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STANDARDIZATION OF BLASTOCYSTIS HOMINIS DIAGNOSIS USING DIFFERENT STAINING TECHNIQUES

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Abstract

Background: *Blastocystis hominis* is one of the most frequently encountered intestinal protozoa worldwide, yet its diagnosis remains challenging due to its pleomorphic nature and lack of standardized detection methods.

Objective: The primary objective of this study was to evaluate and standardize different staining techniques for the detection of *Blastocystis hominis* in stool samples.

Methodology: This cross-sectional analytical study was conducted on 85 patients presenting with gastrointestinal complaints. Stool samples were collected and examined using multiple staining techniques, including trichrome, iron hematoxylin, Giemsa, and modified Ziehl–Neelsen stains. Results were compared against a composite reference standard, defined as positivity by at least one staining method.

Results: Out of 85 samples, *Blastocystis hominis* was detected in 34 (40.0%) cases by at least one staining method. Trichrome stain demonstrated the highest detection rate (36.5%), followed by iron

hematoxylin (32.9%), Giemsa (28.2%), and modified Ziehl–Neelsen (11.8%). Diagnostic accuracy analysis showed trichrome to have a sensitivity of 91.2% and specificity of 96.1%, while iron hematoxylin had 82.3% sensitivity and 96.1% specificity. Inter-observer agreement was strong across all stains, with kappa values ranging from 0.86 to 0.91. No statistically significant association was observed between gastrointestinal symptoms and *Blastocystis* positivity.

Conclusion: It is concluded that trichrome staining is the most sensitive and practical method for routine detection of *Blastocystis hominis*, while iron hematoxylin remains a reliable but technically demanding alternative. Giemsa provided moderate results, whereas modified Ziehl–Neelsen was unsuitable for routine diagnosis.

Keywords: *Blastocystis hominis*, trichrome stain, iron hematoxylin, diagnostic accuracy, stool microscopy, parasitology.

Introduction

Blastocystis hominis is among the most commonly encountered intestinal protozoa worldwide, with prevalence estimates ranging from 10–60% in developing regions and 5–15% in industrialized nations[1]. The organism belongs to the genus *Blastocystis*, which includes multiple subtypes infecting both humans and animals. Its transmission is primarily fecal–oral, facilitated by contaminated water, food, and person-to-person contact. The high carriage rate of *Blastocystis* in asymptomatic individuals has historically fueled debate over its pathogenic role. However, accumulating evidence suggests that certain subtypes may be more virulent, contributing to symptoms such as chronic diarrhea, abdominal discomfort, flatulence, fatigue, and even extra-intestinal manifestations[2]. Understanding the burden of infection is thus highly dependent on the reliability of diagnostic techniques. The organism exhibits marked pleomorphism, appearing in vacuolar, granular, cystic, and amoeboid forms, which complicates recognition under the microscope[3]. The vacuolar form is most commonly seen in stool samples, but intermittent shedding and morphological overlap with other fecal debris can lead to misinterpretation. Conventional direct wet mount microscopy, while simple and inexpensive, demonstrates poor sensitivity, especially in cases with low parasite load. This diagnostic gap has prompted efforts to optimize staining and concentration methods to enhance detection[4].

A range of staining methods has been evaluated for *Blastocystis hominis*. The trichrome stain, widely used in parasitology laboratories, provides good visualization of nuclear and cytoplasmic structures but requires considerable technical skill and processing time. Iron hematoxylin stain, though historically regarded as a gold standard for intestinal protozoa, is less commonly employed today due to its complexity. Modified Ziehl–Neelsen stain, typically used for coccidian parasites, has shown limited utility for *Blastocystis*. Other techniques such as Giemsa and acridine orange stains have been reported, with varying sensitivity and specificity. Differences in fixation procedures, staining duration, and interpretation criteria across laboratories have resulted in inconsistencies, making standardization an urgent priority. In addition to staining, culture methods using Jones' medium or Locke–Egg serum have been employed to increase yield[5]. Cultures, however, are time-consuming and not practical for routine diagnostics. More recently, molecular assays, particularly polymerase chain reaction (PCR), have been recognized as the most sensitive approach, enabling not only detection but also subtype differentiation. Despite their advantages, molecular diagnostics remain limited to research centers and specialized laboratories due to cost, equipment requirements, and the need for trained personnel. Consequently, in many endemic regions, microscopy and staining remain the mainstay of diagnosis[6-7].

The clinical implications of missed or delayed diagnosis are significant. Patients with unexplained gastrointestinal symptoms may undergo multiple investigations or receive empiric treatments without clear etiology. Accurate diagnosis of *Blastocystis* is essential not only for patient management but also for epidemiological surveillance and understanding the organism's role in gastrointestinal disease[8-9]. Moreover, standardizing diagnostic methods allows comparisons across studies and populations, contributing to more reliable global prevalence estimates. Previous

comparative studies have highlighted that staining techniques can differ markedly in diagnostic yield. For instance, trichrome staining has been reported to detect *Blastocystis* in up to 30% more samples than wet mount preparations, while iron hematoxylin has shown slightly higher sensitivity but poorer practicality in routine use[9]. The choice of stain also influences the morphological details observed, with some methods enhancing nuclear features while others emphasize cytoplasmic vacuoles. Without harmonization, laboratories risk underreporting or misidentifying infections, leading to significant variability in reported prevalence data. Standardization involves not only identifying the most effective staining method but also establishing uniform protocols for specimen preparation, fixation, staining time, and interpretation. Such efforts are critical for developing guidelines that can be implemented in both resource-limited and advanced laboratory settings. By comparing different stains head-to-head under controlled conditions, researchers can provide evidence-based recommendations for laboratories worldwide[10].

Objective

The primary objective of this study was to evaluate and standardize different staining techniques for the detection of *Blastocystis hominis* in stool samples.

Methodology

This was a cross-sectional analytical study conducted at over a period of one year. A total of 85 stool samples were collected from patients presenting with gastrointestinal complaints who met the inclusion criteria.

Inclusion Criteria

- Patients aged ≥18 years.
- Individuals presenting with gastrointestinal symptoms such as diarrhea, abdominal pain, bloating, or flatulence.
- Patients who provided informed consent and submitted stool samples for parasitological examination.

Exclusion Criteria

- Patients who had received antiparasitic therapy within the last four weeks.
- Individuals with incomplete clinical or laboratory data.
- Samples with insufficient quantity or poor preservation.

Sample Collection and Processing

Fresh stool samples were collected in clean, wide-mouthed, sterile containers. Each specimen was divided into two parts: one preserved in 10% formalin for staining procedures and another stored without preservatives for direct wet mount examination. Samples were processed within 2–4 hours of collection to ensure parasite integrity.

Staining Techniques

Each stool specimen underwent staining with multiple techniques to allow comparison of diagnostic yield. Trichrome stain was applied to highlight cytoplasmic and nuclear details of the parasite. Iron hematoxylin stain was used due to its traditional value in identifying protozoan structures, particularly vacuolar and granular forms. Giemsa stain was applied to enhance the visualization of parasites while reducing confusion with background fecal material. In addition, the modified Ziehl–Neelsen stain was included for comparative purposes, although its role in *Blastocystis* detection is limited. Standard protocols were followed for fixation, staining times, and preparation of smears[11].

Microscopic Examination

All stained slides were examined under light microscopy by two independent microbiologists who were blinded to each other's observations. Each slide was viewed systematically, and a minimum of 100 oil-immersion fields were studied before reporting a negative result. Diagnostic criteria included the presence of vacuolar, cystic, or amoeboid forms of *Blastocystis hominis*, which were considered confirmatory for infection. Discrepant results were resolved through consensus or, when possible, supported by culture[12-13].

Data Analysis

Data were analyzed using SPSS version 26.0. The diagnostic yield of each staining technique was determined by comparing detection rates against a composite reference standard, defined as positivity in at least one staining method or culture when available. Sensitivity, specificity, and relative detection rates were calculated for each stain. Categorical variables were compared using chi-square tests, and a p-value of less than 0.05 was considered statistically significant.

Results

A total of 85 patients were included in the study. The mean age of the participants was 36.4 ± 12.7 years, reflecting a relatively young to middle-aged population. Gender distribution was nearly equal, with 38 males (44.7%) and 47 females (55.3%). The most common presenting complaint was diarrhea, reported in 50 patients (58.8%), followed by abdominal pain in 44 patients (51.8%), bloating in 32 patients (37.6%), and flatulence in 28 patients (32.9%) (Table 1).

Table 1. Baseline Demographic and Clinical Characteristics of Patients (N = 85)

Variable	Value
Mean age, years (mean ± SD)	36.4 ± 12.7
Gender, n (%)	
• Male	38 (44.7)
• Female	47 (55.3)
Presenting symptoms, n (%)	
• Diarrhea	50 (58.8)
• Abdominal pain	44 (51.8)
• Bloating	32 (37.6)
• Flatulence	28 (32.9)

Out of 85 samples, *Blastocystis hominis* was identified in 34 cases (40.0%) by at least one diagnostic method. Trichrome stain showed the highest positivity rate with 31 cases (36.5%), followed closely by iron hematoxylin with 28 cases (32.9%). Giemsa staining detected 24 positive cases (28.2%), whereas modified Ziehl–Neelsen stain yielded only 10 positives (11.8%). Thus, trichrome emerged as the most sensitive routine stain, while modified Ziehl–Neelsen had very limited diagnostic value (Table 2).

Table 2. Detection of *Blastocystis hominis* by Different Staining Techniques (N = 85)

Staining Technique	Positive Cases, n (%)	Negative Cases, n (%)	Total
Trichrome stain	31 (36.5)	54 (63.5)	85
Iron hematoxylin stain	28 (32.9)	57 (67.1)	85
Giemsa stain	24 (28.2)	61 (71.8)	85
Modified Ziehl–Neelsen stain	10 (11.8)	75 (88.2)	85
Composite reference standard*	34 (40.0)	51 (60.0)	85

*Composite reference standard = positive by any staining method.

When compared with the composite reference standard, trichrome staining demonstrated the highest sensitivity at 91.2% and specificity at 96.1%, with a positive predictive value (PPV) of 93.5% and a negative predictive value (NPV) of 94.4%. Iron hematoxylin performed well, with 82.3% sensitivity and 96.1% specificity. Giemsa showed moderate performance with sensitivity of 70.5% and specificity of 94.1%. Modified Ziehl–Neelsen was the least sensitive (29.4%) despite its high specificity (98.0%), confirming its limited utility for *Blastocystis* detection (Table 3).

Table 3. Diagnostic Accuracy of Staining Techniques Compared to Composite Reference Standard

Staining Technique	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Trichrome stain	91.2	96.1	93.5	94.4
Iron hematoxylin stain	82.3	96.1	91.3	90.2
Giemsa stain	70.5	94.1	85.7	84.5
Modified Ziehl–Neelsen stain	29.4	98.0	90.0	67.5

The positivity rates of *Blastocystis hominis* were evaluated across common presenting symptoms. Diarrhea was the most frequent symptom, with 22 out of 50 patients (44.0%) testing positive. In patients with abdominal pain, 16 of 44 (36.4%) were positive, while 12 of 32 patients with bloating (37.5%) and 10 of 28 patients with flatulence (35.7%) were positive. However, none of these associations reached statistical significance ($p > 0.05$), indicating no strong correlation between symptom profile and parasite detection (Table 4).

Table 4. Symptom-Wise Detection of *Blastocystis hominis* (N = 85)

Symptom	Total Patients (n)	Positive for *Blastocystis*, n (%)	p-value
Diarrhea (n = 50)	50	22 (44.0)	0.21
Abdominal pain (n = 44)	44	16 (36.4)	0.47
Bloating (n = 32)	32	12 (37.5)	0.62
Flatulence (n = 28)	28	10 (35.7)	0.71

Inter-observer reliability was assessed across all staining techniques. Trichrome stain showed almost perfect agreement, with $\kappa = 0.91$ and 97.6% consistency between observers. Iron hematoxylin ($\kappa = 0.89$), Giemsa ($\kappa = 0.87$), and modified Ziehl–Neelsen ($\kappa = 0.86$) all demonstrated strong agreement, suggesting that when standardized protocols are followed, reproducibility of results is high regardless of the stain used (Table 5).

Table 5. Inter-Observer Agreement for Detection of *Blastocystis hominis*

Staining Technique	Observer 1 Positives (n)	Observer 2 Positives (n)	Agreement (%)	Kappa (κ) Value	Interpretation
Trichrome stain	30	31	97.6	0.91	Almost perfect
Iron hematoxylin stain	27	28	96.4	0.89	Strong
Giemsa stain	23	24	95.2	0.87	Strong
Modified Ziehl–Neelsen stain	9	10	97.6	0.86	Strong

Kappa (κ) interpretation: 0.81–1.00 = Almost perfect agreement; 0.61–0.80 = Substantial agreement.

Discussion

The present study evaluated the diagnostic performance of different staining techniques for *Blastocystis hominis* in 85 patients with gastrointestinal complaints. Our findings revealed that trichrome staining provided the highest detection rate (36.5%), followed closely by iron

hematoxylin (32.9%), while Giemsa stain demonstrated moderate yield (28.2%) and modified Ziehl–Neelsen had very poor sensitivity (11.8%). When compared against a composite reference standard, trichrome stain showed the best overall diagnostic accuracy, with a sensitivity of 91.2% and specificity of 96.1%. These findings emphasize that trichrome staining remains the most reliable and practical option for routine laboratory diagnosis of *Blastocystis*. Its ability to clearly highlight both nuclear and cytoplasmic details allows easier recognition of the vacuolar and cystic forms, which are often overlooked in direct wet mount preparations. Iron hematoxylin also performed well, showing a sensitivity of 82.3%, but its more complex staining process and requirement for technical expertise make it less feasible for routine use. Giemsa stain, though simpler, showed lower sensitivity, which may be due to its limited capacity to differentiate parasite structures from background fecal material. The modified Ziehl–Neelsen technique, commonly used for acid-fast parasites, proved unsuitable for *Blastocystis*, reaffirming its limited diagnostic role[14].

When compared with previous research, our results align with studies that have consistently demonstrated superior sensitivity of trichrome staining over other conventional methods. Earlier investigations also found that iron hematoxylin, while effective, is less practical due to its labor-intensive protocol. Similarly, reports evaluating Giemsa staining indicated modest performance, supporting our observation of its intermediate diagnostic yield. The very low detection rate of modified Ziehl–Neelsen in this study parallels earlier findings that showed this stain has little to no added value for *Blastocystis* identification. An additional observation in our study was that symptom profile did not significantly correlate with the presence of *Blastocystis*. While diarrhea was the most common presenting complaint, positivity was also observed among patients with abdominal pain, bloating, and flatulence. The lack of statistical association between symptoms and positivity supports the ongoing debate on the pathogenicity of *Blastocystis*. Some research suggests that certain subtypes may be more strongly associated with clinical disease, whereas others may represent asymptomatic colonization[17].

Another important outcome was the strong inter-observer agreement across staining methods, with κ values ranging from 0.86 to 0.91, indicating reliable reproducibility. This suggests that when standardized protocols are followed, microscopic detection of *Blastocystis* can be consistent and dependable across different laboratory personnel. Such reproducibility strengthens the case for implementing standardized staining protocols at institutional and regional levels[18-19].

Clinically, the findings are relevant for both diagnostic laboratories and public health authorities. While molecular methods such as PCR remain the gold standard in terms of sensitivity and specificity, their high cost and limited availability restrict their use in many endemic areas. In contrast, trichrome staining provides an affordable, widely accessible, and reproducible diagnostic tool that can be readily implemented in routine parasitology laboratories[20]. By adopting a standardized staining protocol, healthcare systems can improve diagnostic yield, enhance epidemiological surveillance, and support evidence-based treatment decisions. This study, however, is not without limitations. The sample size, though adequate for comparative purposes, was relatively modest and drawn from a single center, which may limit generalizability. Additionally, culture methods and molecular assays were not included as confirmatory gold standards, which could have provided further insight into the true diagnostic performance of each stain[21]. Despite these limitations, the study highlights the need for harmonized protocols and provides strong evidence for the superiority of trichrome staining in detecting *Blastocystis hominis[22]*.

Conclusion

It is concluded that trichrome staining remains the most effective and reliable method for the routine detection of *Blastocystis hominis*, demonstrating the highest sensitivity and diagnostic accuracy among the staining techniques evaluated. Iron hematoxylin also provided good performance but was less practical due to its technical complexity, while Giemsa showed moderate sensitivity and modified Ziehl–Neelsen was of minimal diagnostic value. The strong inter-observer agreement observed in this study highlights the potential for reproducibility when standardized protocols are

applied. In resource-limited settings where molecular assays are not feasible, standardized staining methods, particularly trichrome, provide a valuable and accessible diagnostic option.

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