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Use of certain chemicals for easy and quick detection of *Nosema mylittensis* Spores infecting tropical tasar silkworm, *Antheraea mylitta* Drury (Saturniidae: Lepidoptera)

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Abstract
Among diseases of tropical tasar silkworm, *Antheraea mylitta* Drury pebrine caused by *Nosema mylitensis* is highly contagious and more prevalent due to its transovarial transmission causing vertical infection directly from parents to offspring leading to death of worms. The aim of the present investigation was to develop a chemical reagent for easy and quick identification of *N. mylittensis* spores during mother moth microscopic examination under light microscopy to check the spread of disease through vertical infection. Various chemicals which are known for cleaning and cell lyses were tested. Of the tested chemicals, sarcosyl (2.0%), Sodium dodecyl sulphate (1.0%) and surf excel quick wash (2.0 %) have shown promising results. The visibility in microscopic field was very clear, no cellular debris observed, spores clearly visible and easy to identify. Liberation of spore from infected tissues was also much high (7,72,000 to 8,53,000 spores/cm^3). These promising chemicals were combined to form the chemical reagent for efficient detection of pebrine spores. The treatment with chemical reagent increased the visibility and clarity in microscopic field and quantum of spores was also higher (9, 12, 000 spores/cm^3) and easy to identify. This chemical reagent is showing promising results in the tasar grain ages across the country in enhancement of production of disease free layings (DFLS) and thus helps in doubling of farmer's income through heathy silkworm rearing.

Keywords: chemicals, *Nosema myllitensis*, *Antheraea mylitta*

1. Introduction
Tropical tasar silk is produced by the larvae of *Antheraea mylitta* Drury (Saturniidae: Lepidoptera). Being wild in nature and larvae reared outdoor on its primary food plants *Terminalia tomentosa*, *T. arjuna* and *Shorea robusta.* Silkworm often suffer from various diseases causing heavy losses to the economy of the silk industry. Of the biotic constraints, pebrine, virosis, muscardine and bacteriosis are the commonly encountered diseases caused respectively by different pathogens *Nosema mylittensis* (Microsporidia), *Antheraea mylitta* Cytoplasmic Polyhedrosis Virus (AmCPV), a reovirus, *Penicillium citrinum* and *Penicillium varioti* (Fungus) and different types of bacteria. The diseases in tasar silkworms are primarily due to the pathogens and certain stress factors, which promote the disease development during silkworm rearing. In India, the extent of tasar crop loss due to the silkworm diseases is nearly 40% [1, 2]. Of the various diseases, pebrine, virosis, bacteriosis and muscardine contribute to 20-25, 25-30, 10-15 and 2-5% respectively of total crop loss [2]. About 25-30% crop loss is attributed due to Pebrine disease with occasional crop failure [3]. This disease of silkworms seems to be the primal constraint in enhancing the production of raw silk. Pebrine infection has been reported from almost all the tissues of the silkworm [4]. This disease is more violent/severs due to its transovarial transmission causing vertical infection directly from parents to offspring and death of worms due to primary infection [5, 6, 7, and 8]. Secondary infection occurs by feeding on contaminated leaves. In this case, if larvae succeed to reach cocoon stage, pupal and adult stages will be affected and thus can't produce disease free layings (Dfls).
Pebrine free generation of silkworm, i.e. dfls are produced through mother moth examination [3, 7]. 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. The presence of fat globules, debris of body cells, other non pebrine artifacts in tissue sample and improper liberation of spores from infected tissues make difficulty to identify the infective stage, i.e. spore during microscopic examination. Hence, in the present study, a successful attempt was made to develop a chemical reagent for easy and quick identification of pebrine spores during microscopic examination of mother moth.

2. Materials and Methods

2.1 Selection of chemicals: The chemicals which are known for cleaning and cell lyses were selected and procured for the study. The chemicals used are Sodium Dodecyl Sulphate (SDS), $(CH_3(CH_2)_{11}SO_4Na$, Sodium Lauryl Sarcocinate (Sarcosyl) $CH_3(CH_2)_{10}CON(CH_3)CH_2COONa$, Potassium Bi Carbonate (K_2CO_3),Potassium Hydroxide (KOH), Lauryl Dimethyl Amine Oxide ($C_{14}H_{31}NO$), Octoxynol (Triton 100) ($C_{14}H_{22}O(C_2H_4O$, Sodium Dodecyl Benzene Sulfonate (SDBS) ($CH_3(CH_2)_{11}C_6H_4SO_3Na$), Lauryl Ethoxylate ($C_{12}H_{25}(OCH_2CH_2)_nOH$), Hydrchloric Acid (HCl), Acetic Acid (CH_3COOH), from Sigma-Aldrich, Cween 20 $C_{58}H_{114}O_{26}$, Cween 80 ($C_{64}H_{124}O_{26}$), from Central Drug House and Surf Excel quick wash detergent from commercial store.

2.2 Preparation of chemicals solution: The chemicals in solid state were used as weight/volume and chemical in liquid state as a volume/volume for preparation of stock solutions. 5.0% stock solution were prepared by dissolving 5.0g or 5.0ml chemical in 100 ml double distilled water. Further the required concentrations (0.5 and 1.0%) of the solution of the particular chemical were prepared by dilution method from the stock solution.

2.3 *Antheraea mylitta* mother moth examination: The lower middle portion of abdomen (4 to 7 segment) of moth was cut with the help of a scissors and homogenized with the help of mortar and pestle by adding KOH (2%) and K_2CO_3 (0.5%) in equal volume. A drop of the homogenate was put on the clean glass slide and covered it with a clean cover glass and observed at 600 X magnification. Then the same sample of homogenate was observed with mixing a drop of above chemical solutions separately. Further a chemical reagent was prepared in combination of promising chemicals and examined, following the same process for quick and easy detection of *N. mylittensis* spores in the homogenate. Liberated spore count is carried out by haemocytometer. The entire experimental setup was carried out at Silkworm Pathology Laboratory, Central Tasar Research & Training Institute, Ranchi.

3. Results

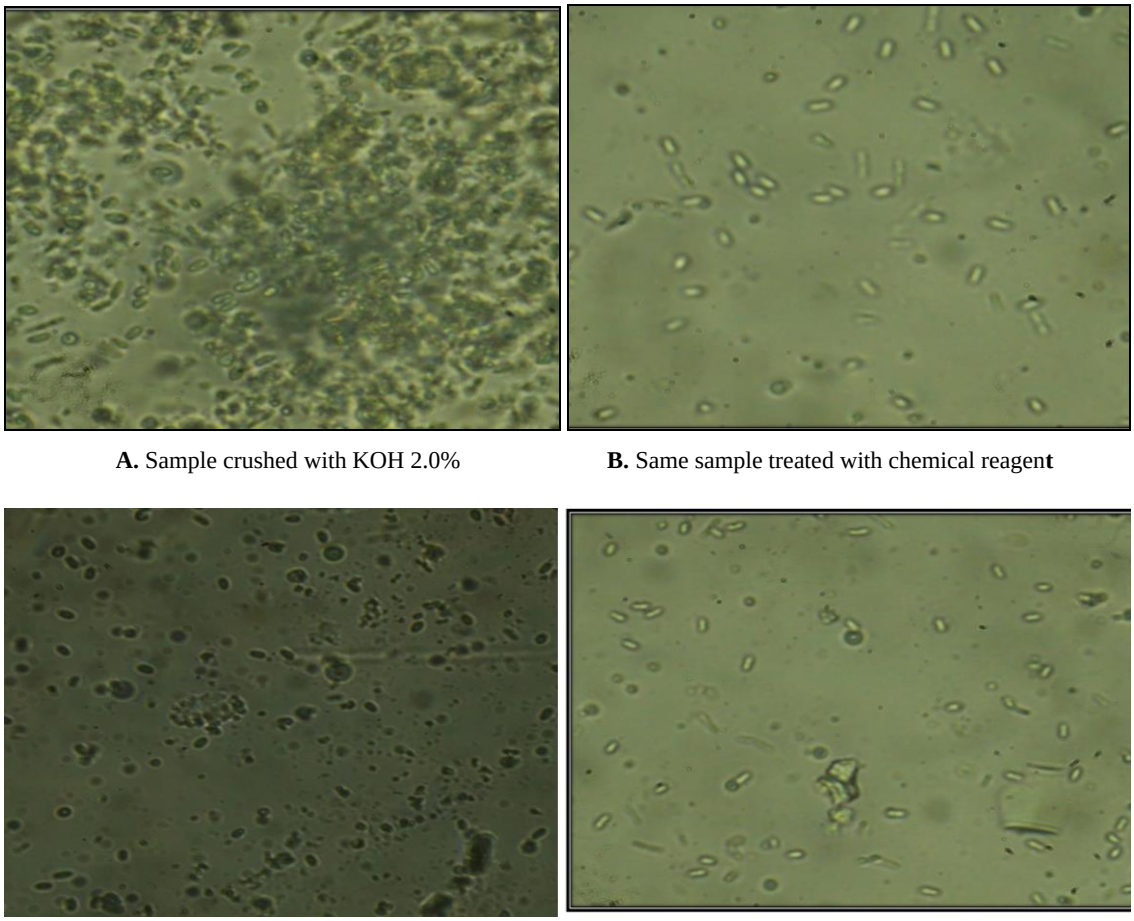
Results of visuals observation of tasar mother moth smear treated with different chemicals are represented in Table 1. Treatments of Sodium Dodecyl Sulphate (SDS), Sodium Lauryl Sarcocinate (Sarcosyl) with concentration 1.0 and 2.0% has shown encouraging results (Table 1). The visibility in microscopic field was very clear, no cellular debris observed, spores clearly visible and easy to identify. Liberation of spore from infected tissues was much high 7, 72, 000 and 8,12,000 spores/cm^3 with the treatment of Sarcosyl and 8,25,000 and 8,53,000 spores/cm^3 with the SDS treatment at 1.0 and 2.0% respectively.

Further a chemical reagent was prepared in combination of promising chemicals i.e. Sarcosyl 2.0%, SDS 1.0% and surf excel 2.0% and tested for identification of *N. mylitensis* spores. The treatment of the chemical reagent made the smear much clear, cellular debris, lipid particles and other artifacts are digested/removed/dissolved, spores were clearly visible and easy to identify; liberation of spore was much high 9,12,000 spores/cm^3 than the other treatments and control. The treatment of chemical reagent in one tooth pick quantity for 10-15 seconds is sufficient for easy and quick identification of *N. myltitensis* spores. The chemical reagent is found to be superior than the existing chemicals K_2CO_3 and KOH. The efficacy of the chemical reagent has been confirmed at Central Tasar Research & Training Institute laboratory (2017-2018 grainage) as well as at tasar seed production centres across the country. The In-charges of seed production centers observed and validated the chemical reagent performance and recommended for use in mother moth examination.

Table 1: Visual observation of moths homogenate treated with different chemicals.

#	Name of the Chemical	Conc. %	Observation	Liberation of spores No./Cm3
1	Lauryl Dimethyl Amine Oxide	1.0	Field was clear, cellular debris observed, liberation of spores normal	425000
		2.0	Field was clear, cellular debris observed, liberation of spores normal	449000
2	Octoxynol (Triton 100)	1.0	Field was not clear, cellular debris observed, liberation of spores poor	238000
		2.0	Field was not clear, cellular debris observed, liberation of spores poor	327000
3	Sodium Lauryl Sarcocinate (Sarcosyl)	1.0	Field was clear, no cellular debris, spores clearly visible and easy to identify, liberation of spore was high.	772000
		2.0	Visibility increased, no cellular debris, spores clearly visible and easy to identify, liberation of spore was increased.	812000
4	Sodium Dodecyl Sulphate (SDS)	1.0	Field was clear, no cellular debris, spores clearly visible and easy to identify, liberation of spore was much high.	825000
		2.0	Visibility increased, No cellular debris, spores clearly visible and easy to identify, liberation of spore was increased.	853000
5	Sodium Dodecyl Benzenes Salfonate (SDBS)	1.0	Field was not much clear, liberation of spores was normal	342000
		2.0	Field was not much clear, liberation of spores was normal	412000
6	Lauryl Ethoxylate	1.0	Field was not much clear, liberation of spores was normal	228000
		2.0	Field was not much clear, liberation of spores was normal	347000
7	Cween 20	1.0	Field was not much clear, liberation of spores was normal	251000
		2.0	Field was not much clear, liberation of spores was normal	313000
8	Cween 80	1.0	Visibility in microscopic field was poor, liberation of spores was normal	324000
		2.0	Visibility increased, liberation of spores was normal	352000

9	Hydrochloric Acid	1.0	Clump formation of cellular debris was observed, spores not clearly visible	175000
		2.0	Clump formation of cellular debris was observed, spores not clearly visible	198000
10	Acetic Acid	1.0	Clump formation of cellular debris was observed, spores not clearly visible	110000
		2.0	Clump formation of cellular debris was observed, spores not clearly visible	115000
11	Surf Excel detergent	1.0	Field was clear, no cellular debris, spores clearly visible, liberation of spores was poor.	125000
		2.0	Visibility increased, no cellular debris, spores clearly visible, liberation of spores was poor.	136000
12	Sarcosyl + SDS + surf excel	2+1+2	Visibility in microscopic field was very clear, no cellular debris observed, spores clearly visible and easy to identify, liberation of spore was much high.	912000
	Control (1) Potassium Bi Carbonate	0.5	Poor visibility, cellular debris observed, liberation of spores normal	426000
	Control (2) Potassium Hydroxide	2.0	Poor visibility, cellular debris observed, liberation of spores normal	352000



A. Sample crushed with KOH 2.0% **B.** Same sample treated with chemical reagent

C. Sample crushed with K2CO3 0.6% **D.** Same sample treated with chemical reagent

Fig 1: Chemical reagent for identification of pebrine spore in moth's tissues under microscope

4. Discussion

Identification the pebrine disease by microscopic technique is typically in practice in Tasar sericulture. This method of identification is laborious, time consuming, interference of non-pebrine tissues/artifacts which sometimes lead the confusion in identification of spores and non-release of spores from infected tissues. To unravel these hurdles, need was felt to investigate and to develop a solution which can disintegrate the debris/tissues without affecting the pebrine spores. Interestingly, an ideal chemical-combination formulation was developed which works as reagent for easy and quick identification of *N. mylittensis* spores during microscopic examination of mother moth to check the spread of disease through vertical infection. Out of tested chemicals/ concentrations/combinations, Sarcosyl, Sodium dodecyl sulphate and surf-excel quick wash have shown promising results. Sarcocyl is an ionic surfactant derived from sarcosine used as a foaming and cleansing agent in shampoo, shaving foam, tooth-paste, and foam wash products. It is known that SDS and sarcosyl bind tightly to (mostly unfolded) proteins via their hydrophobic tail. The major difference between these detergents is that SDS has a negative charge and sarcosyl is zwitterionic (at pH> 5.5). Both these detergents have role in cell-lysis and release of pebrine spores which creates comparatively very effective visualisation than traditional KOH. Use of these chemicals for pebrine visualization was not documented earlier. Our trial results indicates that this developed reagent helps in acquiring the smear clear by disintegration of cellular debris and other artifacts, spores are clearly visible and easy to identify, liberation of spore from tisues/cells was also much higher in contrast to earlier methods. It is assumed that this chemical reagent is playing key role in cell membrane lipids solubilization and cleaning other cellular debris. Similarly [9,

[10, 11] stated that SDS is widely used detergent/ surfactant in the areas of Biotechnology, Biochemical analysis, surfactant chemistry and polymer technology. It solubilizes the cell membrane, lipids and release the cell contents in to the solution. The commercially available surf excel detergent is having detergent properties. The exact mode of action is not clear. The present reagent saves the time, labour and thus helps in the production of DFLs. Liberation of spore from infected tissues was also much high (7, 72,000 to 8, 53,000 spores/cm^3). These promising chemicals were combined to form the chemical reagent for efficient detection of pebrine spores. The treatment of chemical reagent increases the visibility and clarity in microscopic field and quantum of spores was also higher (9, 12,000 spores/cm^3) and easy to identify. This work is frst of its kind in tasar industry for visualizing the pebrine spores. This robust reagent will help the industry for dfls production, there by healthy crop and thus doubling the tribal farmer's income.

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