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IDENTIFICATION OF *ENTAMOEBA HISTOLYTICA* IN DIARRHEAL PATIENTS USING REAL TIME PCR AT WASIT PROVINCE / IRAQ.

Dr. Abdulsadah A. Rahi* and Raheek Faris

Department of Biology, College of Science, Wassit University, Kut, Iraq.

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***Corresponding Author**
Dr. Abdulsadah A. Rahi
Department of Biology, College of Science, Wassit University, Kut, Iraq.

ABSTRACT

Entamoeba hisolytica is a parasitic protozoan causing common diarrhea. The present study aimed to determine the prevalence of *E. histolytica* parasite from a total of 100 patients suffered from diarrhea attended to Al Al-Karamah Teaching Hospital and Al-Zahra'a Teaching Hospital at Wasit province / Iraq during the period from November 2015 to March 2016. Their ages were ranged from under 1 year – 60 years. Stool samples were collected and examined in direct wet smear method using normal saline, the results showed that 39 of samples were positive for *E. histolytica*. The age group (<1–10) years showed the highest rate of infection (63.88%), while patients aged (31 – 40) years showed the lowest rate infection (14.28%). The infection rate in male was higher (53.84%) than in female (46.16%). Results of *E. histolytica* were subjected to Real Time PCR appeared 35 samples out of 39 samples (35%), as well as 7 samples out of 61 (7%) samples negative in direct method, gave positive results. The results showed that 42 samples out of 100 samples were gave positive in Real Time PCR.

KEYWORDS: *E. histolytica*, Diagnosis, Real-time PCR.

INTRODUCTION

Parasitic infections are major health problem, particularly intestinal parasites which are most common and with high prevalence in Iraq.[1,2] Parasitic disease are incriminated in causing more than 33% of global deaths of which intestinal parasitic infections are believed to take the major share.[3,4] Amoebiasis caused by the intestinal parasite *Entameoba histolytica,* has an estimated worldwide prevalence of 500 million infected people and is responsible for

40000 – 100000 deaths each year. It is an important health problems, especially in developing countries.[5] *E. histolytica* is a pathogenic parasite for which humans are the primary reservoir.[6] The clinical presentation can range from asymptomatic carriage to gastrointestinal disease and invasive disease. *E. histolytica* is morpholo-gically identical to the nonpathogenic species *E. dispar* though differences have confirmed their separation into independent species.[7] Several recent diagnostic tests are now available which surpass the microscopic detection of these parasites and facilitate a more accurate diagnosis. These approaches include ELISA and PCR.[8] Antigen and antibody detection by ELISA is becoming the standard method for diagnosis of *E. histolytica* infection.[9]

MATERIAL AND METHODS

Study Subjects

A total of 100 stool specimens were collected from diarrheal patients who were admitted to Al-Karramah Teaching Hospital and Al-Zahra'a Teaching Hospital at Wasit province / Iraq during the period from beginning of November 2015 to end of March 2016. The patients aged from under 1 year – 60 years.

Microscopical examination

Small amount of fresh stool was mixed with normal saline (0.9% NaCl) on glass slide then examined by the light microscope, 40X.[10]

DNA extraction

Genomic DNA was extracted from positive stool specimens in direct method using (AccuPrep® stool DNA extraction kit, Bioneer, Korea). The extraction was done according to company instructions using stool lysis protocol method with proteinase K. Then,the extracted gDNA was checked by Nanodrop spectrophotometer (Thermo, USA), and measured the purity of DNA through reading the absorbance at (260/280 nm.), then stored at -20ºC at freeze until used .

Real Time – Polymerase chain reaction (RT-PCR)

It was performed for detection *E. histolytica* based on subunit ribosomal RNA gene (ITS 1 region) from human stool samples. This method was carried out according to method described by.[11]

Real Time – PCR master mix preparation

qPCR master mix was prepared using (GoTaq®qPCR Master mix) and this master tube contains freeze – dried pellet and PCR reaction prepared according to company instructions in 20 µl total volume by added 5 µl of purified genomic DNA, 1 µl of 10 pmol of forward primer, 1 µl of 10 pmol of reverse primer, 1 µl of 20 pmol of hsp probe, 12.5 µl of qPCR master mix and 4.5 µl of deionizer PCR water and briefly mixed by exispin vortex centrifuge (Bioneer, Korea) at 3000 rpm for 3 minutes. The reaction was performed in a thermocycler (BioRad, USA) by set up the following thermocycler conditions; initial denaturation temperature of 95 ºC for 3 min.; followed by 40 cycles at denaturation 95 ºC for 10 Sec. anneling for 60 ºC for 30 sec. and extention 60 ºC for 60 sec.

Real Time PCR data analysis

qPCR data analysis was performed by calculation the threshold cycle number (CT value) that presented the positive amplification of ITS gene for *E. histolytica* in Real Time cycle number.

RESULTS AND DISCUSSION

1- Prevalance of *Entamoeba histolytica* according to age

In the present study, the patients aged from under 1year – 60 years (table 1).

It was observed out of 100 samples, 39 cases were given positive results (39%) and 61 cases were given negative results (61%). The results found that the number of patients was higher in age group (<1–10) years, reaching (63.88%). While the lowest number in age group (31–40), reaching (14.28%). The present results were almost similar to those obtained by Al-Abodi[12], Al-Kaeebi and Al-Difaie.[13]

The reason of high prevalence may be attributed to the low immunity against various pathogens as these age group are comparatively less resistant to disease as described in previous studies.[14,15]

Table 1: Distribution of positive and negative cases according to age groups.

Age group	Number of Samples	Positive Samples	%	Negative samples	%
< 1 – 10	36	23	63.88	13	36.12
11 – 20	17	5	29.41	12	70.59
21 – 30	13	4	30.76	9	69.24
31 – 40	7	1	14.28	6	85.72
41 – 50	13	2	15.38	11	84.62
51 – 60	14	4	28.57	10	71.43
Total	100	39	39	61	61

2- Prevalence of *E. histolytica* according to gender

The results showed that 21 out of 39 (53.84%) and 18 (46.16%) positive samples in male and female respectively (table 2), the result of our study seems similar to the results of other studies done in Iraq and the world can interpretation of this engagement on basis of the behavior of male with their surroundings than female. This finding is in agreement with the result of a study done in Babylon[16] and in Al-Qadisiyah provinces.[13]

Table 2: Distribution of *E. histolytica* among patients according to the gender.

Gender	No. of patients	%
Male	21	53.84
Female	18	46.16
Total	39	100

3- Detection of *E. histolytica* by RT-PCR

Table 3 shows the comparison of direct and Real Time PCR methods. The results showed 35 samples out of 39 samples (89.74%) positive in direct method were given positive in Real Time PCR, while 7 samples out of 61 samples (7%) were given positive in Real Time PCR that negative results in direct methods.

Table 3: Comparison of Direct Method and RT-PCR for detection of *E. histolytica.*

Results	No. of samples (%)	Direct methods (%)	Real Time PCR (%)
***Entamoeba histolytica* positive**	39 (39%)	39 (39%)	35 (35%)
***Entamoeba histolytica* positive**	61 (61%)	0 (0%)	7 (7%)
Total	100 (100%)	39 (39%)	42 (42%)

The Real Time PCR technique offers the highest combination of sensitivity and specificity and is considered the gold standard by many laboratories.[17] However, all positive and negative samples in direct method does not provide positive results in Real Time PCR. As a results, after application of Real Time PCR technique showed to be more sensitive than the direct method. The results of Real Time PCR were agreed with.[12,18] The difference between two methods may be attributed to it was possible that false positive results of microscopic examination may be due to misidentification between *E. histolytica* and another non pathogenic amoeba such as *E. dispar, E. hartanni and E. moshkovskii*; the quantity of the pathogen in stools: stool with a low number of cysts, may be negative in direct microscopic examination and may yield positive results with the Real Time PCR test.[19] Additionally, the time between samples receipt and assay. In that stool samples was placed into buffer on the day of receipt and assayed within one week. That stored for long time up to a year. However,

many of the DNA extracts used for PCR were made from stool samples have been stored in frozen at – 20 ºC (for four months and 12 months) before DNA extraction.[20]

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