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Comparative immunological study of children infected with *Entamoeba* spp. parasites in Thi-Qar province

Iman Adhab Ali, Zainab Abdali Mohammad

Department of Biology, College of Education for Pure Science, Thi-Qar University, Thi-Qar, Iraq

ABSTRACT

Background. Amoebiasis is one of the major public health problems, it is caused by the genus *Entamoeba* which includes several species, such as *E. histolytica, E. dispar* and E. moshkovskii.

Purposes. The present study aimed to differentiate among *Entamoeba* spp. by using molecular method (PCR) and to identify characteristic serum markers (IgG and IL-6) according to species.

Methods. About 697 stool samples were collected from children under five years old, and blood samples (3 ml) of venous blood were collected from patients with positive microscopic examination to evaluate some immune parameters (IL-6, IgG) for children infected with the parasite using the ELISA test. DNA extraction was carried out from fecal samples using a special stool DNA extraction kit and Multiplex PCR primers were designed to detect *E. histolytica, E. dispar* and *E. moshkovskii.*

Results. About 100(14.34%) were infected with *Entamoeba* spp. by microscopic examination and 68 samples DNA-samples were positive. Fifty samples 50% were infected with *E. histolytica,* and 12% with a mixed infection. A significant increase ($p \leq 0.05$) in the concentration of IgG immunoglobulin in the sera of children infected with the *E. histolytica* parasite. A significant increase ($p \leq 0.05$) in the concentration of interleukin-6 in the sera of patients groups infected with *Entamoeba* spp., the highest significant increase ($p \leq 0.05$) was recorded in patients group infected with the *E. moshkovskii.*

Conclusion. Molecular tests have the ability to detect and distinguish between the *Entamoeba* species infecting humans. Amoebiasis caused an increase in the concentration of IgG and interleukin-6.

Keywords: PCR, *Entamoeba* spp., IL-6, IgG, Thi-Qar province, amoebiasis

INTRODUCTION

This illness, which is caused by different species of the genus *Entamoeba,* is a major public health concern in many nations. It is thought of as amoebiasis. This disease is particularly prevalent in underdeveloped nations due to a combination of factors, including a lack of sanitation infrastructure, polluted water supplies, and a general lack of awareness about the prevalence of parasite infections [1]. Parasites such as Amoebiasis can infect humans through the gastrointestinal tract. The most common species, *E. histolytica,* is responsible for the illness, although other species, such as *E. dispar* and *E. moshkovskii,* can also infect humans [2]. In humans, *E. dispar* colonizes the intestines in a manner similar to that of *E. histolytica.* It is now known to be an entirely separate species, completely devoid of any potential for invasion [3]. Since its discovery as a separate but closely related protozoan species, this non-invasive organism has significantly impacted the field of amoebiasis epidemiology; it causes most asymptomatic infections worldwide [4].The cyst and trophozoite forms of *E. moshkovskii* are visually identical to those of *E. histolytica* and *E. dispar*, with whom it shares a common ancestor [5]. While microscopic analysis of stool samples is the gold standard for human *Entamoeba* spp. laboratory diagnosis, it cannot differentiate between these species [6]. Because of the high risk of patient mistreatment due

Corresponding author:
Iman Adhab Ali
E-mail: aqapiadil25@gmail.com

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to morphological similarity among *Entamoeba* species, an accurate diagnostic method is required. One such method is molecular diagnosis, which makes use of the polymerase chain reaction (PCR) technique and has found application in various parts of the world [7]. The process of cellular immune response is initiated when the trophozoite parasite penetrates the host's intestinal tissue. In order to form colonies, the parasite tries to attach to the epithelial cells lining the large intestine, which causes inflammation. As a result, these cells produce and release cytokines, which in turn serve as proinflammatory factors. Systemic immune cells, including macrophages, neutrophils, and mononuclear cells, then arrive at the site of inflammation and attack the parasite [8]. The humoral immune response is also activated when *E. histolytica* is infected. There was a noticeable rise in the levels of antibodies like IgG, IgM, and IgA [9]. The pathogenicity of *Entamoeba histolytica, E. dispar,* and *E. moshkovskii* differs significantly, despite the fact that they have comparable appearance, gene profiles, and protein profiles. Infection with *Entamoeba histolytica* may lead to amoebic dysentery and liver abscess, but infection with *E. moshkovskii* produces moderate diarrhea. While the pathogenic *E. histolytica* has been the subject of numerous investigations into the functions of the host immune system, data on *E. dispar* and *E. moshkovskii* is still in short supply.

This study set out to use polymerase chain reaction (PCR) to distinguish between different species of *Entamoeba* and to find serum markers (IgG and IL-6) that are specific to each parasite type in infected individuals.

METHODS

About 697 stool samples were collected from cases under five years old, who suffer from diarrhea and complain of abdominal pain in the Bint Al-Huda Educational Hospital for Women and Children and Mohammed Al-Musawi Hospital in Thi-Qar Province for the period of October 2022- August 2023, stool samples were examined by direct microscopic method. About 3 ml of venous blood were collected from patients with positive microscopic results and 50 blood samples were collected from healthy children as control group. The venous blood was drawn using a wine-wine syringe in a test tube and left to clot for half an hour, in order to obtain the serum. Then it was placed in the centrifuge, where it was stored. The serum is in the refrigerator at-20 degrees for the purpose of later use in measuring some immune parameters (IL-6, IgG) by using the ELISA test.

DNA extraction: DNA extraction was carried out from fecal samples with positive microscopic results by using a special stool DNA extraction kit prepared (Geneaid), and the extraction was carried out according to the company's instructions.

Primers: Multiplex PCR primers were designed to detect *E. histolytica, E. dispar* and *E. moshkovskii* in this study using NCBI-Genbank and Primer 3 Plus. These primers were provided by Macrogen Korea. (Table 1).

Prepare a multiplex PCR mixture: Prepare the reaction mix (Go Taq Green PCR Master Mix), and this master mix was made according to the company's instructions (Table 2).

TABLE 2. Components of the multiplex PCR mixture

Master mix mPCR		Size
Template DNA		ML 5
Forward primer 20pmol	*Entamoeba histolytica*	1 ML
	Entamoeba dispar	1 ML
	Entamoeba moshkovskii	1ML
Reverse primer 20pmol	*Entamoeba histolytica*	1 ML
	Entamoeba dispar	1 ML
	Entamoeba moshkovskii	1 ML
Go Taq Green Master mix		12.5 ML
water PCR		1.5 ML
Total size		25 ML

The main PCR mixture component mentioned above was then transferred to the Exispin vortex centrifuge at 3000 rpm for 3 minutes and then placed in the PCR machine.

Thermocycle conditions program for polymerase chain reaction: All thermal cycles of the polymerase chain reaction were carried out using a PCR Thermocycle device according to the conditions shown in the Table 3.

Electrophoresis agarose gel: DNA samples are detected using the electrophoresis agarose gel technique and the results of the PCR technique.

TABLE 1. Primers used in the Multiplex PCR technique and the size of the PCR test product

GenBank code	Product size	3-5 Sequence		Initiator
OM780326.1	521 bp	TTCTAAGGAAGGCAGCAGGC	F	*Entamoeba histolytica*
		AAATGCTTTCGCTCTCGTGC	R	
AB282661.1	356 bp	AACTGCGGACGGCTCATTAT	F	*Entamoeba dispar*
		GGTAATTTACGCGCCTGCTG	R	
MN53649 4.1	645 bp	ATTGGAGGGCAAGTCTGGTG	F	*Entamoeba moshkovskii*
		GTGCCCTTCCGTCAATTCCT	R	

TABLE 3. Heat cycles for multiplex PCR

PCR step	Temp	Time	Repeat
Initial Denaturation	95°C	5 min	1
Denaturation	95°C	30 sec	cycle 35
Annealing	58°C	30 sec	
Extension	72°C	2 min	
Final extension	72°C	5 min	1
Hold	4°C	Forever	–

Study of immunological parameters: Interleukin 6 concentration was measured by using Human Interleukin 6 (IL-6) ELISA kit and the concentration of immunoglobulin G antibodies IgG was measured by using Human immunoglobulin G (IgG) ELISA kit.

Statistical analysis: the data of current study were statistically analysis by using SPSS at p. value <0.05.

RESULTS

The results of current study showed that 100 out of 697 (14.34%) were infected with *Entamoeba* spp. by microscopic examination (Figure 1).

Molecular method using polymerase chain reaction (PCR) technique

Extraction of deoxyribonucleic acid DNA: Deoxyribonucleic acid (DNA) was extracted from positive samples according to the direct microscopy method and electrophoresed on an agarose gel at a concentration of 0.8%, Figure 2.

PCR Technology

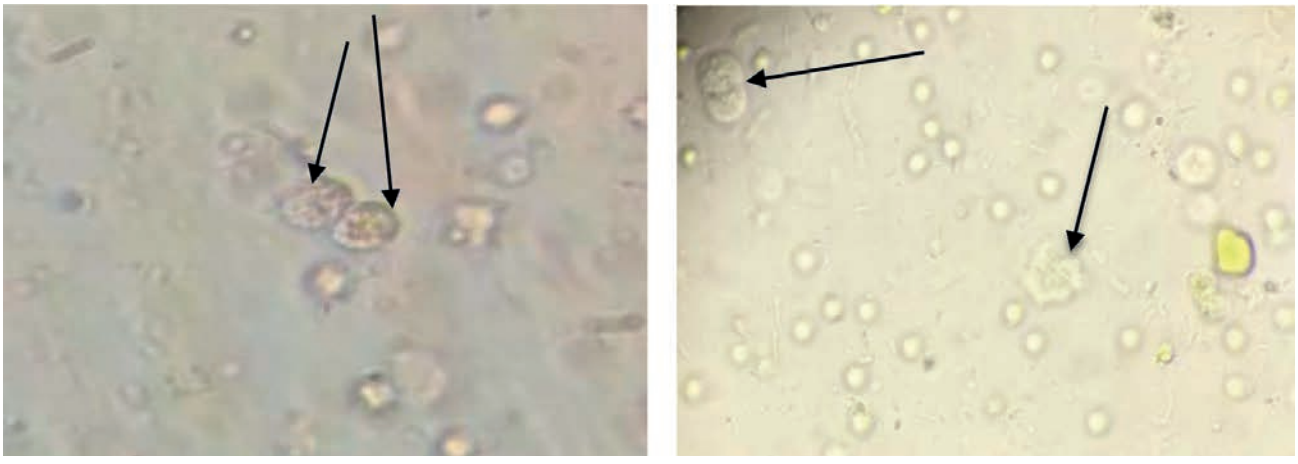
The data showed that 68 samples out of 100 (68%) DNA-Samples were positive for infection with *Entamoeba* spp. parasites according to PCR technique by using special primers for each species. A significant differences ($p \leq 0.05$) were recorded among the identified species. Fifty samples 50% were infected with *E. histolytica,* and 12 samples 12% with a mixed infection (*E. histolytica* + *E. dispar*), 2 samples 2% were infected with E. dispar, 4 samples 4% were infected with the *E. moshkovskii* (Table 4, Table 5).

TABLE 4. The number and percentage of positive stool samples by PCR

No. samples	No. positive samples	%
100	68	68

Infection with *E. histolytica:* 50 samples 50%, were infected with the *E. histolytica* by PCR technique, the size of the parasite's DNA was 521 bp, compared to the DNA ladder (Figure 3).

Infection with E. dispar: 2 samples 2%, were infected with the E. dispar by PCR technique, the size



A B

FIGURE 1. A – Cyst; B – Trophozoite of *Entamoeba* spp. under light microscope (100X)

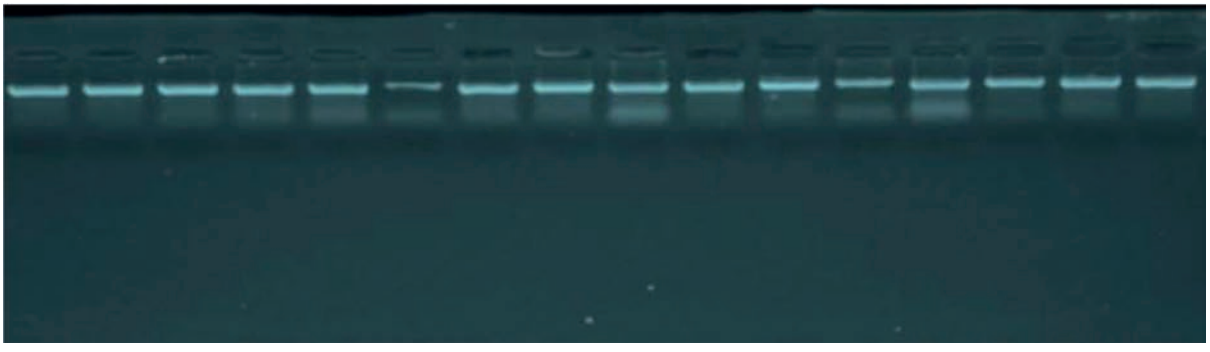


FIGURE 2. Electrophoresis of DNA samples extracted on a 0.8% agarose gel

TABLE 5. Percentage of infection with *Entamoeba* spp. by PCR technology

Entamoeba spp.	NO. positive samples	%
E. histolytica	50	50
E. dispar	2	2
E. moshkovskii	4	4
Mixed *(E. histolytica + E. dispar)*	12	12
TOTAL	68	68

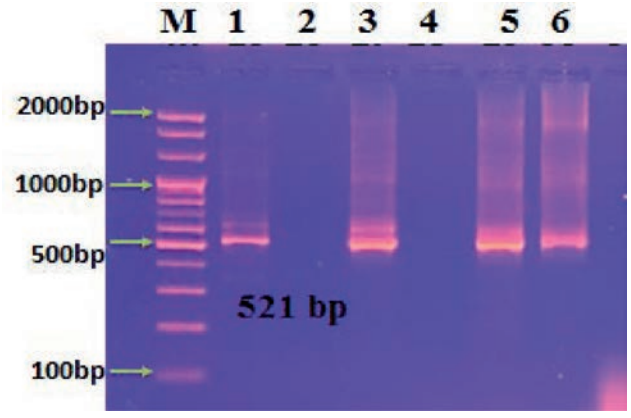


FIGURE 3. Electrophoresis of DNA isolated from samples infected with the *E. histolytica*. The size of the parasite's DNA is 521 bp compared to the DNA ladder

of the parasite's DNA was 356 bp, compared to the DNA ladder (Figure 4).

Infection with *E. moshkovskii:* 4 samples 4%, were infected with the *E. moshkovskii* by PCR technique, the size of the parasite's DNA was 645 bp, compared to the DNA ladder (Figure 5).

Mixed infection: 12 samples 12%, were infected with *E. histolytica* + *E. dispari* by PCR technique (Figure 6).

The data recorded a significant increase ($p \leq 0.05$) in the concentration of IgG immunoglobulin in the sera of children infected with the *E. histolytica* parasite compared to the control group, and a significant rise also for this group compared to the rest of the groups of patients infected with other species of *Entamoeba* (*E. dispar* and *E. moshkovskii*) and the group of patients with mixed infection *(E. histolytica + E. dispar)* (Table 6).

The results of the current study recorded a significant increase ($p \leq 0.05$) in the concentration of interleukin-6 in the sera of patients groups infected with Entamoeba spp. compared with the control group, the highest significant increase ($p \leq 0.05$) was recorded in patients group infected with the E. moshkovskii compared with the rest of the patients groups infected with the parasites *E. histolytica, E. dispar,* and mixed infection (Table 7).

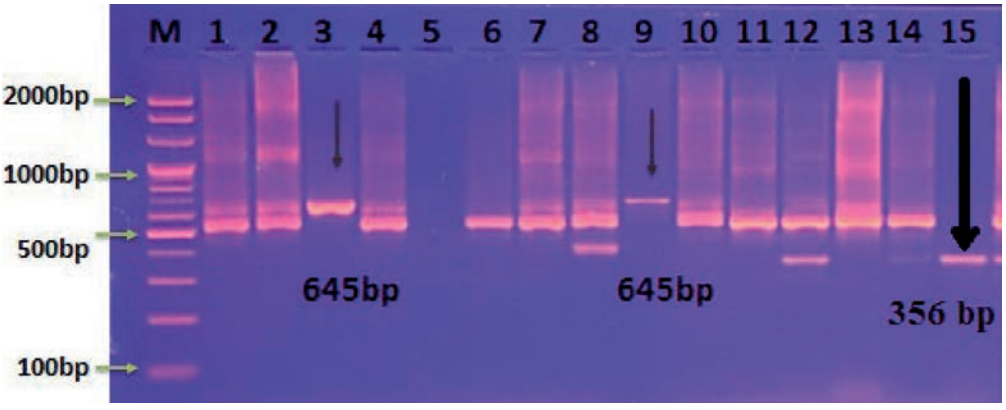


FIGURE 4. Electrophoresis of DNA isolated from samples infected with the E. dispar. The size of the parasite's DNA is 356 bp compared to the DNA ladder

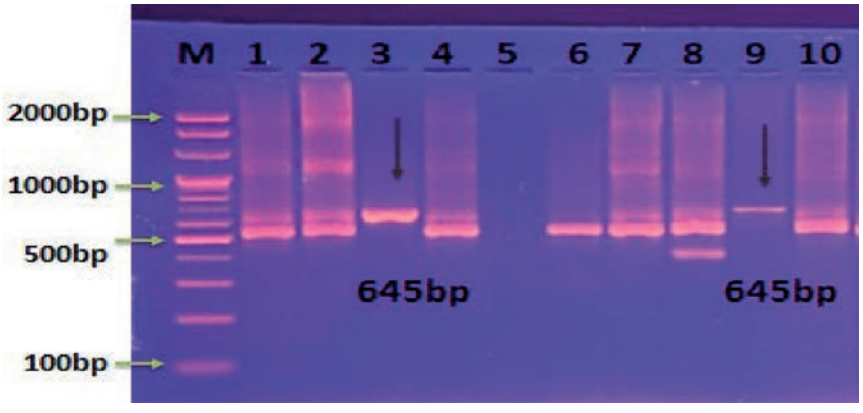


FIGURE 5. Electrophoresis of DNA isolated from samples infected with *E. moshkovskii.* The size of the parasite's DNA is 645 bp compared to the DNA ladder

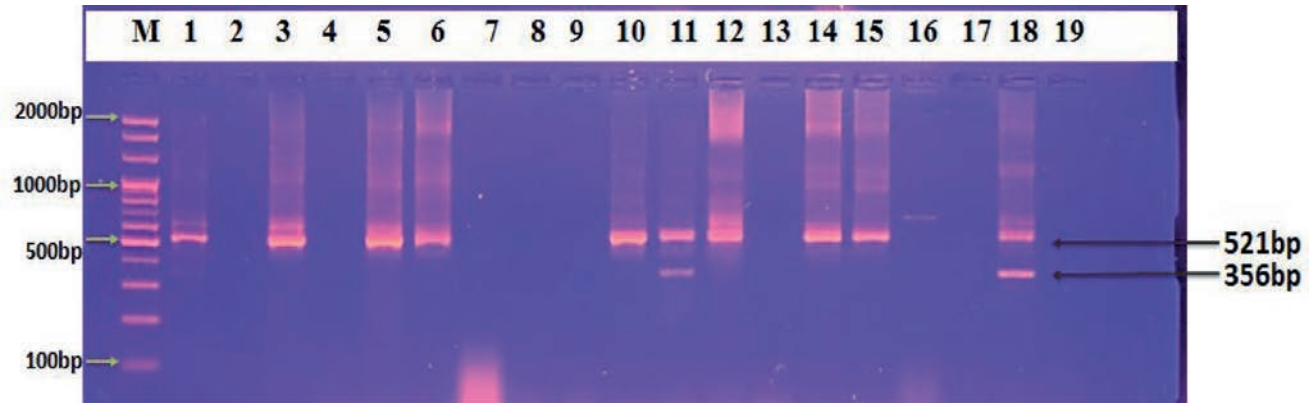


FIGURE 6. Electrophoresis of DNA isolated from samples infected with *E. histolytica* + *E. dispari*

TABLE 6. Effect of infection with *Entamoeba* spp. on the concentration of IgG

Immune parameter	*E. histolytica* (n=50)	*E. dispar* (n=2)	*E. moshkovskii* (n=4)	Mix *(E. histo + E. dispar)* (n=12)	Control (n=50)
IgG mg/dl	0.037 ± 0.012[a]	0.020 ± 0.013[b]	0.018 ± 0.013[b]	0.017 ± 0.011[b]	0.017 ± 0.008[b]

TABLE 7. Effect of infection with *Entamoeba* spp. on the concentration of IL-6

Immune parameter	*E. histolytica* (n=50)	*E. dispar* (n=2)	*E. moshkovskii* (n=4)	Mix *(E. histo + E. dispar)* (n=12)	Control (n=50)
IL-6 pg/ml	31.5 ± 5.29[d]	37.8 ± 3.94[b]	40.4 ± 3.33[a]	35.1 ± 5.27[c]	23.7 ± 2.69[e]

DISCUSSION

Since it is not possible to distinguish between Entamoeba species by direct microscopy, a very sensitive approach is to use the polymerase chain reaction (PCR) technique. PCR provides a more precise picture of the epidemiology of infection produced by several species of *Entamoeba.*

Out of 697 participants, 100 (14.34%) tested positive for Entamoeba spp. After carefully examining the samples under a microscope, those that tested positive were subjected to polymerase chain reaction (PCR) in order to distinguish between different species of Entamoeba. The primers utilized in this study were derived from the central region of the small-subunit rRNA gene, which is conserved across all organisms and used for species identification and classification due to its distinct characteristics [10]. When tested for infection with *Entamoeba* spp., 68 out of 100 (68%) DNA-samples came back positive.

With a prevalence of 50%, *E. histolytica* was the most common, followed by a mixed infection of 12% with *E. dispar* and 4% with *E. moshkovskii.* Using specific primers for each species, the PCR method was able to effectively differentiate the three species.

AL-Abodi, [11] in Al-Qadisiya province, several species of *Entamoeba* was found to have varying prevalence rates. When looking at prevalence, *E. histolytica* (74%), E. dispar (26%), and *E. moshkovskii* (7%), was the most common. Another study in Al-Qadisiya province reported *E. histolytica* 47%, *E. dispar* 31% and *E. moshkovskii* 19% AL Khafaji, [12]. AL-Yaquob, [13] Different prevalence rates were noted for these three species in Basrah: *E. dispar* at 42%, mixed infection with *E. histolytica* and *E. dispar* at 30%, and no infection with *E. moshkovskii.* The prevalence of *E. histolytica* and *E. dispar* was 39.29% in Thi-Qar province, according to the study [14]. AL-Hamdani et al. [15] recorded high prevalence of *E. dispar* 53% compared with prevalence of *E. histolytica* 18.6%.

According to research, Iran had the greatest occurrence of *E. dispar* at 58% and the lowest prevalence of *E. moshkovskii* at 7%. That was Bahrami et al. [16]. According to Shimokawa et al. [17], the prevalence of *E. histolytica* infections was 4.63%, *E. dispar* infections were 0.35%, and *E. moshkovskii* infections were 2.95% in Bangladesh. Ngobeni et al. [18] found 8.5% *E. histolytica* prevalence in South Africa, but no cases of *E. moshkovskii* infection were reported.

One possible explanation for discrepancies in PCR results is that various stool samples or different ways of extracting DNA from them yield varied parasite densities. Furthermore, the samples may have varied in age, transportation, and preservation methods utilized in each study, all of which contribute to the observed diversity [19,20].

If the wrong type of *Entamoeba* is diagnosed, patients may undergo needless therapy, which might

lead to drug resistance in the parasite. Therefore, it is crucial to distinguish between invasive and non-invasive *Entamoeba* species [21,22].

Immune cells' cytotoxic activity and their capacity to produce various cytokines, such as interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin (IL) 2, recruit the inflammatory response against the parasite, making the immune and inflammatory responses pivotal in fighting *E. histolytica* infection [23,24].

The results showed that compared to the control group, children infected with other *Entamoeba* species (*E. dispar* and *E. moshkovskii*) and those with a mixed infection (*E. histolytica* + *E. dispar*) had a significantly higher concentration of IgG immunoglobulin in their serum ($p \leq 0.05$), these results agreed with study of Kaur et al. [25] that recorded a significant increase in the concentration of IgG immunoglobulin four times in sera of patients infected with *E. histolytica* when compared with the control group. The impact of the IgG anti *E. histolytica* antibodies was shown to be statistically significant ($P < 0.05$) when compared to the control group, according to AL-Rwi et al. [26]. Even if the concentration of anti-amoebal IgG in serum is raised, Haque et al. [27] found that it does not provide protection against repeated infections. According to the results of the IgG test in reference [28], both severe amoebic patients and amoebic carriers exhibited elevated levels of IgG specific to *E. histolytica.* Severe amoebic patients also exhibited the highest levels of IgA, IgG, and IgG2 titers against the glycolipid *E. histolytica* lipophosphoglycan. Trigger a cascade of events that culminate in the synthesis of the interleukin IL-6 by mast cells, dendritic cells, and T-cells [29]. An uptick in IL-6 and other cytokines is indicative of inflammation [30]. IL-6 is a pleiotropic proinflammatory cytokine [31].

In comparison to the control group, the groups of patients infected with various types of *Entamoeba* spp. showed a significant rise ($p \leq 0.05$) in interleukin-6 concentration in their sera. Among these groups, the patients infected with *E. moshkovskii* showed the highest significant increase ($p \leq 0.05$), followed by those infected with *E. histolytica*, *E. dispar*, and mixed infection. While, in other study mouse immunization with *E. moshkovskii* trophozoite cells was shown to induce higher and faster total-antibody response than *E. histolytica* [5]. Comparing amoebic carriers to *E. dispar* infected persons, high cytokine levels of interleukin (IL-4) were found, similar to sever amoebic patients, but IL-6 was only raised in sever amoebic patients. Amoebic carriers had lower levels of IL-10 compared to patients with severe amoebiasis [28]. Mohammed et al. [32] found that infected and control people did not differ significantly in the immunological evaluation of IL-17, but there were substantial and very significant differences in the evaluation of IFN-γ and TNF-α, respectively.

CONCLUSIONS

Molecular tests have the ability to detect and distinguish between the *Entamoeba* species infecting humans when compared with microscopic examination. Amoebiasis caused an increase in the concentration of IgG and interleukin-6, and this increas affected by *Entamoeba* species.

Authors' contribution:
Iman Adhab Ali: conceptualization, methodology, software, validation, investigation, resources, data curation, writing original draft, writing-review & editing, visualization.
Zainab Abdali Mohammad: conceptualization, methodology, software, validation, investigation, resources, data curation, writing original draft, writing, review & editing, visualization, supervision.

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REFERENCES

1. Watanabe K, Petri WA Jr. Molecular biology research to benefit patients with Entamoeba histolytica infection. *Mol Microbiol.* 2015 Oct;98(2):208-17. doi: 10.1111/mmi.13131
2. Roure S, Valerio L, Soldevila L, Salvador F, Fernández-Rivas G, Sulleiro E et al. Approach to amoebic colitis: Epidemiological, clinical and diagnostic considerations in a non-endemic context (Barcelona, 2007-2017). *PLoS One.* 2019 Feb 21;14(2):e0212791. doi: 10.1371/journal.pone.0212791
3. Uslu H, Aktas O, Uyanik MH. Comparison of Various Methods in the Diagnosis of Entamoeba histolytica in Stool and Serum Specimens. *Eurasian J Med.* 2016 Jun;48(2):124-9. doi: 10.5152/eurasianjmed.2015.0074
4. Al-Areeqi MA, Sady H, Al-Mekhlafi HM, Anuar TS, Al-Adhroey AH, Atroosh WM et al. First molecular epidemiology of Entamoeba histolytica, E. dispar and E. moshkovskii infections in Yemen: different species-specific associated risk factors. *Trop Med Int Health.* 2017 Apr;22(4):493-504. doi: 10.1111/tmi.12848
5. Khomkhum N, Leetachewa S, Pawestri AR, Moonsom S. Host-antibody inductivity of virulent Entamoeba histolytica and non-virulent Entamoeba moshkovskii in a mouse model. *Parasit Vectors.* 2019 Mar 13;12(1):101. doi: 10.1186/s13071-019-3363-5
6. Parija SC, Mandal J, Ponnambath DK. Laboratory methods of identification of Entamoeba histolytica and its differentiation from look-alike Entamoeba spp. *Trop Parasitol.* 2014 Jul;4(2):90-5. doi: 10.4103/2229-5070.138535
7. Bahrami F, Haghighi A, Zamini G, Khademerfan M. Differential detection of Entamoeba histolytica, Entamoeba dispar and Entamoeba moshkovskii in faecal samples using nested multiplex PCR in west of Iran. *Epidemiol Infect.* 2019 Jan;147:e96. doi: 10.1017/S0950268819000141

8. Vivanco-Cid H, Alpuche-Aranda C, Wong-Baeza I, Rocha-Ramírez LM, Rios-Sarabia N, Estrada-Garcia I et al. Lipopopeptidephosphoglycan from Entamoeba histolytica activates human macrophages and dendritic cells and reaches their late endosomes. *Parasite Immunol.* 2007 Sep;29(9):467-74. doi: 10.1111/j.1365-3024.2007
9. Haque R, Mondal D, Duggal P, Kabir M, Roy S, Farr BM et al. Entamoeba histolytica infection in children and protection from subsequent amebiasis. *Infect Immun.* 2006 Feb;74(2):904-9. doi: 10.1128/IAI.74.2.904-909.2006
10. 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 Sep;44(9):3196-200. doi: 10.1128/JCM.00778-06
11. Al- Abodi H R J, Phylogenetic sequenceing for species Entamoeba histolytica, E.dispar, E. moshkovaskii in Al-Qadisiya Povince. Ph.D. thesis. College of Education. University of Al-Qadisiya. 2015; 1-120.
12. Al-Khafaji A S. Molecular characterization of Entamoeba moshkovskii as the new recording in Diwaniya by using single round polymerase chain reaction PCR. MSc.thesis. college of medicine. Al-Qadisiya Univ. 2014;1:150.
13. Al-Yaquob A J. Diagnostic study on the causative agent of amoebiasis by PCR technique and ability of culturing it in Basrah province. M.Sc.thesis, college of Education. University of Basra. 2008;1:67.
14. Al- Abady FAM, and Al-Saidy MKK. Molecular investigation of the parasite Entamoeba spp. among children with diarrhea in Thi Qar province. J. 2017;12(4):16-30.
15. Al-Hamadani AH, Dawood KA, and AlAumashi GA. Molecular identification of Entamoeba histolytica/E. dispar using nested polymerase chain reaction. Al-Yarmouk. 2011;J.2:234-23.
16. Bahrami F, Haghighi A, Zamini G, Khademerfan M. Differential detection of Entamoeba histolytica, Entamoeba dispar and Entamoeba moshkovskii in faecal samples using nested multiplex PCR in west of Iran. *Epidemiol Infect.* 2019 Jan;147:e96. doi: 10.1017/S0950268819000141
17. Shimokawa C, Kabir M, Taniuchi M, Mondal D, Kobayashi S, Ali IK et al. Entamoeba moshkovskii is associated with diarrhea in infants and causes diarrhea and colitis in mice. *J Infect Dis.* 2012 Sep 1;206(5):744-51. doi: 10.1093/infdis/jis414
18. Ngobeni R, Samie A, Moonah S, Watanabe K, Petri WA Jr, Gilchrist C. Entamoeba Species in South Africa: Correlations With the Host Microbiome, Parasite Burdens, and First Description of Entamoeba bangladeshi Outside of Asia. *J Infect Dis.* 2017 Dec 19;216(12):1592-1600. doi: 10.1093/infdis/jix535
19. Parija SC, Mandal J, Ponnambath DK. Laboratory methods of identification of Entamoeba histolytica and its differentiation from look-alike Entamoeba spp. *Trop Parasitol.* 2014 Jul;4(2):90-5. doi: 10.4103/2229-5070.138535
20. López-López P, Martínez-López MC, Boldo-León XM, Hernández-Díaz Y, González-Castro TB, Tovilla-Zárate CA, Luna-Arias JP. Detection and differentiation of Entamoeba histolytica and Entamoeba dispar in clinical samples through PCR-denaturing gradient gel electrophoresis. *Braz J Med Biol Res.* 2017 Apr 3;50(4):e5997. doi: 10.1590/1414-431X20175997
21. Santos HL, Peralta RH, de Macedo HW, Barreto MG, Peralta JM. Comparison of multiplex-PCR and antigen detection for differential diagnosis of Entamoeba histolytica. *Braz J Infect Dis.* 2007 Jun;11(3):365-70. doi: 10.1590/s1413-86702007000300013
22. Dinoop KP, Parija SC, Mandal J, Swaminathan RP, Narayanan P. Comparison of nested-multiplex, Taqman & SYBR Green real-time PCR in diagnosis of amoebic liver abscess in a tertiary health care institute in India. *Indian J Med Res.* 2016 Jan;143(1):49-56. doi: 10.4103/0971-5916.178592
23. Ito Y, Miyauchi A, Kudo T, Oda H, Yamamoto M, Sasai H et al. Trends in the Implementation of Active Surveillance for Low-Risk Papillary Thyroid Microcarcinomas at Kuma Hospital: Gradual Increase and Heterogeneity in the Acceptance of This New Management Option. *Thyroid.* 2018 Apr;28(4):488-95. doi: 10.1089/thy.2017.0448
24. Paul WE, Zhu J. How are T(H)2-type immune responses initiated and amplified? *Nat Rev Immunol.* 2010 Apr;10(4):225-35. doi: 10.1038/nri2735
25. Kaur U, Sharma AK, Sharma M, Vohra H. Distribution of Entamoeba histolytica Gal/GalNAc lectin-specific antibody response in an endemic area. *Scand J Immunol.* 2004 Nov;60(5):524-8. doi: 10.1111/j.0300-9475.2004.01512.x
26. Al-Rwi L I F, Alakori M M H, and Abdulghafour K H. Prevalence of Entamoeba Histolytica infection in patients with colitis (Ulcerative and Infective). *Fac Med Bagh J.* 2016;58(3):283-8.
27. Haque R, Mondal D, Duggal P, Kabir M, Roy S, Farr BM et al. Entamoeba histolytica infection in children and protection from subsequent amebiasis. *Infect Immun.* 2006 Feb;74(2):904-9. doi: 10.1128/IAI.74.2.904-909.2006
28. Bernin H, Marggraff C, Jacobs T, Brattig N, Le VA, Blessmann J, Lotter H. Immune markers characteristic for asymptomatically infected and diseased Entamoeba histolytica individuals and their relation to sex. *BMC Infect Dis.* 2014 Nov 25;14:621. doi: 10.1186/s12879-014-0621-1
29. Hemphill A, Müller N, Müller J. Comparative Pathobiology of the Intestinal Protozoan Parasites Giardia lamblia, Entamoeba histolytica, and Cryptosporidium parvum. *Pathogens.* 2019 Jul 29;8(3):116. doi: 10.3390/pathogens8030116
30. Ma K, Zhang H, Baloch Z. Pathogenetic and Therapeutic Applications of Tumor Necrosis Factor-α (TNF-α) in Major Depressive Disorder: A Systematic Review. *Int J Mol Sci.* 2016 May 14;17(5):733. doi: 10.3390/ijms17050733
31. Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. *Cold Spring Harb Perspect Biol.* 2014 Sep 4;6(10):a016295. doi: 10.1101/cshperspect.a016295
32. Mohammed HS, Ali SAK, Mohammed LO, Mohammed MS. Prevalence of Amoebiasis and Estimation of Certain Cytokines (IL-17, IFN-γ and TNF-α) in Children with Amoebic Infection in Sulaimani Province/Iraq. *Iraq Med J.* 2022;6(1):6-15. doi: 10.22317/imj.v6i1.1148