ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

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

Ghaidaa Abbas Jasim[1], Musafer Hindi Alardi[2]

1 Al-Qadisiya unv. - Veterinary collage

2 Al-Qadisiya unv. - Science collage

Manuscript Info

Manuscript History:

Received: 15 October 2015
Final Accepted: 22 November 2015
Published Online: December 2015

Key words:

Entamoeba bovis , RT-PCR , SREHP , Genotyping , melting curve analysis .

Corresponding Author

Ghaidaa Abbas Jasim

Abstract

The purpose of this study was planned to diagnose *Entamoeba* spp.in Cattles and Sheep By using the PCR method , for identified some of these species used the Nested -PCR, and identified genotypes of *E. histolytica* by RT-PCR and SYBER GreenI dye .

Collected (48)Samples (24 from all cows and sheep) that positive with microscopic test ,then examined with PCR to detection *Entamoeba* spp., species identified by Nested –PCR , then we determined genotypes of *E. histolytica* by amplification of SREHP gene used RT-PCR with SYBR GreenI dye and melting curve analysis, for the period from the beginning October 2014 until the end of March 2015 .

This study recorded infection of cows and sheep by (*E.histolytica; E.dispar ; E. bovis*) with infection ratio as (78.5% , 28.5% , 21.5%) respectively and detected five genotypes (I , II ,III , IV , V) of *E. histolytica*, all genotypes infected cows but sheep infected with (II , V) only .

Method of genotyping using SREHP gene and Melting curve analysis easy way and give guaranteed results, presence of five genotypes for SREHP in cows while two genotypes in sheep and the possibility of infect them with *E.histolytica* ; *E.dispar* and *E.bovis* which the first molecular recorder in Iraq .

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 were wide hereditary differences between them in Human and animals (1).

Recognize the fact that *E. histolytica* and *E. dispar* are similar but are not identical species was effected on the correct diagnosis of infection and Search of Amoebiasis (2).

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 or a symptom (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 by it (4).

SYBR green dyes used in many scientific research in the diagnosis of parasites *Entamoeba* spp. (5; 6) ,*Cryptosporidium parvum* (7) and *Microsporidia* sp. (8).

The aim of the study are to diagnosis and detection the genotype of *Entamoeba* spp. in cattles and sheep .

Materials and methods

Samples collection

The study was conducted in laboratory of Parasites and Microbiology Department Veterinary Medicine College Al- Qadisiya University were collected (48) specimens as (24) from cows and (24) from sheep which suffer from diarrhea .

These samples were collected from different areas of the Al- Qadisiya province.

The samples examined by microscope , then the positive samples for *Entamoeba* spp. were examined by PCR method for confirmative *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.

Primer		Sequence	Amplicon	Ref.
E gene	E1	TTTGTATTAGTACAAA	900 bp	(10)
	E2	GTA (A/G)TATTGATATACT		
E.histolytica gene	EH1	AATGGCCCATTCATTCAATG	550 bp	(11)
	EH2	TTTAGAAACAATGCTTCTCT		
SREHP	F	GCTAGTCCTGAAAAGCTTGAAGA AGCTG	549 bp	(9)
	R	GGACTTGATGCAGCATCAAGGT		
nSREHP	F	TATTATTATCGTTATCTGAACTAC TTCCTG	450 bp	(9)
	R	TGAAGATAATGAAGATGATGAAG ATG		
SREDP	F	GAGGATCCATGTTCGCATTTTTAT TGT	729 bp	NCBI*
	R	GAGGATCCTTAGAAGACAATTGC CA		
E.bovis	F	AAACTGCGGACGGCTCATTA	174bp	NCBI*
	R	CGCGGCATCCTTTTTCACAA		

* National Center Biotechnology information

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 (12, 11).

Real-Time PCR

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

Melting curve analysis for genotyping detection

After completion of 40 cycles of PCR, the PCR products were melted by raising the temperature from 40C° to 95C° 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.

Results

proportion of infections by PCR.

The present study recorded overall infection (58.3%) of *Entamoeba* spp. in 14 of 24 fecal samples of cows were positive microscopic examination and the same number of samples in sheep when study recorded proportion of infections reached to (58.3%), (14 of 24) samples were positive (Fig.1), (Table1).

infection rate by Nested-PCR

22 From 28 samples were positive into PCR were examined by Nested-PCR and recorded total proportion of *E.histolytica* infection estimated (78.5%)(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 type of infection while types 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 (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* (I; II; III; IV; V) .

The result was Showed five genotypes of *E. histolytica* distributed as follows 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).

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%), while recorded genotype (V) ratio in less proportion in samples of Cows and Sheep feces together as (8.3% ; 12.5%) respectively(Table 4).

Table (1) total infection By *Entamoeba* spp. using PCR

Negative		Positive		Animals
Percent %	**No.**	Percent %	**No.**	
41.6	**10**	**58.3**	**14**	**Cows**
41.6	**10**	**58.3**	**14**	**Sheep**

Table (2) total infection By *Entamoeba* spp. using Nested PCR

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

big latters for vertically direction.

small letters for horizontally direction.

Table(3) different melting temperature of *E.histolytica*

Percent %	No:	Genotypes	Tm 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 No: 8		Cows No: 12		Genotypes
NO:	Perc. %	NO:	Perc. %	
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

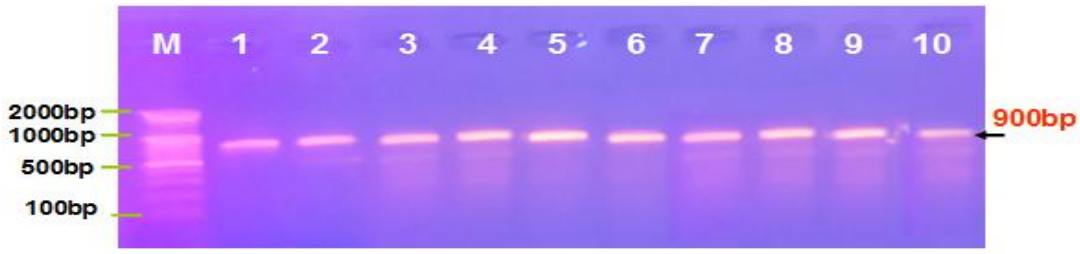

Fig 1: Agarose gel electrophoresis image that showing the PCR product analysis of small subunit rRNA gene in *Entamoeba* spp. positive stool samples. Where M: marker (2000-100bp), lane (1-10) *Entamoeba* spp. positive stool samples at (900bp) PCR product.

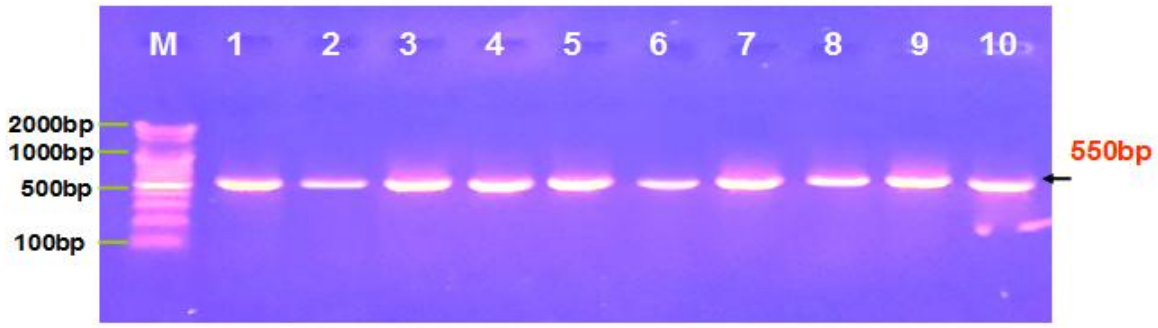

Fig 2: Agarose gel electrophoresis image that showing the Nested PCR product analysis of small subunit rRNA gene in *E. histolytica* positive stool samples. Where M: marker (2000-100bp), lane (1-10) *E. histolytica* positive stool samples at (550bp) nPCR product.

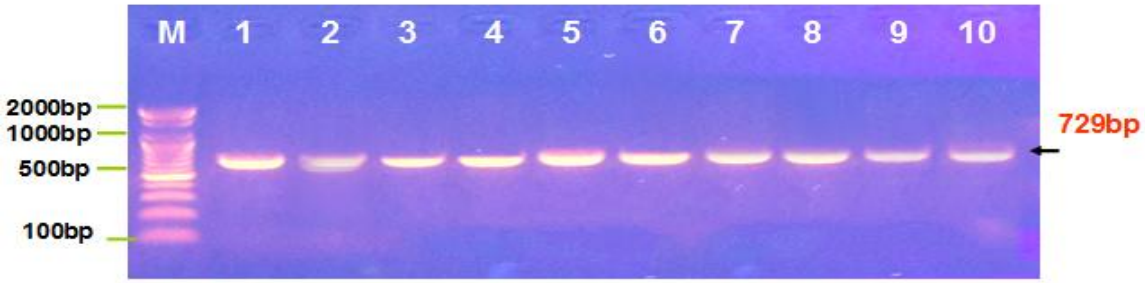

Fig 3: Agarose gel electrophoresis image that showing the PCR product analysis of small subunit rRNA gene in *E. dispar* positive stool samples. Where M: marker (2000-100bp), lane (1-10) *E. dispar* positive stool samples at (729bp) PCR product.

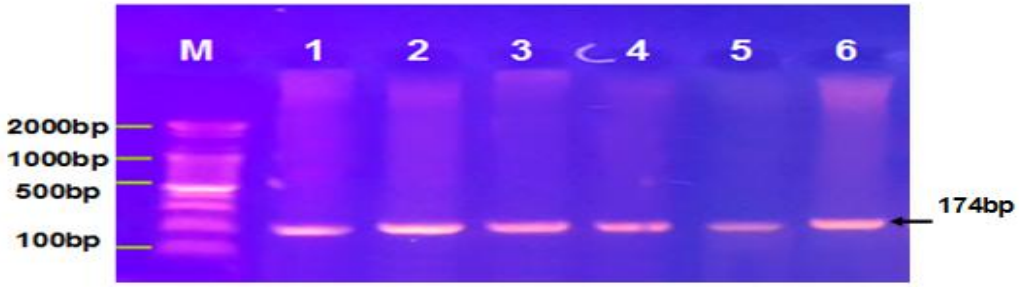

Fig 4: Agarose gel electrophoresis image that showing the PCR product analysis of small subunit rRNA gene in *E. bovis* positive stool samples. Where M: marker (2000-100bp), lane (1-6) *E. bovis* positive stool samples at (174bp) PCR product.

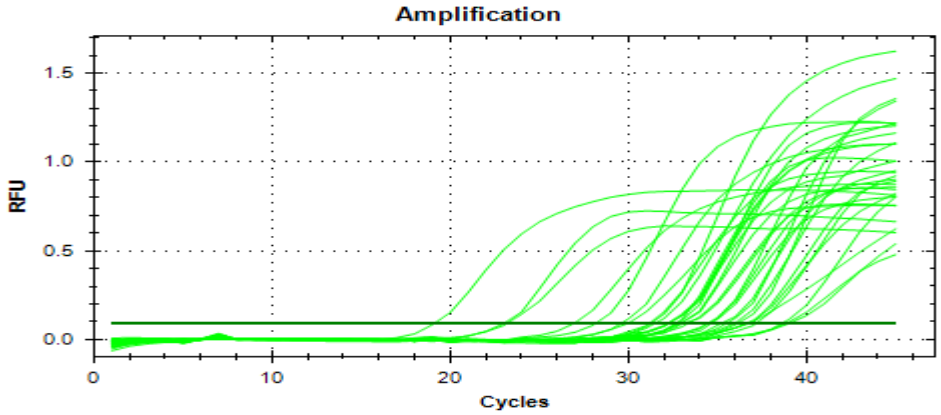

Fig 5: Real-Time PCR amplification plots that showing amplification plots of nested SREHP gene of *E. histolytica*.

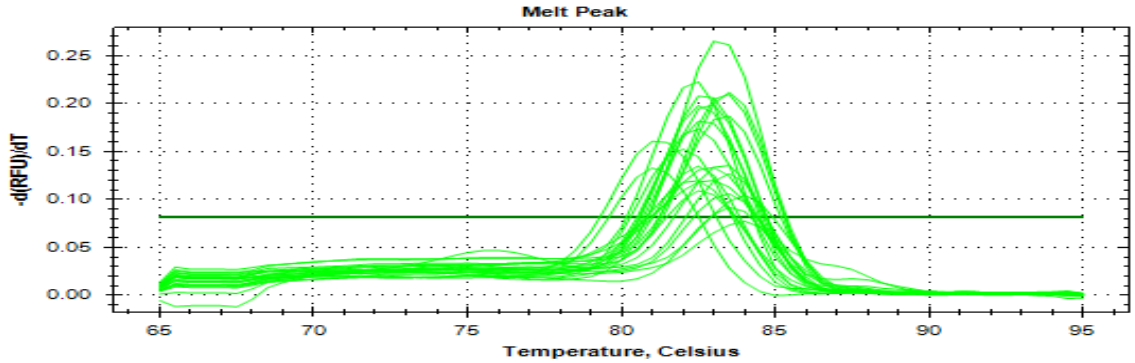

Fig (6): Display Real-Time PCR Melt peak that showing the melting point for nested SREHP gene of *E. histolytica*.

Discussion

infection rate by Nested-PCR

The study records presence of *E.histolytica* by Nested-PCR (85.7% ; 71.4%) and *E.dispar* (21.45% ; 35.7%) in samples of Cows and sheep 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 the normal host (Cows) and in other abnormal host (Sheep) as (28.5% ; 14.2%) respectively, as confirmed previous studies which reveal to possibility of the presence and the isolation of Entamoeba spp. from cows , deer and sheep (14) due to the use of contaminated water or use animals the same areas for grazing .

The result of this study matches the record of (15) in the National Park in Ivory Coast, who recorded infection ratio of *Entamoeba* spp. at 53.27% (16) recorded 57.17% infection rate of *E.coli* in horses and donkeys in Diwaniya - Iraq , while (17) recorded less 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 higher ratio 71.5%.

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 50 C º 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).

The result was five genotypes of *E. histolytica* distributed as follows (genotype: I; II; III; IV; V) which corresponds to melting temperatures (84, 83, 82, 81, 79) Cº respectively, these findings agree with (10).

The highest percentage (50%) of Genotype II in cows and sheep reflects the high potential of this genotype to spread and transmission between different hosts and refers to the high virulence of it , especially with the presence in each host that examined contrary to Genotypes (I , IV) that the presence of the least ratio (5%) among sheep and cows.

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 sheep, there are significant difference between genotypes ratios in $P \geq 0.05$.

Conclusions

Method of genotyping using SREHP gene and Melting curve analysis easy way and give guaranteed results, presence of five genotypes for SREHP in cows while two genotypes in sheep and the possibility of infect them with *E.histolytica* ; *E.dispar* and *E.bovis* which the first molecular recorder in Iraq.

Reference

1- Suzuki J , Kobayashi S , Murata R , Yanagawa Y , Takeuchi T (2007). Profiles of a pathogenic *Entamoeba histolytica* like variant with variations in the nucleotide sequence of the small subunit ribosomal RNA isolated from a primate. (De Brazza's guenon) J. Zoo Wildl. Med ., 38:471–474.

2- Zaki M (2002). Characterizations of polymorphic DNA and its application to typing of *Entamoeba histolytica* and *Entamoeba dispar* . Ph D thesis London School for Hygienl and tropical medicine London University ., UK.

3- Ali IK, Mondal U , Roy S, Haque R, Petri W A (2007). Evidence for a link between parasite genotype and outcome of infection with *Entamoeba histolytica* . J. Clin. Microbiol ., 1: 285–289 .

4- Gilchrist CA and Petri WA (2009).Using differential gene expression to study *Entamoeba histolytica* pathogenesis. Trends. Parasitol ., 25:124–131.

5- Rasti S, Haghighi A , Kazemi Z , Rezaian M (2006).Cloning and Characterization of Serine-rich *Entamoeba histolytica* Protein Gene from an Iranian *E. histolytica* Isolate. Pakistan. J. Bio. Sci .9: 654-658.

6- Gomes T, Garcia M , de Macedo F , Peralta J , Peralta R (2014). Differential Diagnosis of *Entamoeba* spp .In Clinical Stool Samples Using SYBR Green Real-Time polymerase Chain Reaction. Scien. Wor. J .,Vol(2014):1-8.

7- Tanrıverdi S , Atila Tanyeli A, Bas l ¸ lı B, Ko¨ksal F , Kılınc¸ Y, Feng BG, Tzipori S , Giovanni W G (2002). Detection and Genotyping of Oocysts of *Cryptosporidium parvum* by Real-Time PCR and Melting Curve Analysis. J. Clin. Microbial.,40: 3237–3244.

8- Polley S D , Boadi S, Watson J, Curry A , Chiodini Peter L (2011).Detection and species identification Of Microsporidial infections using SYBR Green real-time PCR. J. Clin. microbial .,60: 459–466 .

9- Rahman SM , Haque R , Roy S , Mondal M (2006). Genotyping of Entamoeba histolytica by real-time polymerase chain reaction with SYBR green I and melting curve analysis. Bangl . J. Vet. Med., 4: 53–60.

10-Haque R, Huston, Christopher D, Hughes M , Houpt E ,William, A, Petri Jr (2003). Amebiasis . N . Engl. J. Med., 34(8):1565-73.

11-Yee Ling Lau , Anthony C , Fakhrurrazi Siti A, Ibrahim J, Init I , Mahmud R (2013) Real-time PCR assay in differentiating *Entamoeba histolytica*, *Entamoeba dispar*, and *Entamoeba moshkovskii* infections in Orang Asli settlements in Malaysia. Para. and Vec ., 6:250 .

12-Levecke B , Dreesen L , Dorny P , Verweij J J ,Vercammen F , Casaert S , Vercruysse J , Geldhof P (2010) . Molecular identification of *Entamoeba* spp in Captive Nonhuman Primates . J . Cli. Microbiol., 48: 2988–2990 .

13-Wienken C J , Baaske P, Duhr S and Braun D (2011). Thermophoretic melting curves quantify the conformation and stability of RNA and DNA". Nucl. Ac. Res ., 39: 1-10.
14-Stensvold C R , Lebbad M , Clark C G (2010). Genetic characterization of uni-nucleated cyst producing *Entamoeba* spp from ruminants . Intel. J. parasitol ., 40:775-778
15-Kouassi R , McGraw S W , Yao1 P , Abou-Bacar A, Julie Brunet J , Pesson B, Bonfoh B (2015). Diversity and prevalence of Gastrointestinal parasites in seven non-human primates of the Taï National Park Côte d'Ivoire. Paras ., 22:1-12.
16-Wannas H Y , Dawood Kh , Jassem Gh (2012). Prevalence of Gastro-intestinal Parasites in Horses and Donkeys in Al Diwaniyah Governorate. AL-Qadisiya J. of Vet. Med. Sci.,11:148-155.
17-Regan C S , Yon L , Hossain M , Elsheikha Hany M (2014). Prevalence of *Entamoeba* species in captive primates in zoological gardens in the UK. Peer.J .,2: 492.
18-Nicolas L, Milon G , Prina E (2002). Rapid differentiation of Old World Leishmania species by Light Cycler polymerase chain reaction and melting curve analysis. J. Microbiol. Med., 51: 295– 299.
19-Soliman R , Othman AA (2009).Evaluation of DNA Melting Curve Analysis Real-Time PCR for Detection and Differentiation of *Cryptosporidium* Species. P.U.J.2: 47 – 54.