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Gender Influenced Spore Dimorphism in *Nosema bombycis* Nageli Causing Pebrine Disease in Mulberry Silkworm, *Bombyx mori* L.

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Abstract

Nosema bombycis is a pathogen causing pebrine disease of the mulberry silkworm, *Bombyx mori*. The disease spreads mainly through transovarian transmission of environmental spore and secondarily through contaminated food, rearing appliances, *etc*. by primary spores. Ultra-structure studies using Scanning and Transmission Electron Microscopy of the two spores revealed differences in the primary spore which contained a Short Polar Tube (ST) with a thin wall (< 200 nm), and the environmental spore which contained a Long Polar Tube (LT) with a thick wall (> 200 nm). It is observed that the yield of spore with LT is highest in female moths, whereas, it is spores with ST are highest in male moths. Besides ultra-structures, the development pattern of the two types of spores is also different. It is an interesting finding in the present study that, spores of *N. bombycis* produced two types of spores and multiplied in different gender under the influence of the host's reproductive role and physiology for transmission of disease. The detailed study on ultra-structure of disporous *N. bombycis* in both the sexes of *B. mori* along with their development in the life cycle stages of silkworms with special reference to the inoculum concentration of spore is discussed.

Keywords: *Nosema bombycis*, polar filament, primary spore, environmental spore, gender specificity

Introduction

Pebrine is a deadly disease of the mulberry silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae) caused by the parasite, *Nosema bombycis* Nageli (Microsporidia: Nosematidae). It is primarily transmitted through the eggs and secondarily through the feeding of contaminated leaves, rearing tray, rearing bed, layings as well as alternate hosts. Cross infection with other microsporidia is reported from silkworms. In spite of advance research, in most sericultural countries, the loss due to the pebrine disease is yet to be fully eliminated. One of the pioneer scientists made a detail study on the growth and multiplication of pathogen and detected the transmission of pebrine through the eggs *i.e*., transovarial transmission [1]. However, pebrine disease in silkworms has been kept under control within a reasonable limit, but not been eradicated completely. About 36 % crop loss is due to pebrine disease with occasional crop failure in India [2].

Mother moth examination is the only reliable technique available to detect pebrine spore infection to check the spread of the disease, and thereby control the disease to date. This method is being followed throughout the country and other parts of the world wherever sericulture is practiced. The reason for mother moth examination after oviposition under light

microscope is to observe whether the female moth is pebrine infected, if so, the eggs are ignored and rejected for commercial rearing. It is the standard method to control the disease in vogue for the last 145 years [1]. Male moths won't contribute to the vertical transmission of pebrine owing to fact that the spore can't get transmitted through sperm as it is bigger than sperm. Pebrine spores are disporous and dimorphic, producing primary and environmental spores. The ultra-structure of pebrine spore found in male and female moths is entirely different. Besides, the concentrations of spore harvest are also different in two sexes even though both of them are contaminated from the same source and the same inoculum concentration. However, such a type of gender specific character of *N. bombycis* is lacking in the literature though some preliminary study on spore concentration have done by some workers [3]. Therefore, the ultra-structure of *N. bombycis* spores produced by male and female moths with special reference to the role of the spore in different sexes in transmission of the disease were undertaken to find out the gender specific character of pebrine spores.

Materials and methods

Spores of *N. bombycis* were isolated from live infected moths of *B. mori* and purified by centrifugation at 3,000 rpm for 10 min using percoll cushions (PVP coated silica particles, Sigma chemicals Co., USA) following a standard procedure [4]. After centrifugation, the spores were suspended in 0.85 % NaCl and stored at 4 °C. To obtain fresh spores, third instar '0' hour larvae were *per orally* inoculated with microsporidia (1×10^6 spore ml^{-1}). Spores were counted using a Neubauer haemocytometer under light microscope (×600) and the inoculum concentration determined following a standard method [4] and used as a stock solution. 1.5 ml fresh, purified, concentrated (2.5×10^8 spore ml^{-1}) mature spores were suspended in distilled water and centrifuged at 6,000 rpm for 10 min for scanning electron microscope (SEM) study and the pellet containing mature spores were primary fixed with 2.5 % glutaraldehyde followed by post fixation with 1 % osmium tetroxide following a standard technique [4]. 2.5 ml of fresh, purified, concentrated (2.5×10^8 spore ml^{-1}) mature spores were suspended in distilled water and primary fixed with 2.5 % glutaraldehyde followed by post fixation with 2 % osmium tetroxide hardened in 1.5 % molten agar (w/v) following the standard technique for transmission electron microscopy (TEM) study [4]. Only golden and grey sections were stained with uranyl acetate and lead citrate.

The silkworm (Nistari breed) rearing was conducted as per package of practices [5]. The experiment was conducted for three seasons during November - December (Season-1) and February - March (Season-2) (favourable seasons for silkworm) and May (Season-3) (unfavourable season for silkworm). Eighteen replications with 60 3rd stage '0' hour larvae were used for each treatment. Microsporidia purified from silk moths were prepared in three different concentrations (1.5×10^6, 1.5×10^7 and 1.5×10^8 spore ml^{-1}). Three replications each with 60 larvae were maintained for each concentration. Each dose of microsporidian aqueous suspension (1.5 ml) was thoroughly mixed with three freshly prepared mulberry leaf dishes (28.3 cm^2), dried and fed to the 3rd stage '0' hour larvae for a period of six hours. The mulberry leaves smeared with distilled water were fed to the larvae of the control group. The spores were isolated from pooled sample of the three day old infected pupae and two day old virgin moth before copulation of both sexes and purified and counted using a Neubauer haemocytometer under light microscope (×600) following a standard procedure [4].

Results and discussion

The present SEM study shows a mature spore of *N. bombycis* with a smooth and distinct surface wall (**Figure 1**). The primary spore has ST with a thin wall and is the non-resting spores and round / oval in shape and measured 3.78 ± 0.5 µm in length and 2.18 ± 0.5 µm in width (Volume = 9.20 μm^3, **Figure 2**). It germinates immediately after maturation to further disseminate the infection within the host. It germinates within the cytoplasm of the host cell by protruding the sporoplasm like the germs into neighboring cells. They are secondary infective forms meant to spread the infection within the host [6]. The environmental spore has LT with a thick wall and is the resting spore and oval in shape and measured 4.10 ± 0.5 µm in length and 2.78 ± 0.5 µm in width (Volume = 16.64 μm^3, **Figure 3**). These are resistant forms with a thick wall, spores commonly recovered from the mother moth or dead

silkworms. Normally *per oral* infection of silkworm larvae with *N.bombycis* starts in the midgut epithelium and surrounding muscles [4]. The present TEM study indicates that spore of *N. bombycis* exhibits dimorphism, one group of spores having 3 - 4 coils and another group having 12 - 14 coils (**Figures 4** and **5**). A spore with a thin wall (< 200 nm) and ST with 3 - 4 coils, is produced in the early sporogonial sequence as well as a spore with a thick wall (> 200 nm) and LT with 11 - 14 coils, is produced during the late sporogonial period [12]. The sporogonial sequence of microsporidian depends on the kind of parasites, species of the host and their developmental stage *viz*., embryonic, larval, pupal and adult. However, sporoplasm of LT penetrates through the midgut and arrives directly in the body cavity [7]. In the case of ultra-structural observation in the present study, it is observed that the spores in female pupa and moths mostly contain LT, whereas, it is ST in male pupa and moths. This is an interesting finding in the present study. It supports the view that spores containing ST multiplied at a higher rate and in male moths only. But sometimes the spores with ST are not detected under light microscope (×600) as it contains a thin wall (< 200 nm) and short in size (3.78 ± 0.5 µm × 2.18 ± 0.5 µm). This is an interesting finding in the present study. Besides, 1 - 2 to 10 - 12 spores harvested from male moths contain LT compare to 6 - 7 to 17 - 20 spores contain ST are observed with increasing inoculums concentrations (1.5×10^6 to 1.5×10^8 spores/ml) in 200 µm^2 TEM field. However, 10 - 12 to 20 - 22 spores contain LT compared to 2 - 3 to 10 - 12 spores with ST in female moths with the same increasing inoculum concentrations and in the same area (**Table 1**, **Figures 6 - 8**). This adds immense value to the new findings.

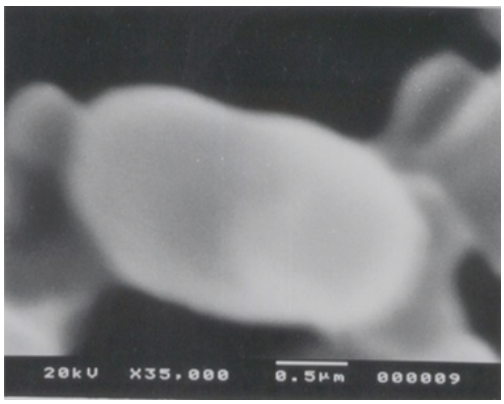

Figure 1 *Nosema bombycis* SEM photographs of a mature spore.

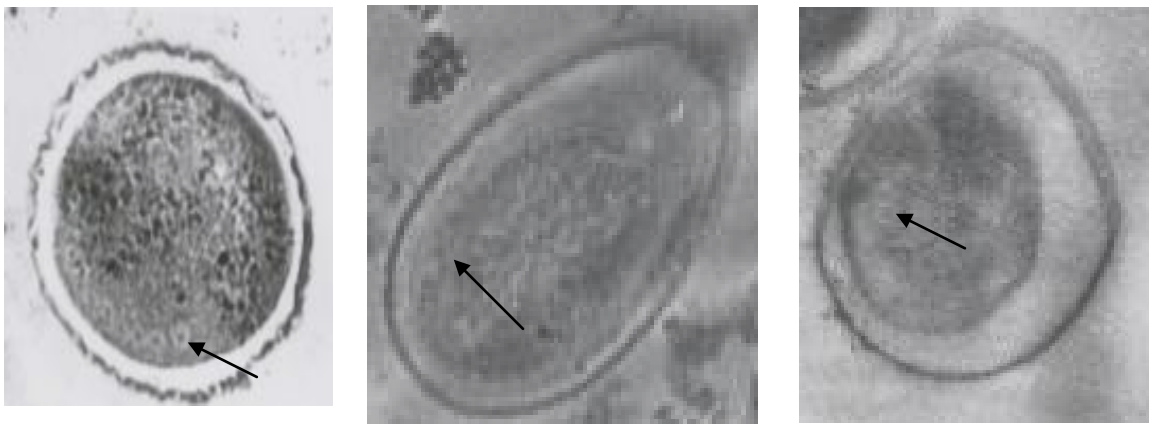

Figure 2 *Nosema bombycis* TEM photograph with short polar tube (ST) indicated by arrow.

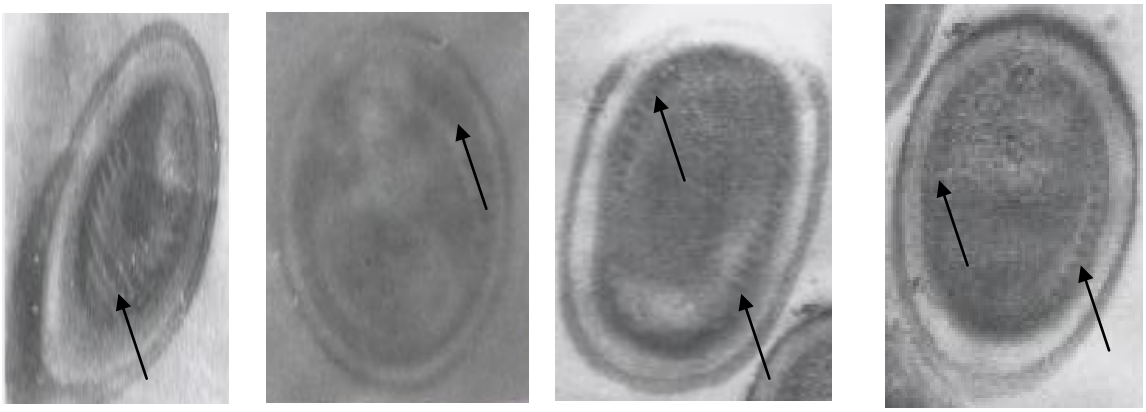

Figure 3 *Nosema bombycis* TEM photograph with Long Polar Tube (LT) indicated by arrow.

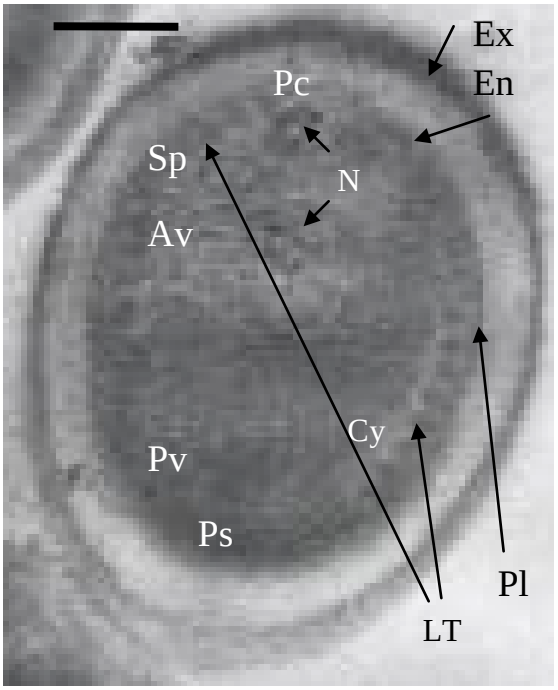

Figure 4 *Nosema bombycis* Enlarged view of TEM photograph with Long Polar Tube (LT) showing Nucleus (N), Exospore wall (Ex), Endospore wall (En), Anterior vacuole (Av), Posterior vacuole (Pv), Polar cap (Pc), Sporoplasm (Sp), Cytoplasm (Cy), Polar sac (Ps) and Plasmalemma (Pl). Bar represents 1 µm.

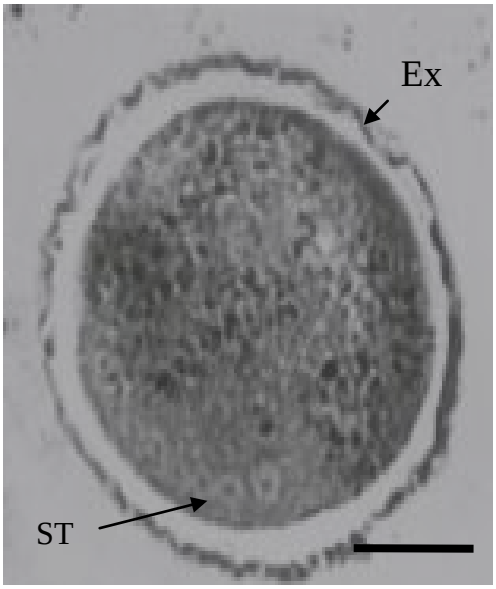

Figure 5 *Nosema bombycis* TEM photograph with Short Polar Tube (ST). (Enlarge view) Bar represents 1 µm.

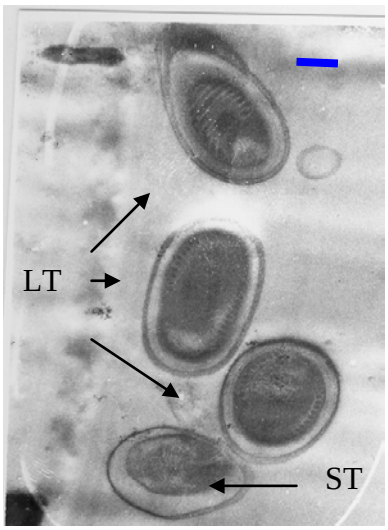

Figure 6 *Nosema bombycis* TEM photograph Spores with 3 Long Polar Tube (LT) and 1 Short Polar Tube (ST), harvested from female moths in 200 µm^2 EM field. Bar represents 1 µm.

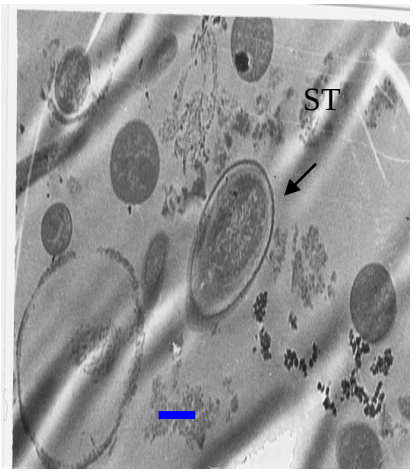

Figure 7 *Nosema bombycis* TEM photograph Spores with only 8 Short Polar Tube (ST), harvested from male moth (Inoculum concentration is 1.5×10^8 spores/ml) in 200 µm^2 EM field. Bar represents 1 µm.

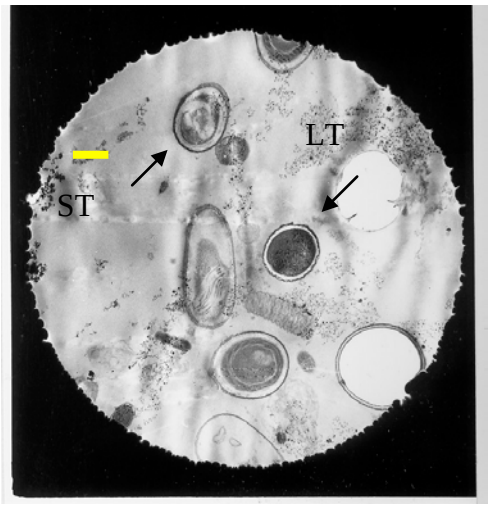

Figure 8 *Nosema bombycis* TEM photograph Spores with one Long Polar Tube (LT) and 05 Short Polar Tube (ST) from male moth (Inoculum concentration is 1.5×10^7 spores/ml) in 200 µm^2 EM field. Bar represents 1 µm.

Similar findings are also available on spores of *N. bombycis* from moths of the lawn grass cutworm, *Spodoptera depravata* Butler and from cell culture of *Bombyx mori* [6]. In one type of spore, the coil number was characterized by 3.8 turns (ST = short tube or FC = Few coils), while in the other type it was 11.8 turns (LT = long tube or MC = Multiple coils). *Nosema* could produce two spores from sporont [7]. Such a type of spore dimorphism in Microsporidia including *Nosema bombycis* and *Nosema* sp. is well established [8]. Sporont of *N. bombycis* usually produces two sporoblasts [9]. The appearance of two distinct spore forms within the same species has also been documented [10]. Spore dimorphism can be considered in *N. apis* as the adaptation of the

parasite to different needs during their life cycle. Spores, which appear during the early development of the parasite, apparently have the ability to germinate intracellularly. Intracellular extrusion of the relatively short polar tube can serve as a means to deliver sporoplasm in neighboring cells [6]. Spore dimorphism and developmental cycle was also studied in *N. apis* and the first population of spores mainly cause the spread of the parasite in the epithelium. The second population of spores is formed later on and are adapted to survive outside the host [11]. Early primary and environmental spores are found immature but variants of the early and environmental spores normally occur in different tissues of the host. The number of coils of polar filament in the spores of 15 microsporidian species ranges from 3 - 4 coils in *N. cuniculi* to 44 in *N. apis* with the majority of species having 11 coils [13]. In the present study, the dimorphic spores developed in separate patterns in different sexes in the later metamorphic stages of the silkworm i.e., from pupal stage for adaptation of the parasite to different needs during its life cycle. There is a huge scope for investigations on the physiology of both the host and pathogen with special emphasis on the reproductive physiology & energy budgeting and its influence on spore development and spore dimorphism.

It is observed that the spore harvest from pupa of both sexes are almost same (7.53×10^7 - 9.17×10^7 spore ml^{-1}) though the inoculums concentration varied (**Table 1**). The time taken for establishment of the parasite for completion of its life cycle and production of spores depends on inoculum load and other environmental factors [14]. However, in the present study, a significantly higher amount of spore production was recorded in the male moth over the female moth and is a new finding. The spore harvest from the moths of both sexes was higher (8.83×10^6 - 8.87×10^8 spore ml^{-1}) and increased with the increase in inoculum concentration (**Table 1**). The difference in the spore yields from male and female moths varied significantly at both lower and higher inoculum loads but, not at the moderate dose.

Table 1 Gender specificity study of pebrine spore in pupa and moth of silkworm.

Gender	Inoculum dose (Spores/ml)	Larval stage of inoculums	Spore harvest (Spores/ml)		Spore harvest (Spores/ml)		Spore harvested from moth/200 μm² TEM field	
			Stage	Spore/ml	Stage	Spore/ml	ST	LT
♂	1.5×10^6	3rd	Pupa	7.53×10^7 (7.8×10^6)	Moth	3.70×10^7 (6.6×10^6)	6 - 7	1 - 2
♀	1.5×10^6	3rd	Pupa	7.73×10^7 (1.5×10^6)	Moth	8.83×10^6 (3.9×10^5)	2 - 3	10 - 12
♂	1.5×10^7	3rd	Pupa	7.77×10^7 (3.7×10^6)	Moth	1.50×10^8 (1.7×10^7)	10 - 12	3 - 4
♀	1.5×10^7	3rd	Pupa	7.63×10^7 (2.9×10^6)	Moth	1.37×10^8 (3.3×10^7)	8 - 10	15 - 20
♂	1.5×10^8	3rd	Pupa	8.3×10^7 (2.4×10^6)	Moth	8.87×10^8 (3.9×10^7)	17 - 20	10 - 12
♀	1.5×10^8	3rd	Pupa	9.17×10^7 (6.6×10^6)	Moth	1.67×10^8 (2.0×10^7)	10 - 12	20 - 22
ANOVA				NS		1.14×10^8		
CD @ 1%								
CV				30.206		19.617		

(Data in parenthesis indicates the Standard Error of mean)

The data reveals (**Table 1**) that spore harvest was more in male moths compared to that in female moths though the inoculum concentration, source of pathogen and the rearing environments were the same. 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 spores of *N. acridiophagus* and *N. cuneatum* in larvae of *Melanoplus asnguinipes* after 20 days of inoculation with 10^4 and 10^6 spores compared to lower spore yields which continued in the spore multiplication stage when inoculated with 10^2 spores [15]. If development is allowed to proceed to the stationary phase, multiplication of the parasite is greater with lower concentrations than with higher concentrations [16]. 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 factors [17]. A particular concentration is effective for multiplication of spore and mortality [18] and below that threshold level the concentrations do not cause any larval mortality [19]. 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 [18]. In the present findings, spore production reached the stationary phase in female during pupal stage to moth stage which continued to progress in male pupal stage to moth stage till the time of harvest. It is an interesting phenomenon of *N. bombycis* which needs to be investigated further for detailed understanding and for the findings to assume importance for taxonomic classification.

It is also observed in the present study that, multiplication of pebrine spores continued with the progress of metamorphosis from larva to pupa and moth in males, while it remained almost constant in female pupa and moths without further multiplication. Spores containing ST are virulent and found mainly in male therefore, male moths are short lived. However, spores containing LT are principally found in female moths and are responsible for the long lived and transovarian transmission of the disease. Higher and lower inoculum doses produced significant variations in spore harvest among the sexes which is not evident at the moderate inoculum dose. The pathogen multiplies maximally to ensure certain percentage of pathogens to get entry into the next generation through transovarial transmission. It is assumed that the higher rate of infection of the adult would result in an increased rate of transmission to the next generation [15].

Parasitic development occurs in male larva, producing massive infections, which usually prove fatal to the host during the 4^{th} larval stage. In females, parasitic development is suppressed or delayed and restricted to the oenocytes. Female larvae pupate normally and emerge as apparently healthy adults, which transmit the parasite transovarially to the progeny, when mated with healthy mates [3]. Females have higher enzyme activities than males which might also be a cause for inducing difference in the spore yields as well as spore dimorphism although this needs to be ascertained. Similarly, the impact of higher nutritional requirements of female larvae over males towards the parasites reproductive biology needs attention. Hence the higher amino transferase activity in female is indicative of higher conversion of amino acid pool and subsequently more protein synthesis.

Conclusions

From the above study, it may be concluded that the pathogenicity of, Nosema bombycis is gender specific and the pathogen multiplies according to their need. Virulent spores containing ST in males multiplied for the secondary contamination and spread the infection horizontally through the food, rearing appliances, room, and air; while the spores with LT in females spread the infection through the eggs using transovarian mode of vertical transmission. Detailed studies are required about the mechanisms and factors responsible for triggering the spore to identify the sex of the host to pave the way towards completely eradicating the pebrine menace in silkworms from the host.

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