Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

J E Z S Journal of Entomology and Zoology Studies

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2019; 7(3): 1388-1390

Received: 24-03-2019
Accepted: 28-04-2019

Gajendra Pal Singh
Central Tasar Research and Training Institute, Central Silk Board, Piska Nagri, Ranchi, Jharkhand, India

Mohammed Muzeruddin Baig
Central Tasar Research and Training Institute, Central Silk Board, Piska Nagri, Ranchi, Jharkhand, India

Jitender Singh
Central Tasar Research and Training Institute, Central Silk Board, Piska Nagri, Ranchi, Jharkhand, India

Jay Prakash Pandey
Central Tasar Research and Training Institute, Central Silk Board, Piska Nagri, Ranchi, Jharkhand, India

Karambeer Jena
Central Tasar Research and Training Institute, Central Silk Board, Piska Nagri, Ranchi, Jharkhand, India

Alok Sahay
Central Tasar Research and Training Institute, Central Silk Board, Piska Nagri, Ranchi, Jharkhand, India

Correspondence
Mohammed Muzeruddin Baig
Central Tasar Research and Training Institute, Central Silk Board, Piska Nagri, Ranchi, Jharkhand, India

Chromotrope-2r and naphthalene blue black stains for easy detection of *Nosema mylittensis* spore infecting tropical tasar silkworm, *Antheraea mylitta* D

Gajendra Pal Singh, Mohammed Muzeruddin Baig, Jitender Singh, Jay Prakash Pandey, Karambeer Jena and Alok Sahay

Abstract
Pebrine disease caused by *Nosema mylittensis* in tropical tasar silkworm, *Antheraea mylitta* D. is highly contagious and more prevalent due to its transovarial transmission and death of worms. Pebrine detection during mother moth examination in grainages is strenuous. Staining of pebrine for easy detection in tasar sericulture is largely unexplored. The aim of the present study was to identify suitable stains for easy identification of *N. mylittensis* spores during microscopic examination of mother moth. The stains which are used for staining and identification of pathogens in microbiological field and clinical laboratories were selected and used. Among sixteen stains used, six stains viz Wright stain, Nigrosin, Amido black, Bismark brown, Chromotrope-2R and Naphthalene blue black have shown the staining of *N. mylittensis* spores. However, Chromotrope-2R and Naphthalene blue black found more suitable because the spores took prominent red and blue colour respectively and easy to identify from cellular debris and other non pebrine artifacts. Other stains used have shown partial staining of *N. mylittensis* spores and the spores are very difficult to differentiate from debris and other artifacts in the smear. Hence, Chromotrope-2R and Naphthalene blue black may be used for staining and easy identification of *N. mylittensis* spores during mother moth examination.

Keywords: Tasar silkworm, *Nosema mylittensis*, stains, mother moth examination, pebrine

1. Introduction
Tropical tasar silk is produced by the larvae of *Antheraea mylitta* Drury. Being wild in nature and reared outdoor on the primary food plants like *Terminalia tomentosa*, *T. arjuna* and *Shorea robusta* larvae often suffer from various diseases causing heavy losses to the economy of the silk industry. Of the biotic constraints, virosis, pebrine, muscardine and bacteriosis are the commonly encountered diseases caused respectively by different pathogens *Antheraea mylitta* Cytoplasmic Polyhedrosis Virus (AmCPV), *Nosema mylittensis* (Microsporidia), *Penicillium citrinum* and *Penicillium varioti* (Fungus) and different types of bacteria. About 25-30% crop loss is attributed due to Pebrine disease with occasional crop failure [1]. This disease is more violent due to its transovarial transmission causing vertical infection directly from parents to offspring and death of worms due to primary infection.
Currently, abdominal portion of female mother moth is crushed with Potassium bicarbonate (K_2CO_3) or Potassium hydroxide (KOH) and examined under microscope at 600 x magnification for pebrine spore examination. The presence of fat globules, debris of body cells, other non pebrine artifacts in tissue sample make difficulty to identify the infective stage, i.e. spore during microscopic examination in grainages. Hence, the present investigation aims to identify suitable staining technique for easy identification of *N. mylittensis* spores during microscopic examination of mother moth.

2. Materials and Methods
2.1 Selection of chemicals for staining: Sixteen Different chemicals and stains used in different microbiology fields for identification of pathogens were selected and used for staining and identification of *Nosema mylittensis* spores (Table 1). All chemicals and stains were procured from Sigma Aldrich, Merck. The Chemical stains used are Congo Red ($C_{32}H_{22}N_6Na_2O_6S$), Bromophenol Blue ($C_{19}H_{10}Br_4O_5S$), Methyl Red ($C_{15}H_{15}N_3O_2$),

2.2 Wright Stain (Mixture of eosine red and methylene blue), Malachite Green ($C_{23}H_{25}Cl\ N_2$ (chloride)), Geimsa Stain, Thymol Blue ($C_{27}H_{30}O_5S$), Naphthalene Blue Black ($C_{22}H_{14}N_6Na_2O_9S_2$), Bismark Brown ($C_{18}H_{18}N_8.2HCl$), Amido Black ($C_2H_{14}N_6Na_2O_9S_2$), Potassium Permanganate ($KMnO_4$), Methyl blue ($C_{16}H_{18}Cl\ N_3S$), Leishman stain, Nigrosin stain, Chromotrope-2R ($C_{16}H_{10}N_2O_8S_2$) and Crystal Violet.

2.3 Preparation of chemicals solution and staining

The chemicals in solid state were used as weight/volume and chemical in liquid state as volume/volume for preparation of stock solution. 2.0% stock solutions were prepared by dissolving 2.0g or 2.0 ml chemical in 100 ml double distilled water. Further the required concentrations (0.5 and 2.0%) of the solution of the particular chemical were prepared by serial dilution method from the stock solution.

2.4 Mother moth examination

The lower middle portion of the abdomen (4 to 7 segment) of pebrine infected moth was cut with the help of a scissors and homogenized with the help of mortar and pestle by adding 3-4 drops K_2CO_3 (0.5%). A drop of the homogenate was placed on the clean glass slide, smeared in a thin layer, air dried and covered it with staining solution for 1 to 2 minutes and then washed gently in water for 5 seconds, dried and examined without cover glass using oil immersion under microscope using 1000X magnification. Entire experiment setup is replicated thrice and visual observations were documented.

3. Results

Among the fifteen stains used for staining of *Nosema mylittensis* spores, six stains were shortlisted (Wright stain, Nigrosin, Amido black, Bismark brown, Chromotrope-2R and Naphthalene blue black. The Wright stain at the higher concentration of 2.0% showed better staining to spores. Similarly, Nigrosin, Amid Black and Bismarck Brown at 1.0% concentration showed promising results. Staining with Chromotope-2R and Naphthalene blue black at lower concentration of 0.5% have shown best results for staining and identification of *N. mylittensis* spores (Table 1 and Figure 1). The pebrine spores takes red colour and blue colour prominently with the Chromotrope-2R and Naphthalene Blue black stains respectively. With these stains the pebrine spores can be easily identified from the other artifacts in the smear (Figure 1).

4. Discussion

During microscopic examination, identification of *Nosema mylittensis* spore in homogenate of mother moth without centrifugation, filtering and staining is very difficult due to the presence of fat globules, debris of body cells, other non pebrine artifacts in the tissue sample. Identification of pebrine spores during mother moth examination requires a simple staining method which can be utilized for easy identification of *Nosema* spores from other artifacts and contaminants.

Methyl Red, Wright stain, Nigrosin, Amido black, Bismark brown and combination of potassium permanganate + crystal violet as suitable stains for the easy identification of pebrine spores in the presence of artefacts and contaminants during mother moth examination in tasar silkworm grainages [7]. In present study, though six stains viz. Wright stain, Nigrosin, Amido black, Bismark brown, Chromotrope-2R and Naphthalene blue black have shown the staining of Nosema spores but Chromotrope-2R and Naphthalene blue black found more suitable because the spores take red and blue colour prominently than background and easy to identify from other artifacts and contaminants. The present finding is supported by earlier workers who described Chromotrope -2R as a suitable stain for easy identification of microsporidia in clinical samples [2, 3]. Geimsa stain has not performed well for staining and identification of *N. mylittensis* spores in homogenate of tasar silk moth in our study. Similarly [4, 5], reported that the microsporidia are partially stained by Geimsa stain and the spores are very difficult to differentiate from debris and other artifacts in the smear. $KMNO_4$ and Crystal violet have not shown promising results for staining and identification of spores in the present study. $KMNO_4$ and Crystal violet have been reported suitable stains for *N. bombysis* spores [6]. This may be due to difference in spore wall protein structures in different spores of microsporidia.

Table 1: Visual observation of *Nosema mylittensis* spores treated with different Stains

S. N.	Stains used	Final Conc. (%)	Observations on staining of spores
1	Geimsa Stain	1.0	Spores partially stained and difficult to differentiate from debris and other artifacts
2	Congo Red	1.0	Spores stained but difficult to differentiate from debris and other artifacts
3	Bromophenol Blue	1.5	Spores partially stained and difficult to differentiate from debris and other artifacts
4	Methyl Red	1.5	Spores partially stained and difficult to differentiate from debris and other artifacts
5	Wright Stain	2.0	Spores stained but difficult to differentiate from debris and other artifacts
6	Malachite Green	1.0	Spores partially stained and difficult to differentiate from debris and other artifacts
7	Thymol Blue	1.0	Spores partially stained and difficult to differentiate from debris and other artifacts
8	Naphthalene Blue Black	0.5	Spores takes red colour prominently and easy to identify from cellular debris and other artifacts
9	Bismark Brown	1.0	Spores partially stained and difficult to differentiate from debris and other artifacts
10	Amido Black	1.0	Spores partially stained and difficult to differentiate from debris and other artifacts
11	Potassium permanganate	2.0	Spores not stained
12	Methyl blue	1.0	Spores partially stained and difficult to differentiate from debris and other artifacts
13	Leishman stain	1.0	Spores partially stained and difficult to differentiate from debris and other artifacts
14	Nigrosin	1.0	Spores partially stained and difficult to differentiate from debris and other artifacts
15	Chromotrope-2R	0.5	Spores takes red colour prominently and easy to identify from cellular debris and other artifacts
16	$KMNO_4$ and Crystal Voilet	1.0	Spores partially stained and difficult to differentiate from debris and other artifacts

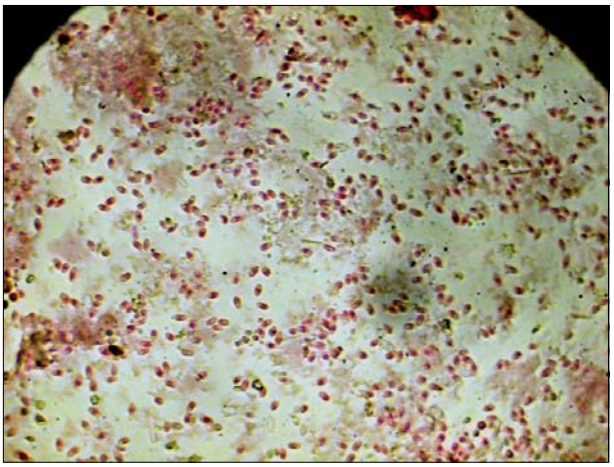

Fig 1: Pebrine spores stained with Chromotrope-2R

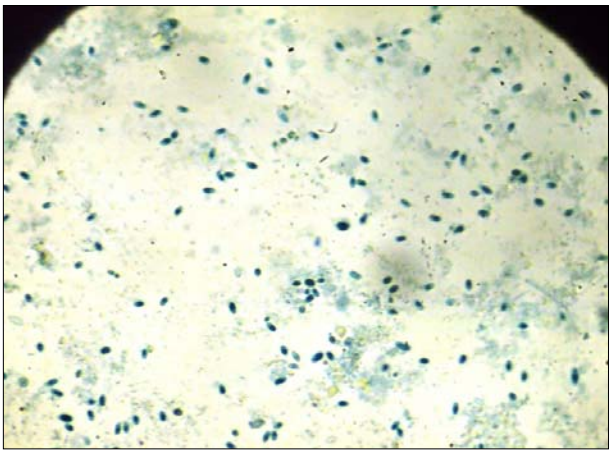

Fig 2: Pebrine spores stained with Naphthalene Blue Black

5. Conclusion

We conclude from the present study that Chromotrope-2R and Naphthalene blue black were found as suitable stains that can be used for the easy identification of pebrine spores in the presence of artefacts and other contaminants during tasar silk mother moth examination.

6. Significance statement

This study has demonstrated that Chromotrope-2R and Naphthalene blue black are the stains which made easy identification of *Nosema mylittensis* spore from cellular debris and other artifacts during mother moth examination.

7. Acknowledgement

Authors are thankful to Central Tasar Research and Training Institute, Central Silk Board for providing facilities and support to carry out this work.

8. References

1. Sahay DN, Roy DK, Sahay A. Diseases of tropical tasar silkworm, *Antheraea mylitta* D., Symptoms and Control Measures. In: Lessons on Tropical tasar, Thangavelu, K. (Ed.). Central Tasar Research and Training Institute, Piska Nagri, Ranchi, India, 2000, 104.
2. Moura H, Schwartz DA, Bornay Llinares F, Sodre FC, Wallace S, Visvesvara. A new and improved Quick hot Gram Chromotrope Technique that differentially Stains microsporidian spores in clinical samples, including paraffin embeded tissue sections. Archives Pathology and Laboratory Medical. 1997; 121:888-892.
3. Awadalla HN, Naga IF, el-Temsahi, Negm AY. Detection of Microsporidia by different staining techniques. Journal of Egyptian Society of Parasitology. 1998; 28(3):739-38.
4. Joseph J, Vemuganti GK, Garg, Sharma. Histopathological evaluation of ocular microsporidiosis by different stains. BMC Clinical Pathology. 2006; 6:6-8.
5. Lynne Gracia S. Laboratory Identification of the microsporidia, Journal of Clinical Microbiology. 2002; 40(6):1892-1901.
6. Prasad NR, Govindharaju, Keshawa Reddy. Positive staining method for detection of pebrine spore *Nosema bombycis* in silkworm Bombyx mori. Sericologia. 2000; 40(4):575-583.
7. Madhusudhan KN, Aakash K, Mohitkumar, Tiwari MD, Sinha AK, Kirankumar KP *et al*. Identification of Suitable Stains for Easy Identification of Pebrine Spores Infecting Tropical Tasar Silkworm (*Antherea mylitta* D.). Indian Journal of Biotechnology and Biochemistry. 2013; 1(2):87-90.