Molecular study for the virulence Factor EhCRT gene isolated from atients Infected with Entamoeba Histolytca in Thi Qar Province/Iraq

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KEYWORDS

Entamoeba histolytica; virulence; EhCRT gene; PCR technique; Amoebic dysentery

ABSTRACT

The parasite Entamoeba histolytica is a major public health concern and globally important. Due to the widespread of the E. histolytica infection in Thi Qar province, the aim of this study, was to detect E. histolytica by using polymerase chain reaction (PCR), and to study the pathogenicity of E. histolytica in human through detecting the virulence factor EhCRT gene and comparing the local isolates of this gene with globally genetic sequencing of National Center for Biotechnology Information NCBI. The diagnosis of E. histolytica parasites in human stool based on the diagnostic 18s rRNA gene. About 572 stool samples were collected from patients, who suffer from diarrhea and complain of abdominal pain in the Al-Hussein Teaching Hospital, Bint Al-Huda Educational Hospital for Women and Children and Mohammed Al-Musawi Hospital in Thi-Qar Province during the period from the beginning of December 2023 to the end of May 2024. The results of current study showed 100 out of 572 stool samples according to microscopic examination with prevalence 17.48%. The molecular diagnosis recorded 64 out of 100 samples were positive to diagnostic 18s rRNA gene with prevalence 64%. The virulence factor EhCRT was positive in 50 samples out of 50 examined samples with prevalence 100% by using PCR technology. The genetic sequence of the EhCRT gene was performed and compared with the genetic sequence of virulence factors registered globally at the NCBI. All three local isolates (IQN.No.1-3) exhibited high sequence identity (98.68% - 98.91%) with the USA strain HM-1:IMSS (XM_650149.1), with genetic variations ranging from 1.09% to 1.22%. These findings suggest a high degree of genetic similarity between the local Iraqi E. histolytica isolates and the reference strain, potentially indicating similar virulence profiles.

1. Introduction

Entamoeba histolytica is a protozoan parasite and the main cause of intestinal amebiasis in humans (González et al., 2020). Amoebic dysentery, which caused by the parasite E. histolytica, is one of the most common parasitic diseases after malaria and schistosomiasis. The infection with this parasite can be asymptomatic or develop into a severe infection causing amoebic liver or amoebic colitis. It has been found Amoebic colitis is one of the main causes of severe diarrhea in the world (Zghair et al., 2023). Some cases of amoebic dysentery (untreated) can lead to death, as the World Health Organization (WHO) estimates that the number of infections with this disease around the world is about 50 million infected people every year, and about 100,000 of them die person (Nowak, 2015). Clinical symptoms can vary from one patient to another depending on the severity of the infection (Preativatanyou, 2015). E. histolytica parasite have virulence factors that enable it to enhance infection. These factors are represented by the processes of invasion, colonization, attachment, evasion of the host's immune system, and suppression of the host's immunity. The parasite's ability to resist and adapt to environmental conditions is also a factor of virulence. The virulence of the parasite is generally attributed to its ability to destroy tissues by attaching to and degrading host cells(Faust & Guillen, 2012). The virulence factor calreticulin (CRT) is a calcium-binding protein (Ca2+) located mostly in the endoplasmic reticulum (ER). It is a highly conserved multifunctional protein found across a variety of species. CRT is one of the immune molecules in E. histolytica that stimulates an antibody response in the human host (González Rivas et al., 2011). The role of calreticulin (CRT) in host–parasite interactions has recently become a major area of research. The CRT genes from many parasites (Trypanosoma, Leishmania, Entamoeba, Onchocerca, Schistosoma, and Haemonchus) have been cloned and sequenced(Gonzalez et al., 2018). Previous studies indicated the presence of CRT in E. histolytica, and it was found that EhCRT works to stimulate the immune response in the human host when infected with the E. histolytica parasite, as it was found that more than 90% of patients suffering from amoebic liver abscess (ALA) are infected. With high levels of antibodies in the blood against

EhCRT (González et al., 2002). EhCRT is recognized by IgA and IgG antibodies to amoeba found in the serum and saliva of those infected with invasive E. histolytica at a rate of (>91%). On the contrary, the percentage of antibodies is low in people carrying parasitic cysts without symptoms. It has also been observed that after Following amebiasis patients for 6-12 months, their antibody levels against this protein are reduced by about 70%. This makes the protein potentially useful in diagnosing and detecting new invasive cases of amebiasis in endemic areas (LEZ et al., 2002). Other studies have hypothesized that EhCRT is a receptor for E. histolytica that works to phagocytize dead cells through the process of programmed cell death and red blood cells. Thus, studies have proven that the EhCRT protein is recruited to the cell surface and is localized in the phagocytic cup during interaction with lymphocytes and red blood cells. Regulated overexpression of EhCRT leads to a significant increase in the phagocytosis of E. histolytica lymphocytes, which results in programmed cell death. and red blood cells treated with Ca2+ ionophores, EhCRT specifically binds to both apoptotic cells and human C1q (Vaithilingam et al., 2012). In this study, we diagnosed the presence of the EhCRT virulence factor in the E. histolytica parasite by a molecular method using polymerase chain reaction technology (PCR) , then studied the genetic sequence and evolutionary tree of this factor and compared them with the genetic sequence of virulence factors registered globally at the National Center for Biotechnology Information.

2. Methodology

Collection of stool samples:

About 572 stool samples collected from patients, who suffer from diarrhea and complain of abdominal pain in the Al-Hussein Teaching Hospital, Bint Al-Huda Educational Hospital for Women and Children and Mohammed Al-Musawi Hospital in Thi-Qar Province during the period from the beginning of December 2023 to the end of May 2024. The stool samples were examined by direct microscopic method and the positive samples kept in a refrigerator -20C in a stool container for use in molecular examinations.

Molecular diagnosis:

Extraction of the genomic DNA:

The parasite's DNA extracted from the positive fecal samples of microscopic examination using a special Presto™ Stool DNA Extraction Kit, supplied by Geneaid, and this was done in accordance with the company's instructions. Genomic DNA extracted from stool samples was examined using a Nanodrop spectrophotometer (THERMO. USA), which examines the concentration and purity of DNA by reading the absorbance at (260-280) nm.

The Primers:

The diagnostic primers of *E.histolytica* based on small subunit ribosomal 18s rRNA gene and virulence factor gene calreticulin were designed in this study using NCBI-Genbank and primer 3 plus design. These primers provided from ScientificReserecher.Co.Ltd, Iraq, table (1).

Table (1): The primers used in current study.

Primers	Sequence 5'-3'		Product size	Genbank reference code
ssrRNA gene	F	ACGGGAGAGGTGAAAATCCA	695bp	AB426549.1
	R	TGCGGCCCAAGATGTCTAAG		
EhCRT gene	F	TTCAACATCTTCAGGAAAGGCA	510bp	GU477560.1
	R	TCTTTTGGTGCTAACATATCCCA		

PCR master mix preparation

The PCR master mix was prepared by using (Go taq **Green PCR Master Kit**) and this master mix done according to company instructions.

Table (2). Components of the multiplex PCR mixture.

PCR Master mix	Volume
DNA template 5-50ng	5µL
Forward primer (10pmol)	2µL
Reverse primer (10pmol)	2µL
Green Master mix	12.5µL
PCR water	3.5 µL
Total volume	25µL

PCR Thermocycler Conditions : Initial Denaturation (95C for 5 min), Denaturation (95C for 30 sec) Annealing (58C for 30 sec) Extension (72C for 2 min), Final extension (72C for 5 min) Hold (4°C Forever).

Table (3): Heat cycles for multiplex PCR.

PCR step	Temp.	Time	Repeat
Initial Denaturation	95°C	5min.	1 Cycle
Denaturation	95°C	30sec.	1 Cycle
Annealing	58°C 56°C	30sec.	35 Cycle
Extension	72°C	2min.	35 Cycle
Final Extension	72°C	5min.	1 Cycle
Hold	4°C	Forever	-

PCR product analysis:

The PCR products of was analyzed by agarose gel electrophoresis. then, PCR products were visualized by using UV Transilluminator

3. Result and Discussion

Infection with *E. histolytica*:

Microscopic examination:

The results of current study showed that 100 out of 572 (17.48%) were infected with *Entamoeba* histolytica by microscopic examination, table 4.

Table (4): The prevalence of *E. histolytica* in stool samples according to the microscopic examination.

No. Examined samples	No. Positive samples	No. Negative samples	Prevalence %
572	100	472	17.48

The results of the current study showed that 64 samples out of (100) DNA samples were infected with *E. histolytica* with prevalence 64% according to the polymerase chain reaction (PCR) technique using special primers based on the 18s rRNA gene as in table (5) and figure (1).

Table (5): The prevalence of E. histolytica in stool samples according to the polymerase chain reaction (PCR) technique.

No. Examined samples	No. Positive samples	No. Negative samples	Prevalence %
100	64	36	64

Figure 1: Agarose gel electrophoresis of PCR product show the analysis of small subunit ribosomal RNA gene in *E. histolytica* from Human stool samples. M: marker (2000-100bp). The positive *E. histolytica* samples were showed small subunit ribosomal RNA gene at (695bp) PCR product.

Virulence factor EhCRT gene of *E. histolytica* by PCR:

The virulence factor EhCRT was positive in 50 samples out of 50 examined samples with prevalence 100% by using PCR technology, table (6) and figure (2).

Table (6). The prevalence of virulence factor EhCRT in stool samples according to the polymerase chain reaction (PCR) technique.

No. Examined samples	No. Positive samples	No. Negative samples	Prevalence %
50	50	0	100

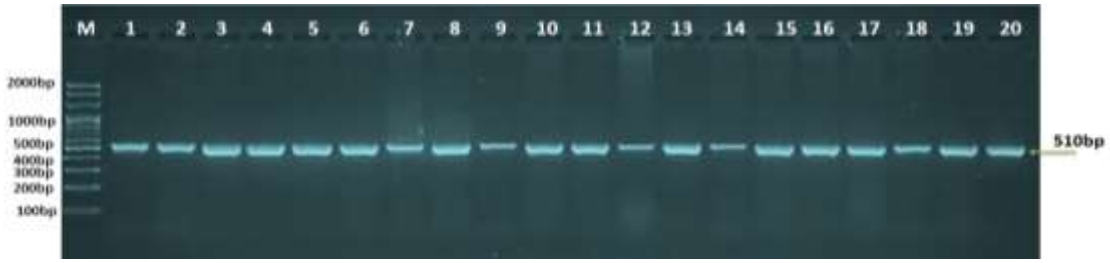

Figure 2: Agarose gel electrophoresis of PCR product analysis of virulence factor Calreticulin gene in *Entamoeba histolytica* positive samples. M: marker (2000-100bp). The positive *E. histolytica* virulence factor EhCRT gene were showed at (510bp) PCR product.

Sequencer of virulence factor EhCRT gene of *E. histolytica*:

DNA sequencing was investigated the genetic characteristics of the calreticulin gene, a virulence factor, in local *E. histolytica* isolates from Iraq (IQN) compared to human isolates and reference strains from GenBank, figure 3.

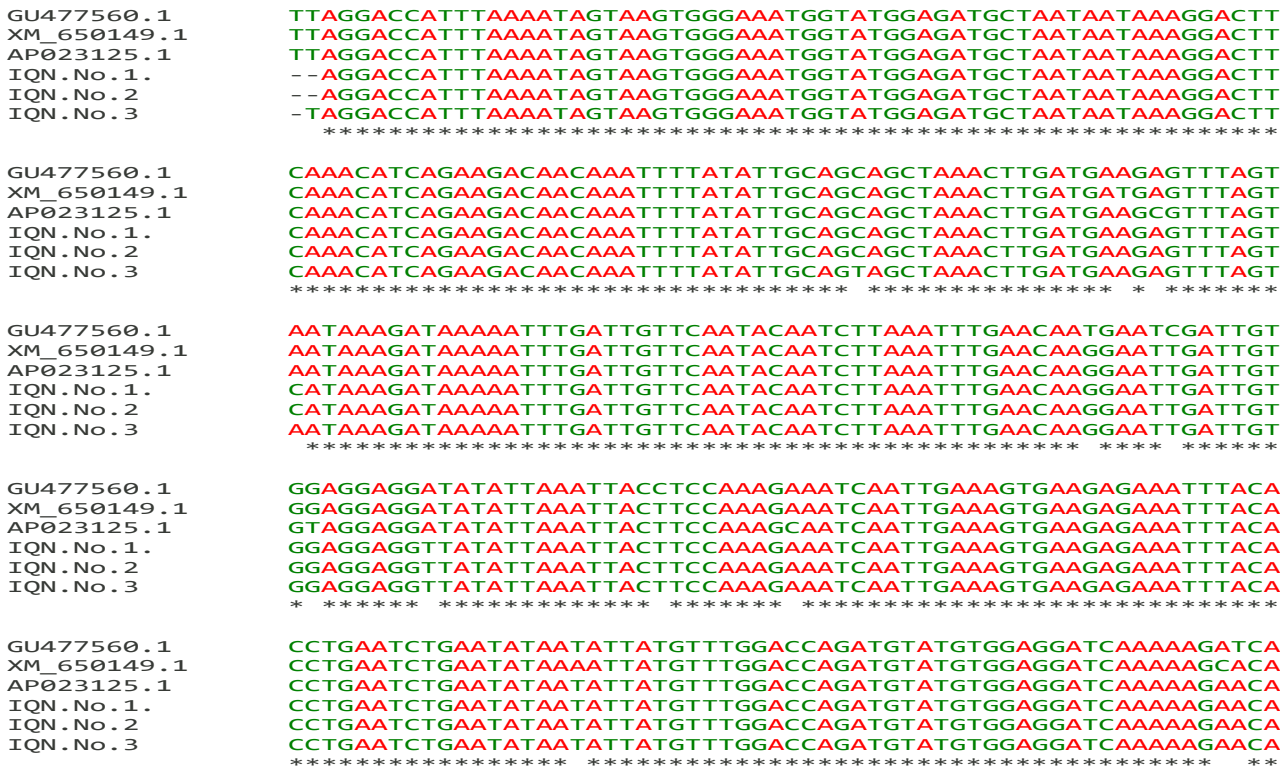

Figure (3): Multiple sequence alignment analysis of virulence factor calreticulin gene in local Entamoeba histolytica IQN. Human isolates and NCBI-Genbank genetic related Entamoeba histolytica isolates. The multiple alignment analysis was constructed using (ClustalW alignment tool. Online). That alignment analysis was showed the nucleotide alignment similarity as (*) and substitution mutations in calreticulin gene between isolates.

Phylogenetic analysis using the calreticulin gene further explored genetic relationships. The UPGMA tree constructed in MEGA 6.0 software and demonstrated a close association between IQN.1-No.3 isolates and the reference strain Entamoeba histolytica HM-1:IMSS (XM_650149.1) with minimal genetic changes (0.0080-0.0020%), table (7).

Table (7) the NCBI-BLAST Homology Sequence identity and genetic variation analysis between *Entamoeba histolytica* isolates and NCBI-BLAST closed genetic related Entamoeba histolytica isolate:

Local isolate	Accession number	Homology sequence identity (%)			
		Genetic related isolate	Country related	Genetic variation (%)	Identity (%)
IQN.No.1	PQ049144	Entamoeba histolytica HM-1:IMSS	USA	1.22%	98.68%
IQN.No.2	PQ049145	Entamoeba histolytica HM-1:IMSS	USA	1.22%	98.68%
IQN.No.3	PQ049146	Entamoeba histolytica HM-1:IMSS	USA	1.09%	98.91%

Furthermore, NCBI-BLAST homology analysis confirmed this close relationship. All three local isolates (IQN.No.1-3) exhibited high sequence identity (98.68% - 98.91%) to the reference strain HM-1:IMSS, with genetic variations ranging from 1.09% to 1.22%.

These findings suggest a high degree of genetic similarity between the local Iraqi E. histolytica isolates and the reference strain, potentially indicating similar virulence profiles, figure (4).

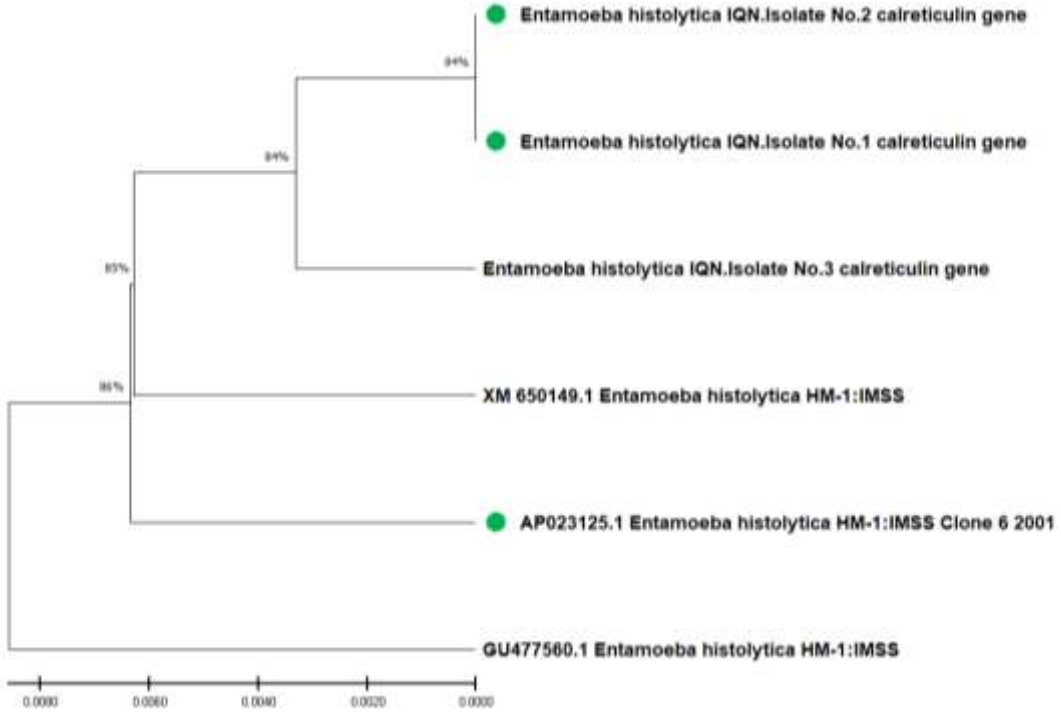

Figure (4): Phylogenetic tree analysis based calreticulin gene partial sequence in local *Entamoeba histolytica* IQN Human isolates that used for genetic relashiposhp analysis. The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). *The Entamoeba histolytica* IQN.1-No.3 isolates showed closed related to NCBI-BLAST *Entamoeba histolytica* HM-1:IMSS (XM_650149.1) at total genetic changes (0.0080-0.0020%).

Discussion:

Intestinal parasitic infections represent a major public health problem on an international scale; the epidemiological research of different types of intestinal parasitic infections in various regions aimed at detecting populations at risk. The results of the current study showed that 100 out of 572 with prevalence 17.48% infected with *E. histolytica* parasites by using the microscopic examination method, the positive samples of microscopic examination were used to molecular diagnostic method.

Several factors have contributed to make diagnosis of *E. histolytica* infection difficult, including the occurrence of asymptomatic carriers and the existence of a morphologically identical, non-pathogenic amoeba: *Entamoeba dispar*(Laughlin & Temesvari, 2005). The results showed that 64 out of 100 (64%) of DNA samples were positive for infection with the *E. histolytica* parasite. This result was lower than that of a study in Al-Diwaniyah province, where the prevalence of *E. histolytica* was 74% (Al-Abodi, 2015). Another study in Al-Diwaniyah province reported that the prevalence of *E. histolytica* parasite was 47%(AS, 2014). A study in Basra recorded the prevalence of *E. histolytica* parasite was 30% (Al-Yaquob, 2008) . A study in Thi-Qar province showed that prevalence of *E. histolytica* parasite was 68%, (Ali, 2023) and this is close to the results of the current study. One study in Bangladesh reported that the prevalence of *E. histolytica* was 4.63%(Shimokawa *et al*., 2012) . A study in South Africa reported that the prevalence of *E. histolytica* was 8.5% (Ngobeni *et al*., 2017). The differences in the prevalence of *E. histolytica* using the polymerase chain reaction (PCR) may be due to differences In methods of extracting DNA from stool samples or due to differences in the number of parasites in stool samples, which may result from differences in the time period in which the samples were obtained, and the method of transportation and preservation used (López *et al*., 2015). In our work, the calreticulin

gene was chosen to study as a virulence factors of *E. histolytica* parasite.

The results of current study showed that the EhCRT gene was positive in 50 samples with prevalence 100% . (Khalaf *et al*., 2022) reported that the EhCRT expressed in 81.8% in Baghdad and they reported that the expression of the EhCRT gene concedes with the duration of diarrhea a virulence factor that plays a role in host pathogenic pathways.

CRT is a multifunctional protein found in all eukaryotic cells except fungi and erythrocytes. Mammalian CRT it is largely restricted to the endoplasmic reticulum, where is acts as a calcium-binding and chaperone protein. In contrast, protozoan and metazoan CRT appear to be secreted proteins. Several protozoan and metazoan parasites, including *Trypanosoma brucei* and *T. cruzi* (the causative agents of sleeping sickness and Chagas' disease, respectively), *Necator americanus* (hookworm) and pathogenic species of schistosome have been shown to secrete CRT and/or express CRT on their surface (Ferreira *et al*., 2004).

Although the functions of CRT are conserved in vertebrates, some CRT functions differ among parasites(Ferreira *et al*., 2004). parasite CRTs bind host C1q (the first component of the classical complement cascade) and inhibit C1q-dependent complement activation. The CRT of *Haemonchus contortus* worms binds host C-reactive protein and C1q(Naresha *et al.*, 2009). Inhibition of the complement system reduces the effectiveness of the host antibody response, and these mechanisms appear to be an integral part of the parasite's strategy to evade the host immune response. It has been shown that trypanosome CRT is potentially useful as a capture antigen in the serodiagnosis(El Aswad *et al.*, 2011).

In human trypanosomiasis, the role of TcCRT as an immune evasion mechanism is easily understood because CRT is located on the surface of the trypomastigote in blood during the acute phase of infection and accessible for binding to C1q (Ximénez *et al*., 2014) .

The ecto-parasite (ticks) *Amblyomma americanum* secretes CRT during feeding, suggesting that the anticoagulant ability of CRT may prevent blood clotting and allows the parasite to feed on the host and induce host antiparasite responses and the cross-reacting of tick CRT with host CRT antibodies raises the possibility for tick antibodies to bind host CRT and delay wound healing (Kim *et al*., 2015).

In *E. histolytica*, the EhCRT induces an important immunogenic response in the human host. More than 90% of patients with amoebic liver abscesses develop high levels of serum antibodies against EhCRT (González *et al.*, 2002).

Gonzalez *et al*. (2018) confirmed that the EhCRT behaves like an amebic immunogenic protein for humans and suggest that the EhCRT participates in the specific stimulation of immune cells and they suggested that the rEhCRT can stimulate human peripheral blood monocular cells proliferation independently of the presence of *E. histolytica* trophozoites, acting as a specific costimulator of the immune response like that induced by ConA. In addition, these results underline EhCRT as a parasitic factor that can modulate the immune response, from the stimulation of proinflammatory cytokines to the immunosuppressive effects.

The Ehcrt gene is located in the nuclear DNA of this parasite, the size of the sequence correlates with the predicted molecular weight of the *E. histolytica* CRT-like protein (González *et al*. 2002). The proteomic analysis of phagosomes isolated from *E. histolytica* parasites using MALDI-TOF mass spectrometry demonstrated the presence of CRT in *E. histolytica* HM1:IMSS strain (Okada *et al*., 2005).

The immunohistochemical assays on trophozoites using monospecific antibodies against recombinant CRT showed that EhCRT is located in the cytoplasmic membrane (Ximénez *et al.*, 2014).

Despite its localization, described primarily in Endoplasmic Reticulum of other parasites (González *et al*. 2002; Ferreira *et a*l., 2004), the CRT can have various isoforms and is found in many sites inside or outside the cell(Michalak *et al*., 2009), suggesting moonlight protein functions during the

pathogenesis as already reported for other parasites(Karkowska-Kuleta & Kozik, 2014).

The BLAST alignment of EhCAR gene in current study demonstrated a close association between IQN.1-No.3 isolates and the reference strain *E. histolytica* HM-1:IMSS (XM_650149.1) with minimal genetic changes (0.0080-0.0020%). Furthermore, NCBI-BLAST homology analysis confirmed this close relationship. All three local isolates (IQN.No.1, No.2 and No.3) exhibited high sequence identity (98.68% - 98.91%) to the reference strain HM-1:IMSS, with genetic variations ranging from 1.09% to 1.22% and these findings suggest a high degree of genetic similarity between the local Iraqi *E. histolytica* isolates and the reference strain, potentially indicating similar virulence profiles.

González *et al*. (2002) reported 95% of dysentery patients and 100% of ALA patients recognized a 51-kDa fraction of *E. histolytica* HM1:IMSS that, once partially sequenced, was shown to be a CRT-like protein. Other study demonstrated a mean identity of amino acid sequences (55%) in comparative analyses between *E. histolytica* and *Acanthomoeba castellanii* (Sánchez *et al*., 2016). The identification of CRT protein homologues in endoplasmic reticulum (ER) or cellular surface of various parasites (Ferreira *et al*., 2004) suggests that this protein could have many conserved roles like parasite and host interactions, phagocytosis, and modulation of host immune response (Mendlovic & Conconi, 2010).

4. Conclusion and future scope

The study revealed a high prevalence of virulence factor EhCRT gene in local Iraqi isolates of E. histolytica parasite and a high degree of genetic similarity between the local E. histolytica isolates and the reference strain, and similar virulence features with the presence of a certain percentage of genetic mutations.

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