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MULTIPLEX PCR FOR DETECTION OF *CYCLOSPORA CAYETANENSIS* IN IMMUNOCOMPROMISED PATIENTS AT WASIT PROVINCE

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Abstract

The present study sought to design and execute a multiplex polymerase chain reaction (m-PCR) for the detection of three parasite infections in immune-compromised patients : *Cyclospora cayetanensis* , *Entamoeba histolytica* and *Cryptosporidium parvum* in patients at Wasit Province, Iraq. The three targeted parasites were effectively identified by the study through the development and use of a multiplex PCR. The results of the current study appeared that 60% of cases were *E.histolytica*, 23% were *C. parvum*, and 17% were *C.cayetanensis*. It was more common in males 62 (62 %) than females 38 (38 %); however, there was no discernible variation in the incidence of *C. cayetanensis* or *C. parvum* between the sexes. As people aged, the frequency of all three parasites dropped. The oldest age group (51–60 years old) having the highest frequency (29%) and the youngest age group (1–10 years old) having the lowest prevalence (4%), the positive percentage gradually increase with age. The results of this study can guide public health initiatives in the area and further knowledge of opportunistic parasite infections in immunocompromised people. In immunocompromised individuals, multiplex PCR provides a sensitive and specific way to diagnose these parasitic infections.

Keywords: Immunocompromised, m-PCR , feces, parasitic infections

Introduction

Cyclospora cayetanensis is a disease that is widely distributed around the world [1]. It is categorized under the Coccidia subgroup of the family Eimeriidae under the phylum Apicomplexa [2]. *C. cayetanensis* is an obligatory intracellular protozoan parasite that was formerly known as cyanobacterium-like body (CLB). It is closely linked to the genus Eimeria. It mostly affects the upper small intestine's epithelial cells, which results in cyclosporiasis [3], a tropical illness that is frequent in tropical nations and a factor in traveler's diarrhea. The parasite is now acknowledged as an emergent pathogen affecting both immunocompromised and immunocompetent individuals globally [4]. The increasing danger of contracting rare and exotic tropical diseases is a result of travel, fresh food consumption, and globalization of the food supply. Fresh vegetables imported into developed countries from underdeveloped nation’s raises the risk of coming into touch with endemic parasites from other areas.

Cryptosporidiosis is an emerging zoonotic disease in humans and animals that contributes to intestinal and extra-intestinal diseases. The major factor controlling the susceptibility and severity

of cryptosporidiosis appears to be the immune status of the host [5]. Those with great risk of infection including the immunocompromised patients [6,7]. They are including AIDS and T.B patients and those with various malignant, cytotoxic drugs receivers, prolonged corticosteroid therapy, the drugs used to prevent organ transplant rejection [4]. Those with chronic diseases and persons who have congenital immunodeficiency's [8,9]. In such patients, cryptosporidiosis is characterized by painful, persistent, frequently cholera-like diarrhea along with extreme abdominal colic, body weight loss of more than 10%, and dehydration [10].The infection is transmitted through the fecal-oral route and results from the ingestion of *Cryptosporidium* oocysts through the intake of food, water or direct contact with individuals or animals. It has been documented that infected individuals shed 108-108 oocysts in a single bowel movement and excrete oocysts for up to 50 days after diarrhea cessation [11].

Entamoeba histolytica is the causative agent of amoebic liver abscess, diarrhea, and colitis. Its severe morbidity and mortality rate accounts for one hundred thousand fatalities every year [12]. Advances in comprehending the biochemical, immunological, and genetic distinctions between *Entamoeba dispar* and *E. histolytica* have resulted in their reclassification as distinct species. Although they are morphologically identical, microscopy cannot discriminate between the two on their own; PCR or antigen detection are required as supplementary techniques [13].

The current study aimed to focus on the development and implementation of Multiplex PCR for identification of three parasite infections; *C.cayetanensis* , *E. histolytica*, and *C.parvum* in immunocompromised patients at Wasit Province, Iraq.

Materials and Methods

Sample Collection

A total of 100 stool samples were taken from immunocompromised patients at the Center for Genetic Blood Diseases (Thalassemia) and Al-Karamah Teaching Hospital at Wasit Province, Iraq. The patients' ages and sexes were taken into account while grouping the samples. Each sample container's label had the patient's name and a unique identification number. The stool samples were collected during October and April, 2023. After that, each stool sample was divided into two parts ; the first was frozen at -20°C for a subsequent multiplex PCR analysis, and the second was direct examined with the Modified ZN stain by microscope [14].

1. Direct Stool Examination: Using the Modified Ziehl-Neelsen stain method, a total of 100 stool samples were examined under a microscope in order to identify the parasites [15].
2. Molecular Diagnosis: *E. histolytica*, *C. parvum* and *C. cayetanensis* were detected using multiplex PCR approach based on ribosomal RNA small subunit.

Primers

Using NCBI-Genbank ; 3 primers were design, the multiplex PCR primers for the detection of *E. histolytica*, *C. parvum* and *C. cayetanensis* based on small subunit ribosomal RNA gene were generated in this work. The following table shows the primers that were given by Scientific Researcher .Co. Ltd, Iraq:

Table 1: PCR primers for *E. histolytica, C. parvum* and *C. cayetanensi*

Primers	Sequence 5'-3'		Product size
E. histolytica	F	GGACAATGCTGAGGGGATGT	389 bp
	R	GTGCCCTTCCGTCAATTCCT	
C. parvum	F	GGGTATTGGCCTACCGTGG	669 bp
	R	AGACTACGACGGTATCTGATCG	
C. cayetanensis	F	TTAATTGCGTGTGTTGGCCC	242 bp
	R	TCCTTGGCAAATGCTTTCGC	

Stool DNA Extraction

Genomic DNA was extracted from stool samples using (Stool DNA extraction Kit, Bioneer. Korea). The extraction was done according to company instructions using stool lysis protocol method with Proteinase K [16]. After that, the extracted gDNA was checked by Nanodrop spectrophotometer, and then stored at -20°C until used in PCR amplification.

Multiplex PCR master mix preparation

The preparation of the Multiplex PCR (mPCR) master mix was conducted utilizing the GoTaq Green PCR Master Mix, following the company's instructions as outlined in the provided table. The first master mix, designed for Multiplex PCR, included a total volume of 50µL. The components comprised 5µL of DNA template (ranging from 5 to 50ng), forward and reverse primers for specific targets (*E. histolytica*, *C. parvum* and *C. cayetanensis*), each at 2µL, and 25µL of GoTaq Green Master Mix. Nuclease-free water was added to achieve a final volume of 50µL. This master mix formulation adheres to the recommended guidelines for conducting Multiplex PCR assays, facilitating the simultaneous amplification of target DNA sequences for the specified pathogens.

Results

Table 2: Prevalence of Intestinal Parasites in Stool Samples

Parasite	Number of positive cases	Total cases	Prevalence (%)
E. histolytica	60	100	60
C. parvum	23	100	23
C. cayetanensis	17	100	17

The table presents a comprehensive overview of the prevalence of three parasitic infections *E. histolytica*, *C. parvum* and *C. cayetanensis* within a sample population. Out of 100 total cases examined, 60 individuals tested positive for *E. histolytica*, resulting in a prevalence rate of 60 %. Similarly, 23 cases were positive for *C. parvum* , representing a prevalence of 23 %. Additionally, 17 cases were positive for *C. cayetanensis*, yielding a prevalence rate of 17 %. These findings underscore the importance of understanding and monitoring the prevalence of these parasitic infections in the studied population.

Table 3: Distribution of intestinal parasite infection according to the sex

Parasite	Males %	Females %	Total (%)
E. histolytica	**38 (38 %)**	**22(22%)**	**60**
C. parvum	**14 (14 %)**	**9 (9 %)**	**23**
C. cayetanensis	**10 (10 %)**	**7 (7 %)**	**17**
Total	**62 (62 %)**	**38 (38 %)**	**100 %**

The frequency of three parasite infections— *C. cayetanensis, C. parvum* , and *E. histolytica*—is shown in the table according to sex. Male make up 62% of the study population, while female make up 38%. The percentages show what percentage of people in each sex category tested positive for the corresponding parasite. Thirty-eight percent of the males tested positive for *E. histolytica,* suggesting that this parasite is significantly more common in the male subgroup. *E. histolytica* was found in 22% of females, which indicates a little lower prevalence than in males. The prevalence of *C. parvum* in the males had 14 %, which suggests that there is a significant incidence in the male population while in females was 9 %, indicating a comparatively less incidence in comparison to males. *Cyclospora cayetanensis* had a moderate presence as shown by the 10% frequency of this parasite in the male while it prevalence in females was 7%, indicating a comparable less incidence to that in males.

Table 4: Prevalence of Three Intestinal Parasites according to the Age

Age groups Years	Positive	Positive percentage
1-10	4	4 %
11-20	12	12 %
21-30	17	17 %
31-40	16	16 %
41-50	22	22 %
51-60	29	29 %
Total	100	100 %

Table 4 summarizes the results of the present study, displaying the prevalence of a specific parasite across different age groups. It shows:

The total positive rate is 100% with the oldest age group (51–60 years old) having the highest frequency (29%) and the youngest age group (1–10 years old) having the lowest prevalence (4%), the positive percentage gradually increase with age. The noted increase in the positive proportion with aging may indicated many factors: Age-related immunity: As people age, they may become more prone to infection due to a degree of immunity they have decreased against the parasite. Effects of cohorts: Various age cohorts could have been exposed to different environmental or social factors that influenced their risk of infection.

Table 5: Statistical Association among *E. histolytica*, *C. parvum* and *C. cayetanensis* Infection and Age Groups

Parasite	Chi-squared statistic	Degrees of freedom	P-value	Result
E. histolytica	7.44	5	0.190	No significant difference
C. parvum	3.61	5	0.607	No significant difference

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C. cayetanensis	**2.44**	**5**	**0.785**	**No significant difference**

The findings of chi-squared tests used to examine the relationship between age groups and parasite infection (*E. histolytica, C. parvum* and *C. cayetanensis*) are summarized in the table 5. A statistical indicator of how strongly age groups and parasite infection are found to be associated.

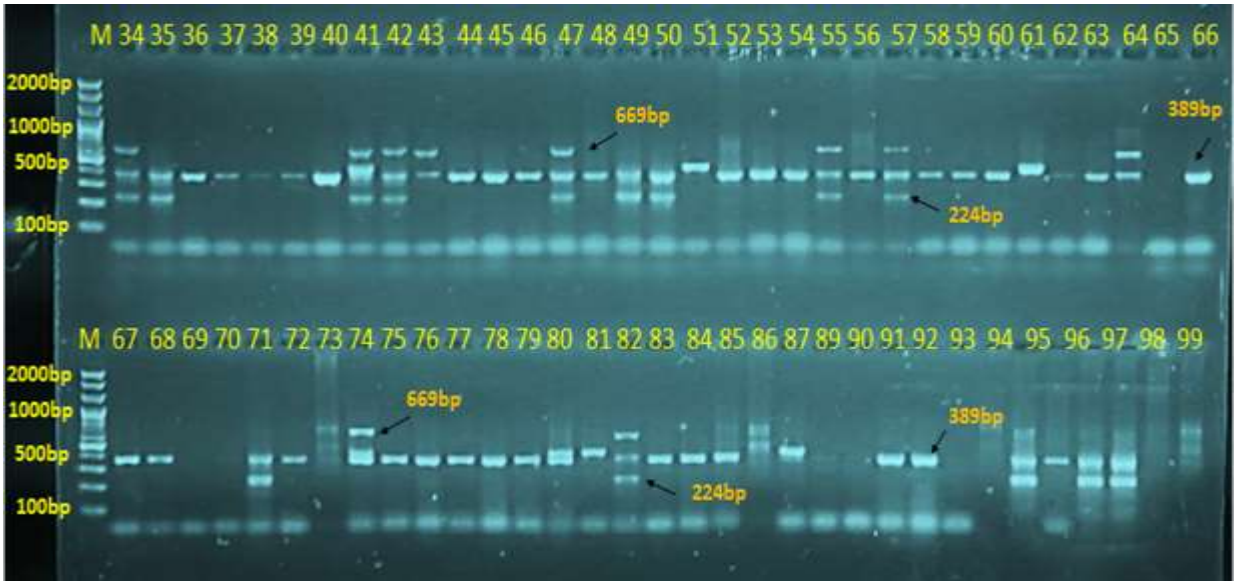

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Figure (1): Agarose gel electrophoresis image that showed the Multiplex PCR product analysis of small subunit ribosomal gene to detect *E. histolytica*, *C. parvum* and *C. cayetanensis* from human stool samples. Where, the Lane (M): DNA marker ladder (100-2000bp) and the Lane (1-99) showed positive *E. histolytica*, *C. parvum* and *C. cayetanensis* at (389bp, 669bp, and 224bp PCR product size) respectively.

Discussion

The results of the current study was appeared that 17 cases were positive for *C. cayetanensis*, yielding a prevalence rate of 17 %. These findings underscore the importance of understanding and monitoring the prevalence of these parasitic infections in the studied population. The *Cyclospora cayetanensis* infection rate found in the present study was in agreement with other earlier studies published in Egypt [17,18] which found that the infection rate was (24.5%) and (25%) respectively utilizing PCR testing. The incidence of cyclosporiasis was first documented in India [15] where it was found that the prevalence rate of *C. cayetanensis w*as 89%. However, Mundaca's study [20] recoded 57.1% in Lima and Peru, indicating that the highest prevalence was found in India due to the endemic nature of *C. cayetanensis* in India. Additionally, it seems to be the more prevalent illness in tropical and subtropical regions, whereas this protozoan is endemic in Nepal, Haiti, Guatemala, Peru, and Peru. Travelers who give more detailed information regarding *Cyclospora* infection have a greater incidence of *C. cayetanensis* in developing nations than in Europe and North America. This parasite causes diarrheal illness in travelers [19, 20].

Cryptosporidium parvum is one of the most important intestinal organism that is a principal cause of digestive diseases in humans. This microorganism often causes chronic, and serious intestinal diseases leads to morbidity and mortality [21], therefore, cryptosporidiosis is a considerable public health problem across the world with a high variety of prevalence. Our results showed statistically significant differences between material status (P=0.005) and type of stool (P=0.001). These findings are similar to Mohaghegh *et al* [22] and Izadi *et al* [23-25]. The prevalence of *Cryptosporidium* infection in immunocompromised patients in Iran was reported as 4.7% in Isfahan [26], 11.5% in Kashan [27], 35.9% in Mashhad [28] and 0.9% in Tehran [29]. Epidemiological studies in Ethiopia, India, Egypt, Cameron, Malaysia, Indonesia, China, Australia, Turkey, Philippines, Iraq and Uganda was 13.2%, 21%, 60.2%, 19%, 12.4%, 4.7%, 8.3%, 2.3%, 4%, 1.9%, 18.9% and 25% respectively [30,31]. It is relatively common in Iraq especially in the southern part. So, cryptosporidiosis among patients can be considered as the main cause of morbidity and mortality among those patients. The mechanisms contributing in the increased vulnerability to infection are splenic dysfunction, decreased IgM levels and defective alternative complement pathway [32].

Numerous studies have been confirmed that *E. histolytica* can infect the males more than females because of their formal structure. During the past three years, an unusual increase in *E. histolytica* was observed between the ages of (10-50) years old. The present study found that the percentage of infected males was higher than that of females. One of the most important reasons

for this is the inability to track children's behavior and toys over time, as well as contamination of food, clothing and even bedding. Moreover, the hormonal effects are different depending on the sex therefore will effect on the immune system and the accuracy of its effectiveness. This can be explained by the possibility of a moderately active immunity that led to a weak response. This view was consistent with other studies which demonstrated a weak immune response of IgA against the CRD active site of *E. histolytica* [33].

Conclusion

It is crucial to recognize the significance of opportunistic parasite infections in immunocompromised individuals. PCR is regarded as an alternate method for diagnosing *C. cayetanensis*, *E. histolytica*, and *C. parvum* in epidemiological investigations. The outcomes demonstrated the PCR's sensitivity in detecting C. *cayetanensis* in stool samples.

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