ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XII Issue XI November 2025

Isolation and Morphologic Differentiation of *Blastocystis* from Insect Vectors Using Staining Media

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DOI: https://dx.doi.org/10.51244/IJRSI.2025.12110145

Received: 27 November 2025; Accepted: 02 December 2025; Published: 20 December 2025

ABSTRACT

The complexity of *Blastocystis* morphology, its presence in various insect vectors, and the need for accurate detection methods warrant further research. This comparative, cross-sectional study aimed to detect *Blastocystis species* in domestic insect vectors, including *Periplaneta americana* (cockroach), *Musca domestica* (housefly), and *Drosophila melanogaster* (fruit fly). The study also evaluated the effectiveness of various staining techniques, namely Acid Fast staining, Gram staining, Methylene blue wet mount, and Direct smear using Lugol's solution, for differential enumeration and morphologic assessment of *Blastocystis cysts*. The results showed that *Blastocystis cysts* were recovered from *Periplaneta americana* and *Musca domestica*. Notably, Methylene blue and Lugol's Iodine in Direct smear yielded higher morphologic scores, suggesting that these staining methods may be more effective for visualizing *Blastocystis* cysts in insect vectors.

Keywords: *Blastocystis*; insect vectors; staining media; external washings; intestinal contents.

INTRODUCTION

Blastocystis species is a unicellular, polymorphic protozoan that inhabits the large intestine of humans and various animals [1]. Its diverse morphological forms, including vacuolar, granular, amoeboid, and cyst forms, contribute to its complex life cycle and pathogenicity [2]. Transmission occurs through the fecal-oral route, often in poor hygienic conditions, and can involve human-human or animal-human transmission [3]. *Blastocystis* is a prevalent parasite in human fecal samples, with higher rates in developing countries [4]. Close contact with domestic animals and livestock can also facilitate transmission [5]. Insect vectors, such as houseflies and cockroaches, can contaminate food and transmit diseases, including parasites like *Blastocystis* [6].

The role of *Blastocystis* in human disease remains debated, with symptoms including diarrhea, abdominal cramps, and nausea [7]. While some studies suggest pathogenic potential, others have raised doubts due to concomitant presence of other pathogens [8].

Various techniques can detect intestinal *Blastocystis*, including DNA probes, PCR, and direct fluorescent antibody methods [9]. However, these methods are often expensive and inaccessible in developing countries. Direct fecal smear microscopy is a widely used and cost-effective method, but requires skilled microscopists to identify the parasite's diverse morphologic forms [10].

Several studies have investigated the sensitivity and specificity of direct fecal smear in detecting *Blastocystis* in human fecal samples, with varying results. Staining methods have been less frequently explored, but notable studies include the standardization of *Blastocystis hominis* diagnosis using Gram and May-Grünwald-Giemsa staining [11]. Giemsa stain was used to diagnose *Blastocystis* in domestic bird species [12]. Khalifa et al. [13] evaluated various staining methods, including Giemsa, trichrome stain, and others, finding Safranin-Methylene blue to be a promising option.

This study differs from previous research in several key aspects. The *Blastocystis* used in this study was obtained from insect vectors, not from humans or a specific *Blastocystis* subtype from a stock culture. While various staining techniques have been explored, there is still no considered ideal method for routine use in detecting

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Blastocystis. The complexity of *Blastocystis* morphology, presence in various insect vectors and detection methods necessitate further research.

Research Gap and Research Problems

The inadequate knowledge about this prevalent parasite, its presence in insect vectors, and the ambiguous findings in its detection using routine microscopy are very compelling reasons to direct the research efforts on this. Hence, this research study was undertaken to answer the following:

1.What is the differential count of *Blastocystis* from insect vectors, namely:
   1.1 *Periplaneta americana* (giant cockroach),
   1.2 *Musca domestica* (housefly), and
   1.3 *Drosophila melanogaster* (fruit fly)?

2. What is the differential count of *Blastocystis* from insect vectors using the various staining media:
   2.1 Gram stain,
   2.2 Acid Fast stain,
   2.3 Methylene blue, and
   2.4 Lugol's direct smear?

3.Is there any significant variation in the differential count of *Blastocystis* from insect vectors when grouped according to the staining media used?

4.What is the effect of staining media to the morphology of *Blastocystis*?

METHODOLOGY

Research Design

This comparative, cross-sectional study involved the detection of *Blastocystis species* from domestic insect vectors: *Musca, Periplaneta* and *Drosophila species* and for its differential enumeration using various staining techniques, namely: Acid Fast staining, Gram staining, Methylene blue wet mount and the Direct smear using Lugol's solution.

Specimen sampling and Locale

Cockroaches were collected overnight using empty jars coated with Vaseline and baited with bread soaked in water, placed in areas they frequent such as kitchens and near garbage cans. Only adult cockroaches with intact bodies were brought to the laboratory. These cockroaches were anesthetized by freezing at 0°C for at least 5 minutes before processing for *Blastocystis* detection.

Flies, on the other hand, were collected using a bait trap made from a disposable plastic water bottle, where the top was cut off and inverted to form a cone leading to the bait inside. The baits used were spoiling fruit or meat, and food residue, which attracted and trapped the flies inside the bottle.

Hundreds of insects were collected randomly from among the localities in Metro Manila, Philippines. A pair of each insect species were sent to the RITM for species identification. Ten each of the randomly selected houseflies, fruit flies, and cockroaches were used for testing. The external washings and intestinal contents were obtained for staining and microscopy.

Process

To obtain the external washings, the insects were washed one by one in sterile saline by manually shaking for 3 minutes in a sterile container. The external washings were centrifuged for 5 minutes at 2,000 rpm, and the filtrate was discarded. The sediment was resuspended in 1 ml of Ringer's solution and mixed for an even distribution. One drop (50 uL) of the specimen was placed on a slide for each of the staining methods. The stained smears

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were microscopically viewed for the presence and quantitation of *Blastocystis*. The morphologic quality of *Blastocystis* was evaluated using the rubric provided.

The intestinal contents were also collected and examined. The insects were placed in flasks rinsed with 70% alcohol for 5 minutes in order to decontaminate the external surfaces. They were transferred to another flask and allowed to dry at room temperature, and finally washed with normal saline for 3 minutes to remove traces of alcohol. Intestinal contents were collected by squeezing the abdomen area of the insects to expel the fecal material. The excreted materials were macerated in 1 ml of Ringer's solution and mixed. One drop (50 uL) of the mixture was used for each of the staining methods.

Data Gathering Procedure

The morphology, identification and enumeration of the parasite were confirmed and evaluated by three experienced Medical Technologists specialized in Clinical Parasitology. The microscopic test was conducted in a single-blind study, where the staining media used were not disclosed to the evaluators to minimize bias.

The following staining media were used: Acid Fast staining, Gram staining, Methylene blue wet mount, and Direct smear using Lugol's solution. The standard Gram staining method and the Kinyoun cold method in AFB were employed for the dry smears, while Methylene blue and Lugol's solution were conducted as wet mounts. Despite the difference in the staining media specimen state – dry in AFB and Gram stain, while wet in Methylene Blue and Lugol's Iodine, each of the slides for staining received an equal amount of one drop (50 uL) of the specimen and examined entirely.

The morphology of the parasite was evaluated using a 4-point rubric scale according to their staining clarity and reaction, cellular and structural differentiation, and over-all visual quality. The number of parasites were counted per smear or specimen drop on slide for the external washings and intestinal contents isolated from insect vectors.

RESULTS AND DISCUSSION

A tabulation of the findings of the differential *Blastocystis* counts (Table I) made through various staining media in three different insect vectors, considering the external washings and intestinal contents as specimens showed that out of the ten cockroaches *Blastocystis* was present in the external washings of only five (5/10) samples; in houseflies, *Blastocystis* was present in eight samples (8/10), whereas, all the samples of fruit flies turned out negative. The intestinal contents of insect vectors revealed that there were seven out of ten (7/10) cockroaches that were positive; similarly, seven out of ten (7/10) houseflies were positive. The fruit flies were all negative in their intestinal contents.

The *Blastocystis* count in *Periplaneta* intestinal contents was notably higher (Gram: 361, AFB: 363, MB: 452, and DS: 443) compared to their external washings (Gram: 100, AFB: 109, MB: 133, and DS: 116). This finding is consistent with previous studies on the role of cockroaches in harboring parasites [14], [15]. In contrast, the *Blastocystis* count in *Musca species* was relatively similar in both external washings (Gram: 201, AFB: 205, MB: 223, and DS: 239) and intestinal contents (Gram: 173, AFB: 185, MB: 191, and DS: 193). This observation aligns with research on the potential of flies as mechanical vectors of parasites [16], [17]. Notably, *Periplaneta* samples had higher parasite counts in their intestinal contents, whereas *Musca* samples had higher counts in their external washings, highlighting differences in parasite carriage between insect species. Whether the surface area of these insects and the volume of their intestinal content play a role in the count is yet to be determined. The p values (Table II) in the differential count of external washings of *Periplaneta* (0.959) and *Musca species* (0.923), and the intestinal contents of *Periplaneta* (0.903) and *Musca species* (0.989), were > 0.05, indicating no significant variation in the differential count of the parasite among the insect vectors.

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Insect Vectors	Staining Media				Control
	Gram stain	Acid Fast Stain	Methylene Blue	Direct Smear	
1.1 External Washings					
Periplaneta species	100	109	133	116	0
Musca species	201	205	223	239	0
Drosophila species	0	0	0	0	0
1.2 Intestinal Contents					
Periplaneta species	361	363	452	443	0
Musca species	173	185	191	193	0
Drosophila species	0	0	0	0	0

Table 1. Differential Count of *Blastocystis* From Insect Vectors

Variables	Statistic ***F***	*p*	Analysis	Decision
2.1 External Washing				
Periplaneta species	0.100	*0.959*	Not Significant	Accept H_0
Musca species	0.160	*0.923*	Not Significant	Accept H_0
Drosophila species	Undetermined			
2.2 Intestinal Contents				
Periplaneta species	0.190	*0.903*	Not Significant	Accept H_0
Musca species	0.04	*0.989*	Not Significant	Accept H_0
Drosophila species	Undetermined			

Table II. Variation in the Differential Count of *Blastocystis* Grouped According to Staining Media

Cockroaches and houseflies are notorious vectors of various parasites, playing a significant role in the transmission of diseases to humans. These insects can pick up parasites from contaminated feces, garbage, or decaying matter and then deposit them onto food, surfaces, or water, facilitating the spread of diseases [18]. Cockroaches, for instance, can carry parasites like *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum*, which cause amoebiasis, giardiasis, and cryptosporidiosis, respectively [19]. One hundred cockroaches (*Periplaneta americana*) were examined in Metro Manila, and 36% of the cockroaches had multiple parasites seen on their external surface. The common parasite observed in the cockroach obtained was the rhabditiform larva (25%) [20]. They can also transmit *Toxoplasma gondii*, a protozoan parasite causing toxoplasmosis, particularly hazardous for pregnant women and immunocompromised individuals [21]. Houseflies, on the other hand, can transmit a range of parasites, including *Entamoeba histolytica, Giardia lamblia, Shigella spp.,* and *Salmonella spp*., causing diseases like shigellosis and salmonellosis [22], [23].

Additionally, houseflies can transmit the eggs of tapeworms like *Taenia saginata* and *Taenia solium*, causing taeniasis [24]. Other parasites carried by these insects include *Blastocystis hominis* and *Dientamoeba fragilis*, which cause gastrointestinal symptoms [25], [26]. Proper hygiene, sanitation, and pest control measures are crucial in preventing the transmission of these parasites.

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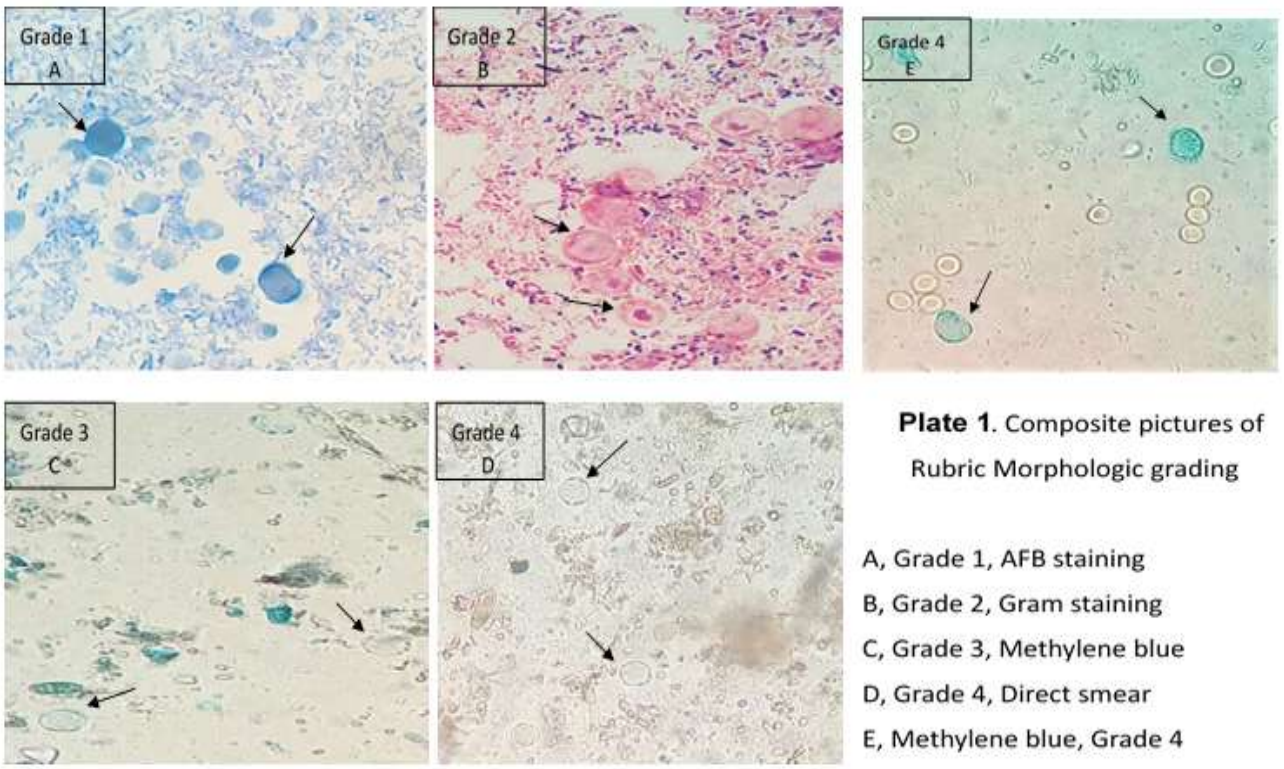

Plate 1. Composite pictures of Rubric Morphologic grading

A, Grade 1, AFB staining
B, Grade 2, Gram staining
C, Grade 3, Methylene blue
D, Grade 4, Direct smear
E, Methylene blue, Grade 4

The morphology of *Blastocystis* (Table III) was consistently rated higher in Methylene blue and Direct smear compared to Gram stain and Acid Fast smear in both external washings and intestinal contents. This is evident in *Periplaneta* external washings, where Methylene blue (X = 1.75) and Direct smear (X = 1.65) outperformed Gram stain and Acid Fast stain (both X = 1.0). Similarly, in *Musca* samples, Methylene blue (X = 2.93) and Direct smear (X = 2.88) showed superior results compared to Gram stain and Acid Fast stain (both X = 1.60). These findings support the use of Methylene blue and Direct smear as effective staining methods for detecting *Blastocystis*, as previously suggested [9], [13].

Considering the morphologic scores obtained in the intestinal contents of *Periplaneta*, Methylene blue (X = 2.53) gave the highest Mean, followed by Direct smear (X=2.40), a notch higher than Gram stain and Acid Fast stain (both with X=1.40). The *Musca* specimen findings weren't different with the Methylene blue (X=2.40) and Direct smear (X=2.38), dominating over Gram stain and Acid Fast stain (both with X=1.40) with lower mean values. This reflected the results of Khalifa et al. [13] who evaluated various staining methods, finding Safranin-Methylene blue to be a promising option.

Blastocystis appears microscopically as a spherical or oval-shaped organism with a central vacuole and peripheral cytoplasm, measuring approximately 5-15 μm in diameter [9]. In Methylene blue staining, *Blastocystis* appears as a blue-stained organism with a distinct central vacuole and peripheral cytoplasm, often with a characteristic "signet ring" appearance [13]. Direct smear with Lugol's iodine staining reveals *Blastocystis* as a brown-stained organism with a central vacuole and peripheral cytoplasm, often with a granular appearance [27]. Gram staining typically shows *Blastocystis* as a Gram-negative organism with a faintly stained central vacuole and peripheral cytoplasm [28]. Acid Fast staining often yields variable results, with *Blastocystis* appearing as a weakly acid-fast organism with a central vacuole and peripheral cytoplasm [29]. The morphology of *Blastocystis* can vary depending on the staining medium and the subtype of the organism [27].

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Insect Vectors	Staining Media			
	Gram stain	Acid Fast Stain	Methylene Blue	Direct Smear
3.1 External washings				
Periplaneta sp.	Visible cell membrane and vaguely delineated intracytoplasmic vacuole	Visible cell membrane and vaguely delineated intracytoplasmic vacuole	Distinct cell membrane, visible large central vacuole and peripheral nuclei	Distinct cell membrane, visible large central vacuole and peripheral nuclei
Mean (X)	1.00	1.00	1.75	1.65
Musca sp.	Distinct cell membrane, visible large central vacuole and peripheral nuclei	Distinct cell membrane, visible large central vacuole and peripheral nuclei	Distinct cell membrane, distinct large central vacuole with thin intracytoplasmic rim, well-defined and dense peripheral nuclei, multiple forms are observed	Distinct cell membrane, distinct large central vacuole with thin intra-cytoplasmic rim, well-defined and dense peripheral nuclei, multiple forms are observed
Mean (X)	1.60	1.60	2.93	2.88
Drosophila sp.	Unobservable	Unobservable	Unobservable	Unobservable
3.2 Intestinal Contents				
Periplaneta sp.	Visible cell membrane and vaguely delineated intracytoplasmic vacuole	Visible cell membrane and vaguely delineated intracytoplasmic vacuole	Distinct cell membrane, distinct large central vacuole with thin intracytoplasmic rim, well-defined and dense peripheral nuclei, multiple forms are observed	Distinct cell membrane, visible large central vacuole and peripheral nuclei
Mean (X)	1.40	1.40	2.53	2.40
Musca sp.	Visible cell membrane and vaguely delineated intracytoplasmic vacuole	Visible cell membrane and vaguely delineated intracytoplasmic vacuole	Distinct cell membrane, visible large central vacuole and peripheral nuclei	Distinct cell membrane, visible large central vacuole and peripheral nuclei
Mean (X)	1.40	1.40	2.40	2.38
Drosophila sp.	Unobservable	Unobservable	Unobservable	Unobservable

Table III. Effects of Staining Media to the Morphology of *Blastocystis*

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Variables	Statistics ***F***	*p*	Analysis	Decision
4.1 External Washings				
Periplaneta species	0.760	*0.524*	Not Significant	Accept H_0
Musca species	***3.62***	***0.022***	***Significant***	***Reject H_0***
Drosophila species	Undetermined			
4.2 Intestinal Contents				
Periplaneta species	1.97	*0.136*	Not Significant	Accept H_0
Musca species	1.74	*0.176*	Not Significant	Accept H_0
Drosophila species	Undetermined			

Table IV. Variation in the Effects of Staining Media to the Morphology of *Blastocystis*

The statistical analysis of *Blastocystis* morphologic findings (Table IV) revealed varying results across staining media. In *Periplaneta* external washings, the F-statistic (0.760) and p-value (0.524) indicated no significant morphologic variation across staining media ($p > 0.05$). Conversely, *Musca* samples showed significant variation in the effects of staining media on *Blastocystis* morphology, with an F-statistic of 3.62 and a p-value of 0.022 ($p < 0.05$). This disparity highlights the importance of considering the specific insect host and staining method when evaluating *Blastocystis* morphology [9], [13]. In the assessment of intestinal contents, both *Periplaneta* ($p = 0.136$) and *Musca* ($p = 0.176$) samples showed no significant variation in the effects of staining media on *Blastocystis* morphology.

CONCLUSION

This study demonstrated that *Periplaneta americana* and *Musca domestica* carry *Blastocystis* parasites on their external surfaces and intestines, whereas *Drosophila species* do not appear to harbor the parasite.

The choice of staining media did not significantly impact parasite counts or morphology in most cases, except for *Musca domestica*'s external washings. Notably, wet mounts using Methylene blue and Lugol's Iodine (Direct smear) provided better microscopic visualization of *Blastocystis* cysts compared to Gram and Acid fast staining methods.

The study supports previous research on the potential of cockroaches and flies as mechanical vectors of parasites, highlighting the need for effective pest control measures to prevent the spread of *Blastocystis* and other parasites.

Disclosure statement
The researchers have no conflict of interest to disclose.

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