: التفاعل التسلسلي للبلمرة – الوقت الحقيقي ، بروتين الغني بالسيرين في الأميبا ، التنميط الوراثي ، تحليل منحنى الانصهار ، *Entamoeba bovis*

Introduction

The molecular tests are the best method to diagnosis of *Entamoeba* spp. and to distinguish it's species from each other or distinguish same species that infect different hosts, as well as the modern molecular tests of the *E. histolytica* showed that there are

wide hereditary differences between human and animals (1), (2) recognize the fact that *E. histolytica* and *E. dispar* are similar but are not identical species is effected on the correct diagnosis of infection and search of amoebiasis. Use tools that enable to discrimination of *E. histolytica* strains important to giving answers to many questions being asked about the virulence of parasites or its attraction to a particular organ of the host's body and to differentiate between genetic models in infections accompanied by clinical symptoms (3). Use of gene SREHP is a promising and important method for 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 (4). SYBR green dyes used in many scientific research in the diagnosis of parasites such as *Entamoeba* spp. (5, 6), *Cryptosporidium parvum* (7) and *Microsporidia* spp. (8). The aim of the study was to diagnosis and detection the genotypes of *Entamoeba* spp. in cattle and sheep.

Materials and methods

Samples collection

The study was conducted on 48 fecal samples (24 of cows, and 24 of sheep suffer from diarrhea), collected from different areas of the Al-Qadisiya province, examined by microscope, and the positive samples were examined by PCR method to confirm the *Entamoeba* spp., after that identified existence of species in these samples by Nested PCR, amplified the Serine-rich *E. histolytica* protein by RT-PCR using SYBR Green I dye and melting curve analysis to identify genotypes exist (9).

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.

PCR and Nested - PCR method

PCR assay was performed using primers of specific genes (18S rRNA) which responsible for diagnosis of *Entamoeba spp.* from animal feces, according to (11, 12).

Real-Time PCR

Real-time PCR technique conducted by using primers of a specific gene (SREHP) which responsible for distinguishing of *E. Histolytica* according to (9).

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 (13).

Statistical Analysis

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

Primers

Primer	Sequence		Amplicon
E. gene (10)	E1	TTTGTATTAGTACAAA	900 bp
	E2	GTA (A/G)TATTGATATACT	
E. histolytica gene (11)	EH1	AATGGCCCATTCATTCAATG	550 bp
	EH2	TTTAGAAACAATGCTTCTCT	
SREHP (9)	F	GCTAGTCCTGAAAAGCTTGAAGAAGCTG	549 bp
	R	GGACTTGATGCAGCATCAAGGT	
nSREHP (9)	F	TATTATTATCGTTATCTGAACTACTTCCTG	450 bp
	R	TGAAGATAATGAAGATGATGAAGATG	
SREDP*	F	GAGGATCCATGTTCGCATTTTTATTGT	729 bp
	R	GAGGATCCTTAGAAGACAATTGCCA	
*E. bovis**	F	AAACTGCGGACGGCTCATTA	174bp
	R	CGCGGCATCCTTTTTCACAA	

***NCBI: National center biotechnology information**

Results

Proportion of infections by PCR.

The study recorded overall infection by PCR reached (58.3%) in 14 out of 24 fecal samples of cows positive in microscopic examination, and same ratio recorded in sheep also (Fig. 1), also the study has recorded the presence of significant difference in the incidence of molecular and microscopic examination in cows only at significant level (P≤0.05) (Table 1).

Table (1): Proportion of total infections by PCR.

Z Value		Negative		Positive		Examined
Table	Calculated	%	No.	%	No.	
0.025	0.096	41.6	10	58.3	14	Cows
	0.008	41.6	10	58.3	14	Sheep

Infection rate by Nested-PCR

From 28 samples were positive by PCR examined by Nested-PCR this study recorded total proportion of *E. histolytica* infection estimated (78.5%) (22 from28 samples) (Fig. 2) and *E. dispar* infection (28.5%) (Fig. 3) *E. bovis* (21.4%) (Fig. 4) from all samples of cows and sheep (Table 2). The study did not show a significant effect of the host in determining the incidence while types of parasite showed a significant effect at (P≤0.05) as well as the significant difference in infection rates between *E. histolytica* and *E. bovis* only at (P≤0.05) (Table 2).

Melting curve analysis for genotyping detection

SREHP genes were amplified in (20 of 22) samples (12 samples from cows and 10 from sheep) of *E. histolytica*, which were positive by microscopic and molecular PCR by Real-Time PCR and using SYBR Green I (Fig. 5) while two samples only failures, the results of the melting curve analysis of the amplification products showed the presence of five different melting temperatures (84.83, 82.81, 79) Cº for SREHP gene for *E. histolytica* (Fig. 6).

Genotypes of the parasite *E. histolytica*

The result was showed five genotypes of *E. histolytica*. It was (I, II, III, IV, V) which corresponds to melting temperatures (84, 83, 82, 81, 79) Cº respectively, where the proportion of the presence of genotypes (5%, 50%, 30%, 5%, 10%) respectively (Table 3).

Genotypes of *E. histolytica* in animal's feces samples

Five genotypes were present in samples of cows feces but deserted sheep feces samples from genotypes (I, III, IV) (Table 4). This study showed (genotype III) in a higher proportion of presence in samples of cows feces (50%) (Table 4). This study recorded genotype (V) ratio of less presence in samples of cows and sheep feces together as (8.3%, 12.5%) respectively (Table 4).

Table (2): Infection rate by Nested-PCR.

*E. bovis**		*E. dispar*		*E. histolytica**		Examined	
Negative	Positive	Negative	Positive	Negative	Positive		
10	4	11	3	2	12	No.	Cows (N=14)
71.4	28.5	78.6	21.4	14.3	85.7	Percent %	
12	2	9	5	4	10	No.	Sheep (N=14)
85.7	14.2	64.3	35.7	28.6	71.4	Percent %	
22	6	20	8	6	22	No.	Total
78.5	21.5	71.5	28.5	21.5	78.5	Percent %	

Table (3): Different melting temperature of *E. histolytica*

%	No.	Genotypes	Temp. C°	Total
5	1	Genotype I	84	46
50	10	Genotype II	83	
30	6	Genotype III	82	
5	1	Genotype IV	81	
10	2	Genotype V	79	

Table (4): Compare genotypes between sheep and cows

Sheep (N=8)		Cows (N=12)		Genotypes
No.	%	No.	%	
0	0	1	8.3	Genotype I
7	87.5	3	25	Genotype II
0	0	6	50	Genotype III
0	0	1	8.3	Genotype IV
1	12.5	1	8.3	Genotype V

Fig. (1): 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.

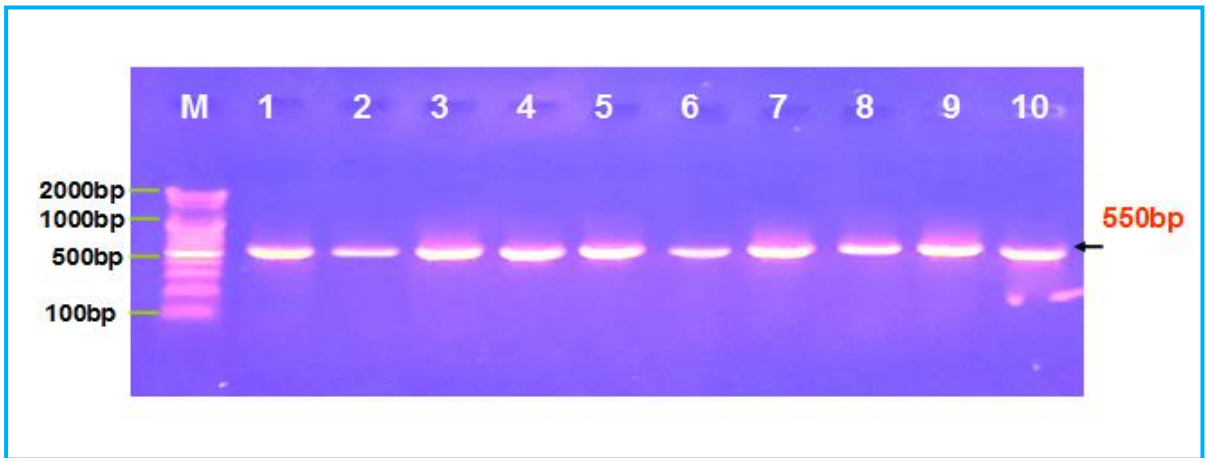

Fig. (2): Agarose gel electrophoresis image that show the Nested PCR product analysis of small subunit rRNA gene in *E. histolytica* positive stool samples. Where M: marker (2000-100bp), lane (1-10) *E. histolytica* positive stool samples at (550bp) nPCR product.

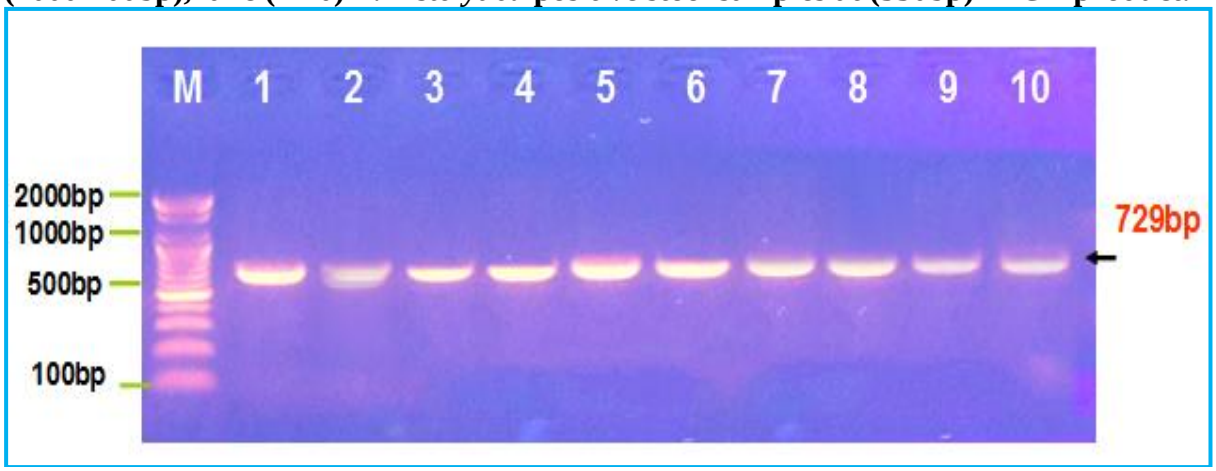

Fig. (3): Agarose gel electrophoresis image that show the PCR product analysis of small subunit rRNA gene in *E. dispar* positive stool samples. Where M: marker (2000-100bp), lane (1-10) *E. dispar* positive stool samples at (729bp) PCR product.

Fig. (4): Agarose gel electrophoresis image that show the PCR product analysis of small subunit rRNA gene in *E. bovis* positive stool samples. Where M: marker (2000-100bp), lane (1-6) *E. bovis* positive stool samples at (174bp) PCR product.

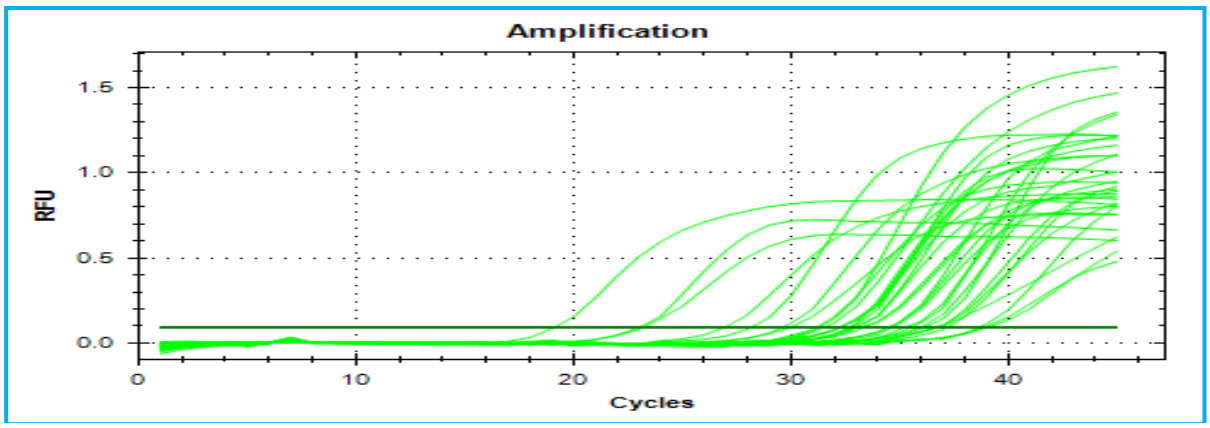

Fig. (5): Real-Time PCR amplification plots that show amplification plots of nested SREHP gene of *E. histolytica*.

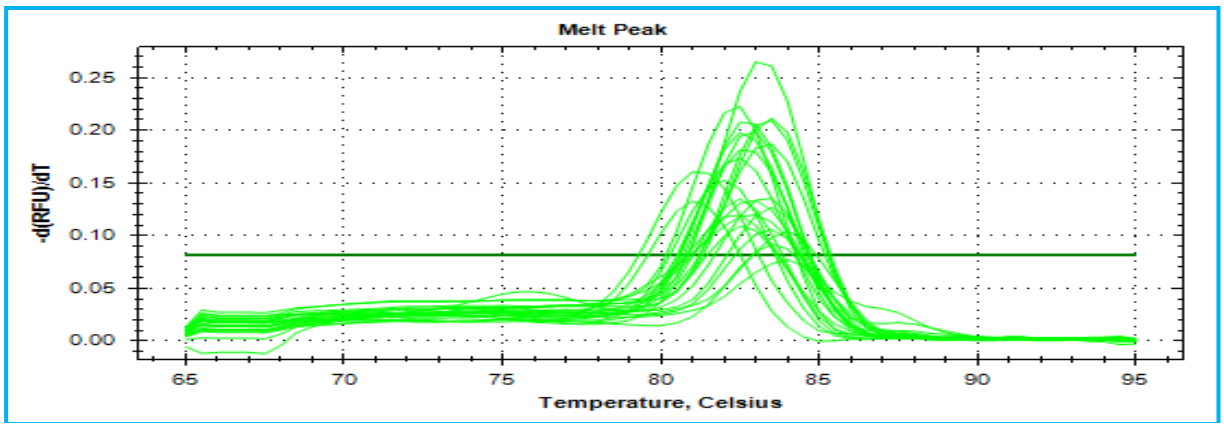

Fig. (6): Display Real-Time PCR Melt peak that shows the melting point for nested SREHP gene of *E. histolytica*.

Discussion

Infection rate by Nested-PCR

The study records presence of *E. histolytica* (85.7%, 71.4%) and *E. dispar* (21.45%, 35.7%) by Nested-PCR in cows and sheep samples respectively, 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 (13) 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 hosts. 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 abnormal host (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 (14). 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 this study matches the record of (15) In the National Park in Ivory Coast, where he recorder infection ratio of *Entamoeba* spp. at 53.27% at study intestinal parasites which spread at the zoo also (16) recorded 57.17% infection rate of *E.coli* in horses and donkeys in Diwaniya–Iraq. Other studies have recorded infection rates less than the current study when (17) 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% *E. dispar*, (13) recorded 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 commit 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.

Melting curve analysis

The study showed presence of five curves represent five 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 (18) 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 50Cº this tide widespread helped to disperse most of the products the process of amplification (19) 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 (10).

Genotypes of the parasite *E. histolytica*.

The result was show five genotypes of *E. histolytica* (I, II, III, IV, V) which corresponds to melting temperatures (84, 83, 82, 81, 79) Cº respectively. These findings agree with (10), in a study conducted in the United Arab Emirates. (20) recorded presence of twelve different genotypes of *E. histolytica* and Gene SREHP also (21) recorded presence of twelve genotypes in Turkey, (5) recorded ten genetic models for the same parasite using the same gene. (22) using DNA microarray method in profiling parasite *E. histolytica* and *E. dispar* recorded four genotypes for *E. histolytica* and two for *E. dispar*. The (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 (Genotypes I, IV) that the presence of the least ratio (5%) among all hosts. The 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. Feces samples of sheep free from genotypes (I, III, IV) may indicate that genotypes can't infect the host, which recorded a significant difference between genotypes ratios in ($P \leq 0.05$). In conclusions: Method of genotyping using SREHP gene and Melting curve analysis easy way and give guaranteed results, presence of five genotypes for SREHP in animals and the possibility of infect animals with *E. histolytica*, *E. dispar* and *E. bovis* which the first molecular recorder in Iraq.

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