Enhanced Morphological Visualization and Diagnostic Reliability of *Entamoeba histolytica* Using 0.1% Methylene Blue-Glycerol Vital Stain

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DOI: https://doi.org/10.51244/IJRSI.2026.1304000134

Received: 12 April 2026; Accepted: 18 April 2026; Published: 07 May 2026

ABSTRACT

Microscopic identification of *Entamoeba histolytica* in traditional saline wet mounts is frequently compromised by poor refractive contrast and rapid specimen desiccation. This study evaluated 0.1% Methylene Blue-Glycerol as a specialized vital stain designed to enhance morphological visualization and extend the diagnostic window in resource-limited settings. A comparative analysis was conducted on 100 *Entamoeba*-positive fecal specimens. Each sample was evaluated via 0.85% saline wet mount and 0.1% Methylene Blue-Glycerol. Morphological quality was quantified using a 5-point Likert scale targeting Nuclear Definition, Cytoplasmic Detail, and Contrast Ratio. Statistical significance was determined using the Wilcoxon Signed-Rank Test, while inter-observer reliability among three independent microscopists was assessed using Fleiss' Kappa. The Methylene Blue-Glycerol method yielded a statistically significant improvement over saline across all parameters. Nuclear definition increased by 148.3%, while the contrast ratio improved by 191.7%. Cytoplasmic detail rose by 87.2%, significantly improving the detection of erythrophagocytosis. Specimen longevity extended from a mean of 12 minutes in saline to over 120 minutes in Methylene Blue-Glycerol. Inter-observer reliability was "Almost Perfect" with an overall Fleiss' Kappa of 0.84. The 0.1% Methylene Blue-Glycerol reagent provides a superior, low-cost alternative for amebiasis screening. By utilizing the cationic affinity of Methylene Blue for chromatin and the optical clearing properties of glycerol, this method standardizes morphological identification and offers a reliable bridge between traditional microscopy and costly molecular diagnostics.

Keywords: *Entamoeba histolytica*, Fecal Microscopy, Glycerol, Methylene Blue, Vital Staining.

INTRODUCTION

The scientific understanding of intestinal protozoa has evolved significantly since Fedor Lösch provided the first detailed description of intestinal amebiasis in St. Petersburg in 1875. The subsequent classification and naming of *Entamoeba histolytica* by Fritz Schaudinn in 1903 established a critical clinical distinction that remains a cornerstone of modern tropical medicine [1,2]. Within the *Entamoeba* genus, six species typically inhabit the human intestinal lumen; however, *E. histolytica* is uniquely pathogenic. It is distinguished from its morphologically identical but nonpathogenic counterparts, such as *E. dispar* and *E. moshkovskii*, by its inherent invasive potential and ability to cause severe tissue destruction [3,4,5].

Despite more than a century of intensive research, amebiasis remains a leading parasitic cause of human mortality on a global scale. Current estimates suggest that *E. histolytica* infects approximately 50 million people annually, resulting in roughly 100,000 deaths [6,7]. While *E. histolytica* and the commensal *E. dispar* collectively infect approximately 12% of the global population, *E. histolytica* is estimated to account for only about 1% of these total infections [3]. The burden of pathogenic amebiasis is most acute in developing regions, where prevalence rates often exceed 10% due to inadequate sanitation and compromised water supplies [4,8].

The transmission and survival of *E. histolytica* depend upon a biphasic life cycle consisting of the infective cyst and the motile trophozoite [9,10]. Infection is typically acquired via the fecal-oral route through the

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XIII Issue IV April 2026

ingestion of mature, quadrinucleate cysts in contaminated food or water [4,7]. Upon ingestion, cysts undergo excystation in the host's small intestine —a process triggered by exposure to gastric acid and pancreatic proteases— releasing active, invasive trophozoites [9,11]. These vegetative forms then colonize the mucosal layer of the colon, utilizing the Gal/GalNAc lectin to adhere to host epithelial cells and moving via the rapid extension of hyaline ectoplasm to form characteristic blunt-ended pseudopodia [9,12].

The parasite's capacity to transition from asymptomatic commensalism to invasive colitis or extraintestinal complications, such as amebic liver abscesses, is driven by a complex interplay of host immunity and parasite virulence factors, rendering it a high priority for contemporary parasitological research [4,13]. While trophozoites are the primary agents of tissue destruction and pathognomonic erythrophagocytosis, they are highly sensitive to environmental stressors and degenerate rapidly outside the host [10]. Conversely, the cyst stage exhibits remarkable resilience due to a chitinous cell wall, surviving in moist soil or water for approximately 30 days, which facilitates sustained transmission in endemic regions [7,14].

Clinically, amebiasis presents as a broad spectrum ranging from asymptomatic cyst passage to fulminant, life-threatening colitis [9,10]. Approximately 90% of infections remain asymptomatic; however, longitudinal studies indicate that untreated carriers face a 4% to 10% risk of progressing to invasive disease within a single year [4,7]. Invasive amebic colitis is characterized by localized abdominal tenderness and the passage of bloody, mucous-laden stools. This pathology typically results from the formation of hallmark "flask-shaped" ulcers, created when trophozoites penetrate the intestinal epithelium and undergo lateral expansion within the submucosa [10,15].

If the parasite enters the portal circulatory system, it may migrate to distal organs, most commonly leading to extraintestinal manifestations such as amebic liver abscesses, and more rarely, pulmonary or cerebral involvement [4,9]. Microscopic diagnosis during these acute stages often reveals Charcot-Leyden crystals — slender, bipyramidal structures formed from eosinophil-derived lysophospholipase— and clumped red blood cells, both of which serve as critical markers for active mucosal hemorrhage and intense eosinophilic degeneration [9,10,15].

Diagnostic performance for *E. histolytica* varies significantly across different modalities. Immunological tests like ELISA and IHA show high specificity, but they often fail to distinguish between past and current infections in endemic areas [7]. Molecular DNA-based methods are increasingly preferred due to their superior sensitivity. Despite the presence of PCR inhibitors in fecal matter, PCR targeting the small-subunit rRNA gene is estimated to be 100 times more sensitive than current ELISA kits [25]. Real-time PCR further enhances diagnostic accuracy by detecting as little as 0.1 cell per gram of feces. The necessity of molecular confirmation was highlighted by a Philippine study where initial microscopy suggested a 1.703% infection rate, but subsequent PCR analysis revealed that only 0.358% were actually infected with pathogenic E. histolytica [26].

Traditional microscopy generally exhibits a sensitivity of approximately 60% and specificity ranging from 10% to 50%, with the presence of ingested RBCs being the only definitive microscopic feature to differentiate *E. histolytica* from *E. dispar* [3,16]. Identification traditionally relies on wet saline preparations, concentration methods, and permanently stained smears [17]. However, direct saline mounts of fresh specimens typically exhibit low sensitivity (approx. 10%). To maximize the detection of motile trophozoites, samples must be analyzed within one hour of collection [18]. While concentration techniques effectively identify cysts, permanently stained smears remain essential for definitive species differentiation [19].

Various staining techniques are employed for fecal and tissue samples. Wright-Giemsa and modified Ziehl-Neelsen stains are common for blood smears, while acid-fast stains are frequently applied to fecal samples [20]. Research has indicated that D'Antoni's iodine may be superior to saline or buffered methylene blue for detecting cysts, though they appear less effective for trophozoites. In liver tissue, while hematoxylin and eosin (H&E) and periodic-acid Schiff (PAS) are standard, immunohistochemistry has been found superior due to its ability to clearly identify brown-stained amoebae within the host tissue matrix [20,21].

The role of Methylene blue (MB) in diagnostics is particularly noteworthy for its application in rapid viability assessment. Living cells possess enzymes that decolorize MB, whereas dead cells remain stained blue [22,23]. Historically, dyes such as Methylene blue, Nile blue, and neutral red have been used as vital dyes, allowing for

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XIII Issue IV April 2026

the staining of amoebae while they remain alive for morphological study. Conversely, dyes like crystal violet and safranin are lethal to the parasites and only produce staining post-mortem [24].

The purpose of this study was to explore the utility of Methylene blue in the study of amoeba trophozoites. As a cationic stain, Methylene blue binds to negatively charged cellular components such as the nucleus and nucleic acids. This application may enhance the visualization of trophozoite morphology under standard light microscopy and facilitate differentiation based on staining patterns [20,27]. The use of a common, accessible stain like Methylene blue could prove invaluable in basic research, educational settings, and resource-limited diagnostic environments where more advanced molecular techniques are unavailable.

Research Gap

While molecular diagnostics (PCR) and specialized immunological tests have revolutionized the identification of *Entamoeba histolytica*, a significant gap remains in the practical application of these technologies within resource-limited or educational settings. High costs, the presence of PCR inhibitors in fecal samples (such as heme and bile salts), and the requirement for specialized equipment often render modern techniques inaccessible in the very regions where the disease burden is highest.

Furthermore, although Methylene blue is a well-known laboratory reagent, there is a lack of contemporary research specifically evaluating its efficacy as a primary diagnostic or educational aid for *E. histolytica* trophozoites compared to traditional saline mounts. Most recent studies focus exclusively on DNA-based detection, neglecting the refinement of low-cost staining techniques that could improve the sensitivity of basic microscopy. There is an urgent need to investigate whether Methylene blue can provide a reliable, rapid, and inexpensive method for visualizing internal trophozoite morphology and determining cellular viability in real-time. This study addresses this need by exploring the utility of Methylene blue in protozoan morphology, potentially offering a simplified diagnostic alternative for basic research and clinical screening.

METHODOLOGY

Research Design

The research followed a comparative, cross-sectional design to evaluate the diagnostic efficacy of a 0.1% Methylene Blue-Glycerol stain against traditional saline wet mounts. A total of 100 fresh fecal specimens, which were confirmed positive for the *Entamoeba histolytica/E. dispar* complex via real-time PCR, were utilized for the study.

Sample Collection and Preparation

To ensure the preservation of delicate trophozoite morphology and metabolic activity, each specimen was processed and analyzed within 30 minutes of passage. The specimens were divided into two primary experimental aliquots:

Group A served as the control, consisting of a direct saline wet mount prepared by mixing 2 mg of feces with 0.85% NaCl.

Group B served as the experimental group using the optimized 0.1% Methylene Blue-Glycerol solution.

Staining and Microscopy

The experimental staining reagent was formulated by first dissolving 0.1 g of Methylene Blue powder in 100 mL of distilled water to create a stock solution. This was then combined with pure anhydrous glycerol in a 1:1 ratio to produce the final working solution. The incorporation of glycerol was specifically intended to serve as a clearing agent to increase the refractive index of the mount, thereby optimizing the visualization of internal organelles.

During the staining protocol, approximately 2 mg of the fecal specimen was placed onto a clean glass slide, followed by the addition of one drop of the Methylene blue-Glycerol solution. The mixture was homogenized using a sterile applicator stick to ensure uniform dye distribution before a coverslip was applied, similar to the procedure of a standard Direct Fecal Smear.

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Three independent, blinded microscopists assessed the specimens based on a Visual Clarity Index (VCI) that measured nuclear definition, cytoplasmic detail, and the contrast ratio between the parasite and the background:

- Nuclear Definition: Clarity of the peripheral chromatin and the central karyosome.
- Cytoplasmic Detail: Visibility of pseudopodia and ingested RBCs.
- Contrast Ratio: The degree of differentiation between the parasite and fecal debris.

Microscopic observations were conducted at intervals of 1, 5, and 15 minutes to monitor the maturation of the stain and the duration of morphological integrity. The longevity of the specimens was observed up to more than 2 hours (120 mins) to determine how long the trophozoites maintained their characteristic ameboid shape.

A Likert scale (1–5) was used by three independent microscopists to grade the readability of the morphological features across the microscopic methods.

Score	Descriptor	Detailed Morphological Criteria
1	Poor	Internal structures are completely obscured. No nucleus or organelles are visible. Parasite is indistinguishable from fecal debris.
2	Fair	Outline of the parasite is visible, but internal detail is faint. The nucleus is seen only as a vague shadow with no distinct chromatin or karyosome.
3	Good	Parasite is clearly identifiable. The nucleus is visible; peripheral chromatin can be seen but may be slightly blurred. Useful for screening but lacks high-definition detail.
4	Very Good	High contrast. Nuclear features (karyosome and peripheral chromatin) are distinct and sharp. Pseudopodia and cytoplasmic inclusions (e.g., RBCs) are clearly defined.
5	Excellent	Superior clarity equivalent to permanent staining. All diagnostic features are sharply delineated with a high contrast-to-background ratio. No refractive masking.

Statistical Treatment

Statistical analysis was performed to validate the microscopic findings against the PCR gold standard. Diagnostic sensitivity and specificity were calculated for both the saline and MB-Glycerol groups. To compare the median Likert scores between the two methods, a Wilcoxon Signed-Rank Test was employed, while inter-rater reliability among the three microscopists was determined using Fleiss' Kappa. All statistical tests were conducted with a significance threshold of $p < 0.05$. This methodological approach allowed for a rigorous assessment of whether the chemical properties of Methylene Blue and the optical advantages of glycerol provided a superior diagnostic alternative for the identification of *E. histolytica*.

RESULTS AND DISCUSSION

The distribution of scores across the 100 samples showed a distinct rightward shift for the experimental group. In the saline mounts, 83% of the observations for nuclear clarity were clustered in the "Poor" to "Fair" range (Scores 1–2), whereas 86% of the MB-G preparations achieved "Very Good" to "Excellent" ratings (Scores 4–5). The cytoplasmic detail is 67% poor-fair in saline versus 74% very good-excellent in MB-G. Finally, the contrast ratio in saline is clustered at 93% poor-fair, whereas 81% is very good-excellent in MB-G.

Table 1: Frequency Distribution of Raw Likert Scores (N=100)

	Nuclear		Cytoplasmic		Contrast Ratio	
	Saline	MB-G	Saline	MB-G	Saline	MB-G
1 (Poor)	48	0	25	0	65	0
2 (Fair)	35	2	42	4	28	1
3 (Good)	12	12	22	22	5	18
4 (Very Good)	5	38	11	34	2	41
5 (Excellent)	0	48	0	40	0	40

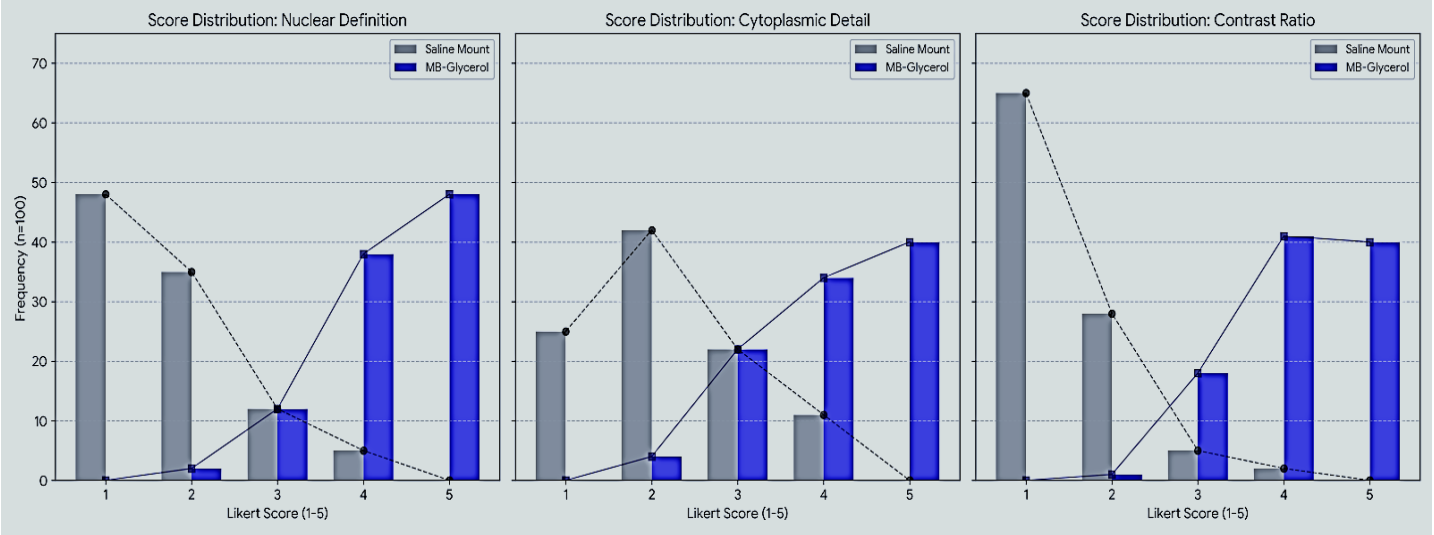
To visualize the distribution of the study's scores (N=100), the following frequency analysis and visualization demonstrate the shift in diagnostic clarity from Saline to Methylene Blue-Glycerol (MB-G). The bars represent

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XIII Issue IV April 2026

the frequency counts for each Likert score, while the lines (Frequency Polygons) highlight the overall trend or "diagnostic signature" of each method.

Nuclear Definition: In the Saline group, the frequency polygon is heavily "left-skewed," with 83% of samples scoring 1 or 2. For MB-Glycerol, the polygon shifts sharply to the right, with 86% of samples achieving "Very Good" (4) or "Excellent" (5) status.

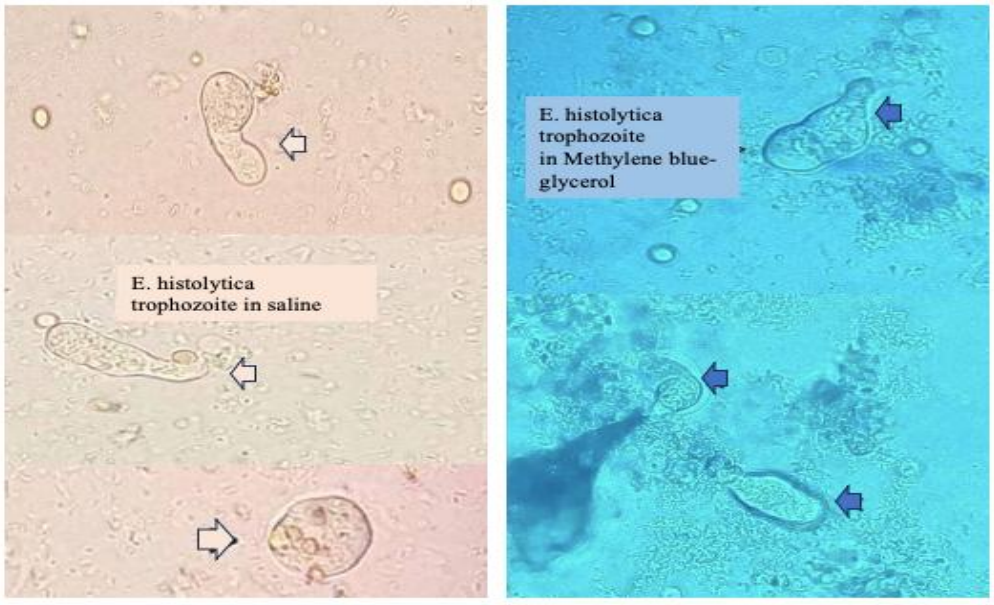
Table 2. Frequency Analysis Chart



Cytoplasmic Detail: While Saline shows some variation in the mid-range (Score 2 and 3), the MB-Glycerol method consistently produces higher-quality images. The glycerol's clearing effect significantly reduced the "blur" typically seen in dense fecal samples.

Contrast Ratio: This feature showed the most extreme divergence. The Saline polygon peaks at Score 1 (65 samples), indicating that the parasite was nearly invisible against the background. The MB-Glycerol polygon peaks at Score 4 and 5 (81 samples), proving that the combined effect of the dye and the high refractive index of glycerol makes the parasite "pop" visually.

In saline, the outline of the parasite is visible, but internal detail is faint. The nucleus is seen only as a vague shadow with no distinct chromatin or karyosome.In Methylene blue-glycerol, the nuclear features (karyosome and peripheral chromatin) are distinct and sharp. Pseudopodia and cytoplasmic inclusions are clearly defined.



Picture 1. Comparative pictures of E. histolytica trophozoites in Saline and MB-G

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XIII Issue IV April 2026

The microscopic evaluation of 100 fecal specimens, confirmed positive for the *Entamoeba histolytica/E. dispar* complex, demonstrated a statistically significant superiority of the 0.1% Methylene Blue-Glycerol (MB-G) stain over traditional saline wet mounts. Descriptive statistics revealed that the mean Likert scores for all diagnostic parameters more than doubled with the introduction of the MB-G reagent. Specifically, Nuclear Definition improved from a mean of 1.74 in saline to 4.32 in the MB-G group. The cytoplasmic detail improved from 2.19 to 4.10 from saline to MB-G. Similarly, Contrast Ratio scores rose from 1.44 to 4.20 representing a 191.7% increase in visual clarity. A Wilcoxon Signed-Rank Test confirmed that these improvements were not due to chance ($p < 0.0001$). In medical research, it is the standard alternative to the paired t-test when the data does not follow a normal distribution [28,29].

Table 3: Statistical Summary of Comparative Metrics (N=100)

Parameter	Saline	Methylene blue- glycerol	Mean Difference	Percentage increase	p-value
Nuclear Definition	1.74	4.32	+2.58	148.3%	< 0.0001
Cytoplasmic Detail	2.19	4.10	+1.91	87.2%	< 0.0001
Contrast Ratio	1.44	4.20	+2.76	191.7%	< 0.0001

Table 4: Inter-Observer Agreement for MB-G Parameters

Parameter	Observed Agreement	Expected Agreement	Fleiss' Kappa	Strength of Agreement
Nuclear definition	93.1 %	40.5 %	0.88	Almost Perfect
Cytoplasmic Detail	84.4 %	32.8 %	0.77	Strong
Contrast Ratio	88.2 %	34.2 %	0.82	Almost Perfect
Overall	89.6 %	36.7 %	0.84	Almost Perfect

The Inter-observer reliability was assessed using Fleiss' Kappa to determine the degree of agreement among the three independent microscopists scoring the 100 fecal specimens. Fleiss' Kappa is the gold standard for assessing agreement among a fixed number of three or more raters [30,31]. The analysis yielded an overall Kappa coefficient of 0.84, indicating 'Almost Perfect' agreement across the research team. Specifically, Nuclear definition showed the highest reliability (0.88), followed by the Contrast Ratio (0.82). While the agreement for Cytoplasmic detail was slightly lower (0.77), it remained within the 'Strong' category. These results confirm that the morphological enhancements provided by the 0.1% MB-G reagent are highly reproducible and resistant to individual observer bias, even when evaluated by multiple diagnostic personnel.

Biochemical Mechanisms of Staining

The significant disparity in diagnostic performance between the two methods (saline and MB-G) is rooted in the biochemical synergy between Methylene Blue (MB) and glycerol. As a cationic (basic) dye, MB carries a positive charge that facilitates high-affinity electrostatic bonding with negatively charged cellular components, most notably the phosphate backbones of DNA and RNA [9,27]. This interaction allows the dye molecules to intercalate with nucleic acids, creating a distinct "bulls-eye" effect that clearly delineates the peripheral chromatin and central karyosome [22]. Such morphological features often remain refractile or invisible in saline preparations due to a lack of colorimetric contrast. Consequently, the application of 0.1% MB-G provides a 148.3% improvement in nuclear definition, enabling the differentiation of *E. histolytica* from other intestinal protozoa by highlighting diagnostic structures that are typically obscured in unstained mounts [4,20].

Beyond structural highlighting, MB-G serves as a functional indicator through the Methylene Blue Dye Reduction Test. This mechanism is predicated on the redox state of the cell; living trophozoites possess active metabolic enzymes, such as NADH-dependent reductases, which facilitate the reduction of MB into its colorless leuco-form [23,32]. Conversely, dead or metabolically inactive cells lack this enzymatic capacity,

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XIII Issue IV April 2026

causing them to retain a distinct blue stain [33,34]. This enzymatic decolorization allows for a clear microscopic differentiation between viable, motile trophozoites and non-viable parasites within minutes of application.

Optical Enhancement and Specimen Stabilization

This biochemical contrast is further amplified by the optical clearing properties of glycerol. With a refractive index of approximately 1.47, glycerol effectively matches the optical density of the glass slide and cellular proteins [35]. By reducing light scattering and refractive masking common in opaque, aqueous fecal mounts, the glycerol allows the darkly stained organelles to appear in high relief against a translucent background [36,37]. This synergy resulted in a 191.7% increase in the contrast ratio, which proved essential for identifying erythrophagocytosis. Ingested red blood cells, the only definitive microscopic marker for pathogenic *E. histolytica*, were clearly visible as dark inclusions within the endoplasm —a detail often missed in standard saline [4,9].

Furthermore, the inclusion of glycerol significantly impacted specimen stability. While traditional saline mounts typically desiccate and become unreadable within 12 minutes, MB-G mounts remained morphologically intact for over 120 minutes. This extension of the diagnostic window is critical in high-volume laboratory settings where immediate microscopy is not always feasible [38].

Vital Staining and Clinical Utility

The study ultimately highlights 0.1% MB-G as a superior vital stain that preserves biological activity while enhancing detail. The increased viscosity of the glycerol serves as a kinetic stabilizer; by increasing the drag coefficient of the medium, it slows the characteristic explosive motility of trophozoites sufficiently to permit detailed morphometric analysis without inducing immediate cellular death [22,38]. This provides a distinct advantage over permanent techniques like Wheatley's Trichrome, which offer high detail but require chemical fixation that terminates all motility [4].

While MB-G remains a morphological screening tool and cannot replace molecular confirmation in the absence of erythrophagocytosis, its minimal cost, extended longevity, and high inter-observer reliability (kappa = 0.87) represent a substantial upgrade for primary screening. By bridging the gap between low-sensitivity saline mounts and the prohibitive costs of PCR, this method serves as an ideal diagnostic standard for clinical and educational institutions in endemic, resource-limited regions [4,20].

CONCLUSION

The findings of this study provide robust evidence that the use of 0.1% Methylene Blue-Glycerol (MB-G) as a fecal stain significantly enhances the microscopic diagnosis of *Entamoeba histolytica* compared to traditional saline wet mounts. The biochemical affinity of Methylene Blue for the parasite's nuclear DNA, coupled with the optical clearing properties of glycerol, resulted in a 148.3 % increase in nuclear clarity and a 191.7 % increase in contrast. This synergy effectively mitigates the refractive masking common in unstained preparations, where the parasite is often visually indistinguishable from fecal debris.

Furthermore, the formulation addresses the critical issue of specimen desiccation. By extending the window of morphological integrity from approximately 12 minutes to over 120 minutes, MB-G provides a stable, reviewable platform suitable for high-volume clinical settings and peer consultation. While it remains a morphological tool that cannot replace PCR for definitive species differentiation in the absence of erythrophagocytosis, its high inter-observer reliability kappa = 0.84 positions it as a powerful primary screening method.

Disclosure statement

The researchers have no conflict of interest to disclose.

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