A Comparison of TPI Gene-Specific PCR and Stool Microscopy in Detecting *Giardia lamblia* Infection in Western, Kenya

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Background and study aim: *Giardia lamblia* is a widespread pathogen of gastrointestinal infection in Kenya, the diagnosis of which is mostly based on stool microscopy. Molecular assays against conserved genes e.g. Triose Phosphate Isomerase (TPI) provide better detection. The objective of the study was to determine the diagnostic accuracy of TPI gene-specific PCR in the identification of *G. lamblia* in symptomatic patients in western Kenya with the colon microscopy used as the comparator.

Patients and Methods: This was a cross-sectional study that was carried out among 147 patients who had presented with gastrointestinal symptoms at Busia County referral Hospital, western Kenya. Direct microscopy of stool samples and TPI gene-specific PCR were used to analyze the samples.

Results: *G. lamblia* was identified in 78 (53.1) of the participants by microscopy and 53 (36.1) of the participants by TPI-PCR. TPI-PCR had sensitivity of 66.7% (95% CI: 55.1-76.9) and specificity 98.6% (95% CI: 92.3 -100). The predictive value (positive and negative) is 98.1 and 72.3 with the overall diagnostic accuracy standing at 81.6. Methods had a great deal of agreement (Cohen kappa = 0.639). The McNemar's test showed that there was significant discordance ($P < 0.0001$). The participants who were positive by microscopy were 136 times more likely to be positive by TPI-PCR as compared to the negative (OR = 136; 95% CI: 17.9-1035.2, $P = 0.00001$).

Conclusion: TPI gene-specific PCR was highly specific, generally diagnostic in its overall performance and showed a high agreement with stool microscopy but sensitivity was moderate.

INTRODUCTION

One of the most prevalent intestinal protozoan parasite in the world and a well-known cause of acute and chronic diarrheal disease especially in low and middle-income countries is Giardia lamblia [1,2]. The major mode of transmission is fecal-oral, which is frequently through the contamination of water, food, or direct person-to-person contact. Giardiasis is a significant cause of gastrointestinal morbidity, malnutrition and slowed growth in children and low productivity in adults in endemic areas [3]. G. lamblia is still a strong health problem in Kenya and in particular the western part of Kenya like Busia County where access to water and sanitation is still uneven [4].

Correct diagnosis of giardiasis is needed in order to manage the case effectively, treat it properly and also to be able to monitor the disease [5]. Traditional stool microscopy is the standard diagnostic modality in most of the clinical laboratories. This technique is based on the direct observation of cysts or trophozoites in feces and is quite cheap and more common [6]. Nevertheless, stool microscopy has significant limitations, such as low sensitivity through intermittent shedding of cysts, reliance on the expertise of the examiner and lower sensitivity in light or chronic infections. Such limitations have been a major cause of under-diagnosis, delayed treatment and subsequent spread in communities [7].

Molecular diagnostic procedures have been found to be useful as an alternative to the traditional parasitological procedures. The polymerase chain reaction (PCR) of conserved genes within the G. lamblia genome provides a better sensitivity and specificity even with low parasite load [8]. Of such targets, the Triose Phosphate Isomerase (TPI) gene has been of special interest since it is highly conserved, is single-copy, and both detectable as well as genotypable [9]. The TPI gene can be detected using PCR, as well as restriction fragment length polymorphism (RFLP), and this technique is not only accurate in the identification of G. lamblia, but also allows the differentiation of genetic assemblage that could have epidemiological and clinical importance [10].

Although the advantages of molecular diagnostics have been demonstrated, stool microscopy is the most commonly used diagnostic method in most Kenyan public health facilities since it is less expensive, has less infrastructure, and technical capacity [11]. There is paucity in local evidence relating performance of molecular method to that of microscopy in normal clinical practice. At Busia County and the general western Kenya area, there is hardly any information available in the added diagnostic value of PCR-based assays of giardiasis.

The objective of the study was to determine diagnostic accuracy of TPI gene-specific PCR using RFLP analysis in the detection of G. lamblia in symptomatic patients in western Kenya using stool microscopy as a comparator. The current work will contribute to the knowledge base in diagnostic strategies, emphasize the gaps in the existing practice, and substantiate the possibility of the implementation of molecular methods into the practice of parasitological diagnosis in the endemic and resource-constrained environment, by assessing sensitivity, specificity, agreement, and overall diagnostic accuracy.

PATIENTS AND METHODS

Study design and study area. It was a cross-sectional lab-based survey on Busia County, Western Kenya. The research was conducted in Busia County Referral Hospital, a large state-owned health institution serving both urban residents and those living in rural areas and having patients with diverse gastrointestinal issues. The G. lamblia laboratory at the hospital was regularly involved in stool microscopy and offered a suitable environment in the comparison of conventional and molecular diagnostic methods .

Study population. The target population was the patients who presented themselves to the hospital with gastrointestinal symptoms that were of intestinal parasitic infection. All persons were eligible to participate, including infants and old-aged persons. Selection bias was reduced through enrollment of participants in the study period in a consecutive manner. Upon laboratory validation with the help of stool microscopy, participants were classified as G. lamblia +(ve) and –(ve)

There were 147 participants that were included in the analysis. The participants varied in terms of their age between 0 and 75 years with a mean age of about 25 years. Both sexes were recruited and both genders showed statistically significant difference in the mixture of the gender between G. lamblia +ve and -ve through the use of microscopy. The clinical manifestations which have been widely reported among the participants were diarrhea, abdominal pain, bloating, foul-smelling stool, mucus in the stool, fatty stool, vomiting, headache, and poor appetite, which depict the wide spectrum of clinical manifestations of giardiasis .

Inclusion and exclusion criteria. The patients were also considered provided they reported with gastrointestinal symptoms and accepted stool sample laboratory analysis. Those individuals who had been treated with antiparasitic treatment in the close past before sample collection or those who failed to provide a sufficient specimen of stool were not considered in the study.

Sample collection and handling. A clean, sterile, airtight container was used to collect a fresh stool sample of each participant. Each sample was identified as having a distinct identifier that can be used to trace and guarantee confidentiality. Stool samples were quickly delivered to the laboratory and done within the day wherever possible. In cases where immediate processing was not viable, samples were refrigerated under suitable conditions to maintain the morphology of the parasites to use in the microscope and to maintain the integrity of the DNA to be used in molecular analysis.

Stool microscopy. The conventional stool microscopy was used as a reference diagnostic procedure. Saline and iodine preparations were used to make direct wet mounts to improve the observation of G. lamblia cysts and trophozoites. The laboratory staff conducted microscopic examination on the basis of typical parasitological procedures. A positive sample was one that had characteristic cysts or trophozoites of G. lamblia. The results of stool microscopy were used to categorize the participants as cases and controls to compare them.

Molecular detection of G. lamblia. The genotyping assays utilized the DNA extracted according to the fecal specimens as previously described [7]. Briefly, Total DNA was extracted from each G. lamblia sample using the QIAamp® Stool mini kit (Qiagen, Germany) following the manufacturer's instructions. To optimize disruption of the cysts, prior to DNA extraction, the samples were subjected to three cycles of freezing and thawing by the following steps: two cycles alternating incubation in liquid nitrogen for five minutes and thawing in water bath at 70°C for five minutes and concluding with a cycle of freezing in liquid nitrogen for five minutes and thawing at 95°C for five minutes. Considering the possibility of false-negative results, negative samples were also processed for DNA extraction.Semi-nested PCR reaction was carried out as mentioned [12]. The first PCR reaction to amplify giardia triose-phosphate isomerase (HQ179643) loci on exon 3 with primers, AL3543 5-AAATIATGCCTGCTCGTCG-3' and AL3546: 5-CAAACCTTITCCGCAAACC-3' into a 605 bp template was followed by the second PCR reaction with primers, AL3544: 5-CCCTTCATCGGIGGTAACTT- Assemblage and sub-assemblage differentiation of the amplicons was done by digesting the amplicons with 1.0 units of endonucleases, BbvI, RsaI, and MnlI (New England Biolabs Inc., USA). Electrophoresis (subcell model 192 electrophoresis system, Bio-Rad, USA) of the digested products was done at 100 volts in agarose gels (Invitrogen, USA) at room temperature under 2 percent ethidium bromide stain. The fragments that had been resolved were subjected to UV light with the help of the gel documentation system (Uvitec, UK) and band size was compared with the positive and negative internal controls (New England Biolabs, USA) [4].

Quality control. The inhibition of PCR was estimated by addition of an internal amplification control in every reaction. Effective amplification of this control verified the fact that there were no inhibitory compounds in the sleep of the processed stool DNA. Sample amplification that failed to amplify or was delayed was deemed to be inhibited and re-extracted or diluted and re-amplified. The sensitivity of TPI-specific PCR to detect low concentration G. lamblia DNA was confirmed by serial dilution of the known positive DNA. The amplification was repeated under low DNA concentration, and this ensured that the assay was sensitive to detect infections with low parasite burden under the optimal conditions.

Data analysis. Structured data collection tools were used in recording demographic, clinical, and laboratory data. Diagnostic performance in the evaluation of diagnostic performance of TPI gene-specific PCR, the gold standard was the stool microscopy. Sensitivity, specificity, positive and negative predictive values, and the overall accuracy were estimated with 95 percent confidence interval. Cohen Kappa coefficient was used in determining agreement between methods, whereas McNemar test was employed in testing the difference in paired proportions. Further tests were performed on confusion matrix and binary logistic regression

RESULTS

Demographic, clinical characteristics and laboratory findings of the study participants

Data in Table 1 is summarized below. The gold-standard diagnostic tool applied was stool microscopy in which 78 participants were found to be infected with G. lamblia whereas 69 participants were found to be negative. Age distributions of microscopy-positive and microscopy-negative were similar with the median ages of 23.9 years (range: 0.01-73.1) and 25.3 years (range: 0.09-74.8), respectively, no statistical significant difference was found between them (P= 0.244). This means that there was no definite age pattern of infection at the time of first diagnosis.

There existed a remarkable correlation between gender and giardiasis that had been microscopically confirmed. The gender representation was characterized in such a way that the proportion of males (62.8%) was higher

than that of females (37.2%), and the proportion of female participants in microscopy-negative (59.4%) included a higher proportion than that of males (40.6%). This was statistically significant (P = 0.008) indicating that males were more burdened by the infection at presentation.

Various clinical manifestations were found to be closely linked to G. lamblia infection which is confirmed by microscopy. There was a great difference in diarrhea between the participants who were infected with 41.0% of microscopy-positive people having diarrhea and only 13.0% of microscopy-negative people having diarrhea (P< 0.0001). Equally, there was a significant association between bloating and infection with 57.1 and 42.9, respectively (P< 0.0001). The prevalence of headache was also important in infected participants (51.1) compared to the uninfected participants (48.9) (P = 0.016.(

The presence of distinct stool was greatly associated with giardiasis that was confirmed by microscopy. Infected participants were found to have foul-smelling stool 64.6 percent versus 35.4 percent of those who were not infected (P = 0.0001). The presence of fatty stool which is a classic characteristic of malabsorption in giardiasis was found in significantly higher numbers in positive cases (72.7) as opposed to negative (27.3) cases (P = 0.018). Mucus in stool was closely related to infection as there were 73.5% of positive cases and 26.5% of negative cases with mucus in stool (P < 0.0001). The coloration of the stool was also highly related to the G. lamblia positivity (whitish vs. 16.7%, P = 0.001).

Microscopic analysis also indicated that the frequency of pus cells in stool was much higher in infected subjects (71.02) compared to the uninfected subjects (29.02) (P< 0.0001), which indicated concomitant intestinal inflammation. There was a distinct stool consistency with the most common being formed stool among microscopy negative (97.7%), loose and semi-formed stool in microscopy-positive cases (P < 0.05 in both comparisons).

Symptoms like poor feeding, vomiting, flatulence, stomach soreness and abdominal pain, in contrast, did not demonstrate any statistically significant relationships with microscopy-confirmed giardiasis (P > 0.05), suggesting that these characteristics are not as specific in initial diagnosis in case stool microscopy is used as the reference method.

Table 1: Demographic and clinical characteristics and laboratory findings of the study participants

Variable	*G. lamblia* Negative (n = 69)	*G. lamblia* Positive (n = 78)	χ^2	*p*-value
Age (years), median (range)	25.3 (0.09–74.8)	23.9 (0.01–73.1)	—	0.244
Gender, n (%)				
Female	41 (59.4)	29 (37.2)	—	**0.008**
Male	28 (40.6)	49 (62.8)		
Clinical features, n (%)				
Poor feeding	65 (68.4)	30 (31.6)	2.333	0.152
Vomiting	23 (59.0)	16 (41.0)	0.569	0.560
Flatulence	23 (65.7)	12 (34.3)	0.062	0.843
Headache	23 (48.9)	24 (51.1)	6.751	**0.016**
Bloating	27 (42.9)	36 (57.1)	21.267	**<0.0001**
Stomach upset	43 (67.2)	21 (32.8)	0.517	0.494
Abdominal pain	61 (66.3)	31 (33.7)	0.593	0.480
Diarrhoea, n (%)				
Yes	9 (13.0)	32 (41.0)	—	**<0.0001**
No	60 (87.0)	46 (59.0)	21.900	
Stool characteristics, n (%)				
Foul-smelling stool	17 (35.4)	31 (64.6)	25.161	**0.0001**
Fatty stool	3 (27.3)	8 (72.7)	6.936	**0.018**
Mucus in stool	9 (26.5)	25 (73.5)	26.942	**<0.0001**
Whitish stool colour	2 (16.7)	10 (83.3)	12.669	**0.001**
Pus cells present	9 (29.0)	22 (71.0)	20.770	**<0.0001**
Stool consistency, n (%)				
Formed	43 (97.7)	1 (2.3)	31.083	**<0.0001**
Semi-formed	27 (50.9)	26 (49.1)	6.078	**0.020**
Loose	24 (49.0)	25 (51.0)	7.141	**0.011**

Table 1: Demographic, clinical, and stool characteristics of study participants based on stool microscopy diagnosis of *G. lamblia*. Data are presented as number (percentage) unless otherwise indicated. Age is presented as median (range). Stool microscopy was used as the gold standard for classification of G. lamblia infection status. Associations between variables and infection status were assessed using the chi-square test. A *P*-value < 0.05 was considered statistically significant.

Specificity, sensitivity, predictive values and accuracy of TPI-PCR in the diagnosis of *G. lamblia*

Table 2 below figures the diagnostic performance of gene-specific PCR of TPI when used against stool microscopy as the gold standard in the detection of Giardia lamblia infection in 147 study participants. Among 78 positive samples on stool microscopy, the TPI PCR was able to identify correctly 52 samples, and 26 samples that were microscopy-positive were not detected. Out of all the 69 samples that were microscopy negative, 68 were rightly classified as negative by PCR, but there was one false-positive. The total positive percentage (36.1) and negative (94) percentage were 53 and 94 respectively.

According to these results, TPI-PCR had the sensitivity of 66.7 (95% CI: 55.1-76.9), which is a moderate ability to identify microscopy-proven infections. The assay, however, had a very high specificity of 98.6% (95% CI: 92.3-100), which is an excellent ability to identify correctly those who are not infected. The positive predictive value was 98.1 (95% CI: 88.1-99.7) which means that PCR-positive outcomes were very reliable and the negative predictive value is 72.3 (95% CI: 65.6-78.1).

TPI PCR had a general likelihood of diagnosis of 81.6% (95% CI: 74.4-87.5). TPI PCR and stool microscopy had high agreement with the Cohen Kappa of 0.639 (CI: 0.523-0.755). The test by McNemar revealed the statistically significant difference between two methods ($P< 0.0001$) and it demonstrated the existence of discordant results of PCR and microscopy. Additionally, Odds Ratio indicated that overall diagnostic performance of TPI-PCR (OR; 136, 95%CI; 17.9 -1035.2 $P<0.00001$). The positive participants by stool microscopy were found to be 136 times more likely to test positive by TPI-specific PCR than a microscopy-negative participant. This suggests that there is a very strong association between PCR positivity and microscopy-confirmed *G. lamblia* infection.

Table 2: Specificity, sensitivity, predictive values and accuracy of TPI-PCR in the diagnosis of *G. lamblia*

TPI-PCR	Stool Microscopy			Sensitivity (95% CI)	Specificity (95%CI)	Predictive value (95%CI)		Accuracy (95% CI)	Kappa (95%CI)	Mc Nemar's (95% CI; Exact Probability)
						+ve Test	-ve Test			
	Positive	Negative	Total							
Positive	52	1	53 (36.1%)	66.7% (55.1-76.9%)	98.6% (92.3-100%)	98.1 % (88.1 - 99.7 %)	72.3% (65.6-78.1%)	81.6% (74.4-87.5%)	0.6388 (0.523-0.755)	17.0% (10.7-23.4; *P*<0.0001)
Negative	26	68	94 (63.9%)							

Table 2: Diagnostic performance of TPI gene–specific polymerase chain reaction (PCR) compared with stool microscopy for detection of *G. lamblia*. Stool microscopy was considered the gold standard. Diagnostic indices include sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), overall accuracy, Cohen's Kappa coefficient for agreement and McNemar's test for paired comparisons. Values are presented with 95% confidence intervals where applicable.

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Diagnostic Performance of TPI Gene–Specific PCR

The agreement between TPI PCR and stool microscopy results is summarized in figure 1b. Out of the 78 positive samples confirmed by microscopy, TPI PCR was able to correctly identify the positive ones, 52 and the negative ones, 26. In the 69 samples with microscopy negative PCR results, 68 samples were correctly identified as a true negative with only one false-positive case detected. This distribution indicates the extremely high specificity and positive predictive value of TPI PCR, and intermediate sensitivity. The confusion matrix also shows that although PCR will hardly misclassify uninfected persons, some microscopy-positive cases were not identified, and this points to the complementary application of molecular techniques and microscopy.

Figure 1

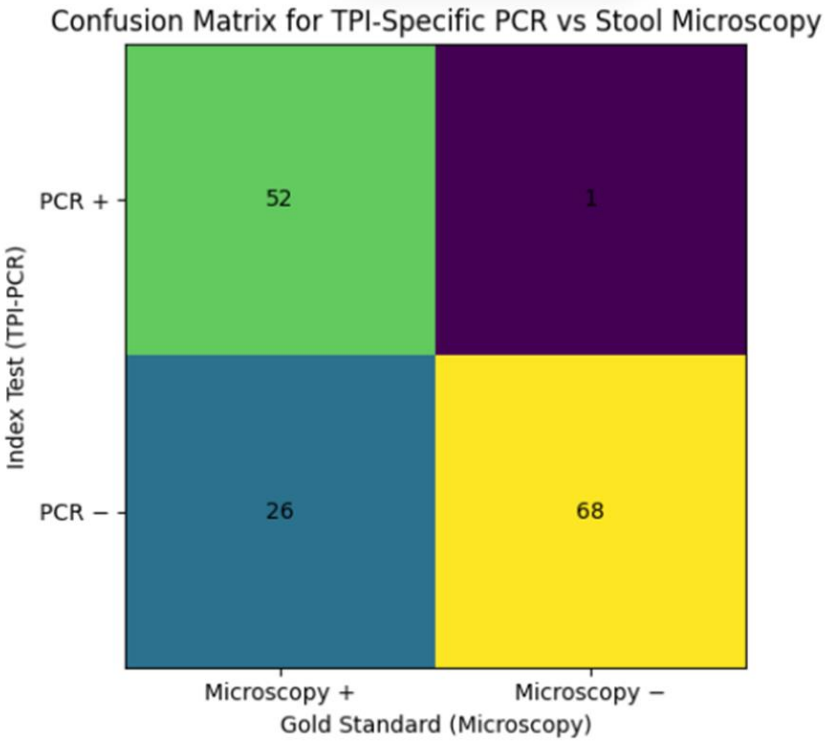

Figure 1: Confusion matrix showing the distribution of true positives, true negatives, false positives, and false negatives between TPI PCR and stool microscopy results.

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DISCUSSION

The aim of the study was to compare the diagnostic efficacy of TPI gene specific PCR to the traditional stool microscopy to identify *G. lamblia* infection in symptomatic patients in western Kenya. The most significant results were that TPI-PCR has an excellent overall diagnostic accuracy and a very large specificity, significant agreement with stool microscopy, but moderate sensitivity. Secondly, giardiasis with microscopic confirmation of the disease was closely related to classical symptoms of diarrhea and typical features of the stool, including a foul smell, mucus, steatorrhea, and changed stool consistency.

Clinically, TPI-PCR has a high level of specificity and positive predictive power, which implies that when the TPI gene is detected, it is a true indicator of G. lamblia infection. TPI gene is a single copy and highly conserved housekeeping gene that participates in the metabolism of energy among parasites, and thus, it is a stable molecular target [13]. It is unlikely to amplify in the absence of the parasite therefore and this is a reason as to why the false-positive rate is very low. The moderate sensitivity, on the other hand, indicates that PCR failed to detect some microscopy-positive infections. This can be explained by the low levels of parasite DNA in feces, unequal localization of the cysts, the existence of the PCR inhibitors, which is typical of fecal samples, or the destruction of the DNA during the work with samples [14]. These are the well-known limitations of stool-based molecular diagnostics and they can hamper the amplification efficiency even in the presence of parasites.

The close correlation between infection as confirmed by microscopy and such symptoms as diarrhea, bloating, foul-smelling stool, fatty stool and mucus supports the pathophysiology of giardiasis. G. lamblia clings on the intestinal mucosa resulting in atrophy of the villi, deficiency of brush border enzymes and lipid absorption [15]. Such a mechanism can describe the prevalence of steatorrhea, malodorous stool, and loose or semi-formed stool in infected people. The positive cases that had pus cells in their stool have a high likelihood of being a manifestation of mucosal irritation and second wave inflammatory reactions especially in patients who have experienced more severe or prolonged infection.

The results of the present study align with the findings of a number of earlier reports indicating that the specificity and the overall diagnostic performance of PCR-based techniques is much better than the microscopy of stool [4,7] . Molecular detection has been found to be reliable in other low- and middle-income environments, where studies have reported PCR specificity values of over 95% when using molecular targets of conserved genes, e.g., TPI, GDH, or SSU rRNA [17,18]. The moderate sensitivity of this study is also consistent with the reports that indicate that the sensitivity of PCR can be highly variable based on the methods of DNA extraction, quality of the samples, and the parasite load [19–21]. It has been reported that PCR is more sensitive than microscopy in some of these studies but these differences may be the result of multiple stool sampling, concentration methods or the application of real-time PCR platforms, which were not used in the current study [22].

Stool microscopy is useful in the routine practice as it is cheap and accessible, especially in the resource-strained environment [23]. Nonetheless, it can be underdiagnosed because of its dependence on the expertise of the operator and low sensitivity in cases of light or intermittent infections [24]. PCR usage particularly in patients with persistent symptoms with negative microscopy findings would result in better detection of cases and direct appropriate therapy. These findings at a policy level can be used to encourage a gradual implementation of molecular diagnostics within the referral and county labs, especially in surveillance, outbreak investigation, and research.

However, the interpretations of the diagnostic performance of TPI-specific PCR must be done with reference to the bias of references standards with the use of stool microscopy as a comparator method. Despite the overwhelming popularity of microscopy as a routine practice in clinical activities, it has a low sensitivity, especially to cases of low parasite burden, intermittent cyst shedding, and chronic infection. As a result, there is a threat of cases of misclassification as uninfected of some of the genuinely infected people. This restriction could have contributed to overestimation of the sensitivity of the PCR and false-positives in PCR positive and microscopy negative results. The bias is further aggravated by microscopy that is operator-dependent and the

use of single stool samples. Hence, discordance between microscopy and PCR as observed is most likely due to methodological bias instead of poor performance of the assays. Although microscopy is a viable reference comparator within a resource-constrained environment, future research must discuss the use of composite reference standards to enhance the accuracy of diagnosis.

CONCLUSION

It was shown in this study that TPI gene specific PCR is a very specific and accurate technique of detection in G. lamblia and it has a good overall diagnostic output and high percentage of agreement with stool microscopy. Although microscopy is vital in the routine diagnosis in a resource-constrained environment, the incorporation of molecular techniques may lead to better giardiasis discovery, patient control, and epidemiological monitoring. The results are part of the increasing numbers of evidence that testify to the complementary use of molecular diagnostics with conventional parasitological techniques in the endemic areas.

Recommendations

In standard clinical practice, especially the laboratories with limited resources, stool microscopy must continue to be the diagnostic approach of choice in the diagnosis of G. lamblia because of its low cost and availability. Nevertheless, the TPI gene specific PCR is recommended to be used as a supplementary diagnostic method, particularly when patients have persistent gastrointestinal symptoms or microscopy results are inconclusive, to enhance the detection of the case.

Basic molecular diagnostic capability including PCR infrastructure and skilled personnel should be developed gradually over time in county and referral laboratories to be able to perform confirmatory testing and enhance disease surveillance. On the policy level, parasitic disease surveillance systems need to incorporate molecular diagnostic information so that improved estimations of the giardiasis burden could be made and used to implement specific types of public health measures.

Further studies ought to concentrate on enhancing molecular assay sensitivity using better technologies like real time PCR, cost effectiveness analysis and genotyping studies to gain a better insight into transmission processes. This should be done in conjunction with enhancing water, sanitation and hygiene measures that will ensure that the giardiasis burden is reduced in the endemic areas.

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Ethical Approval

Ethical approval for the study was obtained from the MMUST institutional ethics review committee protocol number MMUST/ISERC/191/2025, and permission to do the study was sought from NACOSTI number NACOSTI/P/25/658939. Written informed consent was obtained from adult participants and from parents or guardians for minors prior to sample collection. Participant confidentiality was maintained throughout the study by use of coded identifiers and restricted access to study data.

Conflict of Interest

Authors declare no conflict of interest

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Authors' contribution

JRSW & TW: Conceptualization, Ethical approval procedures, literature search, methodology, and writing the manuscript. PKO: Literature search, methodology, coding of the data and data presentation & commenting on results, and writing the manuscript. TW & PKO: consultation and follow up of the research steps and supervision.

All the authors have read and approved the final manuscript.

HIGHLIGHTS

- TPI gene specific PCR had satisfactory overall diagnostic performance, as well as high specificity.
- Classical clinical and stool symptoms such as diarrhea, foul-smelling stool, mucus, steatorrhea and change in stool consistency were strongly linked with microscopy confirmed giardiasis.
- The results suggest that molecular diagnostics can be used as an adjunctary method to stool microscopy to enhance the detection, surveillance, and management of giardiasis in resource-constrained endemic locations.

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