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Molecular detection of Entamoeba histolytica in Iraqi Populations

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ABSTRACT

Entamoeba histolytica is an anaerobic parasitic amoebozoan, part of the genus *Entamoeba*. Predominantly infecting humans and other primates causing amoebiasis, *E. histolytica* has an estimated worldwide prevalence of 500 million infected and is responsible for 40,000-100,000 deaths each years it is an important health problem, especially in developing countries. The present study was conducted to molecular detection of *E. histolytica* by specific primer. A total of 100 stool samples from the patients (60 males, 40 females) age range between 8-55 years from March to August 2020. The collected fresh stool samples were microscopically examined using the direct saline/ iodine wet mount microscopy to detect *Entamoeba trophozoites* and/or cysts. About 0.2 g of each specimen was preserved at –20°C for molecular analysis. The present study was showed the proportion of male patients with diarrheal illness was more than that of female subjects, 60 versus 40, respectively. In the current study, only 24 (34.3 %) were proved to have *E. histolytica* infection out of the 70 cases who were diagnosed based on the morphology. The imminent expansion of the amount genome data will greatly improve our knowledge of the genus and of pathogenic species within it.

1. INTRODUCTION

Entamoeba histolytica is an anaerobic parasitic amoebozoan, part of the genus Entamoeba [1]. Predominantly infecting humans and other primates causing amoebiasis, E. histolytica has an estimated worldwide prevalence of 500 million infected and is responsible for 40,000-100,000 deaths each years it is an important health problem, especially in developing countries [2]. E. histolytica is the causative agent of amebic dysentery and other invasive disease including amoebic liver abscess respiratory system infection, cerebral and genitourinary amoebiasis [3]. Entamoeba has high degree of genomic plasticity and instability. Stool PCR is extremely sensitive, it is considered the gold standard for diagnosis of amebiasis and is becoming readily available through FDA- cleared gastrointestinal panels that simultaneously detect multiple enter pathogens [4]. The application of this new PCR assay as an alternative tool in routine diagnosis and in epidemiological studies of amoebiasis. The sensitivity and specificity of PCR assays exceed what can be accomplished with microscopy and are comparable to those of the antigen test [5]. Tissue invasion associated with tissue arrangement that explanation for the different tRNA-STR genotypes identified in

liver abscess and stool sample for the same infected person infected with Entamoeba. Transposons and repetitive DNA molecules found in great quantity in Entamoeba genome cause reorganization [6]. Transposable elements contribution chromosome instability leading to genomic variation in these parasite [4]. Repetitive elements in Entamoeba species genomes specified copies of LINE (long interspersed elements) and SINE (short interspersed elements). In Entamoeba LINE and SINE elements are class I transposons increased by reverse transcription [7]. E. histolytica contains a number of large multi-gene families. E. histolytica genome does not appear to contain microsatellites like elements, measurement of genetic diversity.

The understanding of the role of the parasite genome in the determination of infection outcome and its severity is still under research and recent paper has been published by [5]. discussing the role of different E. histolytica strains in causation of different clinical features based strain genetic variation. Actually, data concerning the genetic makeup of infecting strains from the endemic areas worldwide is essential to shed light on the role of parasite genome in the outcome of E. histolytica infection [4]. Our country, Iraq, is one main regions of the world that are endemic in E. histolytica, therefore, genetic characterization of this parasite in association with diarrhea is necessary from clinical point of view. For that reason, the current study was planned and carried out in order to accomplish this aim.

2. Materials and Methods

The current study was carried on 100 stool samples from the patients (60 males, 40 females) age range between 8-55 years from March to August 2020. Specimen donors filled out a structured questionnaire about personal status, residency, and source of water supply. The collected fresh stool samples were microscopically examined using the direct saline/ iodine wet mount microscopy to detect Entamoeba trophozoites and/or cysts. About 0.2 g of each specimen was preserved at –20°C for molecular analysis.

Genomic DNA from stool samples was extracted by using AccuPrep® stool DNA Extraction Kit, Bioneer, Korea, and done according to company instructions. Finally, the purified DNA concentrate was eluted from the silica membrane spin column with a low salt buffer. The extracted genomic DNA from stool were estimated by using Nanodrop spectrophotometer (THERMO. USA), that check and measurement the purity of DNA through reading the absorbance in at (260 /280 nm); then, each sample was labeled and stored at –20°C. A conventional PCR was performed. PCR amplification was performed using a thermal cycler (Bioneer, Korea) with 20 µl reaction volumes that consisted of 10 µl Hot Start Master Mix (containing Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, MgCl2, stabilizer, and tracking dye) (Intron, Korea); 2 µl of both the forward and reverse primers (10 pmol for each), 5 µl of DNA template, and 13 µl of PCR water. The PCR cycling and running parameters were defined as one cycle of initial denaturation at 954°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min with a final extension of 72°C for 5 min. The PCR products were electrophoresed in 1%, agarose gels with a 1X Tris-boric acid-EDTA buffer (TBE) and stained with 3µL of ethidium bromide (BioBasic, Canada), with a 100-bp DNA marker ladder (Biolab, UK). The primer used for the amplification of a fragment gene (EhP1) 135bp with the following sequences. F \ CGATTTTCCCAGTTAGAAATTA R\ CAAATGGTCGTCGTCTAGGC.

3. Results

The frequency distribution of patients with diarrhea according to gender is shown in figure (1), were identified the proportion of male patients with diarrheal illness was more than that of female subjects, 60 versus 40, respectively. The frequency distribution of patients with diarrhea according to age is shown in table (1). The comparison of mean age and gender distribution according to results of light microscopic examination for Entamoeba histolytica parasite is shown in table (2), were showed higher rate of positive

with light microscope was detected in males 38 (54.3 %) and the mean age of patients with positive light microscope lower than the mean age of negative light microscope.

3.1 Conventional PCR analysis

DNA was extracted from the 70 positive stool samples; their concentrations ranged from 5 µg/ml to 217 µg/ml, and purity ranged from 2.2–2.8, as measured by Nanodrop spectrophotometer. Figure (2) showed the positive detection rate of conventional PCR in the diagnosis of Entamoeba histolytica parasite out of all positive light microscopy stool samples. In the current study, only 24 (34.3 %) were proved to have E. histolytica infection out of the 70 cases who were diagnosed based on the morphology. These findings may be explained by that it is difficult to identify variuos species of Entamoeba based on morphology using light microsocpical criteria while using specific DNA probe with convneional PCR can identify specifically with high rate of accuracy only those cases having E. histolytica. Figure (3) showed The molecular diagnosis of E. histolytica parasite was based on conventional PCR using common probe (EHP1) for detection of all E. histolytica genotypes.

Table 1: Frequency distribution of patients with diarrhea according to age.

Age (years)	Result
8-9 y, *n* (%)	7 (7.0 %)
10-19 y, *n* (%)	23 (23.0 %)
20-29 y, *n* (%)	32 (32.0 %)
30-39 y, *n* (%)	19 (19.0 %)
40-49 y, *n* (%)	15 (15.0 %)
50-54 y, *n* (%)	4 (4.0 %)
Mean ±SD	27.32 ±12.65
Range	8 -55

Table (2): Comparison of mean age and gender distribution according to results of light microscopic examination for Entamoeba histolytica parasite

Characteristic	**Total** ***n*** **= 100**	**Positive** ***n*** **= 70**	**Negative** ***n*** **= 30**	***p***
Age (years)				
Mean ±SD	27.32 ±12.65	26.41 ±12.64	29.43 ±12.63	0.276 I NS
Range	8-55	8-55	9-52	
Gender				
Male, *n* (%)	60 (60.0 %)	38 (54.3 %)	22 (73.3 %)	0.075 C NS
Female, *n* (%)	40 (40.0 %)	32 (45.7 %)	8 (26.7 %)	

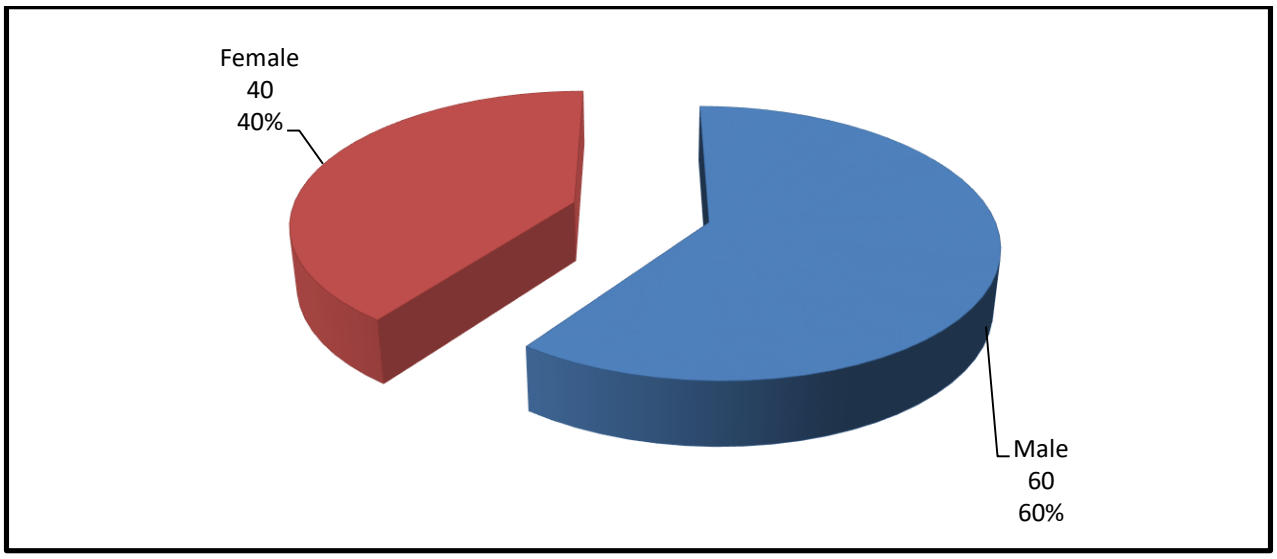

Figure (1): Pie chart showing the frequency distribution of patients with diarrhea according to gender.

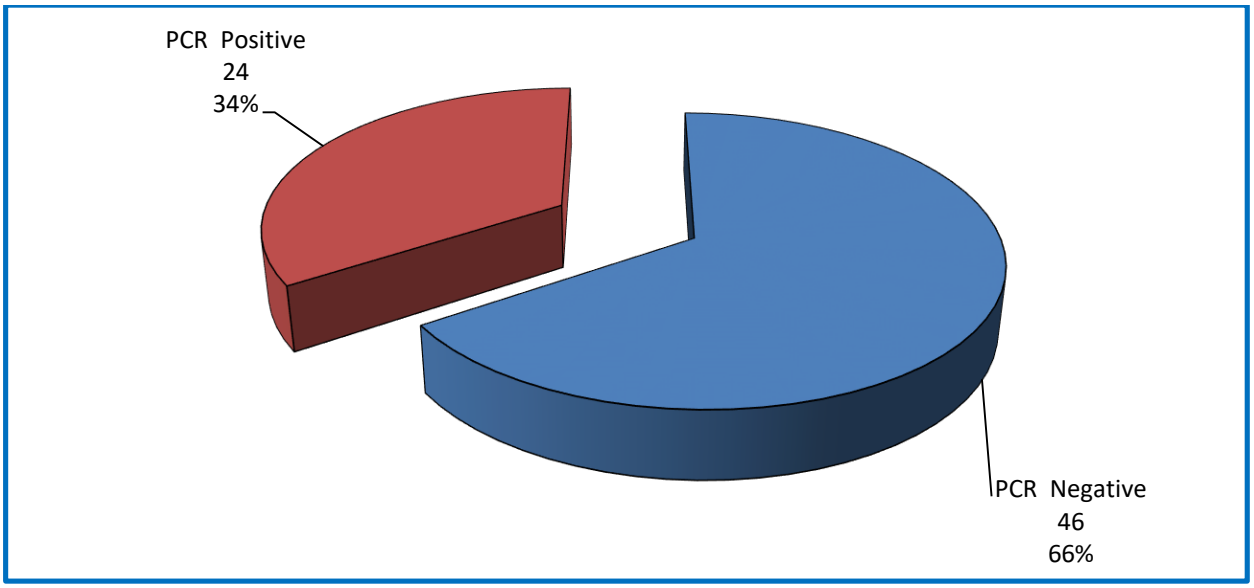

Figure (2): Pie chart showing the positive detection rate of conventional PCR in the diagnosis of Entamoeba histolytica parasite out of all positive light microscopy stool samples.

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Figure (3): Agarose gel electrophoresis image that showed the PCR product analysis of diagnostic EHP gene in *Entamoeba histolytica* from Human stool samples. Where M: marker (1500-100bp) and Lane (1-24) some positive EHP gene was showed at 135bp PCR product.

4. Discussion

The male to female ratio will be 1.5:1 in this study and this implies that males are more prone to diarrheal illness than females. This finding is in line with the observation of [8], who stated that infectious diarrhea was more common among males than in females; however, our results are inconsistent with the results obtained by [9], who found that infectious diarrhea is slightly more common in males than in females. On the other hand, it has been shown that infectious diarrhea attributed to E. histolytica is distributed equally among males and females in the study done by [10]. Indeed the study of [8] and the study of [10], have discussed infectious diarrhea caused by a diversity of pathogens including viruses and bacterial agents in addition to parasites, moreover, the latter study was limited to pediatric age group, therefore, it appears that gender variation with respect to infectious diarrhea is affected by a multitude of host and environmental factors such as lifestyle, hygiene, immune system, water supply and other factors and that the finding of the current study that males are affected more is merely statistical and carry no scientific explanation; however, it has been suggested that that the relative iron deficiency or hormonal factors in women of child bearing age may be a protective factor against disseminated disease [11]. The mean age of patients affected by diarrheal illness in the current study was 27.32 ±12.65 years and there was a wide range of affected age from 8 -55 years. It was observed in this study that the majority of patients were bellow thirty of age. Indeed, this finding is similar to the finding of [12], who stated that E. histolytica revealed that the parasite had highest prevalence (30.82%) in age group of 1 day to 15 years and lowest prevalence (17.34%) in age group of 31 to 45 years. In another study, the prevalence of this infection showed an age- dependency relationship, with higher rates observed among those aged less than 15 years in all ethnic groups studied [13]. Therefore, children, adolescents and young adults are more prone to get infected by E. histolytica than adults and elderly individuals. This may be due to the fact that frequent exposure to the parasite at younger age group may provide immunity in later adult and elderly life. It is very obvious that immunity protozoa parasite needs long time to develop in comparison with viruses or bacteria pathogens [14].

The important limiting step in prevention of parasite invasion is the development of well-organized and strong mucosal immune response and this needs long time and recurrent exposure to the parasite in order to get full development [15]. The rate of E. histolytica in the current study according to light microscopical finding was 70 out of 100 (70 %). It has been shown previously that using fecal antigen detection, E.

histolytica was found to be the most prevalent enteropathogen associated with diarrhea, with a prevalence of 20% [16]. Infection occurs worldwide with a higher prevalence in countries of low socioeconomic status and poor public health. Countries with a high rate of infections include India, Africa, Mexico, and Central and South America. For example, a three-year study conducted in Bangladesh showed there was a 2.2 percent frequency of dysentery cause of E. histolytica in preschool children [17]. In rural areas of Mexico, the seroprevalence of E. histolytica has been reported as high as 42%. Risk factors for infection are mostly related to fecal-oral transmission and have been due to poor hand hygiene, defecation into water sources such as rivers, and being in close proximity with animals. In developed countries such as the United States, amebiasis infections are rare accounting for at least 5 deaths per year and are commonly seen in individuals that have had exposure to endemic areas such as immigrants or recent travelers [2]. Our study has shown that the rate of E. histolytica is very high (70 %) regarding diarrheal illness of all age groups and this need urgent intervention by health authorities to reduce this high rate of endemicity and this can be done by health campaigns aiming at educating people about the risk of unhealthy water and food supplies and the educate people about the health measures and proper sanitation to reduce risk of transmission. In addition, we are in need for a large multicenter national study to identify risk factors associated with transmission of this protozoa parasite. It is well known that Entamoeba includes many species, six of which (Entamoeba histolytica, Entamoeba dispar, Entamoeba moshkovskii, Entamoeba polecki, Entamoeba coli, and Entamoeba hartmanni) reside in the human intestinal lumen [18]. The laboratory diagnosis of E. histolytica currently relies on the direct microscopic identification of the parasite. Other methods of diagnosis include the culture, using Boek and Drbohlav's biphasic amoebic medium, isoenzyme assay using different zymodemes, stool ELISA on monoclonal antibodies to galactose specific adhesin, rapid indirect haemagglutination assay (IHA) to detect serum antiamoebic antibodies and polymerase chain reaction (PCR) nested multiplex PCR targeting 16s like rRNA gene, realine PCR, single round PCR, and PCR-RFLP (restriction fragment length polymorphism) [19].

Of the available diagnostic techniques, the microscopic detection of the morphological forms of the parasite in stool samples is often used in developing countries. Limitation of the microscopic detection is that it is insensitive to differentiate between pathogenic strains of Entamoeba from other nonpathogenic amoebae. Diagnosis by culture, though is much sensitive and specific, is laborious and time consuming which may require several weeks. Amoebic culture can also show false negative results which can be accounted to either delay in processing or probably antiamoebic therapy prior to stool collection. ELISA using monoclonal antibodies (MAbs) directed against pathogen specific epitopes of the galactose adhesin means to diagnose amoebiasis. Detection of antibodies to E. histolytica in patients by using indirect haemagglulination assay (IHA) may fail to distinguish past from present infection [19]. In one study that involved 218 stool specimen, it has been shown that the use and role of PCR in differentially diagnosing pathogenic Entamoeba histolytica from morphologically resembling non-pathogenic E. dispar, that otherwise by routine microscopy cannot be segregated [20]. In their study have examined 130 fecal specimens and demonstrated that molecular method have l00% specificity with respect to differential identification of Entamoeba histolytica and other non-pathogenic amoeba [19]. Advantages and significance of DNA based techniques over other methods in identifying the parasites, quantify and provide important information on formulating and implementing the parasite control programs in human [21]. Of the available diagnostic techniques, the microscopic detection of the morphological forms of the parasite in stool samples is often used in developing countries. Limitation of the microscopic detection is that it is insensitive to differentiate between pathogenic strains of Entamoeba from other nonpathogenic amoebae. Diagnosis by culture, though is much sensitive and specific, is laborious and time consuming which may require several weeks. Amoebic culture can also show false negative results which can be accounted to either delay in processing or probably antiamoebic therapy prior to stool collection [21].

Results of several studies on detection and differentiation of E. histolytica, E. dispar, E. moshkowski and other harmless amoebae in clinical specimen using PCR showed the potential use of molecular methods in the diagnosis of amoebiasis [22]. A recent study which involved 218 stool samples has demonstrated the use and role of PCR in differentially diagnosing pathogenic E. histolytica (51) from morphologically resembling non- pathogenic E. dispar [39], [20], which otherwise by conventional microscopy cannot be differentiated. [24] in their study have evaluated 130 fecal specimens and showed that molecular methods have 100% specificity towards differential identification of E. histolytica and other nonpathogenic amoebae [21]. Significance and advantages of DNA based techniques over other methods in identifying the parasites, quantify and provide important information on formulating and implementing the parasite control programs in both human and animal is highlighted in a recent article by [22]. Diagnosis of amoebiasis is usually performed on clinical grounds alone in most of the endemic countries having limited resources. Microscopic methods, though are cost-effective require well-trained laboratory personnel. This has remarkably affected the estimates of global prevalence of amoebiasis due to E. histolytica. The prevalence and the true epidemiology of amoebiasis are still unclear. Previous studies showing high rates of infection with E. histolytica may not be true as studies reported that E. dispar is about 10 times more common [21].

5. Conclusions

In conclusion, the current study finds most patients with E. histolytica were male. Many questions remain concerning the evolution of Entamoeba species, related to the complex architecture of the genome and to the structure of Entamoeba populations. The ability to rapidly generate whole genome sequences may help to answer some of these questions, both by allowing comparative analyses of genomes to be undertaken within and between species and by identifying genetic markers for use in molecular epidemiological studies. The detection of E. histolytica using specific primers (Ehp1) is more accuracy and specific than using microscopical examination.

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