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Secondary Contamination is the Main Source for Spread of *Nosema bombycis* Resulting in Outbreak of Pebrine Disease in *Bombyx mori* L.

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Abstract

In nature, the population of *Nosema bombycis* (Microsporidia) causing pebrine disease is small and their development is extremely slow and only few ultimately producing spores. Pebrine infected silkworm, *Bombyx mori* larvae collected from sericulture field were alive till 3rd generation though the concentration of *N.bombycis* spore was very high (2.4 - 3.0 x 10^8 spores. mL^{-1}). All larvae were died during 4th generation with extremely high concentration of pebrine spores (3.0 - 4.0 x 10^9 spores. mL^{-1}) and mostly contain long polar tube (LT). Alternately, all larvae were died immediately (at 3rd stage of 1st generation) when it was artificially inoculated with same concentration of *N.bombycis* spores harvested from field (2.4 - 3.0 x 10^8 spores. mL^{-1}) though concentration of spores harvest was very less (3.0- 4.0 x 10^6 spores. mL^{-1}) and mostly contain short polar tube (ST). Artificially pebrine infected male moth when mated with healthy female moth took six generations to develop pebrine disease and all larvae were died at the 2nd stage with very less spore harvest (3.0 - 10.0 x 10^5 spores. mL^{-1}). Survival percentage was increased in all generations (~92.0% at 4th generation) when silkworm rearing was conducted under new integrated disease management system.

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Introduction

Pebrine is a deadliest disease of mulberry silkworm, *B. mori* L. caused by the pathogen, *N.bombycis* Nageli, (Microsporidia: *Nosematidae)*. It is transmitted primarily through eggs i.e., transovarian transmission and through the feeding of contaminated leaf, rearing tray, rearing bed, layings, as well as cross-transmission from alternate host secondarily i.e., transovum transmission (Chakrabarty *et al.,* 2013a). Each oval spore measuring 3 - 4 µm × 1.5 - 2 µm includes a coiled polar filament that may be 100 µm in length. The disease is spread transovarially by environmental spore and secondarily by primary spores. Ultra-structures of the two type of spores are entirely different, primary spore contain short polar tube (ST) with thin wall (< 200 nm); whereas, the environmental spore contain long polar tube (LT) with thick wall (> 200 nm). Pasteur (1870) was the first to detect the transmission of pebrine through the eggs i.e., transovarian transmission and he advised for examination of mother moth. It is the standard method followed to control the disease throughout the country. Transovarian transmission has been reported to be sole mechanism by which transmission of

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the parasites occurs (Kellen *et al.,* 1965, Chapman *et al.,* 1966). It is reported that transmission of microsporidia horizontally by feeding spores produced in male larvae back to larval hosts have been unsuccessful (Kellen *et al.,* 1965) as the size of the spore is larger than the sperm. However, transovum transmission of spore through external surface of genital organ of heavily infected male moths can't be rule out. However, such type of secondary source of contamination of *N.bombycis* is lacking in the literature. Though, some preliminary studies on veneral transmission of spore have been done by some workers (Patil, 1993). However, we identified that *N.bombycis* is capable to specify gender of the *Bombyx mori* and multiplied in male very quickly to spread the infection through transovum transmission (Chakrabarty *et al.,* 2013a).

Therefore, role of transovum transmission of pebrine spore through accessory sex organs / external body surface of male moths in successive generations for transmission of disease was undertaken to find out the sole mechanism of outbreak of disease.

Materials and methods

Rearing of 1st generation of infected silkworm

One hundred live cocoons were collected from one farmer's house at Barunighata village, Birbhum district, West Bengal, India. Fifty cocoons were subjected for isolation of *N. bombycis,* pebrine spore. Spores were isolated from live infected pupae and purified by centrifugation at 3000 rpm for 10 min following new method of pebrine isolation (Chakrabarty *et.al,* 2013b). After isolation, spores were suspended in 0.85% NaCl and stored at 4ºC. Spores were counted using a Neubauer haemocytometer under light microscope (x 600) and determined the inoculum concentration following standard method (Undeen, 1997) and used as stock solution. Fresh spores with 3.0–4.0 × 10^6 spores. mL^{-1} concentration were inoculated (i.e., artificial infection) to 4th stage 1st d larvae (Race: M Con1). Other fifty cocoons were allowed for moth emergence in ambient condition (i.e., natural infection). Whole body tissues of both male and female moths were examined under light microscope for detection of pebrine spores after coupling for male moth and oviposition for female moth. Eggs were incubated properly maintaining temperature and humidity as per standard procedure and allowed for hatching in normal condition. Rearing was conducted as per standard procedure (1st generation). Again, eggs were prepared utilizing moths generated from 1st rearing and rearing of 2nd generation was conducted following previous procedure i.e., 1st generation.

Rearing 2nd, 3rd and 4th generations of infected silkworm

2nd generation

Rearing of 2nd generation were conducted with fifty larvae (Race: Nistari) of 1st brushing (T1) and fifty larvae of 2nd brushing (T2) when eggs were prepared utilizing moths recovered from natural infection of 1st generation. Rearings of 2nd generation were also conducted with fifty larvae of 1st brushing (T3) and fifty larvae (Race: Nistari) of 2nd brushing (T4) when eggs were prepared utilizing moths recovered from artificial infection of 1st generation.

3rd Generation

Rearing of 3rd generation was conducted with 500 larvae when eggs were prepared utilizing moths recovered from natural infection of 2nd generation (T2).

4th Generation

Rearing of 4th generation was conducted with 1000 larvae when eggs were prepared utilizing moths recovered from natural infection of 3rd generation (T2).

Rearing 2nd, 3rd and 4th generations of infected silkworm with protection

All the procedure was same as Rearing 2nd, 3rd and 4th generations. However, these experiments were conducted in two separate batches, one with protection using existing disease management systems, using 'Labex' as bed disinfectant and '5% Bleaching powder' as room disinfectant and other with new disease management system using 'Sericillin' as bed disinfectant and 'fumigant chemicals' as room disinfectant.

Rearing 3rd generations of infected male with healthy female

Here, we have crossed the fully infected male moths (1.0–1.2

Table 1. Comparative performances of *N.bombycis* at different metamorphic stage of *B. mori* L. during artificial and natural infection (data in parenthesis indicates the standard error of mean)

#	Race	Type of study	Stage of silkworm	No. of sample	Intensity of Infection (spores.mL^{-1})	Survival (%)
1	Nistari	Natural infection (1st generation)	Pupa 2nd d	50	2.4-3.0 x 10^{8} (6.0 x 10^{6})	87.2
2	M Con1	Artificial inoculation (1st generation)	Larva at 4th stage 1st d	50	3.0- 4.0 x 10^{6} (1.0 x 10^{5})	1.50
3	Nistari	Natural infection (2nd generation)	Larva under 2nd moult	50	1.0-1.2 x 10^{7} (2.0 x 10^{6})	94.0
4	Nistari	do	Larva at 3rd stage	50	1.0 x 10^{7} (1.0 x 10^{6})	82.3
5	Nistari	do	Larva at 4th stage	50	1.0 x 10^{7} (1.2 x 10^{6})	75.6
6	Nistari	do	Larva at 5th stage	50	x 10^{7} (1.0 x 10^{6})	72.5
7	Nistari	do	Pupa	50	x 10^{7} (1.0 x 10^{6})	67.5
8	Nistari	do	Moth	50	x 10^{7} (1.0 x 10^{6})	62.3

Table 2. Fitness Efficiency Performance of *B. mori* L. during artificial and natural infection

#	Race	Date	Type of study	Survival (%)	Layings (No)	Fecundity (No)	Hatching (%)
1	Nistari	D/C: 24.04.2012	Natural infection (1st generation)	87.2	20	334	88.12
2	MCon1	D/I: 29.04.2012	Artificial inoculation (1st generation)	1.82		All moths died	
3	Nistari	D/B : 15-16.05.12	Natural infection (2nd generation)	62.3	15	249.5	86

× 10^{7} spores.mL^{-1}) recovered from rearing 3rd generations with healthy female moths. We have continued the generations with recording survival percentage and intensity of infection.

Results and Discussion

Nosema could produce two spores from sporont (Ishiwara, 1969). Sporont of *N.bombycis* usually produced two sporoblast (Lai and Canning, 1983). The first populations of spores mainly cause the spread of the parasite in the epithelium. The second population of spore is formed later on for adapting to survive outside the host (Graaf *et al.,* 1994). Early spores and environmental spores are immature and variants of the same spore type, normally occurs in different tissues in the host (Larson, 1999).

In rearing of 1st generation of infected silkworm (*experiment-I*) , all the larvae were died when the larvae were artificially infected with same inoculums concentration of pebrine spore harvested from the moths collected from the field (3.0–4.0 × 10^{6} spores. mL^{-1}). However, the larvae were survived harbouring the same concentration of pebrine spore harvested from the moths collected from the field (3.0–4.0 × 10^{6} spores.mL^{-1}) and larvae completed the life cycle though survival percentage were decreased to 62.3% where intensity of spore were remain almost same concentrations and constant (1.0 × 10^{7} spores. mL$^{-1)}$ for all larval stages, pupal stage and moth (Table 1 and 2). Some of the

Table 3. Comparative performances at different metamorphic stage of *B. mori* L. during different brushing practice at artificial and natural infection (data in parenthesis indicates the standard error of mean)

#	Treatment	Mature single larval wt (g)	ERR (%)	Pupa (%)	Intensity of infection at pupal stage (spores.mL^{-1})	Moth (%)	Intensity of infection at moth stage (spores.mL^{-1})
2nd generation (T2 Natural 2nd brushing)							
1	T1 (Natural infection 1st brushing)	0.990	All larvae died				
2	T2 (Natural infection 2nd brushing)	1.161	22.32	10.28	1.2×10^6 (1.0×10^5)	4.97	$1.0–1.2\times10^7$ (1.0×10^6)
3	T3 (Artificial infection 1st brushing)	1.045	17.34	6.07	1.5×10^6 (1.0×10^5)	1.82	No spore
4	T4 (Artificial infection 2nd brushing)	1.034	4.38	All larvae died			
3rd generation							
1	T2 Natural 2nd brushing	2.032	90	64	1.2×10^6 (1.0×10^5)	46.1	$1.0–1.2\times10^7$ (1.0×10^6)
4th generation							
1	T2 Natural 2nd brushing	All larvae died at 3rd stage with intensity 3.0–4.0 × 10^9 spores.mL^{-1} (8.0×10^7)					

new factor is responsible in the evolution of pathogens as well as variability in host specificity so that their multiplication rate and virulency are restricted in the same host and host of the same origin are only survived. Alternately, artificially infected larvae were died due to loosing the originality of host specificity.

In rearing of 2nd, 3rd and 4th generation of infected silkworm (*experiment-II*)**,** all artificially infected larvae were died in 1st generation. But larvae were died in 4th generation when silkworm rearing was conducted in natural condition. In this case, intensity of infection (3.0–4.0 × 10^9 spores.mL^{-1}) was increased and survival percentage was decreased till 3rd generation and at last all larvae were died in 4th generation. Two contrasting results were observed in this experiment. Intensity of infection was more in naturally infected 1st brushing larvae (3.0–4.0 × 10^9 spores. mL^{-1}) whereas, it is more in 2nd brushing larvae for artificial infection (1.0 × 10^8 spores.mL^{-1}) before their death. Alternately, survival percentage was more in 2nd brushing larvae (ERR~ 22.32 %) infected naturally whereas, it is more in 1st brushing larvae infected artificially (ERR ~ 17.34 %). It was very much interesting to observe that all the larvae were survived in 2nd generation when the larvae contain full of pebrine spore (3.0–4.0 × 10^7 spores.mL^{-1}) when larvae infected naturally and finally, all larvae were died in 3rd generation before resume to 3rd stage and contain full of pebrine spore (3.0–4.0 × 10^9 spores.mL^{-1}) (Table 3).

In rearing of 2nd, 3rd and 4th generation of infected silkworm with protection (*experiment-III*)**,** all the naturally infected larvae were died in 4th generation when rearing was conducted in natural condition and artificially infected larvae were died in 1st generation with existing disease management system. But the survival percentage were increased in all generations when rearing was conducted with new system of management. It was surprised that more than 98% larvae were survived during 4th generation when rearing was conducted with new system of management and no spore was observed in moth under microscope (Table 4).

In rearing of 3rd generation of infected male with healthy male (*experiment-IV)***,** when artificially infected male moth were mated with healthy female then all offspring were died resume from 1st moult at 6th generation and develop pebrine disease though very less spore harvest (3.0–10.0×10^5 spores.mL$^{-1)}$ was recorded. We have not recorded any spore till 5th generation. Besides, all the pre cocoon and post cocoon parameters at 5th generation revealed that the lot was healthy as well as robust considering Effective Rearing Rate (~75%), single mature larval weight (~2.496 g), Shell % (~13.0), Filament Length (~271 m),

Table 4. Comparative performances of management for control of pebrine disease of *B. mori* L. using existing system of management and new integrated system of management.

#	Mature larval wt at 5th stage (g)	ERR (%)	Pupation (%)	Intensity of infection at pupal stage spores.mL^{-1}	Moth (%)	Intensity of infection at moth stage spores.mL^{-1}	No of laying by female (No)	Fecundity (No)	Hatching (%)
3RD GENERATION (Treatment with existing systems)									
1	2.032	90.0	88	1.2 x 10^6	64	1.0-1.2 x 10^7	20	275	96
3RD GENERATION (Treatment with new system of management)									
1	2.173	92.0	90	No spore	85	No spore	42	322	98
4TH GENERATION (Treatment with existing systems)									
1	All worms were died at 3rd stage (3.0 - 4.0 x 10^9 spores.mL^{-1})								
4TH GENERATION (Treatment with new system of management)									
1	2.223	90.0	88	No spore	85	No spore	63	329	98

Table 5. Secondary contamination of pebrine disease in successive generations in *B.mori.*

(Healthy female x Pebrine infected male from 3rd generation of Experiment-II)								
#	Generation	Race	D/B	D/S	Hatch (%)	No of larvae	Larval weight (g)	ERR (%)
1	F1	Nistari	26.08.12	15.09.12	93.56	500	2.163	72.72
2	F2	Nistari	15.11.12	09.12.12	89.35	500	2.154	73.86
3	F3	Nistari	31.01.13	25.02.13	91.68	500	2.176	73.60
4	F4	Nistari	26.03.13	15.04.13	94.24	500	1.988	75.68
5	F5	Nistari	16.05.13	04.06.13	88.45	500	2.496	74.56
6	F6	Nistari	18.07.13	All larvae were died at a time after 1st moulting				

Non-breakable Filament length (~261 m), Denier (1.91) Fitness Efficiency test i.e., Grainage performance (Table 5 and 6) . Besides, the lot performed better in all the favourable and non-favourable seasons.

Time taken for establishment of the pathogen for completion of its life cycle and production of spores depends on inoculum load and other environmental factors (Steinhaus and Huges, 1949). We have observed in our previous study that spore harvest was more in male moth compared to that in female moth though the inoculum concentration, source of pathogen and the rearing were conducted in the same environment (Chakrabarty *et al.,* 2013b). The physiological changes in the insect might have possibly influenced the developmental cycle of the parasite to switch on from a predominantly vegetative stage to sporogony, resulting in an increasing spore production. Spore production had reached a stationary phase and yielded similar amounts of spore of *N. acridiophagus* and *N. cuneatum* in larvae of *Melanoplus asnguinipes* after 20 d of inoculation with 10^4 and 10^6 spores compared to lower spore yields which were continued in spore multiplication stage when inoculated with 10^2 spores (Cali, 1970). If development is allowed to proceed to the stationary phase, multiplication of the parasite is greater with lower concentrations than with higher concentrations (Kawarabata and Ishihara, 1984). But there is an apparent increase in multiplication with increased concentrations during the exponential phase and the difference between the multiplications of spore with the high and low initial spore concentration is narrow. Multiplication of pathogens depends upon the age of silkworm, time and other indirect

Table 6. Intensity of infection and reeling performance of secondary contamination of pebrine disease in successive generations in *B.mori.* (Healthy female x Pebrine infected male from 3rd generation of Experiment-II)

#	Generation	SCW (g)	SSW (g)	Shell (%)	Intensity of infection (spores.mL^{-1})	FL (m)	NBFL (m)	Dr
1	F1	0.983	0.120	12.18	Not observed	265	239	1.96
2	F2	0.972	0.108	11.11	Not observed	259	245	1.98
3	F3	1.169	0.150	12.83	Not observed	273	256	1.60
4	F4	1.065	0.143	13.43	Not observed	255	238	1.73
5	F5	0.919	0.119	13.00	Not observed	271	261	1.91
6	F6	All larvae were died at a time after 1st moulting			3.0 - 10.0 x 10^5			

factors (Loubes, 1999). A particular concentration is effective for multiplication of spore and mortality (Graaf *et al.*, 1994) and below that threshold level the concentrations do not cause any larval mortality (Larson, 1999). Therefore, the number of spores (intensity of infection) that a host can harbour and still function normally is important in determining the role of microsporidia as a parasite in nature (Graaf *et al.*, 1994). We have also found in our previous study that spore production reached the stationary phase in female during pupal stage to moth stage where as it is continued in progress in male pupa to moth stage till the dead of the moth i.e., the time of harvest. It is an interesting phenomenon of *N. bombycis* which needs to instigate further for detailed understanding and the findings assume importance for taxonomic classification (Chakrabarty *et al.,* 2013a).

Conclusion

From the study, it may be concluded that both male and female moths to be examined microscopically during hybrid laying preparation. We could observe only 4th generation from parental generation (P3) to commercial rearing in the field. As there is no chance for 6th generation study in the field, for that reason outbreak of pebrine disease from secondary transmission is not observed. However, we should take care where parental generation (P3, P2 and P1) is maintained to check the secondary transmission. Besides, new management system using 'Sericillin' as bed disinfectant with 'fumigant chemicals' as room disinfectant is required to be adopted, especially where parental generation is maintained i.e., seed production centre, to control the secondary transmission of pebrine disease.

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