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MULTIPLEX PCR FOR DIAGNOSIS OF *ENTAMOEBA HISTOLYTICA* IN IMMUNOCOMPROMISED PATIENTS AT WASIT PROVINCE

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Abstract:

Immunocompromised individuals at Wasit Province, Iraq, face an elevated risk of intestinal parasitic infections, particularly from protozoan pathogens like *Entamoeba histolytica, Giardia lamblia, and Cyclospora cayetanensis.* Conventional diagnostic methods often lack sensitivity, leading to under diagnosis and delayed treatment. This study aimed to investigate the prevalence and distribution of these parasites in immunocompromised individuals, emphasizing the need for accurate detection methods. The three major protozoa causing diarrhea worldwide are Enteric *Giardia lamblia, Entamoeba histolytica, and Cyclospora cayetanensis.* The study collected stool samples from patients at Wasit Province, Iraq, and employed both direct stool examination and molecular diagnostic techniques, such as multiplex PCR, for accurate parasite detection. The prevalence of *E. histolytica* was notably high at 96.00%, emphasizing its significant presence in the studied population. *G. lamblia and Cyclospora cayetanensis.* exhibited lower prevalence rates at 12.00% and 21.00%, respectively, suggesting distinct subpopulations or transmission dynamics. Gender-specific and age-specific analyses revealed variations in parasite prevalence. Females showed a higher prevalence of *E. histolytica*, highlighting potential gender-specific risk factors. Age-specific patterns indicated consistent high prevalence of *E. histolytica* across all age groups, suggesting widespread exposure or shared risk factors. *G. lamblia* exhibited age-related susceptibility, and *C. cayetanensis.* showed a mild downward trend with age.

Multiplex PCR analysis and chi-square tests were conducted to assess possible co-occurrence of parasites. Results indicated independence in parasite prevalence, emphasizing the importance of considering various transmission pathways and demographic vulnerabilities in developing parasite management strategies. The study concludes that immunocompromised individuals in Wasit Province are highly susceptible to intestinal parasitic infections, with *E. histolytica* being the predominant pathogen. The findings underscore the need for tailored prevention and control strategies, taking into account the unique characteristics of each parasite.

Keywords: Modified Ziehl Neelson, multiplex PCR, Stool, Human, Immunocompromised

Introduction

Immunocompromised individuals at Wasit Province, Iraq, face a heightened risk of intestinal parasitic infections, particularly from protozoan pathogens like *E.histolytica*, *G.lamblia*, and *C.cayetanensis* . Unfortunately, traditional diagnostic methods often lack the sensitivity and specificity required for accurate detection, leading to under diagnosis and delayed treatment. This can have dire consequences, as these parasites can cause debilitating symptoms and even life-threatening complications in vulnerable individuals.

Enteric *G. lamblia, E. histolytica,* and *C. cayetanensis*. are the three major protozoa that cause diarrhea worldwide. In wealthier nations, these parasite infections pose a significant threat to public health [1], but they also significantly increase morbidity and mortality rates, especially in children residing in undeveloped nations with limited resources [2-7].These three species of protozoa really account for as much as 70% of the gastrointestinal parasites that are found every year in hospital-based microbiology laboratories across Europe [3]. Moreover, there is growing recognition that these intestinal parasites are important waterborne and foodborne pathogens on a worldwide scale [4].

Intestinal parasite infections continue to be the most prevalent form of parasitic illness and are one of the leading causes of morbidity and death worldwide, particularly in poor nations [6]. The coccidian parasites, which include *Cryptosporidium, Cyclospora,* and *Cystoisospora*, are the most common intestinal parasites worldwide and are responsible for epidemic outbreaks and traveler's diarrhea. Immunocompetent and immunocompromised people have the same risk factors for acquiring these illnesses. immunocompromised patients, however, suffer from a more severe or widespread infection because they are unable to eliminate parasites upon exposure [8,9]. Patients with intermittent constipation who have coccidian infections typically have watery diarrhea with variable degrees of dehydration and malabsorption [8-10].

Because infections can cause life-threatening chronic diarrhea and other clinical symptoms in immunosuppressed patients, they pose a considerable danger to patient health. Despite this, routine detection of these infections is frequently overlooked during chemotherapy or other illnesses [8-11]. Furthermore, prior findings have shown that the incidence of parasite infection in immunocompromised individuals is never low [12,13]. Nevertheless, not enough research has been done on the frequency of these illnesses in infants with compromised immune systems. Thus, the purpose of this study is to identify the prevalence and geographic distribution of intestinal parasites in immunocompromised individuals experiencing diarrhea at Wasit Province. Since it is challenging to identify oocysts using light microscopy, the laboratory diagnosis of intestinal protozoan infections depends on specialized staining procedures, such as modified Ziehl Neelson, modified acid-fast, and Giemsa [14]. The diagnostic success of the usual approach for identifying parasitic infections—microscopic identification—largely depends on the experience of the investigators since the morphological traits, size, and staining affinity of infectious entities vary. Consequently, in order to boost sensitivity and raise the likelihood of an effective diagnosis, other diagnostic methods such polymerase chain reaction (PCR), enzyme immunoassays, and direct fluorescent-antibody tests must be used.

Materials and Methods:

Sample Collection

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The stool samples collected from patients attending Al-Karamah Teaching Hospital at Wasit Province, Iraq. The samples were categorized based on patient gender and age. Each sample container was labeled with a unique identifier and the patient's name. Subsequently, each stool sample was divided into two portions: one was fixed for the Modified ZN stain to examine by light microscope and the other for kept at -22°C for future multiplex PCR analysis [15].

1-Direct Stool Examination: A total of 100 stool samples were examined with a microscope by staining using the Modified Ziehl-Neelsen stain technique [16].

2- Molecular Diagnosis: Multiplex PCR technique was performed for detection *E.histolytica, G.lamblia* and *C. cayetanensis* Based on small subunit ribosomal RNA gene from stool samples.

Primers

The multiplex PCR primers for detection *E. histolytica, G. lamblia, C. cayetanensis,* and based on small subunit ribosomal rRNA genes were designed in this study using NCBI-Genbank and primer 3 plus design. These primers were provided from Scientific Researcher. Co. Ltd.

Table 1: PCR primers for *E. histolytica, G. lamblia and C. cayetanensis*

Primers	Sequence 5'-3'		Product size
Entamoeba histolytica	F	GGACAATGCTGAGGGGATGT	389 bp
	R	GTGCCCTTCCGTCAATTCCT	
Giardia lamblia	F	CCAAGACCGCCTCTGTCAAT	445 bp
	R	GGGCATCACAGACCTGCTAT	
Cyclospora cayetanensis	F	GCTTGTCGCCCTGAATACTTC	288 bp
	R	GCAAGGTAGGCGTTTCCCTA	

Multiplex PCR

Multiplex technique was performed for detection *E. histolytica, G. lamblia,* based on small subunit ribosomal rRNA genes from human stool samples. This method was carried out according to company instructions [3].

Stool DNA Extraction

Stool DNA extraction was performed using the Presto™ Stool DNA Extraction Kit following the manufacturer's instructions. Briefly, the stool sample was lysed in ST1 buffer at 70°C, followed by bead beating and centrifugation. The supernatant was then mixed with ST3 buffer and loaded onto a GD Column. After washing with ST3 and Wash Buffers, the GD Column was centrifuged to dry. Finally, preheated Elution Buffer was added to elute the purified DNA. This concise paragraph effectively summarizes the key steps of the DNA extraction procedure while incorporating the essential details from the provided text.

Results

Table 2: Prevalence of Intestinal Parasites in Stool Samples

Infectious Agent	Number of Examined Samples	Positive Samples (%)
E. histolytica	100	96.00
G. lamblia	100	12.00
C. cayetanensis	100	21.00

Table 2 aimed to determine the prevalence of three common intestinal parasites*: E. histolytica, G. lamblia, and Cyclospora cayetanensis*., in 100 stool samples. The results revealed a striking difference in parasite prevalence: *E. histolytica* exhibited the highest burden, with 96% of samples testing positive. This highlights its significant presence in the studied population. *G. lamblia* and *C. cayetanensis* showed lower prevalence, with 12% and 21% positive samples, respectively. This suggests their presence in distinct subpopulations within the studied groups.

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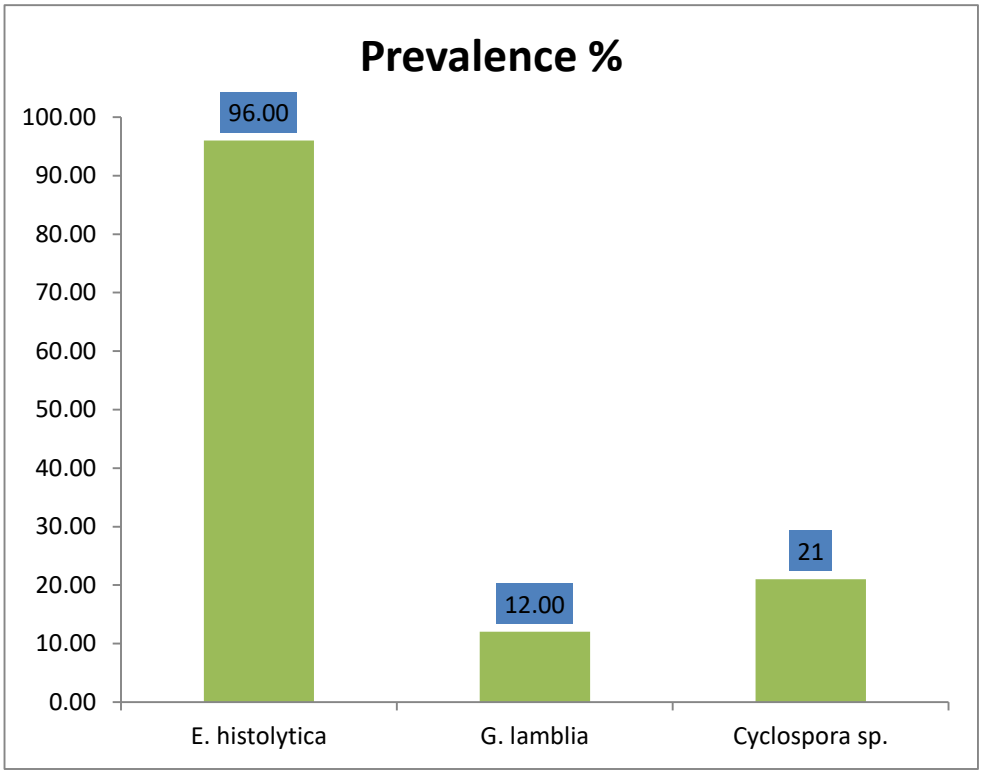

Figure1: Prevalence of intestinal parasites at Wasit province

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Intestinal Parasite	Infected Male (n=40)	Infected Female (n=60)	Total (n=100)	Prevalence (%)
E. histolytica	36 (85%)	60 (10 %)	96	96 %
G. lamblia	5 (12.50%)	7 (11.67%)	12	12 %
C. cayetanensis	8 (20 %)	13 (21.67%)	21	21 %
Co-infection	3 (7.50%)	1 (1.67%)	4	4 %

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

A total of 100 participants (40 male and 60 female), the prevalence and sex-specific distribution of three common intestinal *parasites E. histolytica, G. lamblia, and C. cayetanensis* were examined as shown in table 3. An examination of co-occurrence patterns and infection rates sheds light on possible risk factors and the dynamics of transmission. Where substantially higher than in male (85.00%), indicating the possibility of sex-specific risk factors. *E. histolytica* had the highest overall prevalence of 96.0% along with a noticeable tendency of female dominance. Females had a 100% infection rate with *E. histolytica; G. lamblia* and *C. cayetanensis* had lower prevalence rates (12 % and 21%, respectively). Notably, there were no appreciable sex-based variations in their occurrence, which was comparatively equal in males and females. The percentage of co-infection with several parasites was 0.00%. Male co-infection rates were notably higher (7.50%) than female co-infection rates (1.67%), indicating possible sex-specific interactions between parasites. These results show interesting differences in parasite prevalence and co-occurrence patterns between the genders. The high frequency of *E. histolytica* and the fact that it is more common in females indicate the presence of sex-specific risk factors, which might be linked to hygiene practices or socioeconomic determinants of health.

Parasite	20-29 years	30-39 years	40-49 years	50-59 years	60-69 years	Total %
E. histolytica	12 (92.31%)	16 (81.82%)	21 (87.50%)	16 (80.00%)	14 (82.35%)	96.00%
G. lamblia	1 (7.69%)	3 (13.63%)	2 (8.33%)	1 (4.76%)	1 (5.88%)	12.00%
C . cayetanensis	3 (23.08%)	4 (18.18%)	4 (14.81%)	1 (4.76%)	2 (11.76%)	21.00%
Co-infection	2 (15.38%)	1 (4.55%)	1 (3.70%)	1 (4.76%)	1 (5.88%)	5.26%

Table 4: Distribution of intestinal parasite according age groups

Table 4 explored the prevalence and age distribution of three common intestinal parasites *E. histolytica, G. lamblia, and Cyclospora sp*. in a sample of 100 individuals across five age groups (20-29, 30-39, 40-49, 50-59, and 60-69 years). Examining parasite prevalence within each age group provides insights into potential life-stage susceptibilities and transmission dynamics. *E. histolytica* exhibited a consistently high prevalence across all age groups, ranging from 80.00% to 92.31%, highlighting its widespread presence regardless of age. This suggests persistent exposure or shared risk factors across all age segments. *G. lamblia* and *C.cayetanensis* showed lower and more variable prevalence patterns. *G. lamblia's* prevalence peaked in the 30-39 age group (13.63%) and declined progressively, suggesting potential age-related susceptibility or changing exposure patterns. *Cyclospora cayetanensis* showed a mild downward trend with age, with the highest prevalence in the 20-29 age group (23.08%). Co-infection rates were relatively low overall (5.26%). Interestingly, the highest co-infection rate (15.38%) occurred in the 20-29 age group, suggesting potential age-specific interactions between parasites or increased susceptibility during younger years. These findings reveal a nuanced picture of age-related parasite prevalence patterns. *E. histolytica* consistent presence across ages suggests broad community exposure or age-independent risk factors. *G. lamblia* declining prevalence with increasing age suggests potential acquisition during specific life stages or changes in exposure patterns. *Cyclospora cayetanensis* mild downward trend could indicate age-related changes in susceptibility or environmental exposure. Notably, the higher co-infection rate in younger individuals warrants further investigation into potential age-specific interactions or vulnerabilities. Understanding the age-

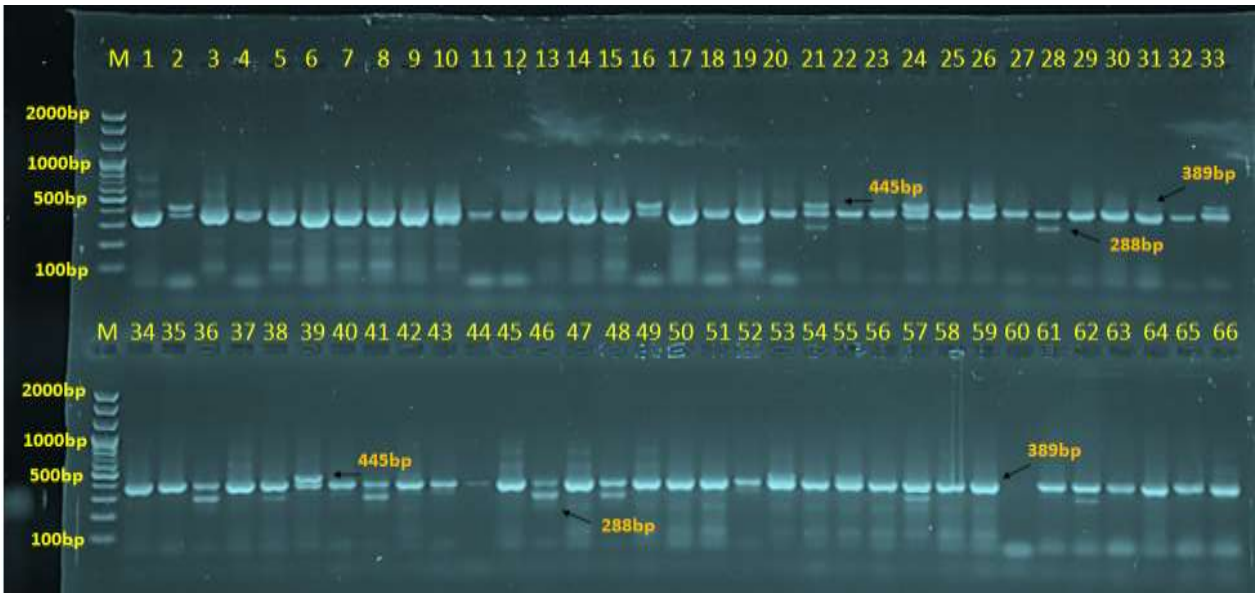
specific dynamics of intestinal parasite prevalence is crucial for targeted prevention and control

Parasite Pair	Feature	Positive	Negative	Total	χ^2	df	p-value	Interpretation
E. histolytica* vs. *G. lamblia	***G. lamblia* Positive**	**12**	**84**	**96**	**0.55**	**1**	**0.42**	**Prevalence independent**
	***G. lamblia* Negative**	**84**	**4**	**88**				
	Total	**96**	**88**	**100**				
E. histolytica* vs. *C. cayetanensis	***C.cayetanensis* Positive**	**21**	**75**	**96**	**1.06**	**1**	**0.29**	**Prevalence independent**
	***C.cayetanensis* Negative**	**75**	**4**	**79**				
	Total	**96**	**79**	**100**				
G. lamblia* vs. *C. cayetanensis	***C.cayetanensis* Positive**	**4**	**17**	**21**	**0.96**	**1**	**0.33**	**Prevalence independent**
	***C.cayetanensis* Negative**	**8**	**71**	**79**				
	Total	**12**	**88**	**100**				

strategies. More cases of *E. histolytica* have been found in the feces of patients with diarrhea.

Table 5 : Independence of the parasite prevalence in Immunocompromised patients

Table 5 examined the possible co-occurrence in a sample of one hundred individuals of three common intestinal parasites*: E. histolytica, G. lamblia, and C.cayetanensis.* We sought to determine if the presence of one parasite affected the presence of the others by utilizing chi-square analysis. 96.00% of the positive samples showed a dominant presence of *E. histolytica*. This demonstrates how common it is in the population under study. *C.cayetanensis* and *G. lamblia* had different patterns of predominance. In 12.00% of the samples, *G. lamblia* was found, suggesting that it was present in a smaller subpopulation. In 21.00% of the samples, *C.cayetanensis* was found, indicating a different mode of transmission or a population vulnerability. Most importantly, chi-square analysis (p-values > 0.05) did not show a statistically significant correlation between any two parasite pairs' prevalence. This implies that the probability of discovering another parasite is not greatly affected by the existence of one. These results depict different parasite environments in the population under study. The prevalence of *E. histolytica* indicates that some environmental or behavioral variables may be helpful in its transmission, which calls for further research to be done on its epidemiology. Significantly, the distinct frequency of *C.cayetanensis* and *G. lamblia* indicates different transmission patterns or population compositions vulnerable to these parasites. This emphasizes the requirement for customized control plans that are unique to every parasite.



The observed independence in parasite prevalence emphasizes how crucial it is to take into account a variety of transmission pathways as well as demographic vulnerabilities when developing parasite management strategies.

Figure (1): Agarose gel electrophoresis image that showed the Multiplex PCR product analysis of small subunit ribosomal gene to detect *E. histolytica, G. lamblia , and C. cayetanensis* from human stool samples. Where, the Lane (M): DNA marker ladder (100-2000 bp) and the Lane (1-99) showed positive *E. histolytica, G. lamblia , and C. cayetanensis* at (389bp, 445bp, and 288bp PCR product size) respectively

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Discussion

Immunocompromised individuals in Wasit Province, Iraq, are at an increased susceptibility to intestinal parasitic infections, notably from protozoan pathogens such as *E. histolytica, G. lamblia, and C. cayetanensis.* Among these parasites, *E. histolytica* exhibited the highest infection rate at 96%. Comparing our findings with those from other studies conducted in Baghdad [22] (23%), (23) (21.8%), Mosul [24] (23.8%), Kirkuk [25] (19.8%), Tikrit [26] (23.3%), Dohok (27) (25.2%), and Erbil [28] (8.15%), the heightened prevalence of *E. histolytica* in this study other than previous studies due to since the samples for this study were collected from immunocompromised patients, and this may constitute a difference in the prevalence the heightened prevalence of *E. histolytica* in our study may be attributed to its rapid transmission through the ingestion of cysts primarily transmitted via the oral-fecal route in contaminated food and water. The role of houseflies in infection transmission is significant, with the incidence of cyclosporiasis among immunocompromised patients being more common in our study compared to similar studies reporting variable *Cyclospora* prevalence ranging from 2.4% to 22.2% in [29, 30, and 31]. The observed disparities may stem from geographical factors and variations in study parameters such as sample size, duration, and recruited patients. Notably, *C. cayetanensis* infection in our study was more prevalent in the immunocompromised group than in other groups, aligning with findings in other studies [32,33]. The findings indicate a clear dominance of *E. histolytica*, potentially reflecting unique environmental or risk factors associated with its transmission in this population this agree with [17]. The presence of *G. lamblia* and *C. cayetanensis*, although lower, suggests diverse routes of infection or specific population demographics susceptible to these parasites [18].

The other most prevalent intestinal identified was *G. lamblia*, exhibiting a prevalence rate of 12%. The occurrence of this protozoan is attributed to factors such as sanitary conditions, personal hygiene, and environmental elements. Additional contributing factors include a shortage of drinking water in schools and contamination of water from pipes. Comparisons with parasitic rates in other Iraqi cities revealed higher prevalence rates in specific areas, such as Baghdad (78%) [22], Kirkuk (53%) [25], and ranges of 40-43% [27]. Data from the annual report of the Center for Control of Communicable Diseases in Iraq [34] indicated rates of 59.98%, with findings in Kirkuk (23.2%), Basra (29.8%), Najaf (24.8%), Sulaimaniya (25% among school children), and Kirkuk (32.88%). Disparities in prevalence rates among these studies may be attributed to variations in factors such as date, sampling techniques, alterations in sanitary conditions, and economic practices. Notably, distinctions were evident in the prevalence of intestinal parasites between middle and high socio-economic districts, with lower socio-economic status and poor hygiene and sanitation conditions contributing to these differences [35].

The discrepancy in research outcomes is attributable to distinct characteristics of the study populations, as delineated in our investigation. Notably, our study focused on individuals with compromised immune systems, a demographic that inherently exhibits heightened susceptibility to parasitic infections. A comparative study in Riyadh, Saudi Arabia, revealed a 39.7% prevalence of parasitic intestinal infections among immunocompromised patients and children [43]. Similarly, a study in Ethiopia reported an overall prevalence of intestinal parasitic infection at 65.5% [37]. These variations can be linked to disparities in demographic attributes, geographic sampling dispersion, diagnostic methodologies, respondents' socioeconomic profiles, sample sizes, sampling methodologies, and sanitary conditions.

Furthermore, our study identified our study is consistent with a study conducted for *E. histolytica* in the north of Baghdad [38]. While PCR is a more precise method for detecting intestinal parasites, its application is often impractical in resource-constrained settings, particularly in peripheral laboratories. Therefore, there is a necessity for a more field-friendly and sensitive approach for on-the-spot identification of intestinal parasite infections [39]. For instance, in direct microscopic examination, distinguishing between morphologically indistinguishable *E.histolytica* and *E.dispar* necessitates specific antigen testing in the stool and microscopic inspection as the preferred diagnostic procedures for a reliable diagnosis of *E. histolytica*.

Our investigation also unveiled a significantly higher prevalence of infection in females compared to males. This finding aligns with a study in Sana'a, Yemen, where girls exhibited a greater infection rate (31.5%) compared to boys (24.6%) among children presenting at the Pediatric Centre [40]. Moreover, females demonstrated a higher prevalence of intestinal parasite infection (41.3%) than males (26.4%), a statistically significant difference ($P < 0.05$) observed in Morocco [41]. Similar trends were identified in Tehran, Iran, where the infection rate was significantly lower in males than females (17.4% versus 19.3%) [42], and in Riyadh, Saudi Arabia, where no significant gender-based difference was found among immunocompromised patients [43]. These gender-related variations may be attributed to differential exposure levels, with females engaging in household chores and adopting lifestyles that lead to increased contact with soil, cultivation of vegetables, and consumption of raw vegetables with prepared food.

Conclusion

The present investigation has demonstrated a heightened susceptibility of immunocompromised individuals to parasitic intestinal infections. Conversely, the prevalence of intestinal parasite infections persists as a significant public health concern in Wasit, with *E. histolytica, G. lamblia, and C. cayetanensis* being the most prevalent causative agents. Through statistical analysis, it has been established that deficient health education and inadequate personal hygiene serve as pivotal predictors for parasitic intestinal infections. In light of the efficacious insights provided by this study to mitigate such infections, a concerted, multi-sectoral approach becomes imperative. Comprehensive preventive measures ought to encompass the promotion of optimal health practices and the implementation of expanded educational health initiatives focusing on personal hygiene for both patients and healthcare providers across all provinces. Raising awareness and enhancing environmental conditions, coupled with improvements in the healthcare system, especially for immunocompromised patients who require specialized care, are critical components of an effective strategy to address this public health issue.

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