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Molecular Epidemiology and Drug Resistance Study of *Entamoeba histolytica* in Clinical Isolates from Tehran, Iran

Arash Hemmati¹, Ali Choopani^{2, 3}, Fatemeh Pourali⁴, Seyed Mohammad Javad Hosseini^{4*}

Abstract

Amoebiasis is a disease caused by *Entamoeba histolytica*, a protozoan parasite. Metronidazole is known as the main drug used for patients suffering from Amoebiasis. Despite the lack of drug resistance in clinical samples, there are scattered reports that are based on the failure of treatment which show the increase of clinical drug resistance against metronidazole. Therefore, the aim of this study was to identify *E. histolytica* by culture and polymerase chain reaction methods, compare them, and assess drug resistance among clinical samples to *E. histolytica*. A total of 1990 samples were collected from patients with dysentery. Positive microscopic samples after staining by lugol's iodine were cultured on biphasic culture medium (HSre+s). The drug sensitivity of clinical isolates and standard reference strain of *E. histolytica* (HM1:IMSS) was evaluated after exposure to various concentrations of metronidazole on the basis of mobility and tonality using 0.01% Eosin. A PCR method was applied to confirm the cultural results. Forty six out of 1990 samples and 41 out of 46 samples were positive for *E. histolytica* by microscopic and cultural methods, respectively. However, only 15 out of 46 samples were positive by PCR amplification using specific primers of *E. histolytica* genome. According to the results of *Entamoeba* growing in the cultures with difference metronidazole concentrations, no resistance was observed at the concentrations higher than 2 mg/ml. The present results indicate the high specificity of the molecular techniques against culture in specific mediums. It also suggests *Entamoeba* isolates in Iran does not seem resistant to the metronidazole antibiotic.

Keywords: *Entamoeba histolytica*, PCR, Culture, Metronidazole, Drug Resistance

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Submission Date: 11/28/2016

Accepted Date: 12/25/2016

Introduction

Amoebiasis as a global expanded disease, caused by a protozoan parasite that named *Entamoeba histolytica*. Contaminated food and water by *E. histolytica* cysts are the main source routes of transferring protozoa to humans [1]. Intestinal and extra-intestinal are the two forms of amoebiasis. Amoebic colitis, abdominal pain and gradual development of mild diarrhea are the symptoms of the intestinal form. Amoebic liver abscess (ALA) is the most common symptom of extra-intestinal form which can occur after an amoebic colitis [2]. There are 40-50 million cases of infection by *Entamoeba* species with the mortality rate of 70,000 to 100,000 people [3]. The genus of *Entamoeba* which exists in intestinal lumen consists of six species which are *Entamoeba histolytica*, *Entamoeba dispar*, *Entamoeba moshkovskii*, *Entamoeba coli*, *Entamoeba polekiand* and *Entamoeba hartmanni* [4-8]. Due to morphological similarities, there is no method for microscopic detection, however, in comparison with the other, only *E. histolytica* is the main pathogenic species to humans [9]. Detection methods include examine for cysts based on microscopic method, antigen and antibody-based detection methods, immunological and molecular methods. Isoenzyme analysis of cultured amoeba was known as the gold standard method in amoebiasis diagnosis prior to development of newer DNA-based techniques [10].

Metronidazole is an antibiotic in nitroimidazole group which is known as the most effective drug for treating amoebic infections) [11]. As a result of high prevalence of protozoan infections, [12] metronidazole has been introduced as an essential drug by the World Health Organization.

Although drug resistance in *E. histolytica* has not appeared as a serious problem, there are scattered reports based on the failure of treatment which show the increase of clinical drug resistance against metronidazole [13]. Re-infection of ALA has been observed even after using metronidazole, and despite adequate treatment, parasitic cysts can survive [14]. However, differences in drug sensitivity between strains of *E. histolytica* has been reported which suggest that there might be low percentage of drug resistance to amoeba; or due to abuse of anti-amoebic drugs, drug resistance strains can suddenly appear [15]. The aim of this study was to identify *E. histolytica* by culture and PCR methods, and assess drug resistance among clinical samples of *E. histolytica*.

Materials and Methods

Clinical samples

A total of 1990 samples were collected from patients with dysentery and examined based on the microscopic method in 2013 and 2014. The microscopic examination was per-



formed by Gram staining for the detection of trophozoites and cysts of *E. histolytica* and *E. dispar*. Microscopic positive samples for *E. histolytica* and *E. dispar* were transferred immediately to molecular biology laboratory for culture and molecular diagnosis.

Preparation of antimicrobial agent

In this study, the applied metronidazole was prepared in standard concentrations (0.5, 1, 1.5 and 2 mg/ml) and stored at 4°C until use.

Culture of *E. histolytica*

Positive microscopic samples were cultured after staining by lugol's iodine on biphasic culture medium HSre+s (Horse serum, ringer, egg and starch rice), according to the method of Dobell and Laidlaw [16]. After preparation of suspension which contains 30-50 mg of dysenteric stool, samples were incubated in sterile condition at a temperature of $35.5 \pm 0.5^\circ\text{C}$ for 48 h. The numbers of living cells were evaluated on the basis of mobility and tonality using 0.01% Eosin and microscopic observation [17].

DNA extraction

Breaking cyst walls was performed by sonication (Hielscher, Germany) including seven shocks, each for 15 s and 0.85 molar sucrose method was used too [18]. The extraction of *E. histolytica* genome was performed by phenol-chloroform method and DNA isolation kit (DNP kit, Cina-gene, Iran) based on the kit protocol [19].

PCR

PCR primers were designed based on 16S rRNA of *E. histolytica*. Primers were designed for amplification of approximately 220 bp of ss1 gene as follows:

5'-CCCAGAGAATAGAAAACCTCTT-3' as forward

5'-TCAAGTATAGTGCACCATCT-3' as reverse.

PCR amplification was performed in a final volume of 25 μl containing 2.5 μl buffer, 1.5 mM MgCl_2 0.8 μl , 0.5 μM of each dNTP, 2 U Taq DNA polymerase (TakapoZist, Iran), 1 μl of each primer (10 mM, TakapoZist) and 2.5 μl DNA template (100-200 ng). The PCR reactions were performed in a Thermalcycler (Eppendorf, Germany) PCR System with the following program: 4 min incubation at 94°C to denature double-strand DNA, 35 cycles of 30 s at 43.5°C (annealing step), 30 s at 72°C (extension step) and 30 s at 94°C (denaturing step). Finally, PCR was completed with an additional extension step for five minutes. The PCR products were analyzed on 1.8% agarose gel in 0.5X EDTA buffer and visualized using ethidium bromide and an UV illuminator. The amplicon sequencing was used to confirm the PCR results.

The standard strain of HM:IMSS was used as the positive control which was prepared at the Department of Parasitology, Shahid Beheshti University of Medical Sciences, and distilled water was used as the negative control.

Results

Forty six (0.23%) out of 19990 samples were positive to *E. histolytica* and *E. dispar* by microscopic method. Forty one (89%) out of 46 samples were positive in the HSre+s medium for the presence of *Entamoeba* spp. (0.2% of initial samples); whereas, only 15 out of 46 samples (33%)

were positive by PCR amplification using specific primers of *E. histolytica* genome (0.07% of all samples) (Table 1).

Table 1. Comparison of PCR and Culture diagnoses methods of *Entamoeba histolytica* cysts.

Detection method	All Samples	Positive samples
Microscopic method	19990	46 (0.23%)
Culture	46	41 (89.13%)
PCR	46	15 (33%)

Then, the numbers of trophozoites were calculated using Neubauer Lam method. The numbers were determined after 24 and 48 h incubation at $35.5 \pm 0.5^\circ\text{C}$ under metronidazole concentrations. As shown in the results of culture, no resistance was observed at the concentrations higher than 2 mg/ml (Table 2, Figures 1 and 2).

Table 2. Metronidazole concentrations effect on growth inhibition of cultured samples after 24h and 48 h.

Metronidazole Concentrations ($\mu\text{g/ml}$)	24 hours (%)	48 hours (%)
0.5	72.33 ± 3.44	96.82 ± 1.1
1	88.82 ± 1.96	99.4 ± 0.6
1.5	95.05 ± 1.85	100
2	100	100

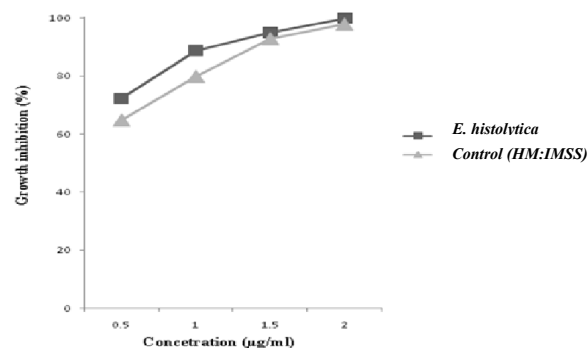


Figure 1. Growth inhibition of *E. Histolytica* (HM1: IMSS) and clinical isolates by metronidazole after 24 h incubation.

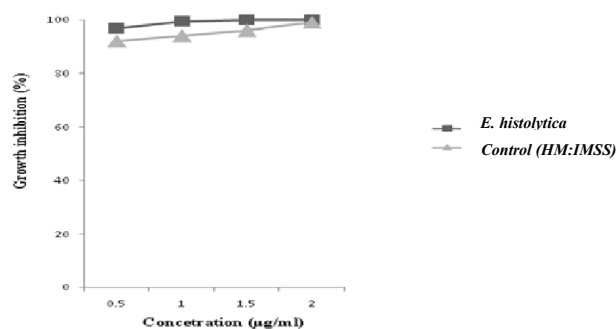


Figure 2. Growth inhibition of *E. histolytica* (HM1:IMSS) and clinical isolates by metronidazole after 48h incubation.

Also, out of the 46 microscopic positive samples, 15 were positive by PCR. Figure 3 shows the bands of 220 bp of positive samples.

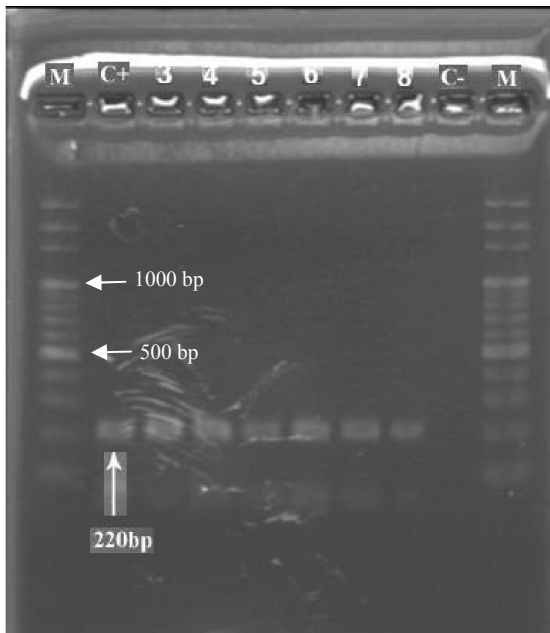


Figure 3. Positive samples of *E. Histolytica*.

Discussion

Amoebiasis is known as a public health problem in most countries of the world, which reflects the worldwide distribution of *E. histolytica*. Infected Polluted waters like rivers and wetlands, fecal-oral transmission and travelling to endemic areas are the main ways of *E. histolytica* cysts transmission [20-22]. In two studies in South Africa in 2002 and 2012, it was reported that the prevalence of *E. histolytica* and *E. dispar* in children under 15 years is up to 18.8%, which may indicate the relationship between age and incidence of *E. histolytica* and *E. dispar* [23, 24]. In the present study, according to the results of culture and PCR, there was 0.07% prevalence of *E. histolytica*, so 99.93% of the samples included *E. dispar* species. The present study results show less prevalence of *E. histolytica* compared to other similar studies. It might be because of the proper sanitation in the Tehran city. Here are some studies conducted in different regions of Iran on the diagnosis of *E. histolytica*. For example, at 2001 in Hamedan province, Iran, isolated 16 samples of *E. histolytica* and *E. dispar* and examined them by PCR; only one of the samples was positive for *E. histolytica* [25]. In another study (2009) in Zahedan city, south east Iran, reported the prevalence of intestinal parasites which were tested by PCR techniques and cultivation methods [26]. 1562 samples were examined microscopically, eight samples were positive for the presence of *E. histolytica* and *E. dispar* by microscopic method, and after PCR any of them were not positive for *E. histolytica*. Human mistakes in the microscopic method are evident as the number of positive samples in PCR and cultural method is too low. Nowadays, the problems in different ways of identifying amoebiasis as a

definitive assessment have made this molecular method a leading method worldwide in identifying *E. histolytica* especially in differentiation of *E. histolytica* and *E. dispar* from each other, while there is no morphological way of diagnosis to separate them. In recent years, the use of molecular

methods together with culture of parasite to identify infectious agents has revealed more clear results in assessment of *E. histolytica* in different communities. Cultivation of *E. histolytica* was first performed by Boeck and Drbohlav about a half century after the discovery of the parasite [27]. Different methods for cultivation of *E. histolytica* have been introduced since then. In 1926, researchers introduced a new medium for cultivation of *Entamoeba* species called Hsre+s which is reported as a useful medium [28]. Hsre+s medium has been known as a diagnostic laboratory method to differentiate between intestinal protozoa and assessment of amoebic infection in different areas and used in biological and immunological studies. In a study performed at 1998 [16] the sensitivity and specificity of Hsre+s medium was reported to be 85 and 100%, respectively, for growth of *E. histolytica* and *E. dispar*. Their results indicated that successful cultivation of *E. histolytica/dispar* in Hsre+s medium was 60.37%. In this study, Hsre+s medium showed 89% sensitivity to *E. histolytica* and *E. dispar*. The results were the same with that of previous studies which showed that Hsre+s medium are suitable mediums for isolation and detection of *Entamoeba* strains. According to a research at 2002, culture of *E. histolytica* is less sensitive than microscopy as a detection method and is not feasible as a routine procedure [29].

PCR results showed that 0.07% (15 out of 19990) of samples were positive to *E. histolytica* species using 220 bp specific primers with 94, 94 and 93% homology with 18S rRNA, 5.8S rRNA (in plasmid) and small-subunit 1 gene, respectively. Many studies in Iran and elsewhere were performed on the molecular detection of *Entamoeba* strains. According to Hamzah *et al.*, study (2006), 30 samples were microscopically positive for the isolation of *E. histolytica* from other intestinal species of *Entamoeba* through PCR and specific primers with 166 bp; PCR was introduced as a sensitive, rapid and effective detection method for the differentiation of these three species [7]. In the present study, less prevalence of *E. histolytica* was observed as compared to similar studies in other parts of Iran.

Metronidazole is an antibiotic in the nitroimidazole group and is currently known as the most effective drug for the treatment of amoebic infections. Metronidazole effects on anaerobic metabolic pathways of parasite and after entry of drug into the vegetative state, is activated by making a reduction in a nitro group by Ferredoxin (or Ferredoxin dependent metabolic processes). The activated Metronidazole acts as a final electron postulate and bounds covalently to macro molecule genomes and cause DNA damage (loss of Ferro hexical). Metronidazole forms a respiration inhibition in the vegetative form of parasite and reduction in activity of metronidazole leads to the production of toxic radicals which react to important components of the parasite cells [30]. Increase in the minimum inhibitory

concentration (MIC) results in the consistent use of pharmaceutical drugs [31]. In recent years, during the assessment of drug resistance among *E. histolytica* patterns, the presence of resistance among a small percentage of Amoebas was revealed [12, 15, 32]. In Iran, metronidazole is known as the forefront drug against amoebiasis. Metronidazole resistance was examined in the laboratory and is associated with the decreased activity of pyrocvate of parasites: ferredoxin oxidoreductase (required for reducing the activity of nitroimidazole) [33]. In the present study, there was no resistance after 48 h and in the concentration higher than 2 µg/ml of metronidazole, which indicates the absence of sufficient resistance in clinical samples against metronidazole. Although, metronidazole resistance has been reported against other pathogenic parasites such as *Trichomonas vaginalis* in Brystan, Australia, [34] *Giardia lamblia* in the United states [35] and *Leishmania donovani* in India, [36] and also induction of resistance of *E. histolytica* under laboratory conditions, [37, 38] according to the present study and other similar studies, there is no significant resistance among the patterns in Iran. Finally, it is suggested that a periodic review of drug-resistance of pathogen strains such as *E. histolytica* using optimal growth conditions and suitable mediums should be performed to have an immediate prescription of an alternative drug.

Conclusion

According to the results, culture method is not suitable for differentiation of *E. histolytica* from *E. dispar*; also parasite culturing is difficult and expensive and has less specificity against molecular method. Cultural method is suitable for drug resistance assays and for laboratories with poor equipment. On the other hand, the results indicate the high specificity of the molecular techniques against culture in specific mediums in differentiation of *Entamoeba* species; although PCR procedure is an expensive and time-consuming detective method used for detection of *Entamoeba* species, their application in routine diagnosis is limited. The introduction of PCR methods has been hindered by difficulties in DNA extraction from fecal samples [39]. Also, there are various PCR-based methods such as real-time PCR and multiplex PCR which can be used in better assessment of *Entamoeba* species. The results of this and other studies in Iran suggest the low prevalence of *E. histolytica* in Iran. According to molecular studies, it seems that amoebiasis due to *E. histolytica* is a rare infection and *E. dispar* is the predominant species especially in northern and central regions of Iran.

Acknowledgements

The authors would like to thank the staff in the Molecular Biology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran.

References

1. Walsh, J., Ravdin, J., Prevalence of *Entamoeba Histolytica* infection. Amebiasis: human infection by *Entamoeba histolytica*. John Wiley and Sons, New York; 1988 1988, Vol. pp. 93-105.
2. Haque, R., Huston, C.D., Hughes, M., Houpt, E., Petri Jr, W.A., Amebiasis. *N Engl J Med*, 2003, Vol. 348, pp. 1565-1573.

3. Walsh, J.A., Problems in recognition and diagnosis of amebiasis: estimation of the global magnitude of morbidity and mortality. *Rev Infect Dis*, 1986, Vol. 8, pp. 228-238.
4. Ali, I.K.M., Clark, C.G., Petri, W.A., Molecular epidemiology of amebiasis. *Infect Genet Evol*, 2008, Vol. 8, pp. 698-707.
5. Clark, C.G., Diamond, L.S., The Laredo strain and other 'Entamoeba histolytica-like' amoebae are *Entamoeba moshkovskii*. *Mol Biochem Parasitol*, 1991, Vol. 46, pp. 11-18.
6. Diamond, L.S., Clark, C.G., A redescription of *Entamoeba histolytica* schaudinn, 1903 (Emended Walker, 1911) separating it from *Entamoeba dispar* Brumpt, 1925. *J Eukaryot Microbiol*, 1993, Vol. 40, pp. 340-344.
7. Hamzah, Z., Petmitr, S., Mungthin, M., Leelayoova, S., Chavalitshewinkoon-Petmitr, P., Differential detection of *Entamoeba histolytica*, *Entamoeba dispar*, and *Entamoeba moshkovskii* by a single-round PCR assay. *J Clin Microbiol*, 2006, Vol. 44, pp. 3196-3200.
8. Haque, R., Ali, I., Akther, S., Petri, W.A., Comparison of PCR, isoenzyme analysis, and antigen detection for diagnosis of *Entamoeba histolytica* infection. *J Clin Microbiol*, 1998, Vol. 36, pp. 449-452.
9. Ngui, R., Angal, L., Fakhurrizi, S.A., Lian, Y.L.A., Ling, L.Y., Ibrahim, J., et al., Differentiating *Entamoeba Histolytica*, *Entamoeba dispar* and *Entamoeba moshkovskii* using nested polymerase chain reaction (PCR) in rural communities in Malaysia. *Parasit Vectors*, 2012, Vol. 5, pp. 187-191.
10. Fotedar, R., Stark, D., Beebe, N., Marriott, D., Ellis, J., Harkness, J., Laboratory diagnostic techniques for *Entamoeba* species. *Clin Microbiol Rev*, 2007, Vol. 20, pp. 511-532.
11. Behnia, M., Haghighi, A., Komeilizadeh, H., Tabaei, S.S., Abadi, A., In vitro antiamebic activity of Iranian *Allium sativum* in comparison with metronidazole against *Entamoeba Histolytica*. *Iran J Parasitol*, 2008, Vol. 3, pp. 32-38.
12. Upcroft, J.A., Upcroft, P., Drug susceptibility testing of anaerobic protozoa. *Antimicrob Agents Chemother*, 2001, Vol. 45, pp. 1810-1814.
13. Orozco, E., Marchat, L.A., Gómez, C., López-Camarillo, C., Pérez, D.G., Drug resistance mechanisms in *Entamoeba Histolytica*, *Giardia lamblia*, *Trichomonas vaginalis*, and opportunistic anaerobic protozoa, in Antimicrobial drug resistance. 2009, Springer. p. 549-559.
14. Pittman, F.E., Pittman, J.C., Amebic liver abscess following metronidazole therapy for amebic colitis. *Am J Trop Med Hyg*, 1974, Vol. 23, pp. 146-50.
15. Burchard, G., Mirelman, D., *Entamoeba Histolytica*: virulence potential and sensitivity to metronidazole and emetine of four isolates possessing nonpathogenic zymodemes. *Experimental parasitology*, 1988, Vol. 66, pp. 231-242.
16. Haghighi, A., Rezaeian, M., Evaluation of five different media for xenic cultivation of *Entamoeba histolytica*. *Parasitol Int*, 1998, Vol. 47, pp. 297-302.
17. Bingham, A.K., Jarroll, E.L., Meyer, E.A., Radulescu, S., *Giardia* sp.: physical factors of excystation in vitro, and excystation vs eosin exclusion as determinants of viability. *Exp Parasitol*, 1979, Vol. 47, pp. 284-291.
18. Walderich, B., Mueller, L., Bracha, R., Knobloch, J., Burchard, G., A new method for isolation and differentiation of native *Entamoeba histolytica* and *Entamoeba dispar* cysts from fecal samples. *Parasitol Res*, 1997, Vol. 83, pp. 719-721.
19. Widmer, G., Carraway, M., Tzipori, S., Water-borne Cryptosporidium: a perspective from the USA. *Parasitol Today*, 1996, Vol. 12, pp. 286-290.
20. Hemmati, A., Hooshmand, E., Hosseini, M.J., Identification of *Entamoeba histolytica* by molecular method in surface water of Rasht city, Iran. *Iran Journal public health*, 2015, Vol. 44, pp. 238-242.

21. Tan, Z., Wong, W., Nik Zairi, Z., Abdullah, B., Rahmah, N., Zeehaida, M., et al., Identification of *Entamoeba histolytica* trophozoites in fresh stool sample: comparison of three staining techniques and study on the viability period of the trophozoites. *Trop Biomed*, 2010, Vol. 27, pp. 79-88.
22. Stanley, S.L., Amoebiasis. *Lancet*, 2003, Vol. 361, pp. 1025-1034.
23. Ouattara, M., N'Guéssan, N.A., Yapi, A., N'Goran, E.K., Prevalence and spatial distribution of *Entamoeba histolytica/dispar* and *Giardia lamblia* among school children in Agboville area (Côte d'Ivoire). *PLoS Negl Trop Dis*, 2010, Vol. 4, pp. e574.
24. Heckendorn, F., N'Goran, E., Felger, I., Vounatsou, P., Yapi, A., Oettli, A., et al., Species-specific field testing of *Entamoeba* spp. in an area of high endemicity. *Trans R Soc Trop Med Hyg*, 2002, Vol. 96, pp. 521-528.
25. Fallah, M., Haghghi, A., Tachibana, H., Preliminary comparative study of *Entamoeba histolytica* and *Entamoeba dispar* by PCR technique in Iran. *Med J Islam Repub Iran*, 2001, Vol. 14, pp. 369-372.
26. Haghghi, A., Khorashad, A.S., Mojarad, E.N., Kazemi, B., Nejad, M.R., Rasti, S., Frequency of enteric protozoan parasites among patients with gastrointestinal complaints in medical centers of Zahedan, Iran. *Trans R Soc Trop Med Hyg*, 2009, Vol. 103, pp. 452-454.
27. Boeck, W.C., Drbohlav, J., The cultivation of *Entamoeba histolytica*. *Am J Epidemiol*, 1925, Vol. 5, pp. 371-407.
28. Diamond, L.S., Lumen dwelling protozoa: *Entamoeba*, *trichomonads* and *Giardia*. Lumen dwelling protozoa: *Entamoeba*, *Trichomonads* and *Giardia*., 1983, Vol. pp. 65-109.
29. Clark, C.G., Diamond, L.S., Methods for cultivation of luminal parasitic protists of clinical importance. *Clin Microbiol Rev*, 2002, Vol. 15, pp. 329-341.
30. Chapman, A., Cammack, R., Linstead, D., Lloyd, D., The generation of metronidazole radicals in hydrogenosomes isolated from *Trichomonas vaginalis*. *Microbiol*, 1985, Vol. 131, pp. 2141-2144.
31. Orozco, E., Guarneros, G., Martinez-Palomo, A., Sanchez, T., *Entamoeba histolytica*. Phagocytosis as a virulence factor. *J Exp Med*, 1983, Vol. 158, pp. 1511-1521.
32. Adagu, I.S., Nolder, D., Warhurst, D.C., Rossignol, J.-F., In vitro activity of nitazoxanide and related compounds against isolates of *Giardia intestinalis*, *Entamoeba histolytica* and *Trichomonas vaginalis*. *J Antimicrob Chemother*, 2002, Vol. 49, pp. 103-111.
33. Wassmann, C., Hellberg, A., Tannich, E., Bruchhaus, I., Metronidazole resistance in the protozoan parasite *Entamoeba Histolytica* is associated with increased expression of iron-containing superoxide dismutase and peroxiredoxin and decreased expression of ferredoxin 1 and flavin reductase. *Journal of Biological Chemistry*, 1999, Vol. 274, pp. 26051-26056.
34. Duchêne, M., *Entamoeba*, in *Molecular Parasitology*. 2016, Springer. p. 325-379.
35. Nash, T.E., Ohl, C.A., Thomas, E., Subramanian, G., Keiser, P., Moore, T.A., Treatment of patients with refractory giardiasis. *Clin Infect Dis*, 2001, Vol. 33, pp. 22-28.
36. Sundar, S., More, D.K., Singh, M.K., Singh, V.P., Sharma, S., Makharia, A., et al., Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. *Clin Infect Dis*, 2000, Vol. 31, pp. 1104-1107.
37. Samarawickrema, N., Brown, D., Upcroft, J., Thammapalerd, N., Upcroft, P., Involvement of superoxide dismutase and pyruvate: ferredoxin oxidoreductase in mechanisms of metronidazole resistance in *Entamoeba histolytica*. *J Antimicrob Chemother*, 1997, Vol. 40, pp. 833-840.
38. Orozco, E., Suárez, M.E., Sánchez, T., Differences in adhesion, phagocytosis and virulence of clones from *Entamoeba Histolytica*, strain HM1: IMSS. *Int j Parasitol*, 1985, Vol. 15, pp. 655-660.
39. Monteiro, L., Bonnemaïson, D., Vekris, A., Petry, K.G., Bonnet, J., Vidal, R., et al., Complex polysaccharides as PCR inhibitors in feces: *Helicobacter pylori* model. *J Clin Microbiol*, 1997, Vol. 35, pp. 995-998.