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

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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 Haemorrhagic 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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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

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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).

Table 1: Nested PCR primers

| Primer       | Sequences (5’-3’)                  | Product size |
|--------------|------------------------------------|--------------|
| Entamoeba sp. | F   TTTGTATTAGTACAAA                | ~900bp       |
|              | R   GAAATTTGATATCT                 |              |
| E. histolytica| F   AATGGCCCATTCCATTCAATG          | 550bp        |
|              | R   TTTAGAAACATGCTTCTCT            |              |
| E. dispar    | F   GAGGATCCATGTCCGCATTTTATGT     | 729bp        |
|              | R   GAGGATCTTTAGAAGACATGGCCA      |              |
| E. bovis     | F   AAACGCGGACGGCTCATT            | 174bp        |
|              | R   CGCGGCATCCTTCTTCACAA          |              |

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, MgCl2, 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 setting up the following thermocycler conditions; Reactions were performed using the following protocol, initial denaturation at 95°C for 4 min, and 35 cycles at 95°C for 1 min, 47°C for 1 min and 72°C for 2 min, and a final incubation at 72°C for 7 min.

The second round Nested PCR master mix that includes *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 5-50 ng/µL    | 5 µL   |
| Entamoeba sp. Primary forward primer | 1 µL   |
| Entamoeba sp. primary reverse primer | 1 µL  |
| PCR water                  | 13 µL  |
| Total volume               | 20 µL  |

Table 3: Nested PCR master mix

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

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

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

After that PCR products were examined by electrophoresis in 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 were 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: GCTAGTCTCGAAAAAGCTTGAAGAGGCTG | 549bp |
|                 | R: GGACTTGATGCAGCATCAAGGT        |              |
| RT PCR-SREHP    | F: TATTATATGTTATCATGTTAAGAGCTTG | 450bp |
|                 | R: TGAAGATAATGAAGATGAGATGAATG |              |

Table 5: RT PCR master mix

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

Table 6: RT PCR master mix

| qPCR step     | Temperature | Time | Cycle |
|---------------|-------------|------|-------|
| Initial       | 95°C        | 3 min| 1     |
| Denaturation  | 95°C        | 10 sec| 45    |
| Annealing     | 55°C        | 30 sec| 1     |
| Detection     | 60-95°C     | 0.5 sec| 1     |
Statistical analysis

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

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 $P \geq 0.05$.

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, 84°C 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, 84°C 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

| Genotype | Melting Tm. | No. | %   |
|----------|-------------|-----|-----|
| I        | 84          | 1   | 9.75|
| II       | 83          | 22  | 53.65|
| III      | 82          | 8   | 19.5|
| IV       | 81          | 4   | 9.75|
| V        | 79          | 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 |
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 four genotypes 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 Diagnostic tools.

Reference

1. Lark CG, Kaffashian F, Tawari B, Windsor J, Fleiner T, Davies M, Mina CG, Blessmann I, Ebert F, Peschel B, Van J, An L, Colin J, Macfarlane L, Egbert T. New insights into the phylogeny of Entamoeba species provided by analysis of four new small subunit rRNA genes. Inter J Syst Evol Microbiol. 2006;56(9):2235-2239. DOI: 10.1099/ijs.0.64208-0
2. Bhattacharya, S, Bhattacharya A. Amoebiasis and Entamoeba species: unexplored liaisons. Trop Gastroenterol. 2013;34(2):55-57. DOI: 10.7869/tg.2012.98
3. Das K, Ganguly S. Evolutionary genomics and population structure of *Entamoeba histolytica.* Comp Struct Biotechnol J. 2014;12(14):26-33. doi.org/10.1016/j.csbt.2014.10.001
4. Okada M, Huston CD, Mann BJ, Petri WA, Kita K, Nozaki T. Proteomic analysis of phagocytosis in the enteric protozoan parasite Entamoeba histolytica. 2005;4(4):827-831. DOI: 10.1128/EC.4.4.827-831.2005
5. Dolabella SS, Serrano LJ, Navarro GF, Cerritos R. Amoebic liver abscess production by *Entamoeba dispar*. Anna Hepatal. 2012;11(1):107-117. DOI: 10.1016/S1665-2681(19)31949-2
6. Ximénez C, Morán P, Rojas L, Valadez A, Gómez A, Ramiro M, Cerritos R, González E, Hernández E, Oswald P, Novelits on Amoebiasis: neglected tropical disease. J Global Infect Dis. 2011;3(2):166-74. doi: 10.4103/0974-777X.81695
7. Ali IK, Mondal U, Roy S, Haque R, Petri WA. Evidence for a link between parasite genotype and outcome of infection with *Entamoeba histolytica*. J Clin Microbiol. 2007;1(45) 285-289. doi: 10.1128/JCM.01335-06
8. Blessmann J, Buss H, Ton N, Phuong A, Dinh BT, Viet N, Quynh T, An Le V, Mohamed D, Abd A, Terry F, Jackson J, Ravdin I, Egbert T. Real Time PCR for detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* in fecal samples. J Clin Microbiol. 2003;40(12):4413-4417. doi: 10.1128/JCM.40.12.4413-4417.2002
9. Costa AO, Gomes MA, Rocha OA, Silva EF. Pathogenicity of *Entamoeba dispar* under xenic and monoxenic cultivation compared to a virulent *E. histolytica*. Revista Instituto Med Trop Sao Paulo. 2006;48(5):245-250. doi.org/10.1590/S0036-46652006000500002
10. Galva MM, Domí MC, Bravo E, Meza I. The interplay between Entamoeba and enteropathogenic bacteria modulates epithelial cell damage. PLOS Negl Trop Dis. 2008;2(7):266. doi.org/10.1371/journal.pntd.0000266
11. Haque R, Huston CD, Hughes M, Houpt E, William A, Petri Jr. Amoebiasis. N Engl J Med. 2003;348(18):1565-73. DOI: 10.1056/NEJMra022710
12. Rahman SM, Haque R, Roy S, Mondal MH. Genotyping of *Entamoeba histolytica* by real-time polymerase chain reaction with sybr green I and melting curve analysis. Bangl J Vet Med. 2006;4(1):53-60. doi.org/10.3329/bjvm.v4i1.15126
13. Yee LL, Lai YL, Anthony C, Fakhurrzaqi SA, Ibrahim J, Ithoi I, Mahmud R. Real-time PCR assay in differentiating *Entamoeba histolytica*, *Entamoeba dispar*, and *Entamoeba moshkovskii* infections in Orang Asli settlements in Malaysia. Parasit Vectors. 2013;6(1):250. doi: 10.1186/1756-3305-6-250
14. Pham DP, Nguyen VH, Hattendorf J, Zinsstag J, Dan CP, Peter OP. Risk factors for *Entamoeba histolytica* infection in a agricultural community in Hanam province, Vietnam. Parasit Vect. 2011;4(1):102. DOI: 10.1186/1756-3305-4-102
15. Levecke B, Dreessen L, Dorny P, Verweij JJ, Vaccammen F, Casarta S, Vercreuyse J, Geldhof P. Molecular identification of Entamoeba spp. in Captive Nonhuman Primates. J Clin Microbiol. 2010;48(8):2988-2990. DOI: 10.1128/JCM.0013-10
16. Stensvold CR, Lebbad M, Clark CG. Genetic characterization of uninucleated cyst-producing *Entamoeba* spp. from ruminants. Inter J. Parasitol. 2010;40(7):775-778. doi.org/10.1016/j.ipara.2010.03.003
17. Sateriale A, Huston CD. A sequential model of host cell killing and phagocytosis by *Entamoeba histolytica*. J Parasitol Res. 2011;2015:1165. doi.org/10.1155/2011/926706
18. Dinoop KP, Parija SC, Mandal J, Swaminathan RP, Narayanam P. Comparison of nested-multiplex, Taqman and SYBR Green real-time PCR in diagnosis of amoebic liver abscess in a tertiary health care institute in India. Indian J Med Res. 2016;143(1):49-56. doi: 10.4103/0971-5916.178592