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Mode of Transmission of a Newly Discovered Microsporidian and Its Effect on Fecundity and Hatching in Silkworm, *Bombyx mori* L.

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The mode of transmission, effect on fecundity, hatching and tissues specificity of a microsporidian (Lb_{ms}) recovered from Lamerin breed of the silkworm *Bombyx mori* L. was studied and compared with standard strain *Nosema bombycis*. Peroral inoculation of Lb_{ms} or *N. bombycis* to zeroday of 4th instar larvae of silkworm was the most suitable method for producing information on development of stage specific mortality, pupation and obtaining infected adults for transovarial transmission studies. It was observed that pupal mortality, the percentage of moths emerged and the percentage of moths infected were significantly high in *N. bombycis* infected batches as compared Lb_{ms} in all the three tested breeds of the silkworm. However no significant difference was observed in larval mortality. The fecundity and hatchability was not affected significantly in Lb_{ms} infected adults, however significant reduction in egg production, fecundity, hatchability and increased egg retention was observed in mother moths infected with *N. bombycis*. The Lb_{ms} is transmitted both horizontally and vertically at lower rate due to its low rate of proliferation. The transovarial transmission of Lb_{ms} to the F_1 progeny generation through eggs averaged only 61.33 ± 5.10% whereas *N. bombycis* was transmitted at 100%. The Lb_{ms} had low oral infectivity and low transovarial transmission in silkworm *B. mori*.

Key words: Generation, Lb_{ms}, Microsporidian, *N. bombycis*, Progeny, Transmission

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Introduction

The microsporidians has been known for many years as pathogen causing a deadliest disease of most animal phyla, including Arthopoda (Wittner and Weiss, 1999; Wasson and Peper, 2000). As the pathogen transmits disease both vertically and per orally, takes a heavy toll of silkworm and sometime even results in total crop loss when the infection is severe. The vertical transmission of pathogen to the silkworm progeny and the destructive effect was clarified by Pasteur (1870). A burning problem in the field of the microsporodiosis is the increasing number of different microsporidians that are being encountered in silkworm crops (Tanaka *et al.*, 1972; Fujiwara 1980; Lim *et al.*, 1982; Ananthalakashmi *et al.*, 1994; Kishore *et al.*, 1994; Samson *et al.*, 1999a, b; Sharma *et al.*, 2003; Nageshwara Rao *et al.*, 2004). These microsporidians have shown to exhibit varying degree of virulence and many of them, though infective and pathogenic have demonstrated low multiplication rate in the silkworm. Most of them have not shown vertical transmission in the host. Only Nosema bombycis and Nosema species M_{11} were reported to be transovarially transmitted (Pasteur, 1870; Han and Watanabe 1988). Microsporidian spore (Lb_{ms}) was isolated from Lamerin breed of the silkworm *B. mori*, its spore shape, size and serological characteristics has been studied, found different from *N. bombycis* (Shabir Ahmad Bhat and Nataraju, 2004). However mode of transmission, its effect on fecundity, hatching and site of infection has not been studied. The present study was conducted to determine the mode of transmission, its effect on fecundity, hatching and site of infection and compared with that of standard strain (*N. bombycis*).

Materials and Methods

Production and purification of microsporidian spores

Microsporidian spore (Lb_{ms}) were isolated from infected larvae / pupae of Lamerin breed of the silkworm *B. mori* and standard strain, *N. bombycis* were cultured in Laboratory reared silkworm larvae at room temperature. Soon after 2nd instar batch of 100 healthy larvae were per orally administered per os with a spore load of 1×10^7. Moths derived from the infected larvae were homogenized and homogenate was purified followed method of Sato and Watanabe (1980) using a gradient of neutralized percoll (Pharmacia) to separate spores from host tissues and immature spores and concentrate spores to make them easier to find.

Larval inoculation and infectivity

In first set of experiment to determine the latency period, the time period between the inoculation of the silkworm and beginning of its infection period (Anderson and May, 1992) and the time period at which horizontal transmission can occur, the following experimental design was chosen. Zero day of 4th instar larvae were allowed to feed on mulberry leaf disc containing 1×10^5 spores/ml of Lb_{ms} or *N. bombycis*. The second normal feeding was given after 18 hrs of post inoculation. Larval and pupal mortality if any due to the infection was recorded during the course of experiment. The number of moths emerged were recorded. Moths obtained from the above exposed larvae were provisionally regarded as infected. After mating and oviposition all moths (male and female) were individually macerated in mortar and pestle and the wet mount were examined for microsporidian infection under phase contrast microscope Nikon (Type - 104). Egg batches from uninfected pairs were discarded and from infected pairs were maintained for next generation. The percentages of infection at moth stage were recorded and the data derived from the above experiment were statistically analyzed by t-test.

Microscopic examination of infected tissues

In second set of experiment assessment of the host tissues that were infected was done. As the microsporidians are obligate intracellular parasites have been observed in different host-cell types (Canning, 1990). Different tissues *viz.*, (gut, malpighian tubule, trachea, silk gland, fat bodies, and gonads) were dissected out individually after 12th day of inoculation and examined for the presence of micosporidian spores using phase contrast microscope and compared with *N. bombycis*. A small sample of tissues was crushed in a drop of water between a slide and a cover slip. Mechanical and osmotic pressure caused the cells to lyse released the spores from the host cells.

Effect of infection on fecundity and hatching

The female moths after mating for 4 hrs were allowed to oviposit for 24 hrs and the eggs laid by infected moths were surface sterilized by immersing in 2% formaldehyde for 5 min at room temperature. The egg laying of Lamerin and CSR2 were acid treated (HCl of Specific gravity 1.075 measured by "CIMCO" hydrometer; at 46.5°C for 5 min in water bath, the temperature of water bath was measured by "ZEAL" thermometer) to terminate the egg diapause and washed in running water to remove the traces of HCl. The treated layings were incubated (25 ± 1°C temperature and 80 ± 1% RH) for normal embryonic development. After head pigmentation stage, layings were covered with black paper till blue egg stage and were exposed to the indirect light for hatching. Fecundity and hatching for every breed under each treatment was assessed.

Mode of transmission

The newly hatched larvae from infected layings were reared as per standard method (Datta *et al.*, 1994) upto 1st instar. A 50-larvae / batch / treatment were subsequently examined under Nikon (Type - 104) phase contrast microscope for the presence of microsporidian spores in their macerated body and the rate of transovarial transmission were calculated.

Results

Larval inoculation and Infectivity

Peroral inoculation of Lb_{ms} to zero day of 4th instar larvae at dosage of 1 ± 10^5 spores / ml resulted in no larval and pupal mortality prior to the adult eclosion (Table 1). Hundred percent of adults were obtained in all the breeds and the percentage of infection was low at moth stage which was 41.2%, 41.4% and 48% in Lamerin, Pure Mysore and CSR2 respectively. However the larval mortality was nil by *N. bombycis* but pupal mortality was 4%, 4% and 6% in Lamerin Pure Mysore and CSR2 respectively. 87.55%, 88.58% and 91.69% of the larvae survived to adulthood were found infected in Lamerin, Pure Mysore and CSR2. In larvae fed with *N. bombycis* 96%, 96% and 94% of emergence was recorded in Lamerin, Pure Mysore and CSR2 breed respectively (Table 1) against 100% in Lb_{ms} infected batches. The result indicated that the Lb_{ms} was less infective to the entire three silkworm breeds tested compared to *N. bombycis*.

Table 1. Effect of microsporidian infection on larval, pupal mortality, moth emergence and moth infectivity following per oral inoculation

Tested silk worm breeds	Microsporidian spores	Mortality due to infection		% moths emerged	% moth infected
		Larva	Pupa		
Lamerin	Lb_{ms}	0 ± 0.00	0 ± 00	100 ± 0.00	41.20 ± 3.49
	N. bombycis	0 ± 0.00	4 ± 0.89	96 ± 0.89	87.55 ± 4.19
	t-test	0	-9.00**	9.00*	-19.01**
Pure mysore	Lb_{ms}	0 ± 0.00	0 ± 00	100 ± 0.00	41.4 ± 5.59
	N. bombycis	0 ± 0.00	4 ± 1.10	96 ± 1.10	88.58 ± 3.84
	t-test	0	-7.76**	7.76**	-15.54**
CSR2	Lb_{ms}	0 ± 0.00	0 ± 00	100 ± 0.00	48 ± 1.58
	N. bombycis	0 ± 0.00	6 ± 2.17	94 ± 2.17	91.69 ± 3.33
	t-test	0	-6.39**	6.39**	-26.52**

Value are mean ± S.D.; **significant at 1% level; *significant at 5% level.

Table 2. Site of infection of host tissues by Lb_{ms} microsporidian isolate and compared with that of standard strain *N. bombycis*

Host tissues	Microsporidian spores	
	Lb_{ms}	*N. bombycis*
Gut	+	++
Malpighian tubule	+	++
Trachea	+	++
Silk gland	+	++
Fat bodies	+	++
Gonads	+	++

+ : Low infection; ++ : High infection.

Tissues infected

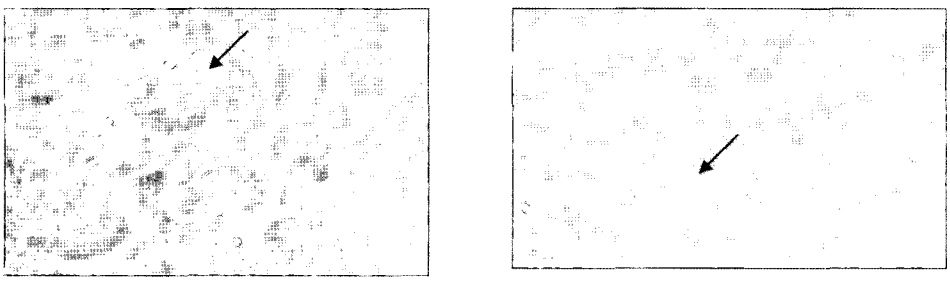
Result indicated that the Lb_{ms} found infective to all susceptible tissues of silkworm larvae as in case of *N. bombycis* (Table 2 and Fig. 1). Infection level in all the tissues examined was very low as compared to *N. bombycis*, where it was high in all tissues. The term "low" and "high" for spore number is qualitative. Low (+) infection which contained only 1 - 5 spores / microscopic field of the smear and the high infection (++) infection contained 10 or more spores / microscopic field of the smear. Lb_{ms} are benign and produce a few spores that only thorough microscopic examination revealed the infection. Only 1 - 5 mature spores could be observed per microscopic field appeared singly or in bundles in different host tissues (Fig. 1). These observations showed that the Lb_{ms} could invade all the tissues but failed to cause any larval or pupal mortality due to low rate of proliferation, and low infectivity.

Effect of infection on fecundity and hatching

The effect of two microsporidians (Lb_{ms} or *N. bombycis*) on the fecundity and hatching differs. The Lb_{ms} could not affect the fecundity and hatching significantly however the infection with *N. bombycis* resulted in the decline of fecundity and hatching in all the three breeds (Table 3).

Mode of transmission

The examination of 2nd instar larvae hatched from eggs



(a) Gut infected with Lb_{ms} (b) Gut infected with *N. bombycis*

Fig. 1. Microphotographs of host gut (at 600 × magnification under phase contrast microscopic camera) infected by Lb_{ms} microsporidian isolate and compared with that of *N. bombycis* (◄—) shows the presence of spores.

Table 3. Effect of infection on fecundity, hatching and assay on transovarial transmission of Lb_{ms} and compared with standard strain (*N. bombycis*) in silkworm

Microsporidian spores	Progeny assayed	Paired moths of different breeds of silkworm infected male × infected female		
		Lamerin	Pure mysore	CSR2
Lb_{ms}	Total eggs / laying	316 ± 12.04	403 ± 6.95	557 ± 21.67
	No. of hatched larvae / laying	238 ± 15.68	386 ± 5.31	528 ± 11.38
	% of hatching	75.38 ± 2.32	95.84 ± 1.64	94.84 ± 1.78
	No. of larvae examined (after 1st molt)	50 ± 0.00	50 ± 0.00	50 ± 0.00
	% of infection in examined larvae	31 ± 2.07	32 ± 3.39	28 ± 29.19
	Transovarial transmission rate	63 ± 4.15	64 ± 6.78	57 ± 4.38
N. bombycis	Total eggs / laying	265 ± 17.37	291 ± 4.93	289 ± 6.02
	No. of hatched larvae / laying	171 ± 10.44	187 ± 17.80	170 ± 12.79
	% of hatching	64.56 ± 1.75	64.91 ± 3.63	58.73 ± 3.34
	No. of larvae examined (after 1st molt)	50 ± 0.00	50 ± 0.00	50 ± 0.00
	% of infection in examined larvae	50 ± 0.00	50 ± 0.00	50 ± 0.00
	Transovarial transmission rate	100 ± 0.00	100 ± 0.00	100 ± 0.00

Note: Each value is mean ± S.D. from 5 pairs of infected moths.

laid by infected females showed that Lb_{ms} could transmit infection transovarially to the F_1 progeny through eggs (61.33 ± 5.10%) whereas *N. bombycis* was transmitted at 100% level in all the three breeds (Table 3). The Lb_{ms} had low oral infectivity and low transovarial transmissiom to the silkworm *B. mori*.

Discussion

In silkworm *B. mori*, the most common mode of entry of microsporidian infection is per os as in case of other Lepidopteran insects. Different microsporidian strains infecting silkworm differed greatly in their virulence and many of them, though infective and pathogenic have demonstrated low multiplication rate in silkworm. The newly discovered microsporidian spore (Lb_{ms}) recovered from Lamerin breed of the silkworm, native of North eastern part of India, was comparatively less virulent could not cause any larval and pupal mortality but produced infection at moth stage, where as *N. bombycis* was virulent and caused pupal mortality in all the three tested breeds and produced chronic infection at moth stage. The Lb_{ms} was less infective to the entire three silkworm breeds tested compared to *N. bombycis* and the percentage of infected moths was less compared to *N. bombycis*. The Lb_{ms} was low in virulence and also low in multiplication in all the silkworm breeds including its host. Reports are available on wide spectrum effects of microsporidians on insect tissues, reproductive potential and fertility (Bansal *et al.*, 1997). The spore concentration was found to be much higher in gonads followed by fat body, gut and malpighian tubules in *B. mori* (Bansal *et al.*, 1997). Heavy concentrations of spores can be seen through the cuticle of some lightly pigmented hosts as white or white yellow cysts when viewed against a black background. These visible sign might also be accompanied by swelling caused by hypertrophy of infected cells at the site of infection. However, sometimes spores can occur in numbers that appear to entirely fill the host without signs of infection.

Dissection of these hosts will often reveal site of infection visible to naked eye. Light infections frequently produce no visible signs, requiring a microscope for diagnosis. Gonadal infection has an important bearing on the vertical transmission of the parasite through transovarial transmission. This aspect is important in sericulture industry since silkworm eggs distributed to farmers are required to be certified "microsporidian free" as microsporidians are obligate intracellular parasites infecting a wide range of insect hosts and transmit infection both vertically and horizontally and have been observed in different host-cell types. The examination of microsporidian infection is usually conducted by examining living organisms for visible signs of infection or by microscopically screening samples of mercerated tissues for spores (Weiser, 1991). Sometimes the prevalence of microsporidia can be quite high, exerting a strong suppressive effect on the host population. Most often however, infection rates are low in natural populations

(< 1.0%) necessitating the examination of a large number of individuals to detect it and even large number to estimate the incidence of infection. The difficulty of this task ranges from easy where there are clearly visible signs of infection to very difficult when infections are extremely light show no outward sign. In the present study the result indicated that Lb_{ms} was observed in all the susceptible tissues (gut, malpighian tubule, trachea, silk gland, fat bodies, and gonads) as in case of *N. bombycis*, but the level of infection was very low in all susceptible tissues as compared to *N. bombycis*, only 1 - 5 mature spores could be observed / microscopic field (Fig. 1). The Lb_{ms} is low in rate of proli- feration, lower oral infectivity and low transoverial transmission as compared to *N. bombycis*. The microsporidians first inhibiting the gut epithelium of the silkworm larvae by sending polar filament into the hosts midgut epithelial cells, injects an infectious sporoplasm and infection is started and latter passes through the gut wall and invades various susceptible tissues of the host (Brook, 1988). Many microsporidians live in the -gut as harmless commensals. Lb_{ms}, which show inapparent infection of all the tissues, was least pathogenic as compared to *N. bombycis*. Both the microsporians (Lb_{ms} and *N. bombycis*) were quiet different in their mode of transmission. Lb_m had shown $61.33 \pm 5.10\%$ transovarial transmission compared to 100% by *N. bombycis*. The mating of Lb_{ms} infected adults showed no significant effect on fecundity and hatchability. However mating of *N. bombycis* infected adults resulted in significantly low fecundity and low hatchability. Therefore it is concluded that Lb_{ms} was a distinctly different microsporidian strain than *N. bombycis* based on the following parameters: (i) low oral infectivity (ii) low transovarial transmission as compared to *N. bombycis* (iii) low virulence and also low level of multiplication in different host tissues and (iv) no significant effect on fecundity and hatching.

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