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AN IMPROVED METHOD FOR THE DETECTION OF PEBRINE (*Nosema bombycis* N.) SPORES IN SILKWORM (*Bombyx mori* L.)

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ABSTRACT

The present paper describes an improved method for the detection of pebrine (*Nosema bombycis*) spores using powdered formulation of Ambistryn-S (antibiotic preparation of streptomycin sulphate) and Transco Trichostar (a biofungicidal preparation of *Trichoderma viridae*) to the larval, pupal and moth suspension after oviposition to check other bacterial and fungal growth. Alternate refrigeration used in this method simultaneously induces the pathogen to complete its intermediate life cycle stages and triggers production of maximum number of *Nosema* spores having long polar tubes (LT) with highly developed coat wall protein that facilitate the observation of pathogen under light microscope. This low-cost method performed successfully in detecting the pebrine spores at very low intensity in small populations, involved minimum skill and gave very quick results.

Keywords: Antibiotic, fungicide, *Nosema bombycis*, pebrine, polar filament

INTRODUCTION

In India, approximately 40% silk crop loss is attributed to the various diseases like pebrine (protozoa), flacherie (bacterium), grasserie (virus) and muscardine (fungus). Most of the commercial silkworm species are highly susceptible to these diseases. Of these, frequent outbreaks of pebrine incidence by *Nosema bombycis* Naegeli (Microsporida: *Nosematidae*), a primitive eukaryotic and pathogenic microsporidian, often leads to low cocoon production. The disease is spread primarily through eggs i.e. transovarian transmission and secondarily through contaminated rearing appliances, room, litter, leaf, etc. The pebrine disease till now has been kept under control within a reasonable limit but not eradicated completely. In India about 36% crop loss is attributed due to pebrine disease with occasional crop failure (Nataraju *et al.*, 2005).

Currently the practice of examining mother moths microscopically after oviposition in a group of 20 or more is followed at the seed production centers and infected seeds summarily rejected. This crude technique is followed for the last 140 years in all silk producing countries to combat disease although many new techniques including biotechnical and molecular biology are in pipe line. Hatakeyama and Hayasaka (2001, 2003) recognized ssu-rRNA gene sequence to be highly useful reliable tools for early diagnosis and identification of silkworm microsporidia and designed specific primers for molecular diagnosis. Several PCR methods based on the amplification of rRNA gene fragments have been reported effective in the diagnosis and species identification of microsporidia (Kawakami *et al*., 1995, 2001). However, they found that since silkworm microsporidia are not competitive in nature so develop strategy for coexistence *in vivo* and *in vitro* (Kawarabata, 2003). The development of new disease-resistant pathogenic strains has made the task of combating pebrine disease difficult. Hence, it is

imperative to develop new techniques that are sustainable in field, require less input and are easily adaptable and eco-friendly. New method using antibiotic and fungicide mixture suspension followed by alternate chilling is useful in detecting the pebrine spores. The present paper reports a suitable need-based modified technique for quick observation of pebrine spores to check the disease.

MATERIALS AND METHODS

The comparative study was made on the moths obtained from artificially inoculated larvae. The moths were inoculated with *Nosema bombycis* spores @ $1x10^6$ spores mL^{-1}. Three methods in vogue viz., in research laboratory method (Undeen, 1997), improved method of mother moth examination (Fujiwara, 1984) and delayed mother moth examination method (Sing *et al.,* 2004) were compared with the present new modified method.

In research laboratory method

N. bombycis, collected from diseased silkworms, were propagated in *Bombyx mori* as per the method of Undeen (1997). Infected moths (20-30), in sterilized water in 0.5% potassium carbonate (K_2CO_3) solution, were crushed in a mortar and pestle to separate spores from host tissues. The suspension was filtered twice through absorbent cotton and washed 2-3 times in endotoxin free (E.F.) water. Then suspension was centrifuged at 5000 rpm for 10 min. and sediments treated with 2% potassium hydroxide (KOH) solution. The *Nosema* suspension was layered over *percoll* cushions and centrifuged at 12000-15000 rpm for 30 min. in an RC5C high speed refrigerated centrifuge (Sorvall) with swing-out-rotor (SH-MT12) to obtain purified *Nosema* spores. Neubaur hemocytometer (German Fine Optik) with Thoma-Zaiss counting slide was used to count *Nosema* spores under compound microscope (15 x 40X) for determining the concentrations.

Improved mother moth examination method

For the examination of mother moths, the standard method of Fujiwara (1984) was followed. The eggs laid by the apparently healthy pebrine-free mother moths were used for the production of nucleus (P_1) and reproductive (P_2) seed. Twenty moths were taken in a mixie cup and 80 mL of 0.6% K_2CO_3 solution was added to it. Mixture was grinded and homogenized for 1-2 min. at 6000-8000 rpm. The homogenate was allowed to settle for 3-5 min. in a beaker and un-macerated tissues and debris were separated and discarded by decantation. Bottom liquid was carefully filtered through thick layer of clean muslin cloth. The filtrate was re-centrifuged for 3 min. at 3000 rpm, the supernatant solution decanted and sediment dispersed in a few drops of 2% KOH solution over a cyclomixer. Two smears from each sample were examined under phase contrast microscope (15 x 40X). The intensity of infection was estimated on the basis of the number of spores/field.

Delayed mother moth examination method

For delayed mother moth examination method (Sing *et al.,* 2004), the moths after oviposition were collected in groups in perforated cardboard boxes/covers and preserved in an isolated, well-ventilated room at 25-30°C for a period of 3-4 days and examined under microscope (15 x 40X) at 4 days interval. Moths were examined as per the procedure described above.

New methods using powder formulation of antibiotic and fungicide mixture

Live larvae/pupa/moths were taken in sterilized 5 mL 0.6% K_2CO_3 solution and crushed in a mortar and pestle to separate spores from tissues. The suspension was filtered through absorbent cotton and followed by muslin cloth once when sample size was 1-2 larvae/pupae/moths and twice if the sample size was 3-10 larvae/pupae/moths. The suspension was kept at room temperature (25-33°C) for 30 min. when sample size was 1-2 larvae/pupae/moths and for 1 hr when sample size was >2 larvae/pupae/ moth. The suspension was decanted when sample size was $\leq$2 larvae/pupae/moth or centrifuged at 2600 rpm for 5 min. followed by 2 washing in distilled water when sample size was >2 larvae/pupae/ moths. The sediment was mixed well with 5 mL 0.8% KOH to dissolve fat body. Then sediment was

kept at room temperature for 30-60 min. and refrigerated at 5±3ºC for 6 hr. The chilled suspension was then washed twice by either decantation or centrifugation at 3800 rpm for 5 min. in E.F. water. The sediment was mixed well with 2 mL distilled water. A powder formulation of Ambistryn-S (broad spectrum antibiotic preparation of streptomycin sulphate) and Transco Trichostar (bio-fungicide preparation of *Trichoderma viridae*) (25 mg each in 5 mL preparation) was added to the suspension and shaken well or short spun by cyclomixer for 1 min. to mix the sample well. The suspension was kept at room temperature (25-30^{o}C) for 15-30 min. and then in a refrigerator at 5±3ºC for 3-24 hr to check the germination and growth of bacteria or fungi. The chilled suspension was then either decanted or centrifuged at 4800 rpm for 8-10 min. and washed in distilled water. The suspension was examined under microscope (600-1000X) and if any pebrine spore was noticed the whole sample was destroyed by dissolving the suspension in 5% formalin or bleaching powder solution. The suspension may be stained with Nigrosin (10%) for spore identification.

RESULTS AND DISCUSSION

The technique developed by Undeen (1997) gave 70-80% recovery of purified spores with very less tissue debris (Table 1; Plate 1). However, the technique is costly than other techniques, requires high technical skill, contains non-viable spores, requires more host tissue (minimum 200 moths) and takes a minimum of 4 days time. Similarly, the effectiveness of the method developed by Fujiwara (1984), the only technique in vogue for reliable diagnosis of pebrine spores, depended on the technical procedures employed for the extraction and concentration of spores and also on the spore intensity in the tissues of mother moth itself. The suspension, prepared through this method, contained more fat bodies and tissue debris, was mixed heavily with bacterial/fungal spores, lead to increase in the density of tissue fluid

Table 1: Comparative study of different techniques

Methods/ techniques	Time taken (hr)	Maximum spores isolated (mL^{-1})	Cost of chemicals (Rs)	Minimum sample size required	Sophisticated equipments	Spore isolation (%) & skill	Remarks on efficiency and quality of spores
Research lab	96	High 1×10^{8}	1250 for one time	200 pupa/ 100 moths	Cooling centrifuge with 15000 rpm cyclomixer	70-80%; high skill	Highly purified spores with very less tissue debris
Improved Method	4-5	1×10^{5}	Negligible	40 pupa/ 20 moths	Simple centrifuge cyclomixer	0-30%; skill	Spores with much fat body and tissue debris mixed with maximum bacterial/ fungal spore
Delayed mother moth	96	1×10^{6}	Negligible	100 moths	Simple centrifuge cyclomixer	30-60%; skill	Spores with much fat body and tissue debris mixed with maximum bacterial/ fungal spore. Larva/pupa is not suitable
New method using antibiotic and fungicide	24-48	1×10^{7}	Negligible	1-10 pupa/ moths	Simple centrifuge cyclomixer refrigerator	60-70%; skill	Suitable for all life cycle stages like larva, pupa and adult moths with minimum size 1-2 and maximum depending upon the capacity of centrifuge machine. Spores with minimum tissue debris/fat body. Less bacterial and fungal spores.

and only 30% spore could be isolated. Seed production centers generally follow this method to microscopically examine the mother moth in different batch/lots after oviposition. If a batch contains even a single spore, the whole lot is summarily rejected and immediately burned.

The delayed mother moth examination method, developed by Sing *et al*. (2004), has advantage of being easy and effective in pebrine detection due to enhanced sporulation in older moths and the fluid mostly contains environmental spores that have LT and is reliable because the pebrine detection is possible under low intensity of infection. Although the technique is useful at basic seed production level upto P_1 seed, the suspension mostly contains more fat bodies and tissue debris that leads to increase in the density of tissue fluid and gives foul smell due to long exposure of bacterial/fungal spores at room temperature. Further, the larvae or pupa are unsuitable and the method takes a minimum of 4 days time, though 60% spores were isolated.

The new method using antibiotic and fungicide mixture performed better in pebrine spore detection than other techniques even at a very low infection rate in a small population. Further, the modified technique is very low in cost, involved

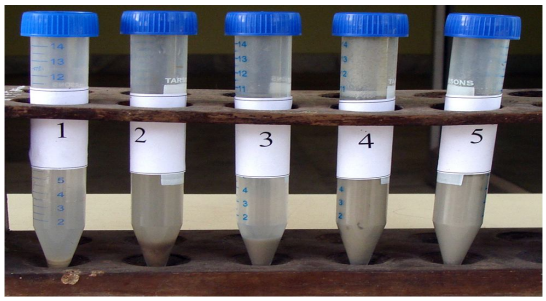

Plate 1: Methods of sample collection 1) In research laboratory 2) Crude homogenate tissue sample 3) Improved method of mother moth examination 4) Delayed mother moth examination 5) New method using antibiotic and fungicide

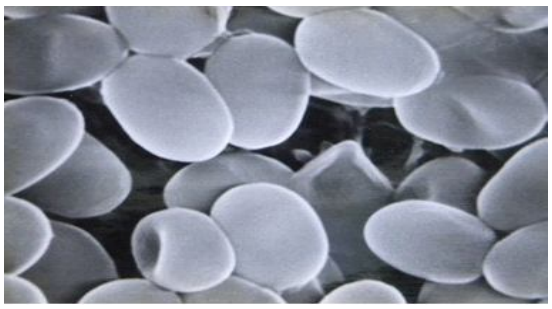

Plate 2. *Nosema bombycis* spore under scanning electron micrograph (x 83000)

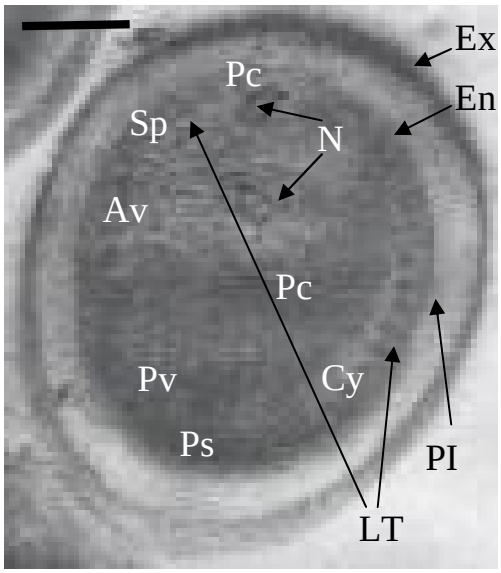

Plate 3: *Nosema bombycis*, enlarged view of TEM photograph with long polar tube (LT) showing nucleus (N), exospore wall (Ex), endospore wall (En), anterior vacuole (Av), posterior vacuole (Pv), polar cap (Pc), sporoplasm (Sp), cytoplasm (Cy), polar sac (Ps) and plasmalemma (Pl); Bar = 1 µm

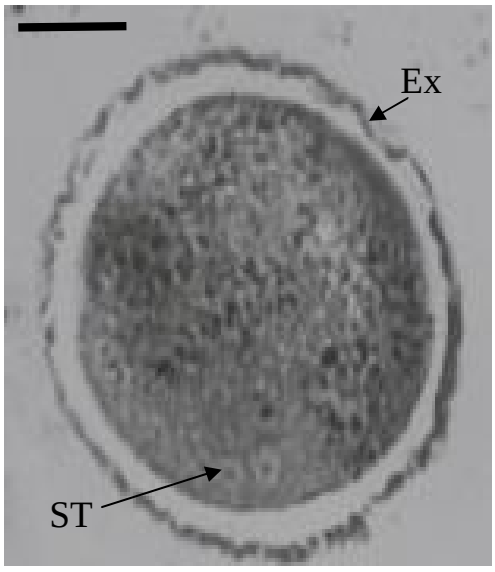

Plate 4: *Nosema bombycis* TEM photograph with short polar tube (ST) (Enlarge view); Bar = 1 µm

minimum skill and gave result in a very short time (24-48 hr). Moreover, the rate of pathogen multiplication (in other words pebrine spore concentration) in the infected larva, pupa and moths showed subsequent increase. The spore harvest was $1x10^7$ spores mL^{-1} in this present method as compared to other methods tested though the inoculum concentration, pathogen source and rearing environment were same. It appears that physiological changes in insect tissue induced the developmental cycle of parasite to switch-over from predominantly vegetative stage to sporogony resulting in enhanced mature spore production. If development is allowed to proceed to the stationary phase, multiplication of parasite is greater with lower concentrations (Lai and Canning, 1983) and pathogen multiplication depends upon the age of silkworm, time and other indirect factors (Solter *et al*., 1989). In present method, the intermediate life cycle stages of pebrine spore i.e. planont, meront/ schizont, sporont, etc. in host tissue continued their development for maturation. The development and sporulation of microsporidian spore is directly correlated with the nutritional status of host, therefore most intermediary life cycle stages of pathogen tend to sporulate. Further, the mature spores with dense coat wall facilitate easy and accurate pebrine spore detection in homogenate. Alternate chilling of tissue homogenate preserved host nutrients to some extent which probably helped the intermediary life cycle stages of pathogen to take host nutrient for some time and utilize the same for maturation. Antibiotic and fungicide use helped in killing other associated pathogens so that entire remaining nutrient was reserve for intermediary life cycle stages of pebrine spore only. The pathogen multiplies maximally to ensure that certain percentage of pathogens get entry into next generation through transovarial transmission. The high infection rate of adults resulted in increased rate of transmission to the next generation (Henry *et al.*, 1973). Female larvae pupate normally, emerge as apparently healthy adults and transmit parasites transovarially to the next progeny, when mated with healthy mates. High amino transferase enzyme activities in females may probably the cause for inducing difference in spore yields.

Ultra-structure studies of spores revealed that mature *N. bombycis* spores exhibit dimorphism; primary spore with thin wall (>200 nm) had 3-4 coils defined as short polar tube (ST) and the environmental spore with thick wall (< 200 nm) had 12-14 coils defined as long polar tube (LT) (Plate 2-4). Primary spores was found in intermediate life cycle stage of host i.e., larvae, pupae and male moth enormous in number. The development pattern of two spore types was different. The *N. bombycis* spores produce two types of spore under the influence of host's reproductive role and physiology for transmission of disease (Chakrabarty *et al.*, 2013).

The primary spore germinates immediately after maturation within the cytoplasm of the host cell by protruding sporoplasm-like germs into the neighboring cells to spread the infection within the host (Iwano and Ishihara, 1991).The environmental spores are resistant forms commonly recovered from live/dead larva/pupa/moth. However, the primary spore is not frequently detected under light microscope as it is short-sized (3.78 ± 0.5 x 2.18 ± 0.5 µm) (Chakrabarty *et al.*, 2013). The findings are supported by Iwano and Ishihara (1991) who isolated *N. bombycis* spores from the moths of lawn grass cutworm, *Spodoptera depravata* and from cell culture of *B. mori*. Sporont *N. bombycis* usually produce two sporoblasts (Kawarabata and Ishihara, 1984). The appearance of two distinct spore forms within the same species has previously been documented (Graaf *et al*., 1994; Loubes *et al*., 1999). Spore dimorphism can be considered as adaptation of parasite to different needs during their life cycle. Spores which appear during early parasite development apparently have the ability to germinate intracellular. Intracellular extrusion of relatively short polar tube can serve as a means to deliver sporoplasm in neighboring cells (Iwano and Ishihara, 1991).

Conclusion: The detection of pebrine disease would be easier by using the present improved method involving use of antibiotic and bio-fungicide formulation to the mother moth suspension suppressing the growth of other microbes. Imposition of alternate chilling treatment hastens the intermediate stages of life cycle of pebrine spore by triggering for the development of maximum spore number contain long polar tube and the cell wall coat protein.

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