# Study *Entamoeba* Species in Livestock by Nested Multiplex PCR in Baghdad City

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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 multiplex PCR. The nested multiplex polymerase chain reaction was conducted using the small subunit 18S rRNA gene , the results show total infection rate of Entamoeba spp was (60%) which include to(30%) in cattle and 30%in sheep. The results second reaction show that the percent of *E.histolytica* was (30%) in cattle and (30%) in sheep

***Keywords****: Entamoeba, Baghdad, PCR, 18S rRNA, gene*

## Introduction

*Entamoeba histolyticais* 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(1). The domestic animals, living in intimate contact with man in rural areas, constitute a high risk for transmission of infection with these protozoal agents to man (2). These protozoa are of public health concern as they may cause infection and severe illness in animals. Infections are mostly self-limiting in people with normal immune system but infection can be life threaten in people who have compromised immune system (3). Symptoms, when present, range from mild abdominal discomfort with diarrhea containing blood or mucous to acute or fulminating dysentery with fever, chills and bloody or mucoid diarrhea(4).Complications of prolonged infection include extra intestinal disease such as amebae or abscesses in the liver, lungs, heart, brain, skin or other organ (5)

*E. moshkovskii*, *E. histolytica* and *E. dispar* are morphologically in distinguishable; it is not possible to differentiate the three species on the basis of traditional microscopic examination. In the identification of *E. histolytica,* new approaches are used, based on detection of *E. histolytica* specific antigen and DNA in stool and other clinical samples Molecular diagnostic tests, including nested PCR, have been developed for the detection and differentiation of *E. histolytica*, *E.dispar*, and *E. moshkovskii* in clinical samples. (6).

## Materials and Methods

### Samples collection

The study was carried out on (50) fecal samples were collected from cattle and (50) fecal samples sheep from different area in Baghdad city in a random way from December 2019 to November2020. Fecal samples will collected in capped fecal containers transported in cold bag to the Parasitology Laboratory, College of Veterinary Medicine , University of Baghdad.

### Molecular study include

### DNA Extraction

The kits were used throughout the study for genomic DNA extraction from stool samples. These are Presto™ Stool DNA Extraction Kit according to the manufacturers instruction. The DNA were stored at -20 C until PCR amplification.

**DNA amplification by PCR**

For molecular identification , nested multiplex PCR targeted (18S rRNA gene) was used to differentiate the DNA of *E.histolytica, E. dispar and E.moshkovskii* [7].In primary reaction, the genus specific primers used were E-1 (5′-TAAGATGCACGAGAGCGAAA-3′) and E-2 (5′-GTA CAAAGGGCAGGGACGTA-3′), was used to amplify about 900 bp of 18S rRNA gene. In secondary reaction of nested multiplex PCR, three pairs of primers: EH-1 (5′-AAGCATTGTTTCTAGATCTGAG-3′) and EH-2 (5′-AAGAGGTCTAACCGAAATTAG-3′); Mos-1 (5′-GAAACC AAGAGTTTCACAAC-3′) and Mos-2 (5′CAATATAAGGC TTGGATGAT-3′); and ED-1 (5′-TCTAATTTCGATTAGAAC TCT-3′) and ED-2 (5′-TCCCTACCTATTAGACATAGC-3′). the reaction conditions were optimized for amplifying species-specific product sizes (439, 553 and 174 bp for *E. histolytica, E. moshkovskii and E. dispar,* respectively).

The first PCR reaction was performed in a final volume of 20nl contain 5nl DNA template and 1nl of Forward primers(10pmol) , 1nl of reveres primers (10pmol) and 13nl PCR water . the reaction were performed in an automatic DNA thermo cycler (THECHNER,USA) for 35 cycles .Each cycle consisted of a denaturing step of 30 sec . at 95 c, and annealing step of 30 sec. at 58 c and 1 min of extension step at 72 c with the final extension step of 72c for 10 min .

The second PCR reaction was performed in a final volume of 20 µl, contain 2.5 µl of the first PCR product , 1 µl for each primer and 11.5 µl PCR water . The reaction conditions for the second PCR were optimised to combine the primers of E. histolytica (EH-1 and EH-2) with E. dispar (ED-1 and ED-2) and E. moshkovskii (Mos-1 and Mos-2) primers in a single reaction mixture under the same conditions.the PCR products were electrophoresed on 1% Agarose gel stained ethidium bromide and visualized by UV transilluminator .

**Sequencing analysis of *Entamoeba spp***

DNA sequencing analysis was performed for confirmative detection of local *Entamoeba spp* and study of phylogenetic relationship tree analysis between local *Entamoeba spp* isolates and NCBI –Blast submission of *Entamoeba spp* as well as submission of local isolates in NCBI-GenBank .Ten PCR positive products of local *Entamoeba spp* isolates were sent to Bioneer Company in Korea in ice bag for performed the DNA sequencing by Applied Biosystem (AB) DNA sequencing system. The NCBI-GenBank submission was carried out using Bankit submission tool.

## Statistical Analysis

The Statistical Analysis System- [8] program was used to detect the effect of difference factors in study percentage. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study.

## Results

**The results of first round.**

One hundred feces samples collected from the cattle and sheep for detection of *Entamoeba spp* ,wherever, all the isolates submit to the polymerase chain reaction test, the prevalence as a table (1). Using specific primers according to the gene of 18S rRNA to using for detection of *Entamoeba spp* (900bp) on electropgoresis agarose gel as fig (1).

**Table (1): Total infection rate of *Entamoeba spp*. in livestock by Polymerase chain reaction.**

| Type | Examined No. | Infected No. | Percentage % |
|---|---|---|---|
| Cattle | 50 | 30 | 60 |
| Sheep | 50 | 30 | 60 |

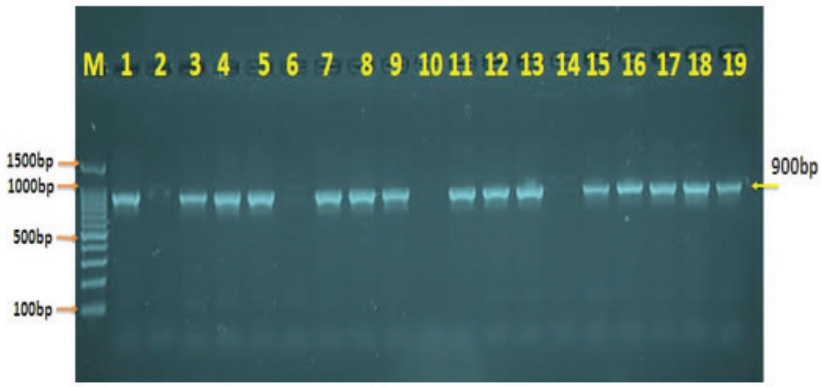


**Figure (1): Agarose gel electrophoresis image showes the PCR product analysis of 18S rRNA gene in Entamoeba sp. from Cattle and sheep feces samples. Where , the Lane (M):DNA marker ladder (1500-100bp) and the Lane (1,3,4,5,7,8,9,11,12,13,15,16,17,18,19 ) were showed positive PCR amplification of 18S rRNA gene in Entamoeba sp. at 900bp PCR product size.**

**-The result of second round**

The result of second round nested multiplex PCR show one species of *Entamoeba histolytica* was recorded 30% in cattle and 30% sheep (30% ,30% ), table ( 2) fig(2).

**Table 2: Result of Entamoeba spp by Nested multiplex PCR for second round.**

| Entamoeba spp | Cattle | | | Sheep | | |
|---|---|---|---|---|---|---|
| | No of examined | No of positive | Percentage % | No of examined | No of positive | Percentage % |
| *E. histolytica* | 50 | 30 | 60 | 50 | 30 | 60 |
| *E. dispar* | | – | | | | |
| *E. moshkovskii* | | – | | | | |

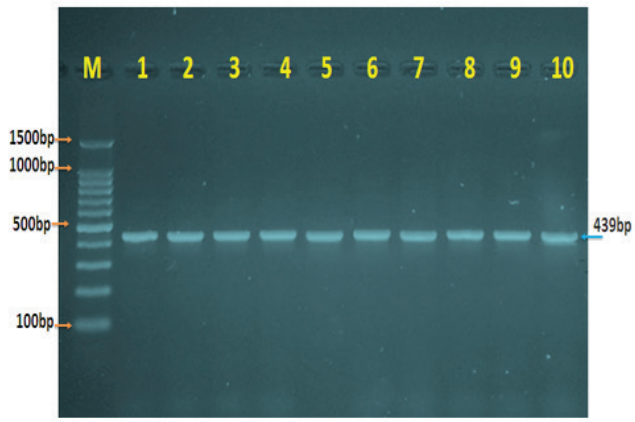


**Figure (2): Agarose gel electrophoresis image that showed the Multiplex Nested PCR product analysis of 18S rRNA gene in *Entamoeba* species from cattle and sheep positive samples. Where , the Lane (M): DNA marker ladder (1500-100bp). The Lane (1-5) were showed positive cow *Entamoeba histolytica* at 439bp PCR product size the Lane (6-10) were showed positive sheep *Entamoeba histolytica* at 439bp PCR product size.**

**Phylogenetic tree analysis**

Ten samples of PCR products out from 60 positive PCR samples were collected randomly were sequenced using forward and reverse primers. The sequences were manipulated in gene bank database NCBI accession numbers, sample No.1 (MK426065), sample No.2 (MK426066), sample No.3 (MK426067), sample No.4 (MK426068), and sample No.5 (MK426069) from cattle and sample No.1 (MK426070), sample No.2 (MK426071), sample No.3 (MK426072), sample No.4 (MK426073), and sample No.5 (MK426074) from sheep (Table 3). Phylogenetic tree analysis based on small ribosomal subunit 18S rRNA gene partial sequence of Entamoeba spp livestock isolates from Baghdad city using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). Showed a very low genetic variation (0.002). The local Entamoeba sheep and cattle isolates (No.1 into No.10) were showed closed related to NCBI-BLAST Entamoeba histolytica (MK332025.1). Whereas, other NCBI Entamoeba species were showed different at total genetic changes (0.140-0.020%) figure(3).

**Table (3) the NCBI-BLAST Homology Sequence identity (%) between local Entamoeba sp. IQ Cattle isolates and NCBI-BLAST submitted Entamoeba species:**

| Local Entamoeba sp. cattle isolate No. | Homology sequence identity analysis | | |
|---|---|---|---|
| | NCBI BLAST Identical Entamoeba sp. | Genbank accession number | Sequence identity (%) |
| Entamoeba sp. cattle isolate No.1 | Entamoeba histolytica | MK332025.1 | 99.48% |
| Entamoeba sp. cattle isolate No.2 | Entamoeba histolytica | MK332025.1 | 99.09% |
| Entamoeba sp. cattle isolate No.3 | Entamoeba histolytica | MK332025.1 | 98.95% |
| Entamoeba sp. cattle isolate No.4 | Entamoeba histolytica | MK332025.1 | 98.72% |
| Entamoeba sp. cattle isolate No.5 | Entamoeba histolytica | MK332025.1 | 98.71% |
| Entamoeba sp. sheep isolate No.1 | Entamoeba histolytica | MK332025.1 | 99.48% |
| Entamoeba sp. sheep isolate No.2 | Entamoeba histolytica | MK332025.1 | 97.09% |
| Entamoeba sp. sheep isolate No.3 | Entamoeba histolytica | MK332025.1 | 98.70% |
| Entamoeba sp. sheep isolate No.4 | Entamoeba histolytica | MK332025.1 | 99.74% |
| Entamoeba sp. sheep isolate No.5 | Entamoeba histolytica | MK332025.1 | 98.84% |

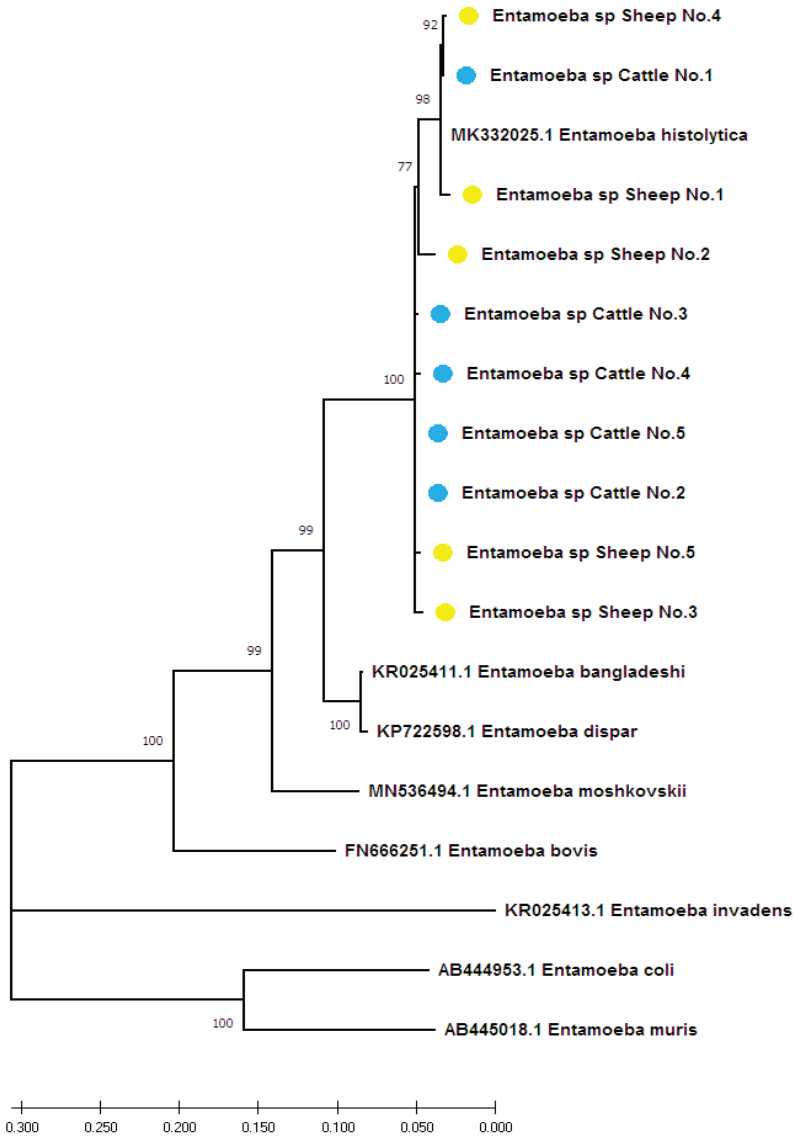


**Figure (3): Phylogenetic tree analysis based on small subunit ribosomal RNA gene partial sequence in local *Entamoeba* sp. isolates that used for genetic *Entamoeba* species identification. The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The local *Entamoeba* sheep and cattle isolates (No.1 into No.10) were showed closed related to NCBI-BLAST *Entamoeba histolytica* (MK332025.1). Whereas, other NCBI *Entamoeba* species were showed different at total genetic changes (0.140-0.020%)**

## Discussion

Amoebiasis is one of the most common infection worldwide[9].The three studies are morphologically similar ,despite genetic and pathogenic differences [10]. *E.histolyticais* generally considered a pathogenic species, while other Entamoeba species are classified as non-virulent[11] therefore, distinguishing of these species is of great significance.

The result in cattle and sheep were agree with [12] when they recorded infection rate of 58.3% *Entamoeba spp* in the cattle and sheep ,the result of the study relative to that recorded [13] In the National Park in Ivory Coast,

which was the injury ratio stood at 53.27% at study intestinal parasites which spread at the zoo also [14] recorded 57.17% infection rate in horses and donkeys in Diwaniya – Iraq.Other studies have recorded infection rates less than the current study by [15] recorded infection rate of *Entamoeba* in goat 20% (10/50). [16] recorded results in center garden Rome infection by (9%) *E.histolytica* , that less than our results.. 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.

## Conclusion

Multiplex conventional PCR method was a rapid and effective in differentiating E. histolytica from E. dispar and E. moshkovskii. This method is an optional tool in the diagnosis and epidemiological studies of amoebiasis

**Ethical Clearance:** The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

**Conflict of Interest:** The authors declare that they have no conflict of interest

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## References

1. World Health Organization. Pan American health organization. UNESCO report of a consultation of experts on amoebiasis. Wkly Epidemiol Rec. 1997;72:97-.
2. Byomi A, Samaha H, Zidan S. Epidemiological studies on some zoonotic enteric protozoa in different areas of Nile delta. JASMR. 2010;5(2):199-207.
3. Cacciò SM, Thompson RA, McLauchlin J, Smith HV. Unravelling cryptosporidium and giardia epidemiology. Trends in parasitology. 2005 Sep 1;21(9):430-7.
4. Al-Shammary AH. Run-off patterns of vancomycin resistant enterococci (VRE clones) in cows raw milk and imported milk powders at Baghdad markets. The Iraqi Journal of Veterinary Medicine. 2019 Dec 28;43(2):61-6.
5. American Academy of Pediatrics, Committee on Infectious Diseases, Pickering LK. Red book: report of the Committee on Infectious Diseases. American Academy of Pediatrics; 2013.
6. Haque R, Huston CD, Hughes M, Houpt E, Petri Jr WA. Amebiasis. New England journal of medicine. 2003 Apr 17;348(16):1565-73.
7. Bahrami F, Haghighi A, Zamini G, Khademerfan M. Differential detection of Entamoeba histolytica, Entamoeba dispar and Entamoeba moshkovskii in faecal samples using nested multiplex PCR in west of Iran. Epidemiology & Infection. 2019;147.
8. Cary N. Statistical Analysis System, User's Guide. Statistical. Version 9. SAS. Inst. Inc. USA. 2012.
9. WHO/PAHO/UNESCO. A consultation with experts on amoebiasis. Epidemiol Bull. 1997;18:13-4.
10. Khairnar K, Parija SC. A novel nested multiplex polymerase chain reaction (PCR) assay for differential detection of Entamoeba histolytica, E. moshkovskii and E. dispar DNA in stool samples. BMC microbiology. 2007 Dec;7(1):1-9.
11. Anuar TS, Al-Mekhlafi HM, Ghani MK, Bakar EA, Azreen SN, Salleh FM, Ghazali N, Bernadus M, Moktar N. Molecular epidemiology of amoebiasis in Malaysia: highlighting the different risk factors of Entamoeba histolytica and Entamoeba dispar infections among Orang Asli communities. International journal for parasitology. 2012 Dec 1;42(13-14):1165-75.
12. Mohammed NQ. Phylogenetic tree analysis of Entamoeba species isolated from goats. Al-Qadisiyah Journal of Veterinary Medicine Sciences. 2017 Dec 30;16(2):38-44.
13. Kouassi RY, McGraw SW, Yao PK, Abou-Bacar A, Brunet J, Pesson B, Bonfoh B, N'goran EK, Candolfi E. Diversity and prevalence of gastrointestinal parasites in seven non-human primates of the Taï National Park, Côte d'Ivoire. Parasite. 2015;22.
14. Wannas HY. Prevalence of gastro-intestinal parasites in horses and donkeys in al diwaniyah governorate. Al-Qadisiyah Journal of Veterinary Medicine Sciences. 2012 Jun 28;11(1):148-55.

15. Mohammed NQ. Phylogenetic tree analysis of Entamoeba species isolated from goats. Al-Qadisiyah Journal of Veterinary Medicine Sciences. 2017 Dec 30;16(2):38-44.

16. Stensvold CR, Lebbad M, Clark CG. Genetic characterisation of uninucleated cyst-producing Entamoeba spp. from ruminants. International journal for parasitology. 2010 Jun 1;40(7):775-8.