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Direct Molecular Detection and Phylogenetic Tree Analysis of Gastrointestinal Protozoan Parasites (*Giardia lamblia*, *Entamoeba histolytica*, *Cryptosporidium parvum*) from Diarrhea Infection in Kut City of Iraq: A Short Communication

D.K. Kadhim [1*], D.A. Alzubaidi [2]

1. Department of Anatomy and Biology, Faculty of Medicine, University of Wasit, Wasit, Iraq
2. Wasit Education Directorate, Wasit, Iraq

HIGHLIGHTS

- *Giardia lamblia* had 99% identity to *G. lamblia* with accession number of DQ157272.1.
- *Entamoeba histolytica* also had 99% identity to *E. histolytica* with accession number of GQ423748.1.
- *Cryptosporidium parvum* had 99% identity to *C. parvum* with accession number of AJ539197.1.

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Acronyms and abbreviations
PCR=Polymerase Chain Reaction

ABSTRACT

Background: The intestinal tract of human can be infected by protozoan parasites. In this short communication, the stool samples were collected from patients with diarrhea referred to Kut hospital, Iraq, and then the parasites (*Giardia lamblia*, *Entamoeba histolytica*, *Cryptosporidium parvum*) were considered for molecular identification.

Methods: Stool samples were collected from 69 patients with diarrhea and then transferred to laboratory. Protozoan parasites were evaluated by Polymerase Chain Reaction (PCR) and phylogenetic tree analysis. Small subunit 18S rRNA region was amplified in the sizes of 514 bp, 409 bp, and 507 pb for *G. lamblia*, *E. histolytica*, and *C. parvum*, respectively.

Results: The results of phylogenetic tree analysis showed that *G. lamblia* had 99% identity to *G. lamblia* with accession number of DQ157272.1 (and total genetic changes of 0.002%); *E. histolytica* also had 99% identity to *E. histolytica* with accession number of GQ423748.1 (total genetic changes of 0.0005%); and *C. parvum* had 99% identity to *C. parvum* with accession number of AJ539197.1 (total genetic changes of 0.05%).

Conclusion: Gastrointestinal symptoms in the individuals with the studied protozoan parasites can be diagnosed directly by molecular detection and phylogenetic tree analysis with satisfying results. As well as, it can be utilized like a target for therapeutic intervention for these enteric protozoans.

Introduction

Intestinal parasites are one of the main causes of public health issues, as well as morbidity and mortality, worldwide (Abubakar et al., 2015). The main intestinal protozoa parasites associated with diarrhea are *Giardia* spp., *Cryptosporidium* spp., and *Entamoeba* spp. (Fletcher et al., 2012). Cyst is the main infectious disease in

* Corresponding author (D.K. Kadhim)
✉ E-mail: thalsarrai@uowasit.edu.iq
ORCID ID: https://orcid.org/0000-0003-0832-5419

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Giardia spp. and *Entamoeba* spp.; and oocyst is the main one in *Cryptosporidium* spp. Consumption of each one with the contaminated food or water is the main cause of infection (Chen et al., 2002). Infection with protozoan parasites is ranged from asymptomatic to severe symptoms, with the major symptom of diarrhea. The infection with *Entamoeba histolytica* may lead to an invasive extra-intestinal amoebiasis (Marie and Petri, 2014). However, *Cryptosporidium* spp. and *Giardia* spp. are mainly responsible for periodic diarrhea in developing countries (Einarsson et al., 2016).

In this short communication, the stool samples were collected from patients with diarrhea referred to Kut hospital, Iraq, and then the parasites of *Giardia* spp., *Cryptosporidium* spp., and also *E. histolytica* were considered for molecular identification.

Materials and methods

Ethical approval

This study approved by Scientific Committee of the College of Medicine, University of Wasit, Iraq.

Fecal samples collection

Stool samples were collected from 69 patients with diarrhea referred to Al-Karama Teaching Hospital, Al-Kut Hospital for Gynecology, Obstetric, and Pediatrics, Al-Kut, Wasit, Iraq from June to December 2021. The samples were transmitted to a dry and clean plastic container and after that were imparted for analysis to laboratory.

Genomic DNA extraction

Genomic DNA was extracted from human feces using stool DNA extraction kit (Bioneer, Korea). The extracted DNA was analyzed using Nanodrop (Thermo Scientific, UK). Then, the samples were stored at -20 °C till next analysis.

Molecular identification

The identification of *Giardia lamblia, E. histolytica*, and *Cryptosporidium* spp. was carried out using Polymerase Chain Reaction (PCR) with the specific primers of the small subunits ribosomal RNA region, including 5'-AGGTGCTTTATCTCGCCGAG-3' and 5'-GAACCCTGATTCTCCGCCAG-3' for *G. lamblia* with the fragment size of 514 bp; 5'-TTCTAAGGAAGGCAGCAGGC-3' as well as 5'-ACATCCCCTCAGCATTGTCC-3' for *E. histolytica* with an amplicon size of 409 bp; and 5'-CGGGTAACGGGGAATTAGGG-3' and 5'-ATGCCCCCAACTGTCCCTAT-3' for *Cryptosporidium* spp. with fragment of 507 bp in length. The reaction solution, in total volume of 20 µl, included master mix buffer (AccuPower® PCR PreMix kit, Bioneer, Korea), 0.2 µM of dNTPs, 1 U *Taq* DNA polymerase, 30 mM of KCl, 10 mM of Tris-HCl (pH 9.0), and 1.5 mM of $MgCl_2$. The purified genomic DNA (100 ng) and the specific primer pair (0.5 mM each)were added. Temperature conditions were set up in a thermocycler (Mygene, Bioneer, Korea) to perform the reaction as following; primary denaturation for 5 min at 95 °C, following 30 cycles for 30 s denaturation at 95 °C, 30 s annealing at 58 °C, and 1 min extension at 72 °C; finally, 5 min extension at 72 °C. The amplification products were assessed via agarose gel electrophoresis (2%) and visualized using Gel documentation. Then, sequencing was done using Sanger method. Sequence analysis was carried out using BLAST and multiple alignments (T-COFFE). The phylogenetic analysis was done using MEGA 7.0 software.

Results and discussion

After conducting PCR assay to identify *G. lamblia*, *E. histolytica*, and *Cryptosporidium parvum* in patients with diarrhea, the results clarified amplicons of 514 bp for *G. lamblia* (Figure 1), 409 bp for *E. histolytica* (Figure 2), and 507 pb for *C. parvum* (Figure 3). Positive and negative samples were used in each run.

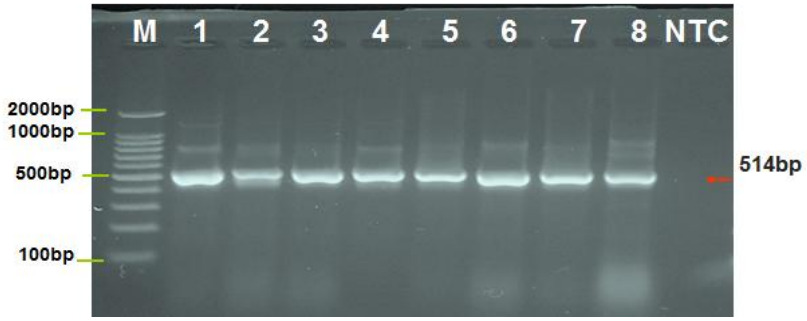

Figure 1: Agarose gel showed small subunit rRNA gene of Polymerase Chain Reaction (PCR) product that using for identify *Giardia lamblia* in human stool samples. Lane M: 100-1,500 bp; lanes 1-8: samples are positive at 514 bp size of PCR product; lane NTC: Non Template Control

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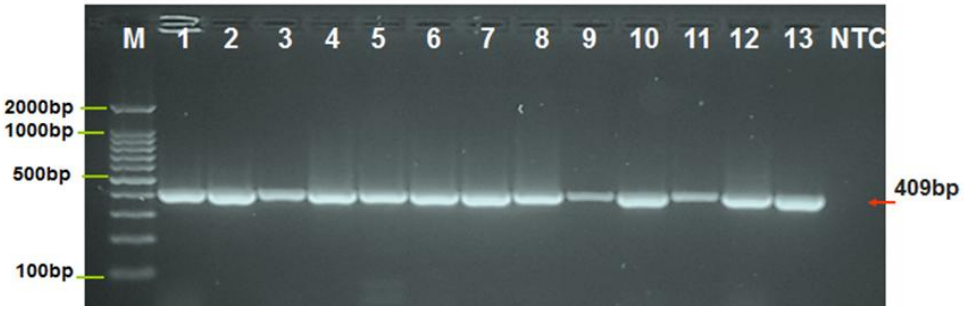

Figure 2: Agarose gel showed small subunit rRNA gene of Polymerase Chain Reaction (PCR) product that using for identify *Entamoeba histolytica* in human stool samples. Lane M: 100-1,500 bp; lanes 1-13: samples are positive at 409 bp size of PCR product; Lane NTC: Non Template Control

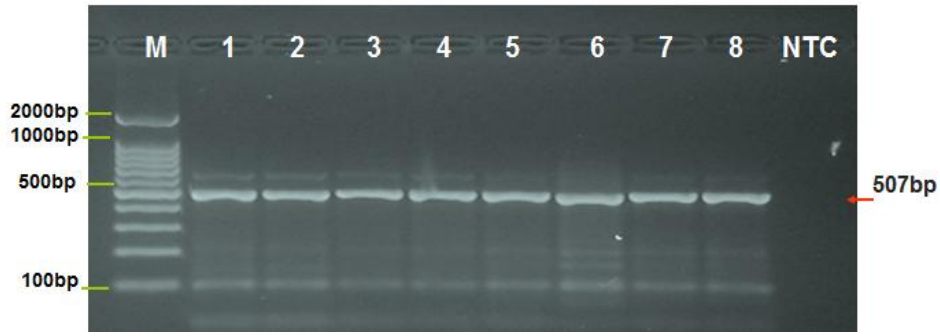

Figure 3: Agarose gel showed small subunit rRNA gene of Polymerase Chain Reaction (PCR) product that using for identify *Cryptosporidium parvum* in human stool samples. Lane M: 100-1,500 bp; lanes 1-8: samples are positive at 507 bp size of PCR product; lane NTC: Non Template Control

Totally, ten positive samples of *G. lamblia* were sequenced and submitted in NCBI, GenBank with the accession numbers of AF1994443.1, AF199444.1, AF199445.1, AF199448.1, DQ157272.1, HQ179632.1, HQ179639.1, HQ179640.1, HQ179642.1, and U09491.1. In addition, eight positive sequences for *E. histolytica* were submitted with the accession numbers of AB197936.1, AB426549.1, GQ423748.1, KC853026.1, KC853039.1, KY823424.1, MF421529.1, and MK332025.1. Moreover, ten positive sequences for *Cryptosporidium* spp. were submitted with the accession numbers of AF015774.1, AF093008.1, AF093009.1, AF093010.1, AF093011.1, AF093015.1, AJ539197.1, AJ539200.1, AJ539201.1, and AJ539205.1.

The sequence analysis with BLAST and multiple alignments showed 99% identity for *G. lamblia* to *G. lamblia* with accession number of DQ157272.1, 99% identity for *E. histolytica* to *E. histolytica* with accession number of GQ423748.1, and 99% identity for *C. parvum* to *C. parvum* strain with accession number of AJ539197.1 (Table 1, 2, and 3).

Anywhere, the analysis of hierarchical cluster clarified that locally parasites isolates (No.1-No.5) closely related to the total genetic changes were 0.002% for *G. lamblia* isolates with the one in GenBank with the accession number of DQ157272.1, 0.0005% for *E. histolytica* isolates with the one with accession number of GQ423748.1, and 0.05% for *C. parvum* isolate with the similar one with the accession number of AJ539197.1 (Figures 4-9).

In addition to viral and bacterial pathogens, gastrointestinal protozoa (*Giardia* spp., *Cryptosporidium* spp., and *Entamoeba* spp.) stay a main reason of enteric sickness in developing countries. The infections with persistent diarrhea specially in children below five years old are significantly associated with *Giardia* spp. and *Cryptosporidium* spp. *E. histolytica* can also cause diarrhea but in less extent (Muhsen and Levine, 2012). Therefore,

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Table 1: Homology sequence of NCBI-BLAST identity (%) between isolates of NCBI-BLAST submitted *Giardia lamblia* and local *G. lamblia*

Giardia lamblia **isolate No.1**	**Homology sequence of NCBI-BLAST identity (%)**		
	Identical *Giardia* intestinalis isolate	Genbank accession number	Identity (%)
G. lamblia IQS No.1 isolate	*G. lamblia*	DQ157272.1	99.19%
G. lamblia IQS No.2 isolate	*G. lamblia*	DQ157272.1	99.38%
G. lamblia IQS No.3 isolate	*G. lamblia*	DQ157272.1	99.17%
G. lamblia IQS No.4 isolate	*G. lamblia*	DQ157272.1	99.55%
G. lamblia IQS No.5 isolate	*G. lamblia*	DQ157272.1	99.22%

Table 2: Homology sequence of NCBI-BLAST identity (%) between isolates of NCBI-BLAST submitted *Entamoeba histolytica* and local *E. histolytica*

Entamoeba histolytica **isolate No.1**	**Homology sequence of NCBI-BLAST identity (%)**		
	Identical *E. histolytica* isolate	Genbank accession number	Identity (%)
E. histolytica IQS No.1 isolate	*E. histolytica*	GQ423748.1	99.12%
E. histolytica IQS No.2 isolate	*E. histolytica*	GQ423748.1	99.18%
E. histolytica IQS No.3 isolate	*E. histolytica*	GQ423748.1	99.16%
E. histolytica IQS No.4 isolate	*E. histolytica*	GQ423748.1	99.33%
E. histolytica IQS No.5 isolate	*E. histolytica*	GQ423748.1	99.45%

Table 3: Homology sequence of NCBI-BLAST identity (%) between isolates of NCBI-BLAST submitted *Cryptosporidium parvum* and local *C. parvum*

Cryptosporidium parvum **isolate No.1**	**Homology sequence of NCBI-BLAST identity (%)**		
	Identical *C. parvum* isolate	Genbank accession number	Identity (%)
C. parvum IQS No.1 isolate	*C. parvum*	AJ539197.1	99.18%
C. parvum IQS No.2 isolate	*C. parvum*	AJ539197.1	99.98%
C. parvum IQS No.3 isolate	*C. parvum*	AJ539197.1	99.19%
C. parvum IQS No.4 isolate	*C. parvum*	AJ539197.1	99.34%
C. parvum IQS No.5 isolate	*C. parvum*	AJ539197.1	99.15%

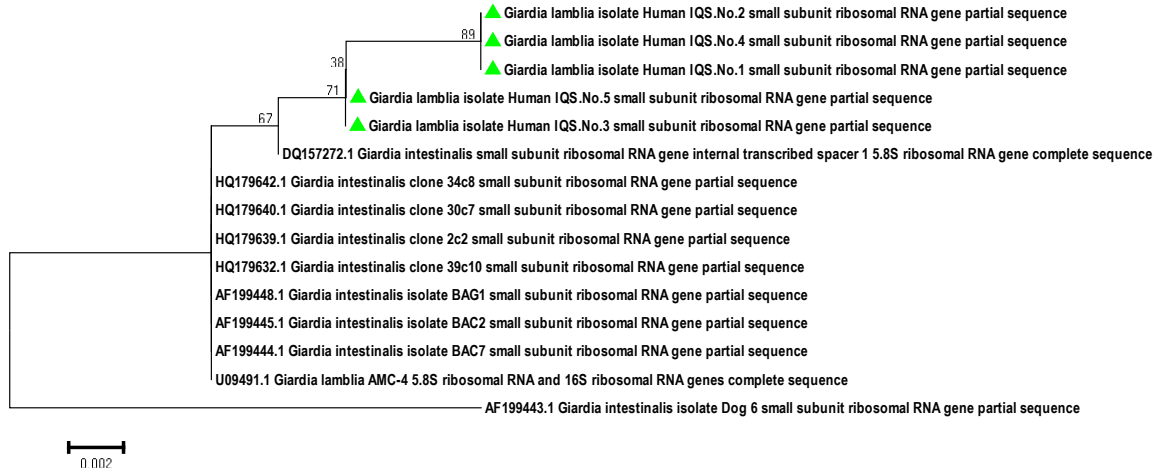

Figure 4: The phylogenetic tree analysis for local *Giardia lamblia* isolates depending on 18S rRNA gene partial sequence. The isolates No.1-No.5 closely related with isolate of *G. lamblia* NCBI-BLAST (DQ157272.1) with (0.002%) total genetic changes

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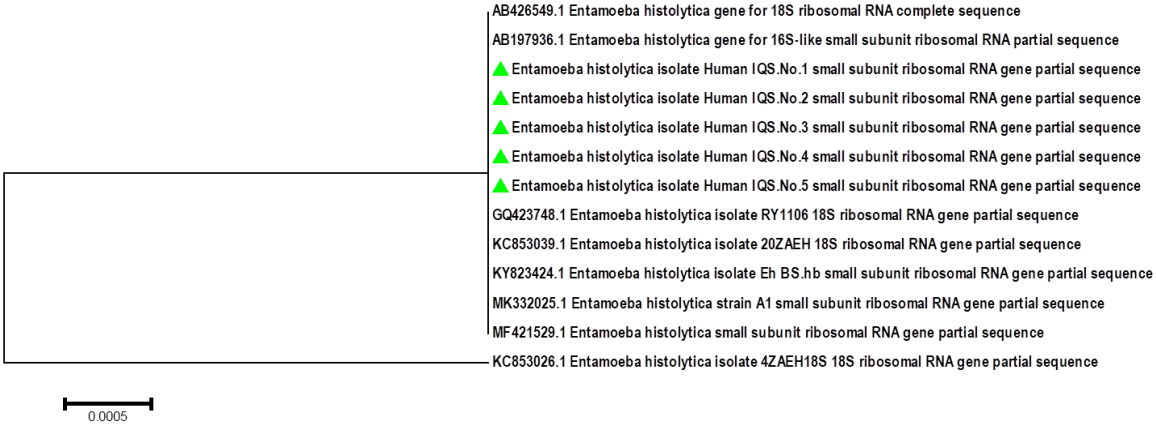

Figure 5: The phylogenetic tree analysis for local *Entamoeba histolytica* isolates depending on 18S rRNA gene partial sequence. The isolates No.1-No.5 closely related with isolate of *E. histolytica* NCBI-BLAST (GQ423748.1) with (0.0005%) total genetic changes

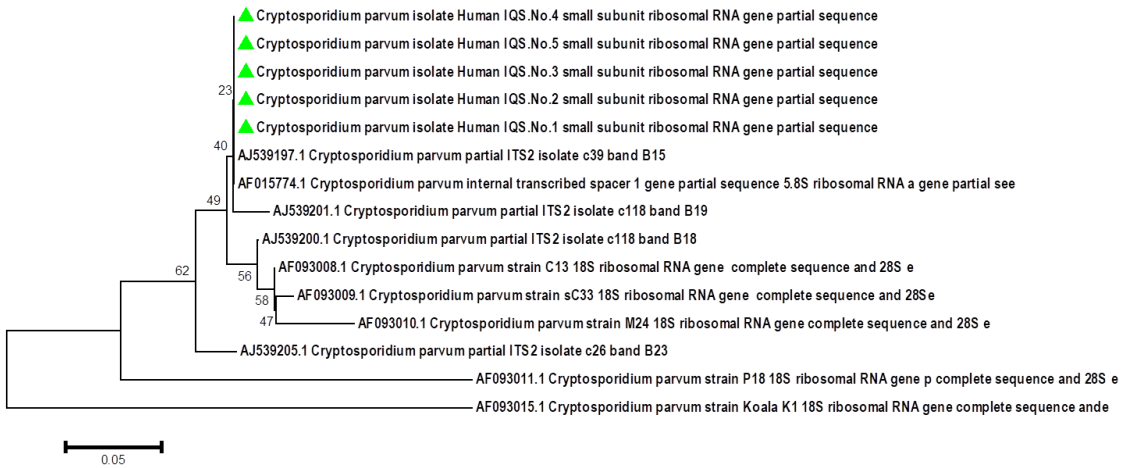

Figure 6: The phylogenetic tree analysis for local *Cryptosporidium parvum* isolates depending on 18S rRNA gene partial sequence. The isolates No.1-No.5 closely related with isolate of *C. parvum* NCBI-BLAST (AJ539197.1) with (0.05%) total genetic changes

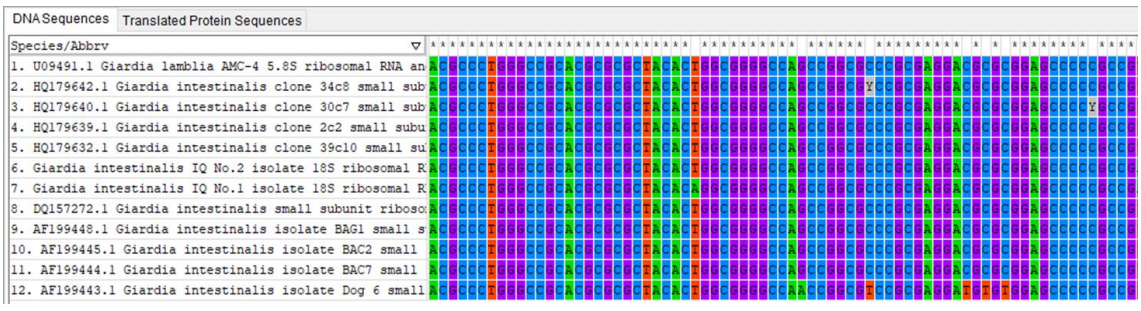

Figure 7: Multiple sequence alignment analysis of the 18S rRNA gene in local human *Giardia lamblia* isolates and the NCBI-Genbank isolate. Multiple alignment analysis was generated using ClustalW's alignment tool (MEGA version 6.0). This showed similar nucleotide alignments as (*) and substitution mutations in the 18S ribosomal gene

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Figure 8: Multiple sequence alignment analysis of 18S rRNA gene in local *Entamoeba histolytica* human isolates and NCBI-Genbank *E. histolytica* isolates. The multiple alignment analysis was constructed using ClustalW alignment tool in (MEGA 6.0 version). That showed the nucleotide alignment similarity as (*) and substitution mutations in 18S rRNA gene

Figure 9: Multiple sequence alignment analysis of 18S rRNA gene in local *Cryptosporidium parvum* human isolates and NCBI-Genbank *C. parvum* isolates. The multiple alignment analysis was constructed using ClustalW alignment tool in (MEGA 6.0 version). That showed the nucleotide alignment similarity as (*) and substitution mutations in 18S rRNA gene

these enteric protozoans are related to impaired childhood progress (Tarleton et al., 2006). In endemic locations, poverty, a lack of or limited access to acceptable water sources and health services and inadequate hygiene and sanitation practices are the main risk factors for these gastrointestinal infections (Berkman et al., 2002).

However, there are several basic data essential to descript a parasites classification such as differences of life-cycle, genetic variations, virulence compatibility, and methods of spread (Verweij et al., 2003). PCR is a fundamental molecular method to alleviate various specificity and sensitivity issues that is conventionally related to the detection of protozoan pathogens. Several PCR-based methods are developed for molecular identification of protozoan infections (Wang et al., 2004).

The direct observation of stool by using light microscope is routine method to detect intestinal protozoan parasites. But this method has less sensitivity; additionally, it depends on expertise (McHardy et al., 2014). Due to the limitations of the microscopic methods, new-substitutional molecular methods are developed based on parasitic DNA or antigens (Verweij and Stensvold, 2014). The molecular tools provide valuable information that help understanding epidemiology, population genetics, and taxonomy. In addition, these methods include several advantages for intestinal parasites diagnosis such as high specificity and sensitivity (Sow et al., 2017).

The 18S rRNA gene is the primary nucleic acid component of the RNA transcription unit of eukaryotes in all parasitic organisms. The sequence of this region examined for a variety of dissimilar organisms that produce a lot of databases about sequence comparison (Hamzah et al., 2006). Moreover, this region is conserved at high levels; therefore, phylogenetic tree depends on it for analysis in the molecular study for enteric protozoan (Malaa et al., 2019). The sequences of 18S rRNA region

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for gastrointestinal Iraqi protozoan isolates in the current study (*G. lamblia*, *E. histolytica*, and *Cryptosporidium* spp.) was used to draw phylogenetic tree with the Iraqi isolations of *Giardia* spp., *Cryptosporidium* spp., and *Entamoeba* spp. deposited in GenBank. The studied isolates were near to *G. lamblia* (DQ157272.1), *E. histolytica* (GQ423748.1), and *C. parvum* (AJ539197.1).

Conclusion

Gastrointestinal symptoms in the individuals with enteric protozoan species (*G. lambilia*, *E. histolytica*, and *C. parvum*) can be diagnosed directly by molecular detection and phylogenetic tree analysis with satisfying results. As well as, it can be utilized like a target for therapeutic intervention for these enteric protozoans.

Author contributions

D.K.K. collected the stool samples and did the experiments; D.A.A. wrote the manuscript. Both authors read and approved the final manuscript.

Conflicts of interest

The authors declared no conflict of interest.

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