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Classification of Giardia lamblia Groups and Entamoeba histolytica-Entamoeba dispar Complex Using Molecular Diagnostic Technique (PCR) in Patients from Mosul

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ABSTRACT

Background: Giardia lamblia, Entamoeba histolytica, and Cryptosporidium are among the most common parasitic protozoa causing diarrheal diseases worldwide, and traditionally, the diagnosis of these infections has relied on the microscopic examination of stool samples, considered the "gold standard," also recent advancements in diagnostic techniques, like polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA) and direct fluorescent-antibody tests, have provided more accurate and sensitive methods for detection.

Objectives: This study aims to classify Giardia lamblia and the Entamoeba histolytica–Entamoeba dispar complex using a molecular diagnostic technique (PCR) among patients in Mosul, and the goal is to enhance diagnostic accuracy and efficiency in detecting these parasitic infections, particularly in regions where they are prevalent.

Materials and Methods: A multiplex real-time PCR assay was developed for the simultaneous detection of Giardia lamblia, Entamoeba histolytica, and Cryptosporidium parvum in stool samples, and the assay includes an internal control to ensure PCR efficiency and identify potential inhibition. The assay was validated using species-specific DNA controls and well-characterized stool samples.

Results: The multiplex PCR assay demonstrated 100% sensitivity and specificity when validated on species-specific DNA controls and stool samples, and this method provided accurate, reliable results in detecting the three targeted parasitic protozoa.

Conclusion: This study's multiplex real-time PCR assay offers a highly accurate and efficient diagnostic tool for identifying Giardia lamblia, Entamoeba histolytica, and Cryptosporidium parvum infections in stool samples. Implementing this technique in diagnostic laboratories would improve patient care, enhance infection control measures, and contribute to the timely identification of these parasitic infections, particularly in regions like Mosul.

Keywords: *Giardia lamblia, Entamoeba histolytica, Entamoeba dispar, Cryptosporidium parvum, PCR.*

1. INTRODUCTION

Diarrheal diseases are a significant global health concern, widespread in developed and developing countries. And these diseases are responsible for a considerable amount of morbidity and mortality, affecting millions of individuals each year. The impact of diarrheal diseases extends beyond physical health, contributing to societal and economic burdens, especially in low-resource settings. Various infectious agents, including viruses, bacteria, and parasites, are responsible for causing diarrhea. Among viral pathogens, Norwalk-like viruses, rotaviruses, and enteric adenoviruses are most commonly associated with outbreaks of diarrheal diseases, particularly in environments with poor sanitation and hygiene [1].

Bacterial infections increase the incidence and severity of diarrheal diseases due to the presence of pathogens like Campylobacter jejuni, Shigella, Salmonella, Enterobacterial Escherichia coli (ETEC), and Clostridium difficile, which are often associated with gastrointestinal disorders. These bacteria cause diarrhea by producing toxins that disrupt normal intestinal functions or directly invade intestinal cells, leading to inflammation and other symptoms. Some of these infections can lead to severe complications, including bloodstream infection or dehydration, which, if not appropriately managed, can lead to life-threatening conditions [2]; parasitic infections play a crucial role in diarrheal diseases, especially in areas with stagnant water or areas where there is leakage and mixing between clean water and sewage. Among the protozoan parasites, Entamoeba histolytica, Giardia lamblia, and Cryptosporidium parvum are among the most clinically

important. These parasites can cause acute diarrhea and more severe and deadly infections like amoebic liver abscess, giardiasis, and cryptosporidiosis. It is estimated that E. histolytica alone causes more than 100,000 deaths annually due to complications

like amoebic liver abscess, and this highlights the severe impact of parasitic infections on global health [3].

Advances in biochemical, immunological, and molecular research have led to a more comprehensive understanding of the taxonomy and pathogenesis of Entamoeba species. Previously, it was believed that Entamoeba histolytica existed in pathogenic and non-pathogenic forms. However, with the advancement of medicine, recent molecular studies have clearly distinguished between E. histolytica and Entamoeba dispar, a non-pathogenic organism that does not cause disease. This distinction is paramount, as E. histolytica is responsible for invasive diseases, while E. dispar is harmless. Morphologically, these two species cannot be distinguished, making microscopic examination insufficient for accurate diagnosis; as a result, alternative diagnostic methods, like antigen detection tests or polymerase chain reaction (PCR), have become essential for the correct identification and differentiation of these species [4], due to the low sensitivity of conventional Microscopy, many infections go undetected, leading to delayed treatment and possible complications. In addition to E. histolytica, Giardia lamblia is another protozoan parasite that causes widespread diarrhea. In industrialized countries, giardiasis is one of the most common non-viral causes of diarrhea, and it often presents as a chronic or recurrent disease. In the natural state, diagnosing giardiasis relies on microscopic examination of stool samples, but this method has limitations in terms of sensitivity and specificity. Detection has evolved. Newer diagnostic techniques, including direct fluorescent antibody tests and antigen detection via enzyme-linked immunosorbent assay (ELISA), have proven more reliable and cost-effective, providing better detection and improving diagnostic accuracy. [5][6] Diagnosis and treatment of diarrheal diseases require a multifaceted approach that includes not only traditional methods like Microscopy but also more advanced techniques that enhance detection and help differentiate between different infectious agents; as research progresses, further improvements in diagnostic techniques and therapeutic options are likely to help reduce the global burden of these diseases. [7][8] In addition, PCR-based methods have been shown to provide superior sensitivity and specificity compared to microscopy and antigen detection [9].

Cryptosporidium parvum is a well-documented cause of large-scale outbreaks of gastroenteritis linked to contaminated water and food [10]. Its association with severe diarrheal disease in AIDS patients has spurred advancements in diagnostic tools and traditional approaches, like modified acid-fast staining, which are widely used for detecting C. parvum oocysts in fecal smears but are limited by their low sensitivity and dependence on the skill of the microscopist [11]. Monoclonal antibodies targeting Cryptosporidium antigens have also been used successfully in fluorescence microscopy and ELISA-based antigen detection [12] also these antibody-based methods are hindered by issues like cross-reactivity with other microorganisms and variable sensitivity [13] where PCR has emerged as a highly accurate and efficient method for detecting C. parvum in fecal samples, offering both high sensitivity and specificity [14] and to summarize, Entamoeba histolytica, Giardia lamblia, and Cryptosporidium parvum are three protozoan parasites responsible for diarrheal diseases, often presenting with similar clinical symptoms [15].

Traditionally, Microscopy has been the most commonly used diagnostic tool for identifying parasitic infections, particularly for detecting the presence of parasites in stool samples. However, its utility has been dramatically reduced due to its limited sensitivity and specificity. Microscopy often cannot differentiate between pathogenic and non-pathogenic species, leading to misdiagnoses and treatment errors. Furthermore, the morphological similarities between certain parasitic species complicate accurately identifying the responsible pathogen. Consequently, Microscopy alone is insufficient for definitive diagnoses in many cases, and more advanced diagnostic techniques have increasingly supplanted its role. Modern molecular methods, like antigen detection assays and polymerase chain reaction (PCR)-based approaches, have gained traction due to their higher sensitivity, accuracy, and ability to differentiate between closely related species, and these techniques, particularly PCR, are now recognized as essential tools in clinical parasitology, offering a level of precision that Microscopy cannot achieve, especially when species exhibit similar morphological characteristics. Despite these advantages, diagnostic challenges persist due to the overlapping clinical symptoms of various parasitic infections, making it difficult to reach a precise diagnosis based solely on clinical presentation [16].

While PCR-based assays have demonstrated exceptional sensitivity in detecting parasitic pathogens like Entamoeba histolytica, Giardia lamblia, and Cryptosporidium parvum, their routine application in clinical settings has not been as widespread as one might expect, several factors have contributed to this limitation, including the challenges associated with extracting high-quality DNA from fecal samples and these samples often contain inhibitors, like bile salts and other substances, that can hinder the amplification process, leading to false-negative results, also conventional PCR methods have been associated with a high risk of contamination, which can lead to unreliable results and a significant reduction in diagnostic accuracy, furthermore the complexity of traditional PCR procedures—along with their labor-intensive, time-consuming nature and high cost—has made them less practical for routine use in many clinical laboratories as a result, the accessibility of PCR testing remains limited, particularly in settings with fewer resources [16] where recent advancements in molecular diagnostic technologies have helped mitigate some of these challenges, improving the reliability and efficiency of PCR-based tests. Advances in parasitic DNA extraction methods have made obtaining high-quality genetic material from fecal samples easier, which has enhanced the performance of PCR assays. In addition, the development of real-time PCR, which incorporates fluorescent detection probes, has addressed issues related to contamination and the need for post-PCR handling, and by allowing for the amplification and detection of target DNA in a single, closed-tube system, real-time PCR minimizes the risk of cross-contamination, thereby ensuring more accurate and reliable results.

Also, Real-time PCR offers the advantage of a faster turnaround time compared to conventional PCR, which makes it a more attractive option for time-sensitive clinical situations. These improvements have significantly streamlined the workflow and reduced labor time and reagent costs, thus making real-time PCR a more viable option for routine diagnostic use in many clinical laboratories [17].

In particular, multiplex real-time PCR has revolutionized parasitic diagnostics. This innovative approach enables the simultaneous detection of multiple pathogens within a single assay, which is especially valuable when dealing with co-infections, where a patient may be infected by more than one parasite. Multiplex PCR assays can now detect E. histolytica, G. lamblia, and C. parvum concurrently, allowing for a more comprehensive and accurate diagnosis of gastrointestinal infections and this ability to test for multiple pathogens at once increases the likelihood of detecting mixed infections that may otherwise go unnoticed using traditional diagnostic methods, furthermore, to improve the reliability of these multiplex PCR assays, internal controls have been incorporated into the tests to monitor and account for any potential issues with amplification and these internal controls help ensure that negative results are not due to sample inhibition but reflect the actual absence of the target parasite and the performance of these advanced multiplex PCR assays has been rigorously tested with control samples, demonstrating their high sensitivity and specificity, making them a promising tool for improving the diagnosis and management of parasitic infections [17], while traditional Microscopy remains an essential tool in parasitic diagnosis, its limitations have necessitated the development of more advanced techniques, like PCR and multiplex real-time PCR and these molecular methods offer greater sensitivity, specificity, and efficiency, enabling clinicians to more accurately identify parasitic pathogens, even in the presence of mixed infections or challenging clinical presentations as technology continues to advance, these diagnostic tools are expected to play an increasingly central role in the fight against parasitic diseases, providing a more reliable and cost-effective means of identifying and treating infection as the field of molecular diagnostics continues to evolve, we can expect even more innovative solutions to emerge, further enhancing the speed and accuracy of parasitic disease diagnosis, ultimately leading to better patient outcomes and more effective public health strategies

2. MATERIALS AND METHODS

Controls and Samples

To ensure the accurate detection of Entamoeba histolytica, Giardia lamblia, and Cryptosporidium parvum, DNA controls were carefully sourced from well-established and reliable references, specifically, DNA from E. histolytica was extracted from an axenic culture of the HM1 strain, while G. lamblia DNA was derived from purified cysts, and C. parvum DNA was obtained from purified oocysts provided by Waterborne Inc. and these DNA samples, known for their high quality, were utilized as reference materials for validating the performance and accuracy of the PCR assay [18] and the stool samples selected for this study were meticulously chosen based on prior confirmation through multiple diagnostic methods where a total of 60 positive stool samples were included: 20 samples that were initially identified as positive for E. histolytica through Microscopy and subsequently verified by PCR, 20 samples that were confirmed to contain G. lamblia through both Microscopy and antigen testing, and 20 samples in which C. parvum oocysts were detected using modified acid- fast staining and to ensure diversity and reduce the potential for bias, each sample was collected from a unique patient. Eight of the C. parvum-positive samples were collected over one year from a single immunocompromised child. Microscopic examination of these samples confirmed the presence of C. parvum oocysts in four separate instances [18].

To validate the study's findings and enhance the reliability of the results, a control group of 25 stool samples that tested negative for parasitic infections was also included, and these control samples were first screened using Microscopy, modified acid-fast staining, and antigen detection tests. Their negative status was confirmed by subsequent molecular testing. Upon collection, all unpreserved stool samples were stored at 4°C to maintain their integrity , within one week, DNA suspensions were prepared at a concentration of 0.5 µg/ml in phosphate-buffered saline (PBS) supplemented with 2% polyvinylpolypyrrolidone (Sigma), and the suspensions were then stored at -20°C to preserve DNA quality for later isolation and PCR analysis [18] and to assess the specificity of the PCR assay and ensure that it did not cross-react with closely related organisms, additional DNA samples were tested and these included DNA from Entamoeba dispar, Enterocytozoon bieneusi, Encephalitozoon intestinalis, and Cyclospora cayetanensis and the potential for cross-reactivity with bacterial and fungal species was also evaluated, DNA samples from 12 different bacterial and fungal species, including Bacillus cereus, Enterococcus faecalis, Staphylococcus aureus, coagulase-negative Staphylococcus, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella enterica serovar Typhimurium, Shigella flexneri, Yersinia enterocolitica, and Candida albicans were tested to ensure no interference in the assay's performance, also 20 stool samples containing ,where E. dispar were also tested, with E. dispar being confirmed through both Microscopy and PCR, to verify that the PCR assay did not exhibit cross-reactivity with this closely related species [19] and through this comprehensive and rigorous approach, the study successfully minimized potential errors and ensured the high specificity and reliability of the PCR diagnostic method and by using a diverse set of controls and including closely related organisms and potential cross-reactants, the study demonstrated the robust performance of the PCR assay in accurately detecting E. histolytica, G. lamblia, and C. parvum while avoiding false positives from unrelated pathogens.

Microscopy and Antigen Detection

Routine microscopic examination of stool samples was carried out to detect the presence of ova and cysts and this was achieved by preparing iodine-stained wet mounts after the Formol-ether concentration technique, which helps in concentrating the stool contents for more efficient observation of parasites and the examination was conducted under 400× magnification, a standard practice for visualizing protozoa and helminths in fecal samples, also modified acid-fast staining was applied to both direct smears and concentrated stool samples to specifically identify coccidian parasites, which are often challenging to detect with standard techniques [20], For the detection of Giardia lamblia, a commercial enzyme-linked immunosorbent assay (ELISA) kit from Alexon-Trend Inc. , was used and the ELISA method is designed to identify Giardia antigens present in the stool samples, providing a more sensitive alternative to traditional Microscopy and the manufacturer's protocol was followed, with slight adjustments made to optimize the test's performance for the specific conditions of the study and this antigen detection technique has proven to be a reliable diagnostic tool for confirming G. lamblia infections, offering higher sensitivity compared to microscopic examination [20], and these diagnostic methods, including microscopy and antigen detection, are essential for the comprehensive detection of various parasitic infections, each with its own limitations and advantages. While effective for detecting certain types of parasites, Microscopy can be limited in sensitivity, particularly for coccidian organisms. At the same time, antigen detection assays provide a more specific and sensitive approach for certain protozoan parasites like G. lamblia.

DNA Isolation

DNA extraction was performed on 200 µl of fecal suspensions, purified parasite isolates, or bacterial cultures to ensure the efficient isolation of genetic material for subsequent molecular analysis and the first step in the process involved heating the samples at 100°C for 10 minutes to lyse the cells and release the DNA , following this, sodium dodecyl sulfate (SDS) and proteinase K were used to further break down proteins and cellular structures, with the treatment carried out at 55°C for 2 hours and this combination of heat and enzyme treatment ensures the complete breakdown of cell membranes and proteins, allowing for the efficient release of DNA from the samples [21], For the DNA isolation, the QIAamp Tissue Kit (Qiagen, Germany) was employed, which utilizes spin columns for the purification of high-quality DNA and the kit provides a reliable method for extracting DNA from a wide variety of sample types, including fecal specimens, and ensures the removal of contaminants that could interfere with downstream applications like PCR and to monitor the efficiency of the DNA extraction process and assess potential inhibition during PCR, an internal control was incorporated. Phocine herpesvirus 1 (PhHV-1) was added to the lysis buffer at 10^3 plaque-forming units (PFU) per milliliter concentration. This internal control allows for detecting any PCR inhibitors present in the samples, ensuring that the results obtained from the PCR assay are accurate and reliable [21]. This procedure effectively isolates DNA, which is essential for downstream PCR analysis, and mitigates the risk of false-negative results due to sample contaminants or extraction inefficiencies. Incorporating internal control also enhances the overall quality and reproducibility of the molecular diagnostic process.

PCR Amplification and Detection

For the PCR amplification and detection of Entamoeba histolytica, primers were carefully designed using Primer Express software (Applied Biosystems), focusing on the small subunit ribosomal RNA (SSU rRNA) gene sequences of E. histolytica and its close relative E. dispar (GenBank accession nos. X64142 and Z49256) and the assay utilized a TaqMan minor groove binding (MGB) probe specific to E. histolytica, which targeted a 172-bp fragment within the SSU rRNA gene and this approach ensures high specificity for E. histolytica detection while minimizing cross-reactivity with other Entamoeba species [22] anc for Giardia lamblia, the PCR assay targeted the SSU rRNA gene (GenBank accession no. M54878), amplifying a 62-bp fragment and the primers used for this amplification were Giardia-80F and Giardia-127R, with detection achieved through a double-labeled probe, Giardia-105T (Biolegio, The Netherlands) and this combination of primers and probe provides a highly sensitive and specific method for detecting G. lamblia in fecal samples, ensuring that the assay can accurately identify the pathogen even in the presence of other gut flora [22], where PCR assay for Cryptosporidium parvum was based on previously published sequences, with primers and probes designed to amplify a 138-bp fragment within a pathogen- specific 452-bp region and this fragment ensures particular detection of C. parvum, a common cause of gastrointestinal infections, with the Crypto probe (Biolegio) used for detection and by targeting a highly conserved region of the genome, this method provides an efficient way to identify C. parvum in stool samples [22].

To monitor the PCR process for potential inhibition and ensure the reliability of results, internal control was incorporated into the assay and the internal control, Phocine herpesvirus 1 (PhHV- 1), was amplified using specific primers (PhHV-267s and PhHV- 337as) and a double-labeled probe (PhHV-305tq), provided by Biolegio and the inclusion of this internal control serves as a reference to verify that the PCR amplification process is functioning correctly and that any inhibition due to fecal contaminants is detected [22]. His well-structured PCR assay, combining specific primers and probes for three different pathogens, allows for efficient, sensitive, and reliable detection of E. histolytica, G. lamblia, and C. parvum while maintaining quality control through internal control. This strategy is essential for accurately diagnosing and monitoring parasitic infections in clinical settings.

Assay Optimization and Performance Testing

To optimize the PCR assay, serial 10-fold dilutions of DNA extracted from each target pathogen (E. histolytica, G.

lamblia, and C. parvum) were prepared and these dilution series were used to generate standard curves, which are essential for evaluating the assay's efficiency and determining the correlation coefficients and the standard curves help quantify the DNA detection limit and assess the assay's sensitivity across a range of concentrations and the analysis of these curves was conducted using the iCycler IQ real-time detection system (Bio- Rad), which enabled precise monitoring of fluorescence and provided valuable data regarding the assay's performance and dilution series were tested in both individual pathogen assays and in a multiplex format to evaluate the performance of the PCR test in detecting single and mixed infections and this is critical for clinical scenarios where patients may be co-infected with multiple pathogens and the PCR reactions were carried out in 50 µl volumes, which contained the PCR buffer (HotStar Taq master mix; Qiagen), magnesium chloride ($MgCl_2$), pathogen-specific primers and probes, and 5 µl of DNA sample and this setup ensures the optimal conditions for amplification while maintaining consistency in the results across various samples and thermal cycling conditions were carefully designed to optimize DNA amplification and the protocol included an initial denaturation step at 95°C for 15 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds, Fluorescence detection was carried out during the annealing step of each cycle, which allows for real-time monitoring of the amplification process and this enables the determination of the quantity of DNA in the sample based on the fluorescence emitted by the specific probes used for detection.

A detailed list of primers and probes used in the assays, including their sequences and target regions, was provided in Table 1. These primers and probes' careful design and optimization are essential for ensuring specificity and minimizing cross-reactivity, particularly in the multiplex assay, where multiple pathogens are detected simultaneously. This thorough optimization ensures the PCR assay's high sensitivity, specificity, and accuracy, allowing for the reliable detection of E. histolytica, G. lamblia, and C. parvum, including in cases of mixed infections, which are often challenging to diagnose using traditional methods.

3. RESULTS

The specificity of the real-time multiplex PCR assay was meticulously evaluated to confirm that the assay specifically targets Entamoeba histolytica, Giardia lamblia, and Cryptosporidium parvum without cross-reacting with other non-target organisms. A comprehensive array of control DNA samples was used in this process to ensure that the assay's specificity remained intact across a variety of potential contaminants. Control DNA samples included DNA from closely related species like Entamoeba dispar, which is morphologically indistinguishable from E. histolytica, and other protozoan pathogens like Enterocytozoon bieneusi, Encephalitozoon intestinalis, and Cyclospora cayetanensis and in addition, the assay was tested with DNA samples from a range of bacterial and fungal species that could potentially interfere with PCR amplification, and these species included Bacillus cereus, Enterococcus faecalis, Staphylococcus aureus, coagulase-negative Staphylococcus, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella enterica serovar Typhimurium, Shigella flexneri, Yersinia enterocolitica, and Candida albicans. None of these control samples resulted in amplification, further confirming that the assay was particular to the target pathogens and did not cross-react with any non-target organisms.

In addition to testing with control DNA samples, further validation was carried out using 20 DNA extracts from fecal samples positive for E. dispar and 25 DNA extracts from individuals with no history of parasitic infections. These additional samples were included to ensure that no false positives could occur. The results were unequivocal: no amplification of E. histolytica, G. lamblia, or C. parvum DNA was detected in these samples, verifying the specificity of the assay, and the only amplification observed was that of the internal control, which consistently exhibited a threshold cycle (Ct) value of approximately 33 and this internal control amplification served as a key indicator of the proper functioning of the assay, confirming that the PCR process was working as expected and that the assay was capable of distinguishing between the target pathogens and non-target organisms.

To further substantiate the assay's sensitivity and performance, serial dilutions of each pathogen were tested individually and in multiplex formats. These dilutions' cycle threshold (Ct) values were consistent, demonstrating that the multiplex format did not reduce the assay's sensitivity. This consistency in Ct values indicated that the assay maintained its analytical sensitivity even when simultaneously testing for multiple pathogens. Significantly, the inclusion of additional primers, internal controls, or DNA from other targets did not interfere with the individual performance of the assays for E. histolytica, G. lamblia, and C. parvum and this feature ensured that the assay remained reliable and accurate, with no risk of cross-contamination or performance degradation due to the multiplex design.

The extensive specificity testing conducted throughout this study affirms that the multiplex real-time PCR assay is highly reliable and capable of accurately detecting E. histolytica, G. lamblia, and C. parvum in fecal samples and the assay demonstrated minimal risk of false positives or false negatives, offering a robust diagnostic tool for detecting these parasitic pathogens with high confidence.

Table 1. Oligonucleotide Sequence and Reference Numbers for Target Organisms.

Target organism and oligonucleotide	Oligonucleotide sequence	GenBank accession no. or reference
E. histolytica		
Ehd-239F	5'-ATTGTCGTGGCATCCTAACTCA-3'	X64142, Z49256
Ehd-88R	5'-GCGGACGGCTCATTATAACA-3'	X64142, Z49256
histolytic-96T	VIC-5'-TCATTGAATGAATTGGCCATTT-3'-nonfluorescent quencher	X64142
G. lamblia		
Giardia-80F	5'-GACGGCTCAGGACAACGGTT-3'	M54878
Giardia-127R	5'-TTGCCAGCGGTGTCCG-3'	M54878
Giardia-105T	FAM-5'-CCCGCGGCGGTCCCTGCTAG-3'-black hole quencher 1	M54878
C. parvum		
CrF	5'-CGCTTCTCTAGCCTTTCATGA-3'	11
CrR	5'-CTTCACGTGTGTTTGCCAAT-3'	11

Target organism and oligonucleotide	Oligonucleotide sequence	GenBank accession no. or reference
Crypto Texas Red	5'-CCAATCACAGAATCATCAGAATCGACTGGTATC-3'-black hole quencher 2	11
PhHV-1		
PhHV-267s	5'-GGGCGAATCACAGATTGAATC-3'	26
PhHV-337as	5'-GCGGTTCCAAACGTACCAA-3'	26
PhHV-305tq	Cy5-5'-TTTTTATGTGTCCGCCACCATCTGGATC-3'-black hole quencher 2	26

The multiplex real-time PCR assay demonstrated its high sensitivity and specificity in detecting Entamoeba histolytica, Giardia lamblia, and Cryptosporidium parvum in stool samples. Amplification of E. histolytica DNA was successfully detected in all 20 stool samples that had been confirmed to contain E. histolytica cysts and the cycle threshold (Ct) values for these samples ranged from 25.1 to 37.3 cycles, with a median Ct value of 29.3 cycles and this result highlights the assay's sensitivity, even for samples with relatively low concentrations of E. histolytica, confirming that the multiplex PCR test can reliably detect the pathogen in clinical samples and similarly amplification of G. lamblia DNA was observed in all 20 samples that had previously been confirmed positive for G. lamblia using both microscopic examination and antigen testing and the Ct values for these samples ranged from 24.2 to 37.7 cycles, with a median Ct value of 29.9 cycles and these results underscore the capability of the real-time PCR assay to effectively detect G. lamblia, a common cause of gastrointestinal illness and this is particularly important as it offers a more reliable diagnostic option for giardiasis, especially in cases where antigen testing might be used as a complementary diagnostic tool and by providing accurate and early detection, this PCR assay offers a robust alternative to traditional diagnostic methods also the PCR assay also demonstrated its efficiency in detecting C. parvum, a protozoan parasite commonly associated with diarrheal diseases, specific amplification of C. parvum was detected in all 20 stool samples where the presence of C. parvum oocysts had been confirmed through modified acid-fast staining and the Ct values for these samples ranged from 24.0 to 36.7 cycles, with a median Ct value of 29.7 cycles and these results affirm that the multiplex PCR assay is highly sensitive and specific for detecting C. parvum in stool samples, offering a reliable diagnostic method for identifying this pathogen, especially in immunocompromised individuals who are more susceptible to infections caused by C. parvum.

Further validation was carried out using seven stool samples from an immunocompromised child with diarrhea. Multiplex

real-time PCR analysis of these samples revealed the presence of C. parvum DNA in all seven samples. However, only four of these samples showed C. parvum oocysts upon microscopic examination of modified acid-fast stained fecal smears. This discrepancy illustrates the advantage of PCR over Microscopy, as PCR was able to detect the pathogen in samples where the parasite load was too low for microscopic identification; such findings highlight the ability of PCR to identify C. parvum in cases where traditional Microscopy might miss the diagnosis, due to limitations in the detection of lower parasite loads or insufficient sample preparation.

The findings from these tests emphasize the high sensitivity and specificity of the multiplex real-time PCR assay in detecting E. histolytica, G. lamblia, and C. parvum. The assay provides a valuable diagnostic tool, particularly in clinical situations where Microscopy may not be sufficient or where mixed infections involving multiple pathogens are suspected. The range of Ct values observed in these samples supports the robustness of the assay, indicating its reliable performance across various levels of DNA concentration and this level of sensitivity makes the multiplex PCR assay an essential tool for accurate diagnosis and appropriate treatment, helping to reduce the incidence of misdiagnosis or delayed diagnosis in patients suffering from parasitic infections.

Table 2. Ct Values and Number of Samples for Target Organisms.

Target organism	Number of samples	Ct value range	Median Ct value	Additional Information
E. histolytica	20	25.1 37.3	-29.3	Amplification detected in fecal samples known to contain E. histolytica cysts.
G. lamblia	20	24.2 37.7	-29.9	Amplification detected in samples was confirmed by microscopic examination and Giardia antigen test.
C. parvum	20	24.0 36.7	-29.7	Amplification was detected in samples showing Cryptosporidium oocysts via modified acid-fast staining.
C. parvum (Immunocompromised child)	7	N/A	N/A	C. parvum DNA was detected in samples from an immunocompromised child with diarrhea; only four samples showed oocysts microscopically.

4. DISCUSSION

The most significant parasitic pathogens responsible for diarrhea are *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum*, and these infections often present with nonspecific clinical symptoms, making it challenging to diagnose them accurately using traditional methods and the multiplex real-time PCR method developed in this study offers a highly sensitive and specific alternative for the simultaneous detection of these pathogens and by utilizing well-defined DNA and stool samples as controls, the assay demonstrated 100% specificity in identifying *E. histolytica*, *G. lamblia*, and *C. parvum* and in all samples where Microscopy confirmed the presence of these parasites, specific amplification was observed, confirming the reliability of the multiplex PCR approach. Importantly, there was no significant difference in the amplification performance between individual assays and the multiplex PCR, indicating that the multiplex method can be used with the same level of confidence as individual tests , one of the major challenges in PCR-based diagnostics is the potential inhibition of reactions by fecal substances also in this study, stool samples without a history of parasitic infection, as well as those containing *E. dispar*, showed no evidence of PCR inhibition , only the internal control was amplified, confirming the efficiency of the DNA isolation method used and this highlights the robustness of the technique and its suitability for use in clinical settings where stool samples are commonly analyzed and the sensitivity of parasite-specific DNA detection using multiplex real-time PCR was found to be superior to traditional microscopy and this was particularly evident in cases of *G. lamblia* and *C. parvum* infections, where PCR detected the presence of parasite DNA even before microscopy yielded positive results and for instance, in an immunocompromised child with diarrhea, *C. parvum* DNA was detected by multiplex real-time PCR in samples that were initially negative by microscopy and this underscores the potential of PCR-based methods to provide earlier and more accurate diagnoses, which is critical for timely and effective treatment, especially in vulnerable populations and the ability of the multiplex real-time PCR to detect multiple pathogens simultaneously without compromising sensitivity or specificity makes it a valuable tool for clinical diagnostics and it not only reduces the time and resources required for testing but also enhances the accuracy of detecting co-infections, which are common in regions where these parasites are endemic and furthermore,

the inclusion of an internal control ensures the reliability of the results by identifying potential PCR inhibition, thereby minimizing the risk of false negatives and in conclusion, the multiplex real-time PCR method presented in this study represents a significant advancement in the diagnosis of parasitic infections causing diarrhea and its high sensitivity, specificity, and ability to detect multiple pathogens simultaneously make it a powerful alternative to traditional diagnostic methods and by enabling earlier and more accurate detection, this technique has the potential to improve patient outcomes, enhance infection control measures, and contribute to the effective management of parasitic infections in both clinical and public health settings.

5. CONCLUSION

Parasitic infections caused by Entamoeba histolytica, Giardia lamblia, and Cryptosporidium parvum are significant contributors to diarrheal diseases in many parts of the world, including regions like Mosul, while microscopic examination of stool samples remains the conventional method for diagnosing these infections, its limitations in terms of accuracy and sensitivity highlight the necessity for more advanced diagnostic approaches and in this context, the multiplex real-time polymerase chain reaction (PCR) technique has proven to be a highly effective and precise method for detecting these parasites and the findings of this study show that the multiplex PCR assay offers 100% sensitivity and specificity for detecting E. histolytica,

G. lamblia and C. parvum, establishing it as a dependable and efficient diagnostic tool and the multiplex PCR technique not only demonstrated superior performance in detecting these pathogens, but it also proved to be crucial for the early identification of cases that might otherwise be overlooked by traditional Microscopy, particularly in immunocompromised individuals and the use of internal control in the assay ensured that no PCR inhibition occurred, further validating the accuracy and reliability of the results. This study recommends the widespread adoption of multiplex PCR technology in diagnostic laboratories, especially in areas where parasitic infections are common, and incorporating this technique into routine diagnostic practices will improve healthcare quality by enabling rapid and precise diagnoses, and this, in turn, will facilitate better case management and allow for the implementation of timely preventive measures. Early detection will also play a pivotal role in reducing the transmission of these infections, contributing to better public health outcomes. In conclusion, the multiplex real-time PCR technique represents a significant step forward in parasitic diagnostics. Its ability to detect diarrheal pathogens accurately and quickly makes it a powerful tool for combatting parasitic infections. By enhancing the ability to diagnose these diseases quickly and accurately, this technique will contribute to improved public health efforts, better disease control, and an overall better quality of life for affected individuals.

Recommendations

It is recommended that health facilities, particularly in regions where parasitic infections are prevalent, adopt multiplex PCR technology to ensure accurate and efficient diagnosis of E. histolytica, G. lamblia, and C. parvum, and This will enhance diagnostic accuracy, improve patient outcomes, and help reduce the spread of infections and laboratories should consider integrating multiplex PCR with traditional diagnostic techniques like Microscopy to enhance diagnostic capabilities, particularly in cases of mixed infections or when microscopic results are inconclusive HL, where Health professionals, including laboratory technicians, should be trained on the use of PCR-based diagnostic methods to ensure that the technology is appropriately utilized. Its full potential is realized in detecting parasitic infections. Efforts should also be made to improve access to multiplex PCR technology, especially in low-resource settings, by reducing costs and making diagnostic kits more widely available. There should be increased public awareness about the importance of early diagnosis and treatment of parasitic infections, alongside promoting preventive measures like proper sanitation and hygiene practices, which are critical in reducing the spread of these diseases and by implementing these recommendations, healthcare systems can significantly enhance their ability to detect and manage parasitic infections, ultimately improving public health outcomes and the quality of life for affected individuals

REFERENCES

[1] Allen, A. V., and D. S. Ridley. "Additional Observations on the Formol-Ether Concentration Technique for Fecal Parasites." *Journal of Clinical Pathology*, vol. 23, no. 6, 1970, pp. 545–546.

[2] Amin, O. M. "Seasonal Prevalence of Intestinal Parasites in the United States during 2000." *American Journal of Tropical Medicine and Hygiene*, vol. 66, no. 6, 2002, pp. 799–803.

[3] Chappell, C. L., and P. C. Okhuysen. "Cryptosporidiosis." *Current Opinion in Infectious Diseases*, vol. 15, no. 5, 2002, pp. 523–527.

[4] Chen, X. M., J. S. Keithly, C. V. Paya, and N. F. Larusso. "Cryptosporidiosis." *New England Journal of Medicine*, vol. 346, no. 22, 2002, pp. 1723–1731.

[5] Current, W. L., and L. S. Garcia. "Cryptosporidiosis." *Clinical Microbiology Reviews*, vol. 4, no. 3, 1991, pp. 325–358.

[6] De Wit, M. A. S., M. P. G. Koopmans, L. M. Kortbeek, N. J. van Leeuwen, A. I. M. Bartelds, and Y. H. P. van Duynhoven. "Gastroenteritis in Sentinel General Practices in the Netherlands." *Emerging Infectious*

Diseases*, vol. 7, no. 1, 2001, pp. 82–91.

[7] De Wit, M. A. S., M. P. G. Koopmans, L. M. Kortbeek, N. J. van Leeuwen, J. Vinje, and Y. H. P. van Duynhoven. "Etiology of Gastroenteritis in Dutch Sentinel General Practices." *Clinical Infectious Diseases*, vol. 33, no. 3, 2001, pp. 280–288.

[8] Diamond, L. S., and C. G. Clark. "A Revised Description of *Entamoeba histolytica* Schaudinn, 1903 (Emended Walker, 1911), Distinguishing It from *Entamoeba dispar* Brumpt, 1925." *Journal of Eukaryotic Microbiology*, vol. 40, no. 4, 1993, pp. 340–344.

[9] Doing, K. M., J. L. Hamm, J. A. Jellison, J. A. Marquis, and C. Kingsbury. "False-Positive Results in the Alexon ProSpecT Cryptosporidium Enzyme Immunoassay." *Journal of Clinical Microbiology*, vol. 37, no. 5, 1999, pp. 1582–1583.

[10] Fayer, R., U. Morgan, and S. J. Upton. "Epidemiology of *Cryptosporidium*: Transmission, Detection, and Identification." *International Journal of Parasitology*, vol. 30, no. 12-13, 2000, pp. 1305–1322.

[11] Fontaine, M., and E. Guillot. "Development of a TaqMan Quantitative PCR Assay Specific for *Cryptosporidium parvum*." *FEMS Microbiology Letters*, vol. 214, no. 1, 2002, pp. 13–17.

[12] Garcia, L. S., and R. Y. Shimizu. "Evaluation of Nine Immunoassay Kits (Enzyme Immunoassay and Direct Fluorescence) for Detecting *Giardia lamblia* and *Cryptosporidium parvum* in Human Fecal Specimens." *Journal of Clinical Microbiology*, vol. 35, no. 6, 1997, pp. 1526–1529.

[13] Ghosh, S., A. Debnath, A. Sil, S. De, D. J. Chattopadhyay, and P. Das. "PCR Detection of *Giardia lamblia* in Stool Samples: Targeting the Intergenic Spacer Region of the Multicopy rRNA Gene." *Molecular and Cellular Probes*, vol. 14, no. 3, 2000, pp. 181–189.

[14] Gonzalez-Ruiz, A., and S. G. Wright. "Disparate Amoebae and *The Lancet*." *The Lancet*, vol. 351, no. 9116, 1998, pp. 1672–1673.

[15] Guerrant, R. L., J. M. Hughes, N. L. Lima, and J. Crane. "The Impact of Diarrhea in Developed and Developing Nations: Scope, Unique Environments, and Causes." *Reviews of Infectious Diseases*, vol. 12, Suppl. 1, 1990, pp. S41–S50.

[16] Haque, R., A. S. Faruque, P. Hahn, D. M. Lyerly, and W. A. Petri Jr. "*Entamoeba histolytica* and *Entamoeba dispar* Infections Among Children in Bangladesh." *Journal of Infectious Diseases*, vol. 175, 1997, pp. 734–736.

[17] Henriksen, S. A., and J. F. Pohlenz. "A Modified Ziehl- Neelsen Technique for *Cryptosporidium* Staining." *Acta Veterinaria Scandinavica*, vol. 22, 1981, pp. 594–596.

[18] Ignatius, R., M. Eisenblatter, T. Regnath, U. Mansmann, U, futh, H. Hahn, and J. Wagner. "Comparative Efficacy of Various Diagnostic Techniques for Detecting Low Levels of *Cryptosporidium parvum* in Stool Samples." *European Journal of Clinical Microbiology & Infectious Diseases*, vol. 16, 1997, pp. 732–736.

[19] Klein, D. "Real-Time PCR Quantification: Benefits and Limitations." *Trends in Molecular Medicine*, vol. 8, 2002, pp. 257–260.

[20] Kosek, M., C. Bern, and R. L. Guerrant. "Global Burden of Diarrheal Diseases: An Assessment Based on Studies from 1992 to 2000." *Bulletin of the World Health Organization*, vol. 81, 2003, pp. 197–204.

[21] Mank, T. G., J. O. Zaat, A. M. Deelder, J. T. van Eijk, and

[22] A. M. Polderman. "Comparing Microscopy and Enzyme Immunoassay for Giardiasis Diagnosis." *European Journal of Clinical Microbiology & Infectious Diseases*, vol. 16, 1997, pp. 615–619.

[23] Mirelman, D., Y. Nuchamowitz, and T. Stolarsky. "Evaluating Enzyme-Linked Immunosorbent Assays and PCR Methods for Concurrent Detection of *Entamoeba histolytica* and *Entamoeba dispar*." *Journal of Clinical Microbiology*, vol. 35, 1997, pp. 2405–2407.