RESEARCH ARTICLE

# Molecular identification of EhCRT gene Calreticulin isolated from children infected with Entamoeba histolytica

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## Abstract

**Objective:** To detect the gene of entamoeba histolytica by polymerase chain reaction and investigate the expression of immunogene entamoeba histolytica calreticulin in stool samples of infected patients.

**Method:** The case control study was conducted at Central Teaching Hospital of Paediatrics and Al Mahmoudia General Hospital, Iraq, from December 30, 2020, to September 1, 2021, and comprised diarrhoeal faecal samples collected from 86 children with age ranging from <1 year to 13 years who were suspected of having been infected with entamoeba histolytica. Microscopically positive samples were then subjected to conventional and real-time polymerase chain reaction for the detection of entamoeba histolytica HM1:IMSS strain using Phage shock protein (Psp) gene sequences and detection of entamoeba histolytica calreticulin expression.

**Result:** Of the 86 patients, 71(82.6%) were found to be infected with entamoeba histolytica; 39(54.93%) boys and 32(45.07%) girls. The remaining 15(17.4%) patients were taken as non-amoebic controls; 8(53.3%) boys and 7(46.7%) girls. There were 36(50.70%) cases and 8(53.33%) controls aged 1-4 years. Among the Entamoeba histolytica gene was detected in 44(62%) of the cases using conventional polymerase chain reaction, and immunogene entamoeba histolytica calreticulin was expressed in 36(50.7%) using real-time polymerase chain reaction. Data was analysed using SPSS 24.

**Conclusion:** Polymerase chain reaction was found to be a useful tool for diagnosing entamoeba histolytica infection in children.

**Key Words:** Entamoeba histolytica, Entamoebiasis, Calreticulin, Polymerase, Diarrhea, Bacteriophages

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## Introduction

Entamoeba (E.) histolytica, a protozoan parasite that causes amoebiasis in humans, infects the intestine. The symptoms include diarrhoea, dysentery and colitis. The third common parasitic cause of morbidity and mortality is amoebiasis[1]. E. histolytica is thought to infect 50 million individuals worldwide each year, causing 70,000 fatalities [2]. While 90% of infected people have no symptoms, the infection can cause serious consequences, like colitis, bloody diarrhoea, liver abscesses and colonic perforation [3].

Amoeba virulence is mediated by a number of mechanisms involving unique molecular interactions at both cellular and molecular levels. These interactions between the host and the parasite take place in a succession of steps. Trophozoites link to intestinal epithelial cells via the parasite's surface Gal/GalNAc lectin, which binds to galactose (Gal) and/or N-acetyl-D-galactosamine (GalNAc) in the host cell membrane[4]. After sticking to host cells, an amoeba uses a variety of cytotoxic processes, including apoptosis, phagocytosis and trogocytosis, to cause cell death and tissue invasion [5].

E. histolytica calreticulin (EhCRT) is one of the immunogenic molecules that stimulate an antibody response in the human host. However, calreticulin interacts with C1q and C1 (Complement 1q and complement 1) complex that inhibits the complement system. EhCRT also affects pathogenesis and the modulation of the host immunological response. EhCRT also works as an immunogenic for the specific activation of peripheral blood mononuclear cells in vitro, causing a Th2 (T helper 2 cells) cytokine profile during the acute phase and a Th1 (T helper 1 cells) profile during the resolution phase. Finally, excess of the CRT gene could constitute a regulatory mechanism that allows the parasite to adapt and survive in host tissues[6].

The current study was planned to detect the gene of E. histolytica by polymerase chain reaction (PCR) and investigate the expression of immunogene EhCRT in stool samples of infected patients.

## Materials and Methods

The case control study was conducted at Central Teaching

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Hospital of Paediatrics and Al Mahmoudia General Hospital, Iraq, from December 30, 2020, to September 1, 2021. After approval form the institutional ethics review committees, the sample was raised from among diarrhoeal faecal samples that were collected from children with age ranging from <1 year to 13 years who presented with diarrhoea and abdominal pain, and were suspected of having been infected with E. histolytica. After microscopic analysis, the samples were transported on dry ice or ordinary ice, and all positive samples were kept at -20°C[7].

The samples were divided into two parts: one part directly treated by the QIAamp deoxyribonucleic acid (DNA) stool Mini Kit (Qiagen, Germany) to obtain DNA for diagnostic identification psp gene of E. histolytica, while the second part of the sample was conserved in TRIzol using ribonucleic acid (RNA) extracted using a purification lit (Direct-zol, RNA MiniPrep, Zymo, United States) to obtain RNA for the diagnosis of EhCRT gene expression. Conversion of RNA to complementary DNA (cDNA) by cDNA synthesis was performed using a real-time PCR (RT-PCR) kit (PrimeScript RT Reagent Kit) as per the manufacturer's instructions )[8].

Psp oligonucleotides were used to amplify DNA. These oligonucleotides amplified an 876-bp product. Amplification was performed in a MultiGene OptiMax Gradient Thermal Cycler (Labnet, US). The amplification conditions were: DNA denaturation for 5 minutes at 95˚C, followed by 45 cycles of denaturation for 30 seconds for alignment at 53˚C, extension for 1 minute at 72˚C, followed by a final extension step of 10 minutes at 72˚C.

For the quantification and expression of the specific oligonucleotides, EhCRT5 TGGACCAGATGTATGTGGAGG and EhCRT3 TGGTGCTTCCCATTCTCCATC primers were used in reverse transcriptase quantitative PCR (RT-qPCR). For qPCR, one-step kit was used (KAPA SYBR FAST, qPCR Master Mix (2X) Kit, KAPA, US). The amplification was developed with 60 cycles with 3 stages; a denaturing stage at 95℃ for 30 sec, a phase of an-nealing to 51℃ for 30 sec, and a phase of extension at 72℃ for 30 sec. Finally, the reaction was finished with 90℃ for 15 sec. The amplification of Eh-α-actin (Entamoeba histolytica α-actin) reference gene (1000-bp; the housekeeping gene) and an E. histolytica DNA control were included[1].

Data was analysed using SPSS 24.

## Results

Of the 86 patients, 71(82.6%) were found to be infected with E. histolytica; 39(54.93%) boys and 32(45.07%) girls. The remaining 15(17.4%) patients were taken as non-amoebic controls; 8(53.3%) boys and 7(46.7%) girls. There were 36(50.70%) cases and 8(53.33%) controls aged 1-4 years. Among the cases, stools of yellow colour was found in 36(50.70%) subjects, mucoid stools 55(77.46%) and 3(4.23%) children had dysentery. The majority of children with amoebiasis were in the acute infection phase 64(90.14%), while 7(9.86%) had chronic infection. The non-amoebic illness was acute in all 15(100%) children. There were 62(87.32%) subjects in the amoebic group and all 15(100%) in the control group complaining of primary episodes of diarrhoea, while 9(12.68%) in the amoebic group appeared to have recurrent infection (Table 1).

**Table-1**:Demographic characteristics.

| | Amoebic group | | Non-amoebic group | | Total | |
|---|---|---|---|---|---|---|
| | N. (71) | % (82.6) | N. (15) | % (17.4) | N. (86) | % (100) |
| **Age groups** | | | | | | |
| ≤ 1 year | 13 | 18.31 | 3 | 20.0 | 16 | 18.60 |
| 1.1-4 years | 36 | 50.70 | 8 | 53.33 | 44 | 51.16 |
| 4.1-8 years | 14 | 19.72 | 4 | 26.67 | 18 | 20.93 |
| 8-12 years | 6 | 8.45 | 0 | 0.0 | 6 | 6.98 |
| > 12 years | 2 | 2.82 | 0 | 0.0 | 2 | 2.33 |
| Total | 71 | 100 | 15 | 100 | 86 | 100 |
| **Gender** | | | | | | |
| Male | 39 | 54.93 | 8 | 53.33 | 47 | 54.70 |
| Female | 32 | 45.07 | 7 | 46.67 | 39 | 45.30 |
| Total | 71 | 100 | 15 | 100 | 86 | 100 |
| **Stool colour** | | | | | | |
| Brown | 29 | 40.85 | 11 | 73.33 | 40 | 46.50 |
| Yellow | 36 | 50.70 | 4 | 26.67 | 40 | 46.5 |
| Bloody | 5 | 7.04 | 0 | 0.0 | 5 | 5.8 |
| Green | 1 | 1.41 | 0 | 0.0 | 1 | 1.2 |
| Total | 71 | 100 | 15 | 100 | 86 | 100 |
| **Stool consistency** | | | | | | |
| Mucoid | 55 | 77.46 | 5 | 33.33 | 60 | 69.8 |
| Liquid | 13 | 18.31 | 10 | 66.67 | 23 | 26.7 |
| Bloody | 3 | 4.23 | 0 | 0.0 | 3 | 3.5 |
| Total | 71 | 100 | 15 | 100 | 86 | 100 |
| **Infection** | | | | | | |
| Acute | 64 | 90.14 | 15 | 100 | 79 | 91.9 |
| Chronic | 7 | 9.86 | 0 | 0.0 | 7 | 8.1 |
| Total | 71 | 100 | 15 | 100 | 86 | 100 |
| **Disease recurrences** | | | | | | |
| Primary | 62 | 87.32 | 10 | 66.67 | 72 | 83.7 |
| Recurrence | 9 | 12.68 | 5 | 33.33 | 14 | 16.3 |
| Total | 71 | 100 | 15 | 100 | 86 | 100 |

Among the cases, E. histolytica gene was detected in 44(62%) using conventional PCR, and immunogene EhCRT was expressed in 36(50.7%) using RT-PCR (Table 2).

Correlation among the study parameters were noted (Table 3).

**Table-2**: Deoxyribonucleic acid (DNA) extraction and conventional and Real-time polymerqse chain reaction (PCR) identification of entamoeba (E.) histolytica HM1: IMSS strain using (Psp) gene sequences and entamoeba histolytica calreticulin (EhCRT) expression in stool samples from amoebic and non-amoebic groups.

| | DNA Extraction | |
|---|---|---|
| | N. | % |
| **Amoebic group** | | |
| Positive | 62 | 72.1 |
| Negative | 9 | 10.5 |
| Total | 71 | 82.6 |
| Non-amoebic group | 15 | 17.4 |
| Total | 86 | 100.0 |
| **PCR identification of E. histolytica** | | |
| **Amoebic group** | | |
| Gene of Eh(HM1: IMSS) + Ve | 44 | 71.0 |
| Gene of Eh(HM1: IMSS) - Ve | 18 | 29.0 |
| Total Eh(HM1: IMSS) +Ve | 62 | 87.3 |
| DNA -Ve | 9 | 12.7 |
| Total | 71 | 100 |
| **Expression of calreticulin (EhCRT)** | | |
| qPCR product (EhCRT) +Ve | 36 | 81.8 |
| qPCR product (EhCRT) –Ve | 8 | 18.2 |
| Total | 44 | 100 |
| Total DNA –Ve and EhCRT –Ve | 27 | 38.0 |
| Total amoebic | 71 | |

**PCR for gene detection**

| | Gene of Eh + Ve | | Gene of Eh – Ve | | Total | |
|---|---|---|---|---|---|---|
| | N. | % | N. | % | | |
| Acute infection | 37 | 84.1 | 18 | 100 | 55 | 88.7 |
| Chronic infection | 7 | 15.9 | 0.0 | 0.0 | 7 | 11.3 |
| Total | 44 | 71.0 | 18 | 29.0 | 62 | |
| Fisher's Exact Test | 0.096 | | | | | |

qPCR: Quantitative polymerqse chain reaction

## Discussion

The current study to the best of our knowledge is the first in Iraq to use EhCRT gene expression as a target for molecular methods to assess the presence of E. histolytica trophozoites in stool samples.

Amoebiasis is a human disease caused by the protozoan E. histolytica, which has been detected globally and causes widespread morbidity and mortality[9]. Environmental, biological, behavioural, social and health-related factors have an impact on parasite infections directly or indirectly[10]. Several studies have investigated the prevalence of E. histolytica in Baghdad governorate [11].

The rate of infection in the current study was highest in the age group 1-4 years, which agrees with earlier findings[12-14]. Also, high numbers of amoebic and non-amoebic infected children appeared to have yellow or brown stools, and majority (90.14%) had acute infection. This age group is more involved in outdoor and indoor activities that may lead to high entamoeba transmission. Besides, insufficient water supply, low educational level of family, and lack of proper health facilities are also contributing factors. High temperature, polluted water and poor nutrition also have a role in this regard[15,16]. A high proportion of children in the amoebic group had mucoid diarrhoea (77.46%), whereas very few children (18.31%) had liquefied stool. E. histolytica has invasive strains and can secrete virulence factors that degrade immunoglobulin A (IgA) in the mucosal mucin layer and lead to lysis of leukocytes. This mechanism of inflammatory activation triggers cell death and the recruitment of immune cells, including neutrophils and macrophages, which lead to the discharge of mucoid stool[17,18].

**Table-3**: Correlation involving PCR gene detection, EhCRT expression and the duration of diarrhoea, disease recurrence and other parameters.

| | Disease recurrence | Duration diarrhoea | Colour | Type of diarrhoea | PCR gene detection | EhCRT expression |
|---|---|---|---|---|---|---|
| **Gender** | | | | | | |
| Correlation Coefficient | 0.041 | 0.094 | 0.075 | -0.029 | -0.250 | 0.498 |
| P-Value | 0.706 | 0.387 | 0.494 | 0.792 | 0.063 | 0.143 |
| **Age** | | | | | | |
| Correlation Coefficient | -0.188 | -0.159 | -.434 | -0.075 | 0.196 | 0.576 |
| P-Value | 0.082 | 0.142 | 0.000** | 0.493 | 0.148 | 0.081 |
| **Disease recurrence** | | | | | | |
| Correlation Coefficient | | 0.127 | 0.029 | -0.025 | -0.031 | -0.234 |
| P-Value | | 0.243 | 0.790 | 0.817 | 0.820 | 0.515 |
| **Duration of diarrhoea** | | | | | | |
| Correlation Coefficient | | | 0.123 | -0.037 | -.280 | 0.019 |
| P-Value | | | 0.259 | 0.736 | 0.037* | 0.960 |

*Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed).

PCR: Polymerqse chain reaction, EhCRT: Entamoeba histolytica calreticulin.

Improvements in molecular diagnostic procedures have led to distinguishing E. histolytica from the non-pathogenic species of entamoeba. Many kinds of entamoeba can produce infection in the human host, and 3 among these species are morphologically identical to E. histolytica. Previous descriptions of E. histolytica infection depended exclusively on microscopy and may have been disguised by these non-pathogenic strains.[19]. The identification of E. histolytica microscopically is generally mistaken and unreliable, specifically when other species of entamoeba that have identical morphology to E. histolytica are present in the stool specimen. Therefore, molecular tools are useful for the specific identification of

entamoeba.

The 86 stool specimens in the current study that were microscopically examined showed 71 to be infected with E. histolytica. From 62 of these specimens, the DNA was extracted and the gene of E. histolytic was detected in 44 samples by using conventional PCR. The result is in line with other studies[20]. By using qPCR, immunogene EhCRL was found in 36 samples that were diagnosed as E. histolytic. However, the differences between the microscopic examination and molecular identification of E. histolytica infection by PCR technique may be due to the inaccurate storage condition that may lead to the destruction of the trophozoites stage of the parasite, or may be due to the existence of other species of entamoeba that have a morphology identical to E. histolytic. Small amount of stool that may contain very low parasitic load, treatment of patients with anti-amoebic drugs, number of cycles for PCR assay and the presence of other entamoeba species inhabiting the human gut are factors affecting the detection of E. histolytica and the expression of EhCRL antigen by PCR technique[21,2].

The result of PCR showed that the gene of E. histolytica was detected in 37 out of 44 (84.1%) children with acute infection. The parasite strains, dose of infection, parasite's mechanisms of immune evasion, host diet and immune responses are factors responsible for the parasite-host relationship. High proportion of infected persons can overcome the acute amoebic colitis without any complication or transform to chronic infection[23].

It was demonstrated that over-expression of immunogenic protein EhCRT Ag (antigen) occurs when trophozoite invades the tissue, adaptation with environment is inappropriate, and human immunity is impaired. Increase in the expression of EhCRT leads to severity of pathogenesis[24].

## Conclusion

E. histolytica is a protozoan parasite highly prevalent among diarrhoeal children, and it is responsible for gastrointestinal amoebiasis in the human host. PCR was found to be a useful tool for diagnosing E. histolytica infection. The expression of EhCRT Entamoeba histolytica chain reverse transcription corresponds with the duration of diarrhoea.

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