Annals of Parasitology 2021, 67(4), 805–812
doi: 10.17420/ap6704.399 Copyright© 2021 Polish Parasitological Society

Original paper

MicroRNAs expression of patients infected with *Entamoeba histolytica* in association with *Entamoeba dispar* isolated from liquid stool

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ABSTRACT. Amoebic dysentery is a parasitic intestinal infection that causes diarrhea containing blood or mucus. A total of 100 amoebic stool samples were collected from patients attending Azadi Hospital in Kirkuk City, Iraq during the period from 1st December 2020 to 15th June 2021. The results showed that out of 100 samples of amoebic infected patients, 88 (88%) were infected with *E. histolytica*, while 12 (12%) were infected with *E. dispar*. There was a non-significant difference between males 46 (46.0%) and females 42 (42%) among *E. histolytica* infected patients, and between males 7 (7%) and females 5 (5%) among *E. dispar* infected patients. According to age groups, the majority of the samples 39 (39%) were reported at the age group 15–44 years, while the lower number 14 (14%) was recorded in the age group 1–4 years. The results of distribution of *Entamoeba* stages showed that the distribution rate of *E. histolytica* trophozoite stage was 25 (92.6%), *E. histolytica* cyst stage was 49 (86%) and both of trophozoite + cyst stages was 14 (87.5%), while the distribution rate of *E. dispar* trophozoite stage was 2 (7.4%), cyst 8 (14%) and the trophozoite + cyst stages was 2 (7.4%), with a highly significant difference (P=0.002). The count of RBCs per field for each *Entamoeba* cell showed that the RBCs were found only in *E. histolytica* infection, while they were not found in *E. dispar* infection with a highly significant difference (P<0.01). Results of observing bacterial activity under microscope showed that there was no relationship between *Entamoeba* infections and bacterial infections (P>0.05). Out of 60 stool samples from amoebic patients, 17 (28.3%) were positive for microRNA-21 of *E. histolytica* with a mean of (8.30±13.34), while 0 (0.00%) of *E. dispar* showed a mean of (1.51±1.91), (P=0.005). Out of 60 stool sample from amoebic patients, 27 (45.0%) were positive for microRNA-22 of *E. histolytica* with a mean of (44.31±61.43), while 0 (0.00%) of *E. dispar* showed the mean (1.43±3.33), (P=0.005).

Keywords: microRNAs, expression, *Entamoeba histolytica*, *Entamoeba dispar*

Introduction

Amoebosis or amoebic dysentery is an intestinal parasitic infection that causes diarrhea containing blood or mucus, with painful stomach and cramps (dysentery). It is caused by members of the *Entamoeba* group. The main source of infection includes ingestion of food or water contaminated with faeces containing *E. histolytica* cyst. Thus, those who travel to developing countries may be infected with amoebosis when they visit endemic areas [1]. Amoebosis is found everywhere in the world [1], although the majority of cases are present in the developing world [2], owing to low sanitation and high faecal contaminations of water supplying facilities [1,2]. About 50 million people in the world contract the infection resulting in the death of 40,000–100,000 people yearly, which makes it the second most common cause of death resulting from infectious parasitic diseases [3]. The first amoebosis case was recorded in 1875, but in 1891 the disease was described in details, which resulted in the terms amoebic dysentery and amoebic liver abscess [4,5]. The scientist Brumpt in 1925, hypothesized that the variations between several asymptomatic amebic infections and individuals with amebic disease may be associated with the presence of two prominent but morphologically identical species, which are, *E. histolytica* (can cause invasive diseases) and *E. dispar* (never causes diseases)

[6–8]. Although *E. dispar* was formerly regarded as a non pathogenic and as a commensal species, intestinal symptoms in patients infected with this species were reported [4,9]. However, it is not clear whether such symptoms included infections with other viral, bacterial or parasitic pathogenic agents [8,9]. Our study aimed to determination of the microRNA-21 and 22 expression.

Materials and Methods

Sample collection

A total of 100 amoebic stool samples were collected from patients who attended Azadi Hospital in Kirkuk City, during the period from 1st December 2020 to 15th June 2021. Stool samples were collected in sterile clean and dry plastic cups with light lids specially made for this purpose. Each cup was given a unique name representing the patient, then each sample was examined by general stool examination. Stool samples were transported very rapidly to the laboratory and examined within 30 minutes of collection of the sample to avoid trophozoite disintegration, also every patient was reported through a specifically prepared questionnaire form including gender and age. The faecal samples were examined with naked eye for appearance, color, odor and presence of blood.

Direct wet mount with normal saline smear

The faecal sample was examined by mixing a small amount of faeces taken by a wooden stick from different sites, especially bloody sites with a drop of saline put on a clean glass slide, then the covered with a cover slide and examined under the microscope. The 10× objective was used first and then by using a high power (40×) objective to detect the trophozoites and cysts of *Entamoeba histolytica.* The 100× objective lens was also used to observe the morphological details.

Direct Lugol's iodine solution smear

The Lugol's iodine was used to stain the glycogen and nuclei of the cysts. Cyst, which tend to predominate in formed stools and trophozoite in diarrheic stools were examined by mixing a small amount of faeces with a drop of iodine placed on a clean glass slide and covered with the cover slip and examined under the microscope. The low-power (10×) objective lens was used first then the high power (40×) objective lens was used for examination [1].

RNA isolation

RNA can be isolated from human faeces and this RNA contains human gene transcripts with ΔCT=CT gene-CT housekeeping gene. We have therefore developed a method for the isolation of total RNA from freshly passed human stools. To unambiguously demonstrate the isolation of RNA from stool, we incubated a mixture of rat cells and control human stool at 37°C for up to 24 h. RT-PCR of the RNA isolated from this sample clearly revealed the presence of rat-specific mRNA. The Quantus^{TM} Fluorometer was used for the detection of concentrations of extracted cDNA so as to detect the goodness of samples for downstream applications. One µl of cDNA was taken and diluted with 199 µl Quanty flor Dye (Promega, USA), which were mixed and then incubated for 5 minutes at room temperature in a dark place, and universal reverse primers were prepared by using the same procedure mentioned above on primers preparation [2].

miR-21-3p-RT
GTTGGCTCTGGTGCAGGGTCCGAGGTATT
CGCACCAGAGCCAACACCCTT
miR-22-3p-RT
GTTGGCTCTGGTGCAGGGTCCGAGGTATTC
GCACCAGAGCCAACACAGTT
RNU43 concentration rang 1-3
miR-22-3p concentration rang 2-4
miR-21-3p concentration rang 2-4

Analysis of microRNA 21 P3, 22 P3 gene expression using Pfaffi method

Primer preparation

Forward and universal reverse primers were prepared by using the same procedure that mentioned above of primers preparation.

GoTaq qPCR master mix components: thermos aquaticus polymerase, $MgCl_2$, DNTPs, Sybr green, reaction buffer

Statistical analysis

Statistical tables including observed frequencies with their percentages, as well as graphical presentation by (bar-charts) were used. Inferential statistics were used to accept or reject the statistical hypotheses which included the Chi-square. The comparison of significance (*P*-value) in any test was as follows:

S=significant difference ($P<0.05$)
HS=highly significant difference ($P<0.01$)
NS=non significant difference ($P>0.05$)

Real-time PCR steps

Master mix components	Stock	Volume	
		1 sample	120 samples
qPCR Master Mix	2x	5 µl	600 µl
$MgCl_2$	25 Mm	0.25 µl	30 µl
Forward primer	10 µM	0.5 µl	60 µl
Reverse primer	10 µM	0.5 µl	60 µl
Nuclease Free Water		2.75 µl	330 µl
CDna	10 ng/µl	1 µl	120 µl
Total volume		10 µl	
Aliquot/single rxn	9 µl of Master mix/tube and added 1 µl of template		
Two master mix were prepared one contain forward and reverse primers for miRNA-22 and the other contain primers for miRNA-21, 22			

Real-time PCR program

Steps	C°	m:s	Cycle
Initial denaturation	95	05:00	1
Denaturation	95	00:15	
Annealing	55	00:15	
Extension	72	00:15	

Results

The results showed that out of 100 samples of amoebic infected patients, 88 (88%) were infected with *E. histolytica*, while 12 (12%) were infected with *E. dispar*.

There was a non-significant difference between males 46 (46.0%) and females 42 (42%) among *E. histolytica* infected patients, and between males 7 (7%) and females 5 (5%) among *E. dispar* infected patients (Tab. 1).

Table 1. Distribution of *Entamoeba* species according gender

Gender	Species	
	E. histolytica	*E. dispar*
Females	42 (42%)	5 (5%)
Males	46 (46%)	7 (7%)

P-value=0.693 Ns

According to age groups, the majority of the samples 39 (39%) were reported at the age group (15–44) years, while the lower number 14 (14%) was recorded in the age group (1–4) years (Tab. 2).

Table 2. Distribution of *Entamoeba* species according age groups

Age groups	*Entamoeba*		
	E. dispar	*E. histolytica*	Total
1–4	1	14	15
5–14	2	17	19
15–44	8	39	47
45–60	1	18	19
Total	12	88	100

P-value=0.006 HS

The results of distribution of *Entamoeba* stages showed that the distribution rate of *E. histolytica* trophozoite stage was 25 (92.6%), *E. histolytica* cyst stage was 49 (86%) and both of trophozoite + cyst stages was 14 (87.5%), while the distribution rate of *E. dispar* trophozoite stage was 2 (7.4%),

Table 3. Distribution of *Entamoeba* stages among the infected samples

Species	Sample No. %	Troph.	Cyst	Troph. + cyst	*P*-value
	No.	25	49	14	
E. histolytica	% of stage	92.60	86.00	87.50	
	% of total	25.00	49.00	14.00	*P*=0.002 HS
	No.	2	8	2	
E. dispar	% of stage	7.40	14.00	12.50	
	% of total	2.00	8.00	2.00	

cyst 8 (14%) and the trophozoite + cyst stages was 2 (7.4%), with a highly significant difference (*P*=0.002) (Tab. 3).

Results of observing bacterial activity under microscope showed that there was no relationship between *Entamoeba* infections and bacterial infections (*P* >0.05) (Tab. 5).

Table 4. Distribution of RBCs with *Entamoeba* infection in each parasite cell

Species		RBCs						
		+1	+2	+3	+4	+5	few	Nil
E. dispar	Count	0	0	0	0	0	0	12
	% within *Entamoeba*	.0%	.0%	.0%	.0%	.0%	.0%	100.0%
	% within RBCS	.0%	.0%	.0%	.0%	.0%	.0%	60.0%
E. histolytica	Count	15	7	2	6	8	42	8
	% within *Entamoeba*	17.0	8.0	2.3	6.8	9.1	47.7	9.1
	% within RBCS	100	100	100	100	100	100	40.0
P-value=0.000 HS								

The count of RBCs per field for each *Entamoeba* cell showed that the RBCs were found only in *E. histolytica* infection, while they were not found in *E. dispar* infection with a highly significant difference (*P*<0.01) (Tab. 4).

Out of 60 stool samples from amoebic patients, 17 (28.3%) were positive for microRNA-21 of *E. histolytica* with a mean of (8.30±13.34), while 0 (0.00%) of *E. dispar* showed a mean of (1.51±1.91)

Table 5. Distribution of active bacterial according to amoeba infections

	No. and %	Bacteria	
		Active	Non-active
	Count	6	6
E. dispar	% within *Entamoeba*	50.0	50.0
	% within bacteria	20.7	8.5
	% of total	6.0	6.0
	Count	23	65
E. histolytica	% within *Entamoeba*	26.1	73.9
	% within bacteria	79.3	91.5
	% of total	23.0	65.0

Table 6. The incidence of microRNA-21 in the study cases

MicroRNA-21	Cases				*P*-value
	E. histolytica		*E. dispar*		
	Count	%	Count	%	
Negative	43	71.7	60	100.0	
Positive	17	28.3	0	0.0	0.005
Total	60	100.0	60	100.0	
Mean±SD	8.30	13.34	1.51	1.91	

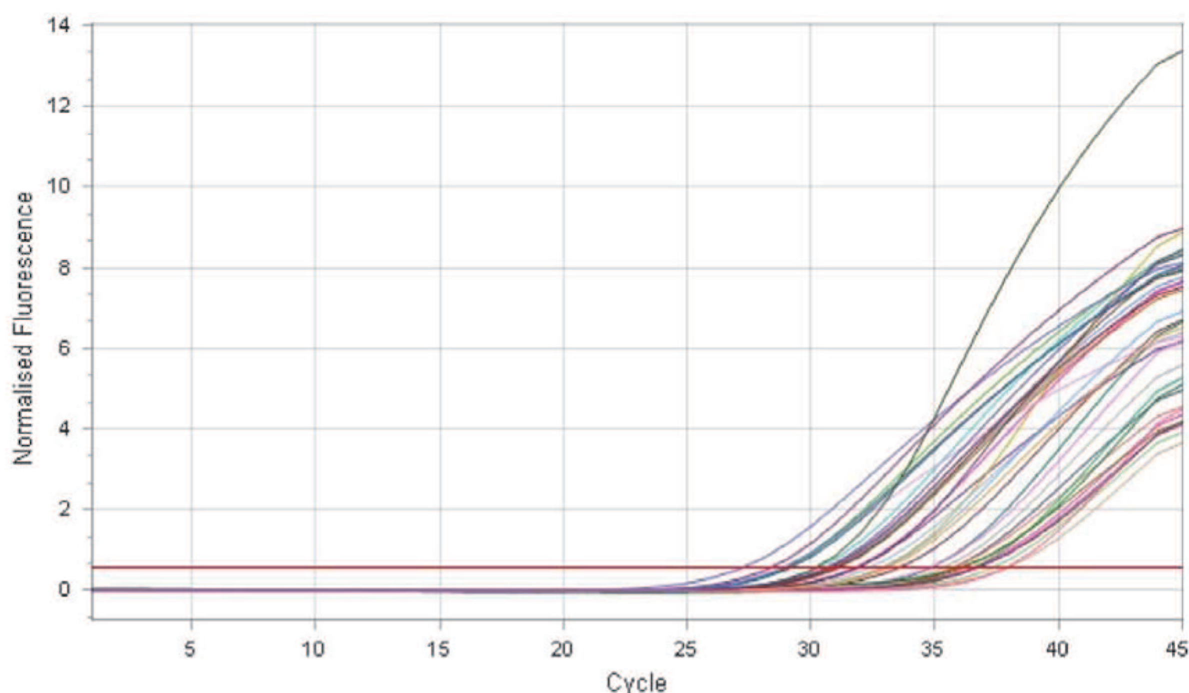

Figure 1. Evaluation of microRNA-21 amoebic infection

(*P*=0.005) as shown in table 6 and figure 1.

Expression of microRNA-21 gene was investigated in *E. histolytica* in liver abscesses and healthy control by using qRT-PCR the findings of amplification were explained in figure 1 that atypical amplification plot. Amplification reaction has an early threshold cycle that consistent with highly levels of microRNA-21 gene and healthy control.

Out of 60 stool sample from amoebic patients,

Table 7. Prevalence of microRNA-22 in the study cases

MicroRNA-22	Cases				*P*-value
	E. histolytica		*E. dispar*		
	Count	%	Count	%	
Negative	33	55.0	60	100.0	
Positive	27	45.0	0	0.0	0.005
Total	60	100.0	60	100.0	
Mean±SD	44.31	61.43	1.43	3.33	

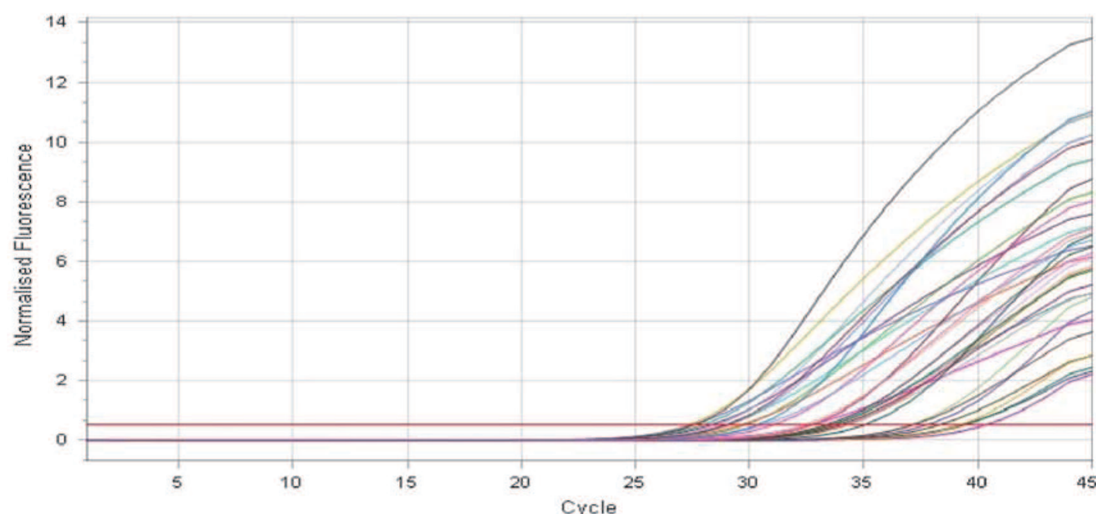

Figure 2. The distribution of microRNA-22 in amoebic infection

27 (45.0%) were positive for microRNA-22 of *E. histolytica* with a mean of (44.31±61.43), while 0 (0.00%) of *E. dispar* showed the mean (1.43±3.33), (*P*=0.005) as shown in table 7 and figure 2.

Expression of microRNA-22 gene was investigated in *E. histolytica* in liver abscesses and healthy control by using qRT-PCR the findings of amplification were explained in figure 2 that atypical amplification plot. Amplification reaction is an early threshold cycle that consistent with highly levels of microRNA-22 gene and healthy control.

Discussion

Entamoeba histolytica is one of the most risky protozoans that infect humans if they become extraintestinal, opposite to *Entamoeba dispar*, which is considered a non-pathogenic protozoan, and only 12 out of 100 samples were infected in our study. These findings agreed with Wang and Kanthan [8] who reported that the most infection with pathogenic amoeba showed that *E. dispar* was not the highest in intestinal infections. Amoebosis within the age group (14–44) years was more prevalent among ages and less common in others. This is consistent with Carrero et al. [10] who stated in his report that these ages are more susceptible to infection, and the reason may be due to their work place conditions or the unhealthy food they eat in those places. The study confirmed that there is a high significant difference in the distribution of stags present in the patient's stool. The study found that the presence of the active trophozoite was more than the cyst, as well as their presence together with the cyst. These results agreed with Kataria et al. [11]. The count of RBCs was found only with *E. histolytica* infection but not with *E. dispar* infection. This proves that it is the only one that consumes blood unlike *E. dispar*. We found the random distribution of red blood cells was within the tissue and amoebic parasite cells, which also proves that *E. dispar* is non-pathogenic [12]. The amoebic infection may be accompanied by bacterial infection that can be diagnosed under the microscope, and these infections may have complications with the original infection, and this leads to the use of a dual-active bacterial and parasite treatment, these findings were in harmony with Mulinge et al. [13] and Saidin et al. [14]. MicroRNA-21 was detected in dysenteric amoebosis with a rate of (28.3%). The RNA extracted from the faeces of infected patients is a mechanism of gene expression, which indicates that the development of infection with this tissue parasite, and extraintestinal amoebosis is able to change its pathological path, and the disease becomes complicated and with-term infection and becomes difficult to be treated. Rosas et al. [15] reported that the microRNAs (miRNAs) are small non-coding RNAs that function as negative regulators of gene expression. Recent evidences suggested that host cells miRNAs are involved in the progression of infectious diseases. The microRNA-22 appeared at a higher rate with the parasitic infection of dysentery amoeba. On the other hand, there is no effect on the microRNAs of

infected people with the parasite *E. dispar*, and these results matched with the findings of Saidin et al. [14] who explained that microRNAs is the best in determining the amoebic infection that causes pathological conditions and are more dangerous when they travel to the vital organs of the body, causing serious damage.

In conclusion, out of the 100 samples of amoebic infected patients, 88 (88%) were infected with *E. histolytica*, while 12 (12%) were infected with *E. dispar*. The count of RBCs per field for each *Entamoeba* cell showed that the RBCs were found only in *E. histolytica* infection. Out of 60 stool samples from amoebic patients, 17 (28.3%) were positive for microRNA-21 of *E. histolytica* with a mean of (8.30±13.34), while 0 (0.00%) of *E. dispar* showed a mean of (1.51±1.91) (P=0.005).

Out of 60 stool sample from amoebic patients, 27 (45.0%) were positive for microRNA-22 of *E. histolytica* with a mean of (44.31±61.43), while 0 (0.00%) of *E. dispar* showed the mean (1.43±3.33)**.**

We recommended the following: to work the genotyping gene sequences of to *E. histolytica* infections; to work the gene expression to another genes like micro-RNA-43 and microRNA-184.

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Received 02 September 2021
Accepted 16 November 2021