Original Article

Combined lactophenol methylene blue-blue glycerol jelly technique as a new approach for helminths staining

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ABSTRACT

Background: Permanent staining of helminths is important for different purposes including taxonomic and prevalence studies as well as teaching. Carmine is one of the most commonly used dyes; however, variable factors affect the quality of the staining outcomes. Therefore, it is essential to find an alternative stain that is efficient, accurate, and less complex.
Objective: The aim of the present study is to evaluate the quality of a combined lactophenol methylene blue-blue glycerol jelly (LMB-BGJ) technique as a potential alternative for staining of helminths compared with carmine-based staining and glycerol jelly mounting techniques.
Material and Methods: *S. mansoni* and *T. spiralis* were obtained from experimentally infected animals. A new staining technique (LMB-BGJ) was used for both parasites in comparison with carmine stain in case of *S. mansoni* and glycerol jelly mounting in case of *T. spiralis*. Comparison was conducted using examination under light microscope and a mean score was given for the structural brightness, and extent of staining of tegument/cuticle, suckers, intestine, as well as reproductive systems.
Results: *S. mansoni* worms prepared with both carmine staining and LMB-BGJ showed moderate staining quality of tegument, suckers, intestine and reproductive organs with a mean total score of 3.3/4 and 3.35/4, respectively. Adult *T. spiralis* mounted with glycerol jelly only showed a mean total score of 3.95/4 for the cuticle, esophagus, and intestine, while the LMB-BGJ showed a staining quality with a mean total score of 3.85/4.
Conclusion: From a practical point of view, LMB-BGJ is a simple and rapid technique that needs less experience for the preparation of semi-permanent slides with satisfactory staining and mounting quality for both trematodes and small nematodes.

Keywords: glycerol; helminths; lactophenol; permanent mount; staining.

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INTRODUCTION

Staining of helminths is one of the essential techniques for parasitologists. Permanent slides serve as documents to prove the correct identification of parasites especially after years following taxonomical changes[1]. Thus, collection of parasites, followed by relaxation, preservation and staining procedures are crucial for scientists for classification of parasites[2]. High quality of permanently stained slides allows differentiation of organs for proper categorization. Preparation of permanent mount is also beneficial for teaching purposes for medical students in order to study the related diseases and the appropriate control and treatment measures of various parasitic infections. In addition, researchers have also used staining techniques to study the prevalence of different helminthic infections[3,4].

Parasites should be collected and fixed directly in the living condition, followed by staining, and mounting. During the preparation of a permanent mount, the worm is exposed to many factors that can greatly affect their taxonomic properties; including chemicals, pressure and temperature changes. Thus, the proper selection of an appropriate method is very important to preserve the worm identity[5]. Several preservatives, and fixatives were used for preservation, fixation, and mounting of helminths; including glycerol alcohol, alcohol-formalin-acetic acid (AFA) and diluted formalin. Helminths were also stained by variable techniques; including hematoxylin, carmine, acetocarmine, eosin, Romanowsky stain, lactophenol cotton blue (LPCB), Lugol's iodine, malachite green...etc[6]. Carmine and hematoxylin are the most frequently used stains for the preparation of permanent slides of different helminths[7]. Variable mounting media can be used in different preparation techniques; including glycerol jelly, glycerin, and Euparal. Notably, staining and mounting approaches vary according to size of specimens, species and stage of development of the parasites[2].

Although, carmine shows good staining properties for different tissues and organs of helminths, the process is time consuming. In addition, the critical step

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in the carmine-based staining of helminths is in the de-staining using acid ethanol and this is greatly related to the type of parasite and its size. If the specimen is not de-stained enough, organs may not be properly differentiated, and if it is de-stained too much, organs may not even be clearly visible[8]. Another staining method for identification of helminths especially flatworms is the mounting and staining with LPCB stain. Nail polish may then be used to seal the cover glass for permanent preparation[9].

Most staining techniques used for identifying flatworms are multi-step procedures that require a long time and much effort, consume many chemicals and needs experienced handling[9]. Likewise, preparation of permanent slides of nematodes especially small ones is a lengthy and time consuming process. Therefore, a more simple technique for staining flatworms is greatly needed[5].

The present study was carried out with the aim of preparing high quality semi-permanent slides for helminths with a simple technique and to save time consumed in the traditional staining techniques. In the study, we evaluated the quality of LMB-BGJ as a potential alternative for staining of *S. mansoni* and *T. spiralis* compared with carmine-based staining and glycerol jelly mounting techniques.

MATERIAL AND METHODS

This case-control study was carried out in the animal house at Theodor Bilharz Research Institute (TBRI), Giza Governorate, Egypt, and the Medical Parasitology Research Unit at Faculty of Medicine Ain Shams University, Cairo, Egypt during the period from January 2021 to January 2022.

Study design: Adults of *S. mansoni* and *T. spiralis* were obtained from experimentally infected animals in TBRI, Giza Governorate, Egypt. Adults *S. mansoni* were divided into two groups, Ia and Ib to be stained by two different methods using carmine-based staining technique and LMB-BGJ, respectively. Adults *T. spiralis* were also divided into two different groups; group IIa to be mounted with glycerol jelly and group IIb to be stained and mounted with LMB-BGJ, respectively. Carmine stain was not preferred for *T. spiralis* owing to the thick cuticle that prevents the stain to be absorbed into this layer and the visceral organs as well[10]. The structural brightness and the extent of staining of different parts of the samples were scored.

Stains and mounting media

- Carmine powder (Merck, Germany), acetic acid glacial (Merck, Germany) and distilled water were used to prepare stock carmine solution. Working carmine solution was prepared by dilution with 70% ethyl alcohol according to Garcia[7].
- Glycerol jelly was prepared from glycerin (El-Gomhouria, Egypt), gelatin powder (El-Gomhouria, Egypt) and distilled water according to Garcia[7].
- The LMB was purchased from (Merck, Germany), and BGJ was prepared according to Perina and Camacho[11]. The prepared glycerol jelly was melted with 2-4 drops of MB according to the amount of glycerol jelly in a 37°C water bath and mixed well until the melted jelly assumed a strong blue color.

Helminths collections: *S. mansoni* and *T. spiralis* worms were collected from already experimentally infected animals.

Fixation[7]: Adults *S. mansoni* were placed in tap water in a refrigerator for 2 h for relaxation. Hot AFA (10 ml formaldehyde, 50 ml 95% ethyl alcohol, 5 ml acetic acid glacial, 45 ml distilled water) was prepared for fixation. The specimens were fixed in the hot AFA (85-90°C when AFA started to steam) for 24 h, and transferred to 70% alcohol until staining. Adults *T. spiralis* were killed with hot water (60 to 63°C) and transferred to 5% alcohol glycerin for at least one day.

Staining methods

Adult *S. mansoni* (group Ia, carmine stain)[7]: Fixed samples were immersed in plates containing carmine solution overnight. The specimens were rinsed in 70% ethyl alcohol then transferred into acid alcohol (2-4 drops of concentrated HCl in 100 ml of 70% ethanol) for several min to few h for removal of excess stain without loss of pigmentation. Female *S. mansoni* needed about 15-30 min, while *S. mansoni* male needed 3-4 h. Samples were rinsed in 70% ethyl alcohol and subsequently dehydrated in 80%, 95%, and 100% ethanol (each step for 15 min), followed by clearance with xylene for at least 15 min. Finally, they were mounted on a microscopic slide using Dibutylphthalate Polystyrene Xylene (DPX).

Adult *T. spiralis* (group IIa, glycerol jelly mounting technique)[7]: Samples fixed in alcohol-glycerin solution were allowed to gradually evaporate the alcohol by partial covering of the container and gradual adding of 1-2 drops/d of glycerin over successive days until specimens sank to the bottom of the container and were ready to be mounted. A drop of liquefied glycerol jelly was placed on a slide, and the specimen was transferred into the drop. A cover glass was placed, and the glycerol jelly was left to solidify in a horizontal position.

New developed LMB-BGJ technique for groups Ib and IIb: A combination technique (two-step technique) was performed for both *S. mansoni* and *T. spiralis* worms. The stain (LMB) was applied over the fixed sample on a microscopic slide until the

organs of the parasite were clearly visible. A filter paper was used to remove the excess stain[9]. For mounting technique[11], a drop of liquefied BGJ was placed over the specimen on a microscopic slide, and a cover glass was applied. All the specimens were left in a horizontal position over night to solidify. Nail polish mixed with 1-2 drops of MB until it turns dark blue was used to seal the edges of the slides.

Evaluating parameters[12]: Two parasitologists independently examined the stained slides under light microscope. Scores were given for each of the following items; tegument/cuticle, suckers, intestine, and reproductive systems (testes in male and ovary and vitelline glands in female) of related helminths. A scale ranging from 1 to 4 points (unstained=1, weak=2, moderate=3 and good=4) was used and the total mean score was calculated. The stained slides were examined microscopically every three months for one year for any possible decrease in quality of staining.

Statistical analysis: Quantitative data were expressed as mean±SD using the statistical package for social sciences, version 20.0 (SPSS Inc., Chicago, Illinois, USA).

Ethical approval: Animal experiments were conducted in the animal house of TBRI according to the international ethical guidelines after approved by the Institutional Review Board of TBRI.

RESULTS

Two techniques were used for staining of *S. mansoni* worms, carmine-based staining (Fig. 1B and 2A), and LMB-BGJ (Fig. 1C and 2B) techniques. Samples stained with carmine (Group Ia) showed some technical difficulties during staining that resulted in 10/17 succeessful trials. After dehydration and clearance of the samples using xylene and just before mounting, blackness of samples occurred that required several trials to overcome. It was solved by adding 1-2 drops of xylene over the sample, while applying DPX for mounting. The score was evaluated for the succeessful trials. Carmine showed a mean staining quality for the tegument (3.2±0.63), suckers (4.0±0.0), intestine (2.9±0.7) and reproductive organs (3.1±0.54), with a mean total score of 3.3±0.42, i.e. in a scale of 4 points (Table 1). In Group Ib, LMB-BGJ technique stained 8 worms out of 8 (100%). Females needed around 10-15 min to visualize the ovary and vitelline glands before mounting. In contrast, males required several trials for determination of the optimum time needed for clear visualization of reproductive organs. Our results showed that overnight staining followed by BGJ mounting gave the best results. This combined technique showed moderate staining quality of tegument, suckers, intestine and reproductive organs with a mean of 3.5±0.5, 4.0±0.0, 2.7±0.46 and 3.2±0.75, respectively and a mean total score of 3.35±0.47, i.e. in a scale of 4 points. (Table 1).

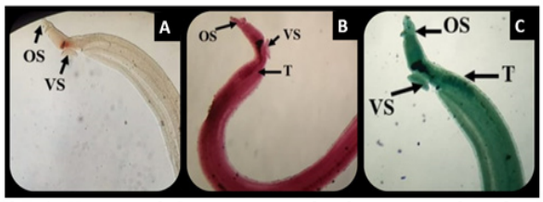

Fig. 1. Unstained *S. mansoni* male showing oral sucker **(OS)** and ventral sucker **(VS) (A).** Male *S. mansoni* stained with carmine showing OS, **VS** and testes **(T) (B).** Male *S. mansoni* prepared with combined LMB-BGJ technique showing OS, VS and T **(C)**. (×40)

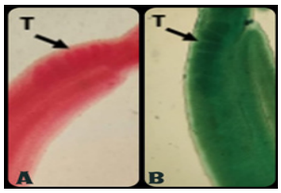

Fig. 2. Testes (T) of *S. mansoni* stained with carmine stain **(A)** and combined LMB-BGJ technique **(B)**. (×100)

Table 1. Semi-quantitative evaluation of *S. mansoni* adult using carmine staining technique and LMB-BGJ technique.

Morphological characteristics	Carmine (Mean ± SD)	LMB-BGJ (Mean ± SD)
Tegument and body tubercles	3.2 ± 0.63	3.5 ± 0.5
Oral and Ventral suckers	4.0 ± 0.0	4.0 ± 0.0
Intestine	2.9 ± 0.7	2.7 ± 0.46
Testes in male/ovary and vitelline glands in female	3.1 ± 0.54	3.2 ± 0.75
Total score (mean)	3.3 ± 0.42	3.35 ± 0.47

Glycerol jelly mounting and LMB-BGJ techniques were used for staining and mounting of the studied nematode; *T. spiralis*. For the glycerol jelly mounting (Group IIa), ten worms were mounted (Figure 3A). Mounting with glycerol jelly showed a mean staining quality for the cuticle (3.9±0.3) and oesophagus and intestine (4.0±0.0), with a mean total score of 3.95±0.05, i.e. in a scale of 4 points (Table 2). Another 10 worms subjected to LMB-BGJ technique were all stained (Group IIb) (Fig. 3B). Adults *T. spiralis* needed around 1 h to visualize the cuticle, oesophagus and intestine before mounting. The combined technique

showed moderate staining quality of cuticle with a mean of (3.8±0.4) and oesophagus and intestine with a mean of (3.9±0.3) and a mean total score of 3.85±0.05, i.e. in a scale of 4 points (Table 2).

All stained slides were followed up. After 3 months, carmine stained slides and glycerol mounted slides showed permanent staining properties while the slides prepared with the combined new technique started to fade in color.

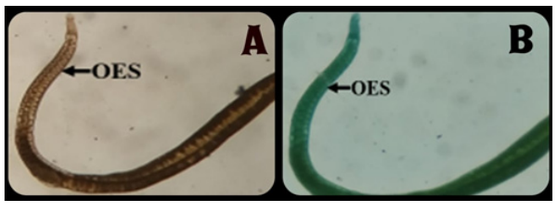

Fig. 3. Female *T. spiralis* mounted with glycerol jelly **(A)** and stained with LMB-BGJ **(B)** showing unicellular oesophagus **(OES)**. (×40)

DISCUSSION

The taxonomy of various helminths depends greatly on microscopic identification of their morphological features including; hooks, spines and suckers that cannot be clearly identified in frozen samples[8]. Therefore, selection of the appropriate method for preparation of a permanent mount is absolutely essential to ensure proper preservation and identification of a parasite[2]. In this study, the preparation of permanent mounts, relaxation, fixation, and staining methods of two helminths have been discussed. Carmine is one of the most important dyes in the stain market. According to many authors it offers a good staining property which can last for years[12]. In the present study, the de-staining process and dehydration of the carmine staining technique were critical steps that required close observation and multiple trials. The need of experienced personnel and variability of staining results from different trials, make it impractical for direct identification of a worm referred to laboratory for clinical diagnosis. This was in agreement with Dapson[13] who stated that carmine stain also showed considerable batch-to-batch variability which causes sometimes frustrations of researchers. Many variables may affect carmine staining; starting from the different manufacturing methods and different methods of dye preparation including heating that may damage the dye molecules and affect the outcome of the staining procedure. Accordingly, previous literature emphasized on the considerable need to find an alternative staining approach that is more efficient, accurate and less complex[14].

On the other hand, LMB is a combined fixative, staining and clearing agent that digests soft tissues and enhances cuticle structures[15]. It offers simple and inexpensive technique that makes it a proper candidate for staining various parasitic helminths. The same was found by Parija *et al*.[16] who used lactophnol cotton blue (LPCB) to stain *E. vermicularis* eggs, *Ascaris* eggs and *Strongyloides* larvae. In this study a combination was done using LMB combined with BGJ that showed a fair potential to stain the selected parasitic platyhelminths and small nematodes. The use of MB twice, one time with the lactophenol and the other time with the glycerol increased the efficiency of the staining technique. It showed advantages over carmine stain, as in this new method, the de-staining was not done. Skipping the de-staining and dehydration processes allowed the new combined technique to be practically used for clinical diagnostic purposes, in addition to saving more time, compared to carmine staining. Moreover, Parija *et al*.[16] claimed that LPCB is a simple and sensitive technique that allowed easy differentiation between parasites and epithelial cells, mucous and other artifacts present in the samples. This was confirmed by Henedi and El-Azazy[9], who used LPCB for staining of platyhelminths.

Table 2. Semi-quantitative evaluation of *T. spiralis* adult using glycerol jelly mounting and LMB-BGJ technique.

Morphological characteristics	Glycerol jelly (Mean ± SD)	LMB-BGJ (Mean ± SD)
Cuticle	3.9 ± 0.3	3.8 ± 0.4
Oesophagus and intestine	4.0 ± 0.0	3.9 ± 0.3
Total score (mean)	3.95 ± 0.05	3.85 ± 0.05

Further advantage offered by LMB-BGJ is solving the problem of blackness that occurred during the dehydration steps. Moreover, LMB-BGJ stains both trematodes and small nematodes, so it can also offer saving resources in laboratories. Fading the color of the slides prepared by the new combined method after the follow up period considers it a semi-permanent staining technique for trematodes and nematodes. However, further drops of MB could be added to the nail polish to avoid discoloration of the preparation[11]. Nail polish was also observed to shrink and crack, so we further recommend using a sealing substance other than nail polish such as paraffin and araldite that may help further evaluation of LMB-BGJ.

In conclusion, LMB-BGJ is a simple and rapid technique for preparation of semi-permanent slides for helminths including both platyhelminths and nematodes, which needs further evaluation to change it into a permanent preparation.

Author contribution: Zahran F proposed the study conception and design, wrote, and revised the manuscript. Nassar DA collected the data and designed the figures. Hanafy MA analyzed and interpreted the results, and shared in writing and revising the manuscript. All authors reviewed the manuscript,

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REFERENCES

1. Ryss AA. A simple express technique to process nematodes for collection slide mounts. J Nematol 2017; 49(1):27–32.
2. Gökçen A. Fixation, staining and preparation of permanent mounts of helminths. Turkiye Parazitol Derg 2008; 32(2):177-181.
3. Rahman M, Islam S, Masuduzzaman M, Alam M, Chawdhury MNU, Ferdous J, *et al*. Prevalence and diversity of gastrointestinal helminths in free-ranging Asian house shrew (*Suncus murinus*) in Bangladesh. Vet World 2018; 11(4):549-556.
4. El-Dakhly KM, Mohamed HI, Kamel AA, Mahrous LN, El-Nahass E, Aboshinaf ASM. Prevalence, distribution pattern and pathological alterations of gastrointestinal helminthosis in domestic ducks in Beni-Suef, Egypt. J Adv Vet Res 2020; 10:1-8.
5. Zahabiun F, Sadjjadi SM, Esfandiari F. Development of a double glass mounting method using formaldehyde alcohol azocarmine lactophenol (FAAL) and its evaluation for permanent mounting of small nematodes Iran. J Parasitol 2015; 10(4):617-624.
6. Aly ME, Dyab AK, Yones DA, Mohamed YM, Bayoumi SA, Elossily NA. Alternative staining of *Fasciola gigantica* using natural herbal dyes. JESP 2020; 50(1):129 -13.
7. Garcia LS. Fixation and special preparation of fecal parasite specimens and arthropods. In Garcia LS (Ed.) Diagnostic Medical Parasitology; 6th edition; ASM Press, USA 2016; Chapter 9; 183-191.
8. Sepulveda MS, Kinsella JM. Helminth collection and identification from wildlife. J Vis Exp 2013; (82):e51000.
9. Henedi AA, EL-Azazy OM. A simple technique for staining of platyhelminths with lactophenol cotton blue. J Egypt Soc Parasitol 2013; 43(2): 419-423.
10. Hanafiah M, Aliza D, Abrar M, Karmil F, Rachmady D. Detection of parasitic helminths in cattle from Banda Aceh, Indonesia. Vet. World 2019; 12(8):1175-1179.
11. Perina G, Camacho AI. Permanent slides for morphological studies of small crustaceans: Serban's method and its variation applied on Bathynellacea (Malacostraca). Crustaceana 2016; 89(10):1161-1173.
12. Marhaba Z, Haniloo A. Staining of parasitic helminths by extracts of *Allium cepa, Juglans regia,* and *Rubia tinctorum*: An approach to herbal dyes. Iran J Parasitol 2018; 13(2):293–300.
13. Dapson RW. The history, chemistry and modes of action of carmine and related dyes. Biotech Histochem 2007; 82(4-5):173–187.
14. Harris TJ, McCormick F. The molecular pathology of cancer. Nat Rev Clin Oncol 2010; 7(5):251-265.
15. Parija SC, Shivaprakash MR, Jayakeerthi SR. Evaluation of lacto-phenol cotton blue (LPCB) for detection of *Cryptosporidium, Cyclospora* and *Isospora* in the wet mount preparation of stool. Act Trop 2003; 85(3):349-354.
16. Parija SC, Sheeladevi C, Shivaprakash MR, Biswal, N. Evaluation of lactophenol cotton blue stain for detection of eggs of *Enterobius vermicularis* in perianal surface samples. Trop Doct 2001; 31(4):214–215.