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Detection of Intestinal Protozoa in HIV Patients: A Comparative Study of Microscopy and Multiplex Real-Time PCR

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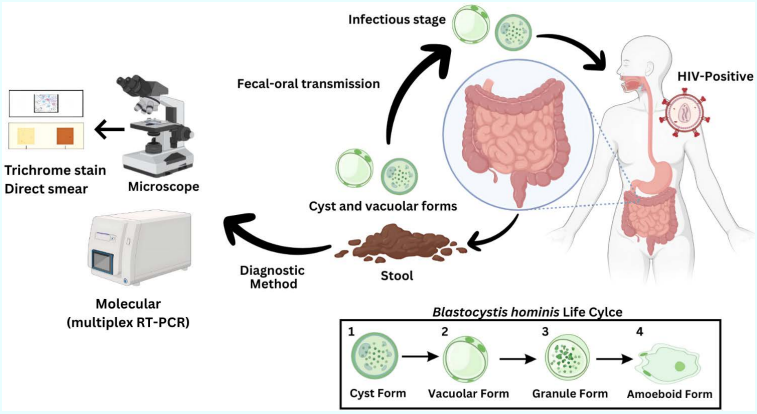
Abstract

Introduction: Intestinal protozoa are traditionally diagnosed through microscopic examination, which is labor-intensive and often requires repeated sampling. Recent molecular approaches, particularly multiplex Real-Time PCR (RT-PCR), provide a faster alternative for parasite detection. This study aimed to compare the sensitivity and specificity of direct smear and multiplex RT-PCR methods against trichrome staining for intestinal protozoa detection.



Methods: A cross-sectional study was conducted using 51 stool samples collected from HIV-naïve patients, HIV patients on therapy, and healthy volunteers at General Hospital Dr. Saiful Anwar, Malang, Indonesia. The samples consisted of 41 HIV-positive individuals (17 HIV-naïve and 24 on therapy) and 10 healthy volunteers. Stool specimens were examined using direct smear, trichrome staining, and multiplex RT-PCR.

Results: Microscopic examination identified *Entamoeba histolytica, Blastocystis hominis*, coccidian parasites, and non-pathogenic amoebae. In contrast, multiplex RT-PCR detected only B. *hominis* in both HIV and non-HIV samples. Compared with trichrome staining, direct smear demonstrated 36.6% sensitivity and 80.0% specificity, while multiplex RT-PCR showed higher sensitivity (63.4%) but lower specificity (40.0%). Differences among methods may be influenced by limited sample size, population characteristics, clinical status, and technical or biological factors affecting microscopy and molecular examinations.

Conclusion: Combining molecular diagnostics with conventional microscopy may improve the diagnostic accuracy of intestinal protozoa infections and support more effective treatment strategies and better clinical outcomes in HIV patients.

Keywords: HIV, Opportunistic infection, Intestinal protozoa, Diagnostic, Molecular detection

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Introduction

Individuals living with Human Immunodeficiency Virus (HIV) are at a heightened risk of acquiring intestinal parasitic infections, which may be caused by well-established intestinal pathogens or opportunistic parasites (1). Immunosuppression, particularly in patients with low CD4+T cell counts, predisposes them to these infections, which can exacerbate morbidity and increase mortality rates (2). Factors such as poor immune status, inadequate sanitation, and low socioeconomic conditions further contribute to the prevalence and severity of these infections (3). Although these parasites are often non-lethal in immunocompetent individuals, they can cause severe complications in HIV-positive patients, particularly those with advanced immunosuppression (4).

The distribution of intestinal parasites among HIV-positive individuals varies geographically, yet commonly identified pathogens include *Entamoeba histolytica*, *Giardia duodenalis*, *Cryptosporidium* spp., and *Isospora belli* (3). A study conducted at the Fann University Hospital in Senegal reported the prevalence of intestinal parasite carriers in patients with acute diarrhea at 20.4%, while in HIV-positive patients with chronic diarrhea, the prevalence was 28.6% (5). Similarly, a study in Yogyakarta, Indonesia, in 2014 found that 81.2% of HIV/AIDS patients had intestinal protozoa infections, with *Cryptosporidium* sp. (61.0%), *Microsporidium* sp. (19.5%), *Entamoeba histolytica* (9.8%), *Cyclospora cayetanensis* (4.9%), *Blastocystis hominis* (2.4%), and *Giardia duodenalis* (2.4%) being the most common (6). As indicated by the aforementioned studies, intestinal protozoa continue to be a pervasive health concern in developing countries. Despite the variation in sample sizes across studies and the outdated nature of the Indonesian data, the findings remain consistent. Moreover, it is noteworthy that both investigations employed solely microscopic examinations. In Indonesia, the examination of intestinal protozoa infections, specifically in HIV-positive populations, predominantly utilizes the microscopy method. To date, there have been no studies comparing the results of molecular and microscopic identification of intestinal protozoa in the HIV-positive population. Thus, this study is important to establish an accurate diagnosis of intestinal protozoa infection and guide appropriate treatment management for HIV-positive patients.

Various diagnostic techniques are available for detecting intestinal parasitic infections in HIV patients, including microscopic examination, serologic testing, and molecular assays (7). Microscopy remains a conventional method, with techniques such as direct smear, formalin-ethyl acetate concentration, and zinc sulfate flotation improving parasite detection (4,8). Additionally, staining techniques like modified acid-fast staining and trichrome staining enhance visualization of protozoan structures. Among these, trichrome staining is considered the reliable method for detecting intestinal protozoa. However, microscopy-based methods suffer from variable sensitivity due to operator dependency, sample variability, and the requirement for skilled personnel, making them resource-intensive and sometimes unreliable (9).

The diagnosis of intestinal parasites in HIV patients remains challenging, particularly in resource-limited settings where laboratory facilities and trained personnel are scarce. Traditional microscopic methods, while widely used, have inherent limitations in accuracy and efficiency. Molecular techniques such as multiplex real-time polymerase chain reaction (multiplex RT-PCR) offer a significant advantage in diagnosing intestinal parasites. This method allows for the simultaneous detection of multiple parasites, even at low concentrations, making it highly sensitive and specific. Multiplex RT-PCR enhances diagnostic accuracy, facilitates co-infection detection, and reduces turnaround time (10). This technology presents an opportunity to overcome the constraints associated with microscopic methods, offering a more reliable alternative for parasitological diagnosis in HIV patients.

Given the diagnostic challenges associated with intestinal protozoa in immunocompromised individuals, there is a critical need for more effective and sensitive testing methodologies. In this study, the utilization of in-house RT-PCR panels, meticulously designed to target specific intestinal parasites, offers a distinctive methodological contribution that has the potential to be adapted by other researchers. These in-house-tailored panels have been successfully validated in previous studies (11). The present study hypothesizes that molecular methods will provide more accurate results in diagnosing intestinal protozoa. This study aimed to analyze the diagnostic efficacy of the direct smear method and multiplex RT-PCR compared to trichrome staining as a standard method in detecting intestinal parasites in HIV-positive patients. It is anticipated that the findings will contribute to the development of more sensitive and specific methods for diagnosing intestinal protozoa during stool examinations in resource-limited settings. Furthermore, the combination of methodological comparison, clinical implications, and relevance to vulnerable populations underlines the importance of this study.

Materials and Methods

Study Design and Setting

A cross-sectional study was conducted at Dr. Saiful Anwar Hospital in Malang, Indonesia, to analyze preserved stool samples from HIV-positive patients (HIV on therapy and naïve-HIV patients) and healthy volunteers (non-HIV). The study spanned two phases: microscopic examination was performed at the Laboratory of Clinical Parasitology between August 2021 and June 2022, while molecular analyses were carried out at the Biomedical Laboratory, Faculty of Medicine, Universitas Brawijaya, and the Laboratory of Parasitology, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, from August 2023 to May 2024.

Patients

A random sampling method was used to recruit the

subjects in this study. The samples were obtained from two groups of subjects: HIV-positive patients who voluntarily sought treatment at Dr. Saiful Anwar General Hospital and patients who were subsequently diagnosed with HIV infection. The HIV group included male and female patients aged 18–60 years with a confirmed HIV diagnosis based on the presence of antibodies to HIV antigens, following the guidelines of the Ministry of Health, Republic of Indonesia, and undergoing a standard six-month regimen of antiretroviral therapy (ART) as per national recommendations (12). The naïve-HIV group comprised patients who had not yet initiated ART and had not received antibiotics, antifungals, or antiparasitic drugs in the two weeks preceding sample collection. The healthy volunteers (non-HIV) group consisted of healthy male and female patients aged 18–60 years, with a confirmed HIV-negative status determined by antibody testing in accordance with national guidelines. Patients were excluded from the study if they had comorbidities such as diabetes mellitus (DM), inflammatory bowel disease (IBD), hepatitis cirrhosis, pregnancy, malignancies, or if they declined participation.

Data Collection

The calculation of the total sample ($n = 51$) size was determined by the confidence interval, with the population estimate of HIV cases in East Java serving as a crucial parameter. Stool samples were collected between August 2021 and June 2022 from three groups: HIV- on therapy, naïve-HIV, and healthy volunteers (non-HIV). Each patient was provided with a sterile, labeled collection cup, and samples were transported in an ice box within 6–8 hours of collection to preserve sample integrity. Each stool sample weighed approximately 15 grams, roughly equivalent to the length of an adult's knuckle. Upon arrival at the Laboratory of Clinical Parasitology, Faculty of Medicine, Universitas Brawijaya, a microscopic examination using the direct smear method was conducted immediately. For molecular analysis, 400 mg of each stool sample was transferred into 1.5-ml microcentrifuge tubes and stored at -20°C. The remaining samples were preserved in polyvinyl alcohol/ PVA (Sigma-Aldrich, St. Louis, MO, USA) for trichrome staining.

Direct Smear Method

The fecal direct smear was performed following a standardized protocol (13). Briefly, a clean, labeled glass slide was prepared by placing one drop each of normal saline and Lugol's iodine (Lugol pro Gram; BIO Analitika, Surabaya, Indonesia) in opposite positions. Normal saline consists of a mixture of sodium chloride (Pro analysi Natriumchlorid.p.A; Merck KGaA, Darmstadt, Germany) and water. Using a sterile applicator stick, stool samples were collected from multiple areas within the specimen cup to ensure representative sampling. The collected stool was thoroughly emulsified in each drop of solution until a homogeneous mixture was achieved. A coverslip was carefully placed over each preparation to prevent air bubbles and ensure uniform distribution. The slides were examined microscopically at 40× and 100× magnifications. This method was employed to identify intestinal protozoa based on their morphological features and motility.

Trichrome Staining

The Wheatley Trichrome technique, a modification of the Gomori stain, was applied to stool samples for microscopic analysis. The object glass with a PVA-treated stool smear was placed in a staining jar containing 70% ethanol (70% Alkohol; CV, Nurra Gemilang, Malang, Indonesia) and iodine (Iodine; Merck KGaA, Darmstadt, Germany) for 10 minutes. The object glass was then immersed in 70% ethanol for 5 minutes, followed by an additional 3-minute soak in the same solution. It was stained with Chromotrope 2R Trichrome Stain (Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes, then briefly de-stained in 90% ethanol and acetic acid (Acetic acid glacial 100%; Merck KGaA, Darmstadt, Germany) for 1-3 seconds. The glass was rinsed several times with 100% ethanol (Ethanol; Merck KGaA, Darmstadt, Germany). Next, the object-glass was transferred to a jar of 100% ethanol for 3 minutes, repeated twice, and then immersed in xylene (Xylene pro analys; Merck KGaA, Darmstadt, Germany) for 10 minutes. Finally, the object-glass was mounted with entellan (Entellan: Merck KGaA, Darmstadt, Germany), ensuring no air bubbles were present, and was observed under a microscope at 100x magnification with immersion oil (Immersion oil; Merck KGaA, Darmstadt, Germany). Trichrome staining was considered the reference standard method for identifying intestinal protozoa based on the morphology of the main life stages.

DNA Extraction of Stool Samples

DNA extraction was performed using a spin-column-based method (QIAamp DNA Mini Kit; Qiagen, Hilden, Germany), as previously described (11). Stool samples were weighed and resuspended in 200 µL of phosphate-buffered saline (Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA) with 2% polyvinylpolypyrrolidone/ PVPP (Sigma, Steinheim, Germany) to reduce PCR inhibitors. After heating at 100°C for 10 minutes, the samples were treated with ATL buffer and proteinase K, followed by incubation at 55°C for 2 hours. Phocine herpesvirus 1 (PhHV-1) was spiked into 400 µL of AL Buffer as an internal control. DNA extraction proceeded according to the manufacturer's spin-column protocol, ensuring efficient purification (11).

Two multiplex RT-PCR panels were utilized for parasite-specific DNA detection. Panel I targeted *Blastocystis hominis*, *Entamoeba histolytica*, and *Entamoeba dispar*, while Panel II targeted *Dientamoeba fragilis*, *Giardia duodenalis*, and *Cryptosporidium spp*. Primer and probe sequences were adapted from a prior study (11). Each PCR run included both negative and positive controls to ensure assay validity. Amplification and analysis were carried out using the CFX96 Touch Real-Time PCR Detection System

(Bio-Rad), and Ct values were analyzed with Bio-Rad CFX Maestro software (Ver. 4.1.2433.1219). DNA loads were categorized based on Ct values: low ($35 \leq Ct < 50$), intermediate ($30 \leq Ct < 35$), and high ($Ct < 30$) (11).

Data Analysis

In this study, the diagnostic performance of direct smear and multiplex RT-PCR was compared with trichrome staining, the reference standard for intestinal protozoa detection in HIV-positive patients (14). Sensitivity was calculated by dividing the number of true-positive test results by the total number of patients confirmed as either positive or negative for the target condition based on trichrome staining. Specificity was determined by dividing the number of true-negative test results by the total number of patients without the target condition. Additionally, the positive predictive value (PPV) was calculated as the proportion of patients with a positive test result who were accurately identified as having the infection, computed by dividing the true positives by the total number of positive test results. The negative predictive value (NPV) represented the proportion of patients with a negative test result who were correctly identified as not having the infection, calculated by dividing the true negatives by the total number of negative test results (15). All statistical analyses, including PPV, NPV, Cohen's Kappa, and Confidence Interval (CI), were performed using MedCalc Software version 23.1.7 (16).

Results

Direct smear method on the prevalence of intestinal protozoa in HIV-positive and HIV-negative stool samples

A total of 51 patients, 41 HIV-positive (17 HIV-naïve and 24 HIV on therapy) and 10 healthy volunteers (non-HIV), were included in the study. Based on the direct smear method, the results of stool examination showed that the HIV-positive group had a higher incidence of intestinal protozoal infection compared to the healthy volunteers (non-HIV) group (Table 1). Among the 17 positive samples by the direct smear method, the HIV-naïve group had a higher prevalence of intestinal protozoan infection than the HIV on therapy and healthy volunteers (non-HIV) groups.

Macroscopic examination revealed that only one sample from the HIV-naïve group exhibited characteristics of bloody diarrhea. The remaining samples displayed normal color and consistency. Microscopic examination showed that the most commonly found intestinal protozoa were coccidian parasites, followed by non-pathogenic amoeba, *B. hominis*, and *E. histolytica* (Table 2; Figure 1).

Table 1. The prevalence of intestinal protozoa in Non-HIV, HIV on therapy, and naïve-HIV groups using direct smear

Groups	Frequency	Direct smear, n(%)	
		Positive (+)	Negative (-)
Non-HIV	10	2 (3.9%)	8 (15.7%)
HIV on therapy	24	2 (3.9%)	22 (43.1%)
Naïve-HIV	17	13 (25.5%)	4 (7.8%)
Total	51	17 (33.3%)	34 (66.7%)

Diagnostic performance of the direct smear method against trichrome staining for detecting intestinal protozoa in HIV-positive and HIV-negative stool samples

The descriptive analysis of the comparison between direct smear and trichrome staining regarding the presence of intestinal parasites is shown in Table 3, with a Cohen's Kappa value of 0.03 and 95% confidence interval (CI) value of -0.23 to 0.23 calculated using MedCalc. Based on the comparison between Trichrome staining and multiplex RT-PCR, the kappa value of 0.03 indicates a very low level of agreement (slight agreement), suggesting that Trichrome staining demonstrates limited reliability for detecting infection when compared with PCR. As the confidence interval includes zero, the result cannot be considered statistically significant, suggesting that the level of agreement between multiplex RT-PCR and Trichrome staining (reference standard) may not exceed that expected by chance. Those results indicated that the number of samples needs to be increased.

The prevalence data demonstrate the risk of intestinal protozoan infection between HIV-positive (naïve-HIV and HIV on therapy) and healthy volunteers (non-HIV) groups in both examination methods.

It is important to note that a single sample may be infected by multiple species of intestinal protozoa. The microscopic findings, obtained through the direct smear and trichrome staining methods, were shown in Figure 2.

The multiplex RT-PCR method for the prevalence of intestinal protozoa in HIV-positive and HIV-negative stool samples

In order to confirm the microscopic examination, multiplex RT-PCR was conducted on all the groups using 51 available samples. The results of molecular examination using multiplex RT-PCR demonstrated a contradictory finding compared to the microscopic examination (Supplementary Data). The highest prevalence of intestinal protozoa infection was observed in the HIV on therapy group, followed by the HIV-naïve

Table 2. Intestinal protozoa species found using direct in HIV-positive and HIV-negative stool samples ($n = 51$)

Species of protozoa	Direct Smear, *n*(%)		
	Non-HIV	HIV on therapy	Naïve-HIV
Blastocystis hominis	0 (0%)	0 (0%)	2 (3.9%)
Entamoeba histolytica	0 (0%)	0 (0%)	1 (2%)
Entamoeba dispar	0 (0%)	0 (0%)	0 (0%)
Non-pathogenic amoeba	2 (3.9%)	0 (0%)	4 (7.8%)
Dientamoeba fragilis	0 (0%)	0 (0%)	0 (0%)
Giardia duodenalis	0 (0%)	0 (0%)	0 (0%)
Coccidian parasite (including *Cryptosporidium* spp. and *Cyclospora* sp.)	2 (3.9%)	2 (3.9%)	7 (13.7%)

Table 3. Diagnostic performance of the direct smear method compared to trichrome staining for detecting intestinal protozoa in HIV-positive and HIV-negative stool samples

Golden standard	HIV-status	Direct smear		Total (n)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
		Positive	Negative					
Trichrome staining	HIV-positive	15 (true positive)	26 (false negative)	41	36.6%		88.2%	23.5%
	HIV-negative	2 (false positive)	8 (true negative)	10		80.0%		
Total		17 (true positive) + 2 (false positive)	26 (false negative) + 8 (true negative)	51	36.6%	80.0%	88.2%	23.5%

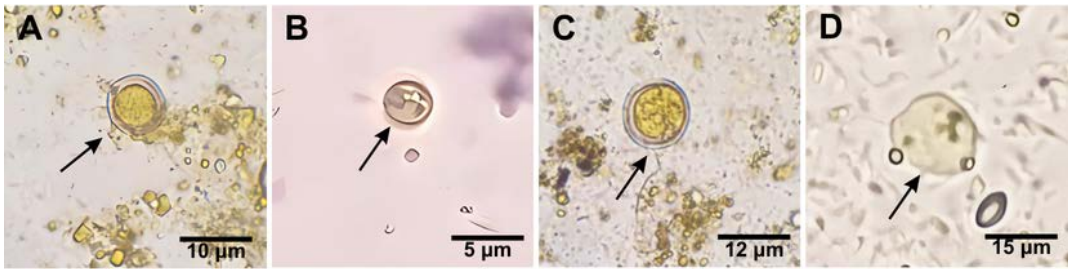

Figure 1. The morphology of parasite species was determined through the direct smear method (observed at 100x magnification). (A) The vacuolated form of *Blastocystis hominis*, as indicated by the arrow, showed a large central vacuole and peripherally located nuclei; (B) The presence of coccidian parasites was detected in the HIV group; (C) A cyst of *Entamoeba histolytica* was suspected in the HIV-naïve group; (D) The presence of a trophozoite non-pathogenic amoeba was also detected using the direct smear method

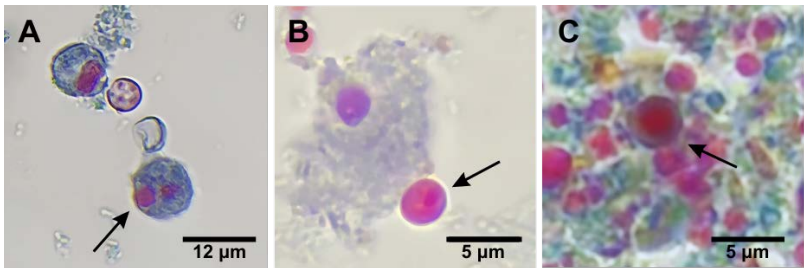

Figure 2. Intestinal parasites were found during microscopic examination using the trichrome staining method (observed at 100x magnification). The parasitic forms are indicated by black arrows, including (A) cyst of *E. histolytica* from a patient with HIV naïve therapy; (B) oocyst of *Cryptosporidium* spp. from a patient with HIV on therapy; (C) cyst of *B. hominis* from a healthy volunteer

and healthy volunteers (non-HIV) groups (Table 4).

The multiplex RT-PCR was performed using two panels, which specifically detected *B. hominis, E. histolytica, E. dispar* (BHD panel) and *D. fragilis, G. duodenalis, Cryptosporidium* spp. (DGC panel). Notable finding, the microscopic examination identified *E. histolytica*, *E. dispar*, and *Cryptosporidium* spp., yet these same samples were subsequently found to be negative based on multiplex RT-PCR analysis (Table 5). Such discrepancies likely reflect technical and biological differences between the methods, including operator-dependent interpretation in microscopy, the presence of PCR inhibitors, or uneven parasite distribution within stool samples.

Diagnostic performance of the direct smear method against trichrome staining for detecting intestinal protozoa in HIV-positive and HIV-negative stool samples

As shown in Table 6, a comparison between multiplex RT-PCR and trichrome staining indicates that the molecular method exhibits sensitivities of 63.4% and specificities of 40.0%, respectively. While the PPV and NPV were 81.3% and 21.1%. The accuracy of multiplex RT-PCR was 58.8%.

Discussion

Microscopic examination using the direct smear method revealed the presence of intestinal protozoa in both the HIV-positive (HIV-naïve and HIV on therapy) and healthy volunteers (non-HIV) groups. Individuals with HIV (immunocompromised) are susceptible to infection by other pathogens, including intestinal protozoa. Research has indicated that the incidence of intestinal protozoan infections in patients with HIV/AIDS can reach up to 81.2% in specific populations (2). The most prevalent pathogens identified in these cases include *Cryptosporidium*, *E. histolytica*, and *G. lamblia* (2,6). The HIV on therapy group exhibited a lower prevalence of intestinal protozoa compared to the HIV-naïve group. A previous study conducted by Woldegeorgis et al., (2023) demonstrated that individuals with HIV who had received therapy (ART) still exhibited a risk of developing opportunistic infections (17). These findings highlight the persistent risk in treated patients; the healthcare providers must maintain vigilant screening for opportunistic infections throughout HIV care, not just at initial diagnosis (18).

The findings of the present study demonstrated that trichrome staining exhibited higher sensitivity

Table 4. The prevalence of intestinal protozoa in Non-HIV, HIV on therapy, and naïve-HIV groups using multiplex RT-PCR

Groups	*n*	Multiplex RT-PCR, n(%)	
		Positive (+)	Negative (-)
Non-HIV	10	6 (11.8%)	4 (7.8 %)
HIV on therapy	24	14 (27.5%)	10 (19.6%)
Naïve-HIV	17	12 (23.5%)	5 (9.8%)
Total	51	32 (62.7%)	19 (37.3%)

Table 5. Intestinal protozoa species found using multiplex RT-PCR in HIV-positive and HIV-negative stool samples ($n = 51$)

Species of Protozoa	Multiplex RT-PCR, n(%)		
	Non-HIV	HIV on therapy	Naïve-HIV
Blastocystis hominis	6 (11.8%)	14 (27.5%)	12 (23.5%)
Entamoeba histolytica	0 (0%)	0 (0%)	0 (0%)
Entamoeba dispar	0 (0%)	0 (0%)	0 (0%)
Dientamoeba fragilis	0 (0%)	0 (0%)	0 (0%)
Giardia duodenalis	0 (0%)	0 (0%)	0 (0%)
Cryptosporidium spp.	0 (0%)	0 (0%)	0 (0%)

Table 6. Diagnostic performance of the multiplex RT-PCR method compared to trichrome staining for detecting intestinal protozoa in HIV-positive and HIV-negative stool samples

Golden standard	HIV-status	Multiplex RT-PCR		Total (n)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
		Positive	Negative					
Trichrome staining	HIV-positive	26 (true positive)	15 (false negative)	41	63.4%		81.3%	21.1%
	HIV-negative	6 (false positive)	4 (true negative)	10		40.0%		
Total		26 (true positive) + 6 (false positive)	15 (false negative) + 4 (true negative)	51	63.4%	40.0%	81.3%	21.1%

and specificity compared to the direct smear method. This emphasizes the reliability of trichrome staining for intestinal protozoa identification. Research has demonstrated that the trichrome stain is a highly effective conventional method for the identification of intestinal protozoa, with a reported accuracy up to 87.93% for the distinction of intestinal protozoa from stool samples (19). The superior performance of trichrome staining over direct smear methods indicates that laboratories should prioritize trichrome staining as their primary diagnostic method for the detection of intestinal protozoa (20). This is of particular importance in settings where accurate parasite identification directly impacts patient management decisions. In the context of systematic screening programs, particularly within immunocompromised populations, trichrome staining has been demonstrated to exhibit superior performance characteristics (21). This renders it the optimal approach for both population-level surveillance and the management of individual patients.

The result of multiplex RT-PCR revealed the absence of intestinal protozoa species, with the notable exception of *B. hominis*. Exceptionally, some species suspected to be positive on microscopic examination, including *E. histolytica* and coccidian protozoa (including *Cryptosporidium* spp.), were found to be negative based on the results of multiplex RT-PCR. Similar to a previous study conducted by Rattaprasert et al., (2021), which reported a discrepancy in microscopy compared to molecular diagnostic methods, using nested PCR during the detection of intestinal protozoa (22). The use of merely standard wet preparation for *Cryptosporidium* was less predictive, resulting in 21.7% misdiagnosis (1.7% undetected plus 20% misdiagnosed as yeast). Yeast and other fungal components are commonly found in stool. Depending on the size and shape, they can be mistaken for a number or protozoan species (23). In this study, we did not run a panel that quantified yeast or other fungal species to support the claim. However, our unreported data showed that *Cryptosporidium* identification using conventional PCR (different primer design compared to the ones used in multiplex RT-PCR) may cross-react with fungal species that are identified through amplicon sequencing.

The presence of *B. hominis* was detected in both HIV-positive (HIV-naïve and HIV on therapy) and healthy volunteers (non-HIV) groups based on the result of multiplex RT-PCR. This result is consistent with the study conducted by Permasutha and Diptyanusa (2024), which shows that the prevalence of *B. hominis* infection is higher in HIV on therapy ($n = 22$) compared to those not undergoing therapy/ HIV-naïve ($n = 4$) (24). Furthermore, a longer duration of antiretroviral therapy (ART) could contribute to immune reconstitution. Patients may experience fluctuations in immune status that could render them susceptible to opportunistic infections, such as *B. hominis* (25). The elevated risk of *B. hominis* infection in HIV patients undergoing prolonged ART is a multifactorial phenomenon, including the patient's immunological status, exposure to environmental factors, hygiene practices, and the management of treatment regimens (26). Previous studies also reported the presence of *B. hominis,* which was identified as pathogenic and non-pathogenic subtypes in the human population (27). A study also showed that the subtype of *B. hominis* may appear in healthy individuals (28). The presence of *B. hominis* in both healthy individuals and HIV patients suggests that certain subtypes may be commensal rather than pathogenic. Additionally, clinicians should exercise caution before initiating treatment for *B. hominis* infections, particularly in asymptomatic patients. The finding suggests that treatment decisions should be based on clinical presentation and symptoms rather than mere presence of the organism, regardless of the patient's HIV

status.

Multiplex RT-PCR has successfully identified a higher prevalence of *B. hominis* in the samples compared to microscopic examination using direct smear and trichrome staining methods. However, this method also showed higher sensitivity but less specificity compared to the trichrome stain. The accuracy of a test is determined by its sensitivity and specificity. High sensitivity signifies the capacity of the test to detect correct cases, while high specificity denotes the ability of the test to rule out false positive results (29). In our case, the low specificity may be attributed to the fact that only one species (*B. hominis*) was positively identified using the molecular method across the samples. While other coccidian parasites, identified as positive in microscopy examination, could not be confirmed using molecular examination due to panel limitations. The result presented in our study challenges the common assumption that molecular methods are uniformly superior. Other studies reported higher sensitivity and specificity of molecular examination compared to microscopic examination (30,31) which opposed the result presented in our study. The observed discrepancies among the studies might be attributed to various factors, particularly during technical and biological processes in microscopy and molecular methods. The presence of uneven staining has been demonstrated to cause artifacts that have the potential to interfere with the observation microscopically of target organisms. Additionally, *B. hominis* manifests pleomorphism, exhibiting multiple morphological forms (e.g., vacuolar, granular, amoeboid, and cystic) that may not all be equally detectable by microscopy. The process of DNA extraction efficiency may vary between different stool sample consistencies. Moreover, the low specificity of PCR may result from biological factors, including the resemblance of conserved sequences. The primers utilized in the multiplex PCR for *Giardia, Entamoeba, Cryptosporidium,* and *Blastocystis* were designed from the conserved 18S rRNA gene. Primers created from this conservative area may attach to DNA fragments of various species, consequently amplifying non-target organisms. In some cases, multiple primer design from various genes is needed in order to correctly identify certain species (20). Hence, this variation has the potential to affect detection rates. A multitude of factors may also contribute to discrepancies, which are not limited to population characteristics, sample size, and clinical status. Population characteristics play a significant role in influencing the prevalence and genetic diversity of intestinal protozoa, which in turn affects detection performance. Sample size is another critical determinant; studies with small cohorts may not adequately represent the diversity of infection patterns, leading to statistical instability and wide confidence intervals in agreement analyses. Limited sample numbers also reduce the likelihood of capturing both positive and negative cases in balanced proportions, potentially biasing sensitivity and specificity estimates. Additionally, the clinical status of immunocompromised individuals, such as HIV-infected patients, altered parasite load, and intermittent shedding can reduce detection accuracy, contributing to inconsistencies between microscopy and molecular assays.

This study is subject to several limitations. The samples collected for this study came from one hospital only, and the number is also limited. These limitations might also contribute to the low diversity of intestinal protozoan species found in this study compared to other previous studies. Moreover, in current study, a specific panel to identify other coccidian parasites (*Cyclospora cayetanensis* and *Isospora belli*) has yet to be developed and optimized. Several factors must be taken into consideration for future research in the development of diagnostic panels using multiplex RT-PCR. These include the consideration of urgency, the availability of positive controls, primer design, and optimal conditions for amplification (32). This study identified additional limitations concerning the validation of negative results in molecular detection. The meticulous execution of the sample collection, processing, and DNA preparation steps was foundational to the successful implementation in molecular detection. Furthermore, the presence of inhibitors in the samples has the potential to influence the outcome of PCR amplification (33). It is important to note that inhibition of parasitic DNA amplification may result in false-negative outcomes (34-40). This emphasizes the importance of incorporating internal amplification controls and optimizing DNA extraction methods to reduce inhibitory effects and improve the reliability of molecular detection in future studies. Taken together, these limitations suggest that the conclusions of this study are context-specific and may not be generalizable to settings that differ in population, methodology, or diagnostic approach.

Nonetheless, molecular examination contributed significantly to reducing the likelihood of misdiagnosis of pathogenic infections in HIV-positive patients. Although multiplex RT-PCR demonstrated higher sensitivity compared to conventional microscopy, its moderate accuracy and relatively low specificity indicate that it should not be regarded as a standard diagnostic tool. Instead, molecular diagnostics are best utilized in conjunction with traditional microscopic methods to enhance the accuracy of intestinal parasite detection, optimize treatment decisions, and ultimately improve patient outcomes.

Conclusion

In summary, this study directly compared three distinct diagnostic tools (direct smear, trichrome stain, and multiplex RT-PCR) in a specific patient population consisting of those who were HIV-naïve, those on therapy for HIV, and those with non-HIV infections. The study revealed that multiplex RT-PCR exhibited higher sensitivity but lower specificity compared to trichrome stain, thereby challenging the prevailing assumption that molecular methods are consistently superior. Nevertheless, the study population was limited to a single

health care facility, which restricts the generalizability of the findings. Variations in patient demographics, prevalence of intestinal protozoa, laboratory practices, and local epidemiology across different regions may influence diagnostic performance. Therefore, larger-scale and multicenter studies are warranted to validate these results, provide more representative data, and strengthen the evidence base for selecting appropriate diagnostic strategies in diverse clinical and epidemiological settings. Additionally, our findings revealed that *B. hominis* was the sole protozoan consistently identified through PCR across all groups, thus prompting inquiries into the clinical relevance and diagnostic accuracy of conventional detection methods in immunocompromised patients. The findings indicate that accurate diagnostics are paramount in settings characterized by a dearth of resources, as such precise assessments facilitate treatment decisions and enhance the well-being of individuals infected with HIV, a matter of particular concern in regions marked by low- and middle-income economies, where the incidence of HIV infection is notably high. The novelty of the study is underscored by its integration of methodological comparison, clinical implications, and relevance to vulnerable populations.

Future study may aim to incorporate additional health centers (hospitals) in order to validate the findings of the present study. Moreover, the development of an additional multiplex RT-PCR panel is imperative for the identification of other parasites.

This study compared direct smear, trichrome stain, and multiplex RT-PCR for intestinal protozoa detection among HIV-naïve, treated HIV, and non-HIV patients. Multiplex RT-PCR showed higher sensitivity but lower specificity, highlighting diagnostic challenges and clinical implications in resource-limited, high-HIV-prevalence settings.

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Competing Interests

The authors declare no competing interests.

Ethical Approval

This study had been approved by the Ethics Commission of General Hospital Dr. Saiful Anwar Malang (Number: 400/106/K.3/302/2021-2 June 2021) and Institutional Review Board of the Faculty of Medicine Universitas Brawijaya (ethical certificate number 253/EC/KEPK/08/2023). The review process started with the submission of research proposal and supporting research documents to institutional ethics committees. The documents consist of research proposal and study design, as well as questionnaire, and informed consent for sample collection. A thorough review of the documents was conducted by the ethics committees to obtain ethical approvals.

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Supplementary Data

The summary of the results obtained through the identification of intestinal protozoa using microscopy and multiplex RT-PCR is available at https://doi.org/10.6084/m9.figshare.28399589

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