MOLECULAR IDENTIFICATION OF ENTAMOEBA HISTOLYTICA IN SULAIMANI PEDIATRIC TEACHING HOSPITAL

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ABSTRACT

Background

Entamoeba histolytica is a unicellular protozoon that conceder as a common cause of dysentery in human especially in the children under 6 years old. This may be transmitted via the ingestion of cystic stage of the parasite.

Objectives

This study aimed to investigate the total rate of *Entamoeba histolytica* infection in children admitted to the Pediatric Teaching Hospital in Sulaimani City, and considering some other aspects such as culturing and detecting subtypes using polymerase chain reaction (PCR).

Patients *and Methods*

This epidemiologic study includes 1005 stool samples collected from children aged 6 months to 6 years old from the 1st of Jun to the 30th of October 2014. Each stool sample was examined and identified by direct wet mount method using normal physiological saline and iodine solution, followed by culturing and isolation of the parasite for 14 days to elongate life span and then genomic amplification applied from the cultured *Entamoeba histolytica* by appropriate PCR based method.

Results

The result of rapid kit occult blood combined with microscopic wet mount (iodine) showed that the infection with *E. histolytica* was (12.9%), in which the rate in male (14.18%) was higher than that female (11.42%), with no significant difference between both genders. cultured *E. histolytica* showed a great ability of this parasite to grow, with detection of the parasite under light microscope (40X). Then two standard kits were used specific primer (1147, F&R), and thus about 1147 bp PCR produced. Then, nested PCR was performed for the same PCR products two different primers (246 F&R) which resulted in fragments of approximately 246bp. This fragment coding for the 16S SSUrDNA gene of HM-1IMSS 16s *E. histolytica* confirms the previous PCR amplification finding. The results of sequence methods identified *E. histolytica* subtype 1 from PCR positive samples by sequencing alignment (99%) similarity with accession number KB823016.

Conclusion

The prevalence rate of infection with *E. histolytica* in Pediatric Teaching Hospital in Sulaimani City was (12.9%). Beef extract medium for culturing of *E. histolytica* showed a great ability for growing of the parasite cystic form. *E. histolytica* subtypel can be detected by sequencing method for the first time in molecular genotype of human E .histolytica in Sulaimani city.

Keywords: *Molecular; Survey scale; Entamoeba histolytica.*

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INTRODUCTION

Amoebae are a unicellular protozoan that forms a large and diverse accumulation of eukaryotes which are characterized by pseudopodia and considered as a third causes of death by parasites, after malaria and Schistosomiasis. According to the World Health Organization (WHO), 500 million peoples are infected with amoebae by which 10% of them diagnosis as pathogenic *E. histolytica*, resulting in 40,000–100,000 deaths per year [1]. Research undertaken in Erbil found that the prevalence ratio of *E. histolytica* was (18.6%) [2]. While in Tikrit city, the average of (23.3%) was recorded in a study conducted between school children [3], after that in Al-Kut city the rate was (23.3%) mentioned in four health centers that suffered from amoibiasis [4]. At the same time a study on intestinal parasite in Basra city established (12.6%) as *E. histolytica* infection [5].

Another study in Dyala (1998) an attributed (13.6%) of infection with *Entamoeba*. However, in Musel, it was shown that the prevalence rate between school children and food factory workers was (10.4%) [6], Also in Babel [7] recorded a ratio of (10.3%) infection between patient attended hospital laboratory, but in Baghdad about (44.4%) for Central Children Hospital and (57.2%) in Ebin-Al-Baladdy, and (23.3%) of *Entamoeba* infection in both Al-Kadhmiya Teaching Hospital and Al-Karkh Hospital recorded respectively [8]. Lastly in Sulaimani, a rate of (38.13%) reported in children presented to the Pediatric Teaching Hospital [9] which that mean its endemic in Iraq compared ro the rest of the world.

Three main strain of Entamoeba have recognized, a pathogenic and even nonpathogenic strain to human and animals, such as *E. histolytica*, that causes amoebiasis, *E. dispar* and *E. moshkovskii*, that morphologically similar to *E. histolytica* have been newly described as different species. Although they have no apparent invasive potential, they exhibit some pathogenicity [10, 11]. The *E. histolytica* lives and multiplies in the intestine forming cysts which passed out with the feces and transmits to other healthy person after consuming contaminated water or food. Mostly infections are asymptomatic, but in immunocompromised or children whose suffered from *E. histolytica* and not treated well can invade the epithelial wall of intestine, causing flask shape ulceration and amoebic dysentery which characterized by bloody diarrhea. If the parasites reached out to blood vessels through the ulcer, they may be spread out extra intestinal sites to the other organs, the most important of which is the liver, where they cause hepatic amoebiasis [12].

The major virulence factors are adhesions, toxins, amoeba pore by protease enzyme which responsible for tissue lysis. For that reason it considered as the most important part of virulence in amoebeasis. There are so many ways for diagnosing this parasite which used for many years but the most proper ones that used now a day is molecular based method for both clinical and epidemiological studies in the developed countries and has been strongly endorsed by the WHO [13, 14]. PCR of the small-subunit rRNA gene (18S rDNA) is reported to be more sensitive than the best ELISA kit currently available [15]. Furthermore investigations for increasing the sensitivity of the PCR assay, a nested multiplex PCR was discovered for the concurrent detection and distinguish of *E. histolytica* and *E. dispar* by extracted DNA from microscopy-positive stool samples [16, 17]. By employing this technique, the sensitivity and specificity were raised to 94% and 100%, respectively [18].

This study aims to determine the total prevalence rate of *E. histolytica* in Pediatric Teaching Hospital of Sulaimani city and also define the frequency of different subtypes of the *E. histolytica* in the infected patients.

PATIENTS AND METHODS

In this study four main procedures was performed for analysis. First stool collection was carried out at the Sulaimani Pediatric Teaching Hospital from the 1st of Jun to the 30th of October 2014. A total of 1005 stool samples were collected by questionnaire file from the patient replaced in a dry clean screw capped plastic bottle and then examined by using wet mount and iodine stain for under microscope testing then occult blood kit used as a rapid kit performed by putting drop of stool on the tester pore [19]. Second step was carried out by culturing 10 samples which recommended by Cleave and Coiller (1930) but with changing the heart infusion to liver infusion in the ingredient .This media is composed of two parts; the upper part which was liquid and the lower part which was solid in the same test tube. Then the third step was conducted which was DNA extraction by employing 10 cultured product sample anticipating Gene aid genomic DNA mini kit (cultured cell) procedure. SSUrDNA gene was Detected by PCR using two sets of different primers (Table 1 and 2); these have been established by [20].

Table 1. Oligonucleotide primers for the detection SSUrDNA gene of *E. histolytica*

Primer name	Nucleotide sequence	PCR product
1147(forward)	5'-ATC TGG TTG ATC CTG CCA GT-3'	1147bp
1147(reveres)	5'-TGA CCA TAC TCC CCC TGA AG-3'	

Table 2. Oligonuclotide primers for Nested PCR detection in SSUrDNA gene of *E. histolytica.*

Primer name	Nucleotide sequence	PCR product
1147-246 (forward)	5'-AAG ATG CAC GAG AGC GAA AG-3'	246bp
1147-246 (reveres)	5'- TGA CCA TAC TCC CCC TGA AG-3'	

The nested PCR was conducted with 1µl of the primary PCR product as the template, and then the final product was electrophoresed in a 0.5% agarose gel and stained with Ethidium bromide. Bands were visualized under UV light, and photos were taken by gel documentation system. Sequencing technique was carried out in Kon-Kuk university ,college of medicine, Korea) by sending second nested PCR product in 50 ml Epindorf tube in freezing condition under 20˚c as done by Lorrezi (2013) [21]. Both SPSS (V.17) and stratigraphic (V.4) software used for statistical analysis depending on t-test and chi-square. $P<0.05$ was considered statistically significant.

RESULTS

The result of examination of 1005 stool samples by using occult blood kit showed double red band which means the presence of the blood cells in the positive stool samples as in Figure 1.

Then total prevalence rate of 12.9% recorded out of 1005 stool samples as in Figure 2 between children's attended hospital at that time.

E. histolytica were distributed among all age groups, by which in male was higher (%14.18) to that of females (%11.42), Table 3.

The testing of stool samples under the microscope showed the ovoid creature with multiple vacuoles inside it, which reveals the appearance of cystic stage of the *E. histolytica* with multi nuclei in both wet mount and iodine stain as in Figure 3.A and 3.B.

10 samples selected to be cultured beef extract media. The first growth of *E .histolytica* appeared after one week of culture increased after every subcultured process. The supernatant was taken and examined under the microscope showed full field cystic form as show in Figure 4.

Then growths of 10 cultured samples were selected to be assessed by PCR. DNA extraction were done for all 10 selected samples and conventional PCR done in which no band appear in the first PCR run, followed by using second sets of primer for next nested PCR run. All the 10 samples yield specific band of 246bp which was specific for SSUrDNA gene as show in Figure 5.

The aligned 1147 bp 16S SSUrDNA gene of the *E. histolytica* HM-1IMSS 16s local isolates showed subtype 1 which was determined by an exact match of 99% similarity as a result of DNA sequencing. The 16S SSUrDNA gene was compared with the same gene in NCBI database using bioinformatics tools (ncbi/ blast alignment program). The 16S SSUrDNA gene sequences obtained in this study has been deposited in GenBank under accession numbers KB823016 [22].

Figure 1. Double red band appearance.

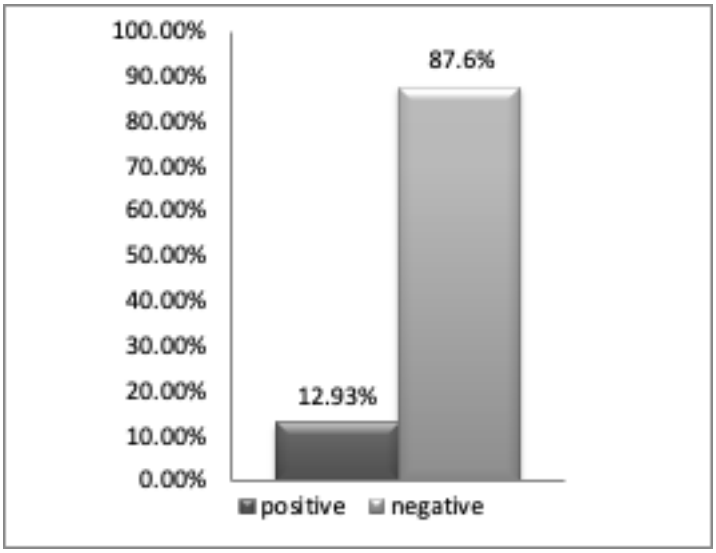


Figure 2. The prevalence rate of *E. histolytica* infection in Pediatric Teaching Hospital in Sulaimani City.

Table 3. Prevalence rate of *E. histolytica* infection according to gender.

Gender	Examined Samples		Positive	
	No.	%	No.	%
Male	550	54.72	78	14.18
Female	455	45.27	52	11.42
Total	1005	99.99	130	12.9

P-value 0.0683

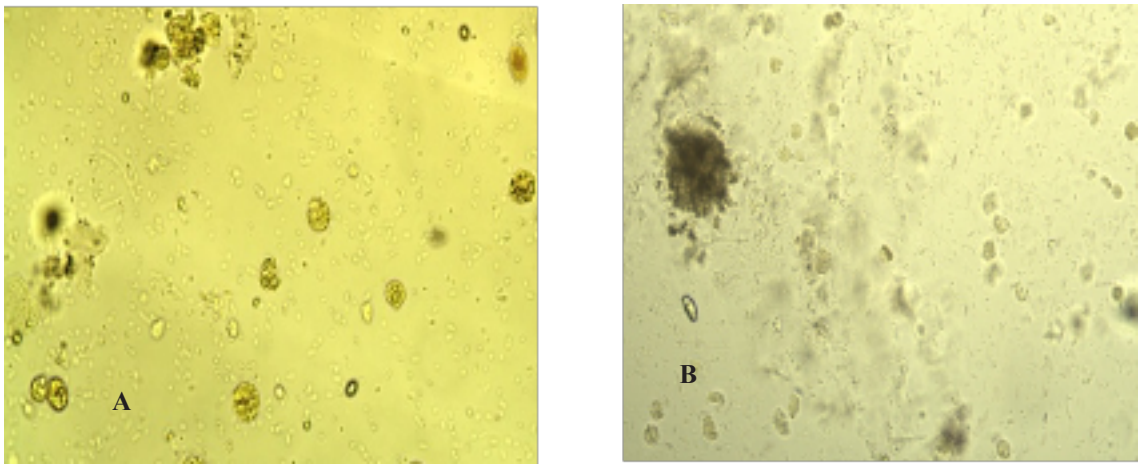


Figure 3. Wet mount (iodine) microscopic examination under (A) 10X and (B) 40X magnification.

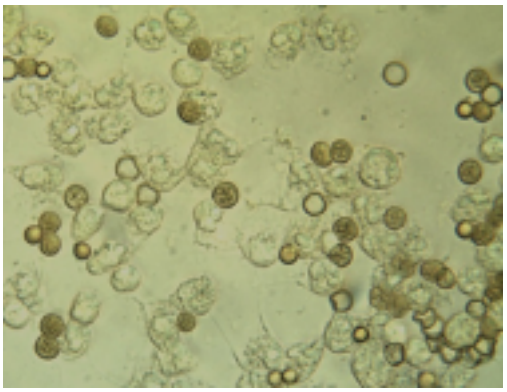
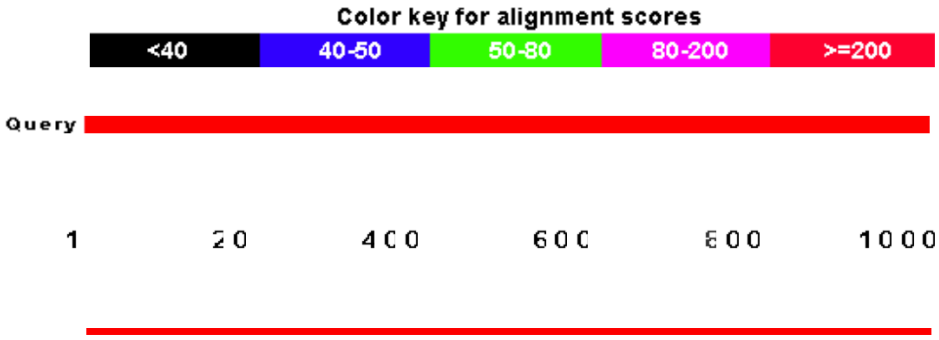


Figure 4. Mature cyst growth in cultured after14 days under magnification 40X for both A and B.

Figure 5. Gel electrophoresis of the nested PCR product (1147-246) bp of the HM-1IMSS 16s strain of SSUrDNA gene of *E.histolytica*.



DISCUSSION

From the result of this study, it was found that the total rate of infection with *E. histolytica* in in Pediatric Teaching Hospital at Sulaimani City was (12.93%). This indicates that Sulaimani City is still endemic with this parasite in comparing with previous study that done by Ali and Mohammed (2010) [9] which recorded a higher rate of infection (38.13%). This difference in the rate of *E. histolytica* infection is due to many factors such as the number of the patient's samples in screening study and the time of sample collection. It also could be due to the contamination of food by fecal rodents, dogs, cats, and sheep that act as reservoirs for this parasite. Besides, water contamination is a great important factor, because chlorinated tap water is available but in low ratio in most of the areas, together with suitable environmental factors such like high temperature which facilitate the spreading and completion of the life cycle of this parasite. In addition socioeconomic condition although using bottle feeding instead of breast feeding which increase the risk of infection among children.

E. histolytica as a protozoan is prevalent more than any intestinal parasite and this may be because of simple life cycle and rapid multiplication and growth with easily acquired by contaminated food and water, with cyst resistance to acidification and chlorination that help its maintains for many weeks [23]. All these factors help *E. histolytica* to be spread. Concerning the gender, males showed a higher rate of infection (14.18%) than females (11.42%) with no significant difference between both genders. This may be attributed to behavioral activities of males at this age for exposing to *E .histolytica* cyst than the females. This was similar to the results recorded by Abd-Al-Husaen(1994) [4]; Al-Saaid, *et al* (1994) [3] and Al-Ganabi (2000) [8].Microscopy is a less reliable method of identifying *Entamoeba* species than antigen detection tests. The sensitivity of microscopy can be poor (60%) and confounded with false-positive results due to misidentification of macrophages as trophozoites, PMNs as cysts especially when lobed nuclei of PMNs break apart [24]. From the culture medium of *E. histolytica* by Cleveland and Collier (1930) the growth did not continue for more than 14 days, and this may be related to the abundance of metabolic products with poisoning material secreted by *E. histolytica* during their reproduction, to increasing activities of the parasite that lead to inadequate component in the medium and to lack of other elements like iron that found in human body in which *E. histolytica* use haemoglobinases enzymes to distract the engulfed red blood cells for obtaining the iron needed by it for growth [25].

Parasite cultures are difficult, expensive, and labor-intensive to maintain in the diagnostic laboratory. Overgrowth of bacteria, fungi, or other protozoan during culture is the main problem encountered, and therefore culture is not recommended as a routine diagnostic procedure for the detection of *Entamoeba* species [26, 27]. This media proved effectiveness for culturing *E. histolytica* which indicates that it was a good media by its component that help growth of this parasite. The result of DNA extraction directly from cultured stool sample performed was to determine genotyping of isolates with two different primer piers were successful because of the pure parasite obtained from the culture media, but the present result was differ from what was recorded by Mukherjee, *et al* (2008) [28] who found that is possible to rapidly identify *E. histolytica* DNA using minute amounts of clinical stool samples, thus eliminating the laborious parasite culturing process. This difference may be attributed to different individual variation of workers in laboratory, while this similar to result that was founded by [26]. The specific gene of *E. histolytica* HM-1 IMSS 16s strain was detected among all 10 cultured stool samples. This result was similar to the previous findings by Joung-Ho, *et al* (2011).

The nested PCR reaction amplified approximately about 246 bp with 1147; 1147-246 primers set from the same ten PCR positive products which confirming the previous finding result for *E. histolytica* genotype, and this may be due to specificity of the primers used. The result agreed with the result found by Cheun , *et al*, (2011)[20] which strongly suggested that the nested PCR method using the 1,147-246 primer set would be effective for detection of *E. histolytica* cysts in clinical samples. The result of Nested PCR product yield specific gene which were send it to sequencing and the result was compared with the gene bank accession numberKB823016 and the similarity between them was 99% the same result was recorded by [29] who describe this gene as a specific of human source. This study is the first investigation in the molecular genotype of human amoebiasis in Sulaimani City and Iraq as a whole, where a majority of entamoeba detection and isolation were done by microscope and culturing respectively from stool in laboratories in hospitals in the country.

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