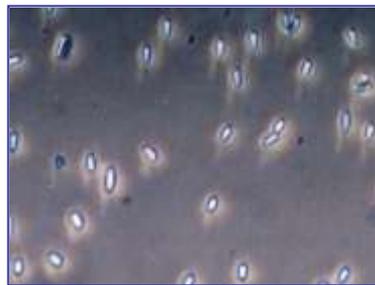


Chapter 2



PATHOGENICITY OF LAMERIN MICROSPORIDIAN TO LAMERIN AND POPULAR MULTIVOLTINE AND BIVOLTINE SILKWORM BREEDS

Pasteur (1870) established that certain corpuscles are the causative organisms of pebrine disease in silkworm. Those corpuscles were the spores of a microsporidia, *Nosema bombycis*, the cause of pebrine disease in silkworm. By the end of 19th century, *N. bombycis* was known as the only pathogen causing pebrine disease in silkworm. At present, several microsporidia are known to cause the disease and among them the most common are *Nosema* sp. (NIS-001, NIS-M11, NIS-M14, and NIK-2r, and NIK-3h.), *Vairimorpha* sp. (NIS-M12 and NIK-4m), *Microsporidium* sp. (NIS-M25), *Pleistophora* sp. (NIS-M27), *Thelohania* sp. (NIS-M32) and *Leptomonas* sp. (Kawarabata, 2003; Singh and Saratchandra, 2003). The microsporidia isolated from the Lamerin silkworm breed may be yet another strain causing the disease in silkworm.

The microsporidians infect silkworm by *per os* means and the process of infection initiates with the germination of environmental spores in the larval midgut. The infection by *N. bombycis*, NIS-001, *Nosema* sp. NIS-M11, *Vairimorpha* sp. NIS-M12, *Nosema* sp. NIS-M14 and *Microsporidium* sp. NIS-M25 in silkworm larvae are systemic and severe (Fujiwara, 1980; 1984a; 1985). These microsporidia form the environmental spores in all the tissues and organs such as muscles, fat body, silk gland, malpighian tubules *etc.* There is also initial development of environmental spores in midgut epithelium by all these microsporidians but it is *N. bombycis* alone form environmental spores in the midgut epithelium. Other microsporidians show initial development of environmental spores in the midgut epithelium (Kawarabata, 2003). *N. bombycis* NIS-001 is also highly gonadotropic while *Nosema* sp. NIS-M11 is moderately gonadotropic (Han and Watanabe, 1988). *Pleistophora* sp. do not form primary spores. They infect only the midgut epithelium of silkworm and form uninucleate environmental spores. (Tanaka *et al.*, 1972; Becnel and Andreadis, 1999). *Thelohania* sp. form uninucleate octospore only in the larval muscles (Fujiwara, 1984).

N. bombycis, NIS-001, *Vairimorpha* sp. NIS M-12 and *Pleistophora* sp. NIS-M27 are most infectious having high infection rate. *Nosema* sp. NIS-M14 and *Microsporidium* sp. NIS-M25 are moderately infectious while *Thelohania* sp. NIS-M32 is low in its infectivity. The pathogenicity of *N. bombycis*, NIS-001 and *Vairimorpha* sp. NIS M-12 are high and usually the larvae die before pupation if the infection is prior to the 4th larval instar. The other microsporidians are moderate or low infectivity viz., *Nosema* sp. NIS-M11, *Nosema* sp. NIS-M14, *Microsporidium* sp. NIS-M25 and *Thelohania* sp. NIS-M32 cause low level of mortality in the larval stages (Fujiwara, 1980, 1984a, b).

Pasteur (1870) reported transmission of the pebrine disease from infected parents to offspring's. Later studies have confirmed the observation and now it is an accepted fact that the *N. bombycis* transmits infection both vertically and horizontally in the silkworm. The primary infection is by vertical transmission with the pathogen transferring the infection directly from the parent to offspring. Horizontal transmission is the transmission of infection from individual to individual, which is accomplished by the ingestion of spores. The percent transovarial transmission differs with different microsporidians infecting silkworm. It is highest with *N. bombycis*. The transovarial transmission of *N. bombycis* and *Nosema* sp. NIS-M11 has been demonstrated in silkworm by Han and Watanabe (1988). The transovarial transmission of *N. bombycis* is also demonstrated in *Diaphania pulverulentalis*, a pest of mulberry by Ramagowda and Geethabai (2005). The rate of transmission of *Nosema* sp. NIS-M11 is lower than that of *N. bombycis* (Fujiwara, 1980; Iwashita *et al.*, 1990). The rate of transmission of *Nosema* sp. NIK-3h is also reported to be low. It transmits the infection to the progeny by only 1.80 ± 0.4 %, while the standard strains *N. bombycis* and *Nosema* sp. NIK- 2r transmit the infection to an extent of 100 % (Ananthalakshmi *et al.*, 1994; Nageswara Rao *et al.*, 2004). There are reports of absence of transovarial transmission in *Vairimorpha* sp. NIS-M12, NIK-4m, *Microsporidium* sp. NIS-M25, *Pleistophora* sp. NIS-N27 and *Thelohania* sp.

NIS-M32 (Tanaka *et al.*, 1972; Fujiwara, 1980, 1984a, b; Ananthalakshmi *et al.*, 1994).

Different silkworm breeds differ in their susceptibility to the microbial infection. Such differences are genetically determined and have been studied extensively involving silkworm viruses (Tanada and Kaya, 1993). In silkworm, large difference exists among various breeds in their susceptibility to infections by *N. bombycis*. (Chinnaswamy and Devaiah, 1984). Resistance to pebrine disease is greater in Chinese breeds, less in Japanese and least in European breeds (Govindian *et al.*, 1998; Singh and Saratchandra, 2003; Nataraju *et al.*, 2005) and multivoltine breeds are relatively more resistant than bivoltine (Patil and Geethabai, 1989). Among bivoltines NB7 was most susceptible followed by NB4D2, KA, (Patil and Geethabai, 1989). Silkworm races such as Pure Mysore (PM), Nistari and C. Nichi have high survival ability than other silkworm races (Devaiah and Krishnaswami, 1975; Devaiah, 1975; Patil and Geetabai, 1989) and the high survival of PM breed has been investigated and it is attributed to the regenerative capacity of their midgut to recover from the infection (Fujiwara, 1993). However, Liu (1984) reports that a silkworm race Baipidan as resistant to *N. bombycis*. Wild silkworm *Antheraea pernyi* and *Platysamia cecropia* are comparatively more resistant to microsporidia than others (Weiser, 1969). In honey bee also resistance to infection by *Nosema apis* is attributed to heterosis and to a polygenic system (Sidorov *et al.* 1975).

It is observed from the results of the chapter 1 that a microsporidia isolated from the Lamerin breed was different from several other microsporidians infecting silkworms. There is an unpublished report that the microsporidia from the Lamerin silkworm breed transmit infection by vertical means without causing significant level of host mortality. There is no information on the mode and site of infection, transmission, virulence etc. In this chapter, the results of investigations on the mode and site of infection, transmission, site of infection, spread of infection in healthy silkworm colony and virulence of the Lamerin microsporidian

isolated from Lamerin breed of the silkworm *B. mori* is presented. Studies were also conducted on the susceptibility of different breeds to Lamerin microsporidia and the results are presented and discussed.

MATERIALS AND METHODS

Mode of infection: Eggs from healthy Lamerin silkworm breed were surface disinfected and incubated following the standard procedure at $25\pm 1^{\circ}\text{C}$ and $80\pm 5\%$ relative humidity. The hatched larvae were reared under hygienic condition and the 2nd instar silkworms were fed with purified spores of the microsporidia isolated from Lamerin silkworm breed. The inoculum was prepared from purified spores of the microsporidia and quantified by standard method using hemocytometer (Cantwell, 1974). One ml of the inoculum containing 1×10^7 spores / ml was smeared on the surface of mulberry (50 sq. cms surface area) and fed to 100 Lamerin breed silkworm larvae immediately after 1st moult (T1) (Table 1). Another set of healthy larvae, immediately after 1st moult were topically smeared with inoculum containing 1×10^7 spores/ml (T2) and kept on sterile surface for 24 h. After 24 h the larvae were transferred to a rearing tray and fed with mulberry leaf. In yet another treatment (T3) the surface of eggs in blue egg stage of Lamerin breed were smeared with 1×10^7 spores/ml. The eggs were incubated till hatching following the standard procedure and larvae hatched from the contaminated layings were reared. Yet another set of larvae (T4) were reared without any inoculation and it formed the control for comparative purpose. There were three replications of 100 larvae each with respect to each treatment. The treatment and control larvae were reared till cocooning. The dead larvae, pupae and moths were homogenized and examined for the spores of the Lamerin microsporidia. The survived moths were allowed to lay eggs and examined for microsporidian infection. Hibernating eggs were treated with hydrochloric acid (HCl) of specific gravity 1.075 at 46.1°C for 5 min to break the diapause. The layings were incubated at $25\pm 1^{\circ}\text{C}$ and $80\pm 5\%$ relative humidity (RH) for 10 days in an incubator. The larvae hatched from the

layings were homogenized and examined for the microsporidian spores. The observations were recorded, tabulated and analyzed.

Infectivity: To determine the infectivity and mode of transmission of the Lamerin microsporidian a popular multivoltine breed- Pure Mysore (PM), a bivoltine breed - CSR2 and the Lamerin breed were perorally inoculated. To compare the results, different sets of larvae from these breeds were inoculated with spores of *N. bombycis*. The inoculum was prepared from purified spores of the respective microsporidia (microsporidia from Lamerin breed and *N. bombycis*) and quantified by standard method using haemocytometer (Cantwell, 1974). The healthy silkworms of the three breeds on the Zero day of 4th instar (immediately after 3rd moult) were allowed to feed on mulberry leaf disc contaminated with one ml of inoculum containing 1×10^5 spores / ml isolated from Lamerin breed of silkworm (T1: Lamerin larvae inoculated with Lamerin microsporidian; T2: PM larvae inoculated with Lamerin microsporidian and T3: CSR2 larvae inoculated with Lamerin microsporidian). Different sets of larvae of these breeds were inoculated with the 1×10^5 spores/ml of *N. bombycis* (T1a: Lamerin larvae inoculated with *N. bombycis*; T2a: PM larvae inoculated with *N. bombycis*, and T3a: CSR2 larvae inoculated with *N. bombycis*). Lower dosage was preferred to ensure survival of larvae to moth stage and egg production. There were also controls with respect to each breed without any inoculation (T1b Control Lamerin larvae without inoculation, T2b: Control PM larvae without inoculation and T3b: Control CSR2 larvae without inoculation). There were three replications of hundred larvae each with respect to control and different treatments.

The silkworms were reared on mulberry leaf after treatment till spinning and allowed to pupate and emerge as moths. The homogenate of faeces, dead larvae, pupa, moth and hatched larva from eggs were examined under phase contrast microscope for the presence of spore of respective inoculated microsporidia and recorded the observation. Larval and pupal mortality due to the infection was also

recorded during the course of experiment and the percent of moths emerged was recorded.

Mode of transmission: Third instar silkworm larvae of Lamerin, Pure Mysore and CRS2 breeds from healthy stock were inoculated with Lamerin microsporidia or *N. bombycis* spores by *per os* means. The inoculum containing 1×10^5 spores / ml of Lamerin microsporidian or *N. bombycis* were prepared from stock inoculums of Lamerin microsporidian or *N. bombycis* spores. One ml of inoculum (1×10^5 spores / ml) were smeared on (50 sq. cms surface area) of mulberry leaf disc and fed 100 larvae immediately after 3rd moult and reared to obtain cocoons and moths. Another set were reared without inoculation till spinning and moth emergence. Moths obtained from the inoculated larvae of different breeds were provisionally regarded as infected and were allowed to pair and lay eggs. Moths obtained from batches without inoculation were provisionally regarded as healthy and were allowed to pair and lay eggs. The pairing of the moths was as follows. These formed the treatments. One set of healthy male moths (HM) was allowed to pair with infected female moths (IF). In another set, infected male moths (IM) were allowed to pair with healthy female moths (HF). Yet another set of infected male moths (IM) were paired with infected female moth (IF). Yet in another set of experiment healthy male moths (HM) were allowed to pair paired with healthy females moths (HF). The breeds and treatments were as follows.

T11: Lamerin healthy male (HM) \times *Lb_{ms}* infected female (IF) moth.

T12: Lamerin *Lb_{ms}* infected male (IM) \times healthy Lamerin female (HF) moth

T13: Lamerin *Lb_{ms}* infected male (IM) \times *Lb_{ms}* infected female (IF) moth.

T14: Lamerin healthy male (HM) \times *N. b* infected Lamerin female (IF) moth.

T15: Lamerin *N. b* infected male (IM) \times healthy Lamerin female (HF) moth

T16: Lamerin *N. b* infected male (IM) \times Lamerin *N. b* infected female (IF) moth.

T17: Lamerin healthy male (HM) \times Lamerin healthy female (Control)

- T21: PM healthy male (HM) × *Lb_{ms}* infected PM female (IF) moth.
- T22: PM *Lb_{ms}* infected male (IM) × healthy PM female (HF) moth.
- T23: PM *Lb_{ms}* infected male (IM) × *Lb_{ms}* infected PM female (IF) moth.
- T24: PM healthy male (HM) × *N. b* infected PM female (IF) moth.
- T25: PM *N. b* infected male (IM) × healthy PM female (HF) moth.
- T26: PM *N. b* infected male (IM) × PM *N. b* infected female (IF) moth.
- T27: PM healthy male (HM) × PM healthy female (HM) (Control).
- T31: CSR2 healthy male (HM) × *Lb_{ms}* infected CSR2 female (IF) moth.
- T32: CSR2 *Lb_{ms}* infected male (IM) × healthy CSR2 female (HF) moth.
- T33: CSR2 *Lb_{ms}* infected male (IM) × *Lb_{ms}* infected CSR2 female (IF) moth.
- T34: CSR2 healthy male (HM) × *N. b* infected CSR2 female (IF) moth.
- T35: CSR2 *N. b* infected male (IM) × healthy CSR2 female (HF) moth.
- T36: CSR2 infected *N. b* male (IM) × infected *N. b* female (IF) moth.
- T37: CSR2 healthy male (HM) × CSR2 healthy female (HM) (Control).

After mating, female moths were allowed to lay eggs on egg sheets and layings were prepared from individual mother moths. The male and female moths were macerated separately after egg laying and the wet mount were examined for the spore of microsporidia and the observation were recorded. The laying laid by moth confirming to the treatment requirements were picked for further study.

The percent transmission of the Lamerin microsporidian was determined based on the number of F1 infected progeny obtained from the secondarily infected parents of Lamerin, PM and CSR2 silkworm breeds. The laying from the different treatments *viz.*, T11-T37 were surface disinfected by soaking them in 2% formalin solution for 5 min at room temperature. The layings of Lamerin and CSR2 were treated with HCl of Specific gravity 1.075 at 46.1°C for 5 min in a hot water bath to terminate the egg diapause and washed in running tap water to remove the traces of

HCl. The layings were incubated for normal embryonic development at $25\pm 1^{\circ}\text{C}$ temperature and $80\pm 5\%$ RH. After head pigmentation stage, layings were covered with black paper till the blue egg stage and were exposed to light to stimulate uniform hatching. The fecundity and percent eggs hatched for each breed was assessed. The dead eggs in each laying were examined for microsporidian spore. The newly hatched larvae from each laying were reared following the standard method and the 1st instar larvae were homogenized individually and examined for microsporidian spores. One hundred larvae / batch / treatment were picked randomly and examined under phase contrast microscope for the presence of microsporidian spores in their macerated body. The percent of transmission with respect to each breed and treatment was calculated following the formula.

$$\text{Transmission (\%)} = \frac{\text{Total number of infected larvae}}{\text{Total number of larvae examined}} \times 100$$

The observations were recorded and analyzed.

Rate of spread infection in silkworm colony: To determine the rate of spread of Lamerin microsporidian infection in healthy silkworm colony of Lamerin, PM and CSR2 silkworm breeds, a specific number of carrier larvae were introduced into a healthy colony of known number of individuals. The carriers were obtained from eggs laid by infected moths and the infection was confirmed during the course of rearing through visual and microscopic examination on death. There were 2 sets of carriers. One set of carriers carried the Lamerin microsporidian infection and another set of carriers carried the *N. bombycis* infection. The larvae of each breed were brushed and reared upto the beginning of 3rd instars following the standard silkworm rearing method and environmental conditions (Datta, 1992). Specific number of Lamerin microsporidian carriers was introduced on the zero day of third instar into the healthy colony of Lamerin, PM and CSR2 breeds. Similarly specific number of *N. bombycis* carriers was introduced on the zero day of third instar into the healthy Lamerin, PM and CSR2 breeds. The numbers of

carriers introduced are 1 in 99, 3 in 97, 6 in 94 and 9 in 91 healthy worms. The different treatments are listed in table 4. Each treatment had three replications of 100 larvae each. Two-control group for each treatment were maintained. Each of them did not carry any carrier. Thus, each colony formed as a group of 100 larvae including the carriers. After introduction of carrier the larvae were reared following the standard procedure till spinning and moth emergence. The dead larvae, pupae and moths were examined for the presence of microsporidian spores and the individual that died due to the infection were included for data analysis. The larvae, pupae and moth died due to other diseases were excluded from the statistical analysis. The live moths were individually examined for infection and recorded. Data in respect of larval and pupal mortality, number of moths infected and total percent infection. The rate of spread of Lamerin microsporidian infection was determined in Lamerin, Pure Mysore and CSR2 silkworm breeds. The result was compared with the rate of spread of *Nosema bombycis* in Lamerin, Pure Mysore and CSR2 breeds.

Site of infection: To determine the site (s) of infection by the Lamerin microsporidian in Lamerin breed of silkworm, the spore of Lamerin microsporidian was inoculated to silkworm and different sites of possible infection were examined for infection. One ml of inoculum containing 1×10^7 spores / ml isolated from Lamerin breed were smeared on to the surface of mulberry (50 sq. cms surface area) and fed to one hundred 4th instar healthy silkworms of Lamerin breed immediately after 3rd moult. The inoculum was prepared from purified spores of the microsporidia and quantified following the standard method using haemocytometer (Cantwell, 1974). The larvae were reared till cocooning.

For comparison of the result of studies on the site of infection in silkworm, different set of Lamerin breed silkworm were inoculated with *N. bombycis*. The Lamerin silkworms were inoculated per orally with inoculum containing 1×10^7

spores of *N. bombycis* / ml. The rearing was continued on uncontaminated mulberry leaf after 24 h of *per os* inoculation till cocooning.

Every day from second day post inoculation, 10- larvae were collected for 15 days, dissected to collect different tissues *viz.*, gut, malphigian tubules, trachea, silk gland, fat bodies and gonads. The tissues were washed in sterilized distilled water and prepared the smear on a clean slide. To prepare the smear, a small sample of tissue was placed with a drop of water between two micro slide, pressed and one of the slide was drawn to form a thin film of smear. Mechanical and osmotic pressure causes the cells to lyses and release the spores from the host cells. The smear was observed under phase contrast microscope with a drop of water and cover slip for the microsporidian infection. At each schedule, 10 - larvae were examined and observations were recorded as positive (+) and negative (-) for gut, malphigian tubules, trachea, silk gland, fat bodies and gonads. The intensity of infection / field (infectivity grade) was recorded as nil: -; low: +; and high: ++.

Ultrastructural observations on infected tissues: To study the ultra structure of infected tissues and developmental stages of the microsporidian spore, the gut and fat bodies collected from final instar Lamerin silkworm breeds inoculated with Lamerin microsporidia were dissected under a dissection microscope (Leica, Wild – M8) and fixed in 3 % glutaraldehyde in phosphate buffer saline (PBS, pH. 7.4). The tissues were kept at 4°C for 24h and post fixed in 1% osmium tetra oxide for 2h. The tissues were washed, dehydrated in an ascending series of alcohol, passed through propyle oxide, and infiltrated with Araldite and propyle oxide for 12h. The samples were centrifuged and the sediment containing the tissue was infiltrated again with fresh araldite by embedding in Araldite and kept at 60°C for 48hr. Semi thin sections (1 μ) were cut with glass knives of microtome (Leica EMUC -6), placed on a hot plate at about 80°C and dried. Ultra thin sections 70 - 80nm (700 – 800 \AA) were double stained with Uranyl acetate and lead citrate, observed and photographed under 60KVA (JEOL 100Cx) electron microscope at different x-magnification.

Virulence: To determine the virulence of the Lamerin microsporidian isolated from Lamerin breed of silkworm, three-silkworm breeds *viz.*, Lamerin, PM and CSR2 were selected. The eggs of these breeds received from germplasm bank of CSRTI, Mysore were surface disinfected and incubated at $25\pm 1^{\circ}\text{C}$ and $80\pm 5\%$ RH. The hatched larvae were reared till the beginning of third instar following standard silkworm rearing practices under hygienic conditions. The silkworm of each breed was inoculated with different concentrations of either Lamerin microsporidian or *N. bombycis* spores. The concentrations identified for inoculation were 1×10^3 , 1×10^4 , 1×10^5 , 1×10^6 , 1×10^7 and 1×10^8 spores / ml. The inoculum was prepared from purified spores by serial dilution and quantified following the standard method using haemocytometer (Cantwell, 1974). Each inoculum concentration forms a treatment and each treatment had five replications of 100 larvae. One set of larvae of each breed were inoculated with different concentration inoculum of Lamerin microsporidian and another by *N. bombycis* spores. One ml of specific concentration of specific microsporidian spores were smeared on the mulberry leaf and fed to the silkworm immediately after 2nd moult.

Observations were recorded daily on mortality due to the Lamerin microsporidian and *N. bombycis* in respective treatments till cocooning. The dead larvae were homogenized and examined for the microsporidian infection under phase contrast microscope. The virulence of the microsporidia was expressed, as the dosage required for killing 50% of the larvae *viz.*, LC_{50} value following Probit analysis (Finney, 1971). The LC_{50} value and fiducial limit were calculated following the Probit method for the microsporidian isolated from Lamerin breed and *N. bombycis*. The virulence of Lamerin microsporidia were compared with *N. bombycis* and discussed.

Susceptibility: To determine the susceptibility of different silkworm breeds to Lamerin microsporidia and *N. bombycis*, seven silkworm breeds *viz.*, CSR2, CSR18, CSR19, NB4D2, Lamerin, Pure Mysore and Nistari were selected. The layings of the selected breeds were received from the silkworm germplasm of

CSR&TI Mysore and incubated at $25\pm 1^{\circ}\text{C}$ and $80\pm 5\%$ RH. The hatched larvae were reared following standard rearing method till the beginning of 3rd instar. The 3rd instar silkworm immediately after 2nd moult were inoculated with the inoculum 1×10^7 spores / ml of either Lamerin microsporidia or *N. bombycis* and reared till cocooning. The inoculum was prepared from the purified stock. The breed formed the treatment and each breed had five replications of 100 larvae. The observation was made on the mortality due to the microsporidiosis and recorded. The dead larvae and pupae were homogenized to determine the cause of the mortality by microscopic examination. Mortality due to the microsporidian infection alone were recorded. The live moths were examined for the infection and data were recorded and analyzed. The susceptibility of these breeds to the Lamerin microsporidia was compared with the susceptibility of these breeds to *N. bombycis*.

RESULTS

Mode of Infection: The observations recorded to determine the mode of infection is presented in Table 1. *Per os* inoculation of Lamerin breed of silkworm through contaminated mulberry with the Lamerin microsporidian spores (T1) resulted in infection of Lamerin silkworms. $9.80\pm 0.84\%$ of silkworm larvae and $3.20\pm 0.84\%$ of pupae died due to the microsporidian infection and the rest of the larvae proceeded to form the cocoon and moth. $59.69\pm 3.37\%$ of moths emerged were found infected with microsporidia. The female moths laid the eggs and $84.21\pm 2.72\%$ of eggs hatched and $16.54\pm 3.42\%$ of eggs remained unhatched. $63.94\pm 2.45\%$ of hatched larvae and $9.32\pm 3.24\%$ of unhatched eggs were found infected. The set of larvae smeared with the microsporidian spore on its integument (T2) did not develop infection. Microscopic observation of homogenate of larva, pupa, moth and larvae hatched from eggs from T2 did not indicate the presence of microsporidian spores. Among the set of larvae hatched from egg surface contamination (T3) treatment 11.80 ± 0.84 and $5.20\pm 0.84\%$ larval and pupal mortality were recorded respectively. $62.29\pm 5.40\%$ moths were found

infected and $84.51 \pm 4.76\%$ eggs hatched and only $9.60 \pm 1.49\%$ were unhatched. $58.13 \pm 5.34\%$ hatched larvae and 10.82 ± 4.23 of unhatched egg were found infected. The larvae, pupa moth and larvae hatched from egg of control (T4) also did not indicate the presence of microsporidian spores and infection.

Infectivity: It is observed from the result of studies on infectivity of Lamerin microsporidia and *N. bombycis* that the Lamerin microsporidia and *N. bombycis* causes infection in all the three tested breeds viz., Lamerin, PM and CSR2. The infectivity of Lamerin microsporidia was compared with the infectivity of *N. bombycis* in different breeds of silkworm viz., Lamerin (T1), PM (T2) and CSR (T3).

It is observed from the symptoms the larvae developed that the *N. bombycis* was comparatively more virulent than the Lamerin microsporidia Table 2 and Fig. 1. Lamerin microsporidian caused $41.20 \pm 5.59\%$ and $48.0 \pm 1.58\%$ infection in Lamerin and CSR2 breed of silkworms respectively. In PM breed the infection was to an extent of 41.20 ± 3.49 . *N. bombycis* caused infection to the extent ranging from $88.58 \pm 3.84\%$ in Lamerin breed to $91.69 \pm 3.33\%$ in CSR2. In PM breed the infection was to an extent of $87.55 \pm 4.19\%$. It is observed that infectivity percentage of Lamerin microsporidia did not differ in different breeds and a comparison with *N. bombycis*, the infectivity of Lamerin microsporidian is significantly lower. Lamerin microsporidian did not cause pupal mortality in any of the three breeds where as *N. bombycis* caused pupal mortality of 3.60 ± 0.89 , 3.80 ± 1.10 and $6.20 \pm 2.17\%$ in PM, Lamerin and CSR2 respectively. 100% moth emergence was recorded in all the three inoculated with Lamerin microsporidia. However 96.20 ± 1.10 , 96.40 ± 0.89 and $93.80 \pm 2.17\%$ moth emergence were recorded in *N. bombycis* inoculated batches of Lamerin, PM and CSR2 breeds respectively. It is also observed that the Lamerin microsporidia causes infection in all the three breeds and the moths obtained from them were also found to be infected and it ranged from 41.20 ± 3.49 – $48.00 \pm 1.58\%$. In case of the breeds inoculated with *N. bombycis* 87.55 ± 4.19 – $91.69 \pm 3.33\%$ of moths had infection

indicating higher rate of transmission. It is observed that the percent infection by two microsporidians differs in silkworm breeds. The Lamerin microsporidia is low in infectivity as compared to *N. bombycis* to Lamerin and other tested breeds.

Mode of transmission: The result of studies on the mode of transmission of Lamerin microsporidian and *N. bombycis* in three silkworm breeds viz., Lamerin, PM and CSR2 is presented in Table 3. The results were compared with mode of transmission of *N. bombycis* in the three breeds. It is observed that larvae hatched from the eggs obtained from infected male × healthy female (IM×HF) in respect of both Lamerin microsporidian and *N. bombycis*, in all the three silkworm breeds (T12, T15, T22, T25, T32 and T35) and healthy male × healthy female (HM × HF) viz., T17, T27 and T37 did not reveal infection in the progeny of the three breeds while the progeny larvae hatched from eggs obtained from healthy male × infected female (HM × IF) viz., T11, T14, T21, T24, T31 and T34 as well as infected male × infected female (IM × IF) viz., T13, T16, T23, T26, T33 and T36 revealed infection. The progeny larval examination of healthy male × Lamerin microsporidian infected female (HM × IF) viz., T11, T21 and T31 of all the breeds revealed infection.

The rate of transmission of Lamerin microsporidian to the F1 progeny in Lamerin, PM and CSR2 breeds were 59.20 ± 2.04 , 58.80 ± 0.98 and $57.20 \pm 3.92\%$ respectively. The F1 progeny larval examination of healthy male × *N. bombycis* infected female (HM×IF) viz., T14, T24, and T34 revealed infection and the rate of transmission was 100% in all the three breeds.

The F1 progeny larval examination of Lamerin microsporidian infected male × Lamerin microsporidian infected female (IM×IF) viz., T13, T23, and T33 revealed infection in all the breeds. The rate of transmission in Lamerin, PM and CSR2 breeds were 62.80 ± 3.71 , 64.00 ± 6.07 and $56.80 \pm 3.92\%$ respectively. The F1 progeny larval examination of *N. bombycis* infected male × *N. bombycis* infected female (IM × IF) viz., T16, T26, and T36 revealed infection and the rate of transmission was 100% in all the three breeds.

The progeny larval examination of Lamerin microsporidian or *N. bombycis* infected male × healthy female (IM×HF) viz., T12, T15, T22, T25, T32, and T35 did not reveal infection in the progeny and the rate of transmission was nil with respect to all the breeds tested. The progeny of the control group (HM × HF) viz., T17, T27 and T32 of all the three breeds with no inoculation with any of the microsporidians (Lamerin microsporidian or *N. bombycis*) did reveal infection and the rate of transmission was nil.

No transmission of either Lamerin microsporidian or *N. bombycis* to the progeny occurred in the healthy female × infected male (IM×HF) crosses, indicating that there was no venereal pathway for either microsporidium. Only the female moth transmits the infection and male moth does not. It is also observed that the hatchability of eggs laid by infected female moths is reduced. It is more conspicuous with female moths infected with *N. bombycis* than the moths infected with Lamerin microsporidia. The hatching percent of eggs laid by *N. bombycis* infected female moths in different breeds ranged from 53.68±6.19–64.91±3.25%. In case of Lamerin microsporidia infected female moth the hatching percent ranged from 92.91±0.90-95.84±1.46% except in Lamerin breed it ranged from 75.38±2.07-76.44±4.29%.

Rate of spread of Lamerin microsporidia: The result of investigation on the spread of infection by Lamerin microsporidian in Lamerin, PM and CSR2 breeds of silkworm and its comparison with infection by *N. bombycis* is presented in Table 4. It is observed from the result that 1, 3, 6 and 9 carriers of the Lamerin microsporidia did not spread infection to an extent to cause larval and pupal mortality in Lamerin and PM breeds. However, 6 and 9 carriers of the Lamerin microsporidia caused spread of infection to cause 0.40% and 1.60% pupal mortality in CSR2 breed.

N. bombycis 1, 3, 6 and 9 carriers however spread infection to the extent to cause mortality in the colony of Lamerin, PM and CSR2 breeds and ranged from 1.00–9.00%; 0.60%-8.80% and 1.00-9.00% respectively. The carriers also caused

pupal mortality in pupae of Lamerin, PM and CSR2 breeds to an extent ranging from 0.20- 0.40%, 0.20-0.40% and 0.60-1.00% respectively.

Though Lamerin microsporidian carriers 1, 3, 6 and 9 did not cause larval / pupal mortality in Lamerin, PM and CSR2 breeds infection is observed in the moth stage of all the three breeds. It ranges from 1.06-9.64%, 1.07-10.39 and 1.08-8.74% respectively. The 1, 3, 6 and 9 carriers of *N. bombycis* however spread infection by the moth stage in Lamerin, PM and CSR2 breeds to an extent ranging from 20.69 – 33.26%, 22.10-31.55 and 43.15-66.41%.

Site of infection: Observations on the site of infection of Lamerin microsporidia in silkworm are presented in Table 5 and Fig. 2 and 3. The spore stage of the microsporidian was first observed on the 9th day of post inoculation in the midgut tissue (Fig. 2a). The intensity of infection was low (+). On the tenth day of the post inoculation, spores were observed in the fat bodies (Fig. 2b) and on 11th day in malphigian tissues (Fig. 2c) followed by gonads (Fig. 2d) on 12th day and trachea (Fig. 2e), silk gland (Fig. 2f) and haemolymph on 13th day. The intensity of infection was comparatively low (+). In case of infection by *N. bombycis*, the spore stage makes first appearance on the 6th day of post inoculation in the midgut, fat bodies and malphigian tubules (Figs. 3a, 3b, 3c) with low (+) level infection. It spread to gonads, trachea and silk gland (Figs. 3d, 3e and 3f) by 7th day with low (+) level infection. The intensity of infection increases in these tissues by 8th and 9th day of post inoculation. Infection in hemolymph is first noticed on 9th of day post inoculation.

The transmission electron microscopic (TEM) observation of sites of infection by Lamerin microsporidian revealed the intracellular presence of early developmental stages and mature spore stage in the gut and fat bodies (Fig.. 4a and 4b). The hypertrophy of infected cells were not noticed which may be due to low level of infection. Meronts, sporonts and mature spore could be observed in the infected tissues, but the whole life cycle was not studied. However single meront was observed in the host cytoplasm (Fig.. 4a). The sporonts showed the same

structure as the meronts except that it was elongated and had the thick osmophillic wall (Fig.. 4b). The meronts and sporonts measured 0.46 ± 0.25 , $1.11 \pm 0.05 \mu\text{m}$ in lengths and 0.32 ± 0.05 , $0.78 \pm 0.10 \mu\text{m}$ in width respectively. The length/width ratio was 1:0.69 meront and 1:0.70 in sporont. The mature spores were broadly ovocylindrical and found singly in their sporophorous vesicle. They measured $4.36 \pm 0.06 \mu\text{m}$ in length and $2.14 \pm 0.01 \mu\text{m}$ in width (Table 6). The length / width ratio of mature spore was 1:0.49.

Virulence: The result of studies on the virulence of Lamerin microsporidian and *N. bombycis* to Lamerin, PM and CSR2 silkworm breeds is presented in Table 7. It is observed that the Lamerin microsporidia caused larval and pupal mortality at a concentration of 1×10^6 spores /ml and above in Pure Mysore and CSR2 breeds. At the concentration 1×10^6 spores / ml the larval mortality was nil in Lamerin and PM breeds while in CSR2 breed it was 2.40%. In Lamerin and PM larval mortality was recorded at concentration of 1×10^7 spores/ml and above. It was 5.40% in Lamerin, 8.20% in PM and 10.40% in CSR2. 1×10^8 spores / ml caused 8.60, 10.00 and 12.40 % larval and 2.80, 5.60 and 7.40 pupal mortality in Lamerin, PM and CSR2 respectively. The individuals survived to the adulthood were not all infected (Table 6). 34.40-44.20% (1×10^3 spores / ml) to 49.60-51.40% (1×10^8 spores / ml) were infected. The total infection (inclusive of mortality in larva and pupa) ranged from 34.40-44.20% (1×10^3 spores / ml) to 62.20-70.80% (1×10^8 spores / ml).

At all inoculum concentrations (1×10^3 - 1×10^8 spores / ml), *N. bombycis* caused mortality at larval and pupal stages in all the breeds tested. The inoculum of 1×10^3 spores / ml caused larval and pupal mortality of 5.80 and 3.40%, 4.0 and 2.80%, 3.20 and 9.40% in Lamerin, PM and CSR2 breeds respectively and all the individuals survived to the adulthood were infected in all the breeds tested. At 1×10^3 - 1×10^7 spores/ml most larvae died due the infection and few adults survived and successfully emerged all were infected. The larvae inoculated with 1×10^8

spores/ml resulted death of 91.41, 97.20% in case of Lamerin and PM however 100% mortality was recorded in CSR2 breed.

The LC_{50} and fiducial limits for Lamerin microsporidia and *N. bombycis* were calculated for larval mortality with regards to Lamerin, PM and CSR2 silkworm breeds following the Probit method and is presented in Table 8. The LC_{50} value estimated for 20days PI for Lamerin microsporidia to Lamerin breed was 8.7×10^9 spores/ml with the 11.990 and 7.889 as Upper and lower fiducial limits. The LC_{50} value of the Lamerin microsporidian to PM breed was 4.7×10^8 spores with 9.145 and 8.191 as upper and lower fiducial limits. The LC_{50} value of the Lameri microsporidian to CSR2 was 2.8×10^8 spores/ml with 8.773 and 8.120 as upper and lower fiducial limits. The LC_{50} value estimate for 20DPI for *N. bombycis* to Lamerin breed was 1.7×10^6 spores/ml with 6.471 and 5.998 as Upper and lower fiducial limits. The LC_{50} value of *N. bombycis* to PM and CSR2 breeds were 9.1×10^5 spores/ml with 6.161 and 5.761 and 1.6×10^5 spores/ml with 5.724 and 4.699 as upper and lower fiducial limits. From the above result, it is observed that the Lamerin microsporidian is low in virulence as compared to *N. bombycis* and its virulence do not differ significantly among the breeds tested. Lamerin microsporidia were 5118, 516 and 1750 times less virulent than *N. bombycis* to Lamerin, Pure Mysore and CSR2 breeds respectively.

Susceptibility: The results of screening of seven breeds screened for susceptibility to Lamerin miosporidian and *N. bombycis* are presented Table 9. It is observed that the breeds inoculated with *N. bombycis* were retarded in growth as compared to the larvae inoculated with Lamerin microsporidia (Fig.5). The larvae CSR2 silkworm breed is most susceptible to Lamerin microsporidia with total mortality of $20.0 \pm 1.0\%$ larvae followed by CSR19 ($19.4 \pm 2.6\%$), CSR18 ($18.6 \pm 1.5\%$), NB4D2 ($15.6 \pm 0.5\%$), Nistari ($13.0 \pm 1.2\%$), PM ($12.6 \pm 1.5\%$). Lamerin breed was the least susceptible with mortality of $10.2 \pm 1.6\%$. However CSR2 was most susceptible to *N. bombycis* with mortality of $75.0 \pm 3.0\%$ followed by CSR19 ($74.6 \pm 5.5\%$), CSR18 ($73.8 \pm 1.1\%$), NB4D2 ($71.4 \pm 3.8\%$), Nistari

(63.6±2.9%), PM (53.2±31%) and Lamerin 47.6±3.0%. Lamerin breed is also least susceptible to *N. bombycis* as it is to Lamerin microsporidia. However the total infection with respect to Lamerin microsporidian in CSR2 was 57.0±2.9% and it was followed by CSR18 (55.9±3.1), Lamerin (54.3±1.2%), PM (53.1±1.4%), CSR19 (50.9±2.7%), NB4D2 (48.8±1.6%) and Nistari (46.6±1.2%) *N. bombycis*, however caused 100% infection in all the three breeds. The result indicated that different breeds respond differently to Lamerin microsporidian infection but Lamerin is least susceptible to both Lamerin microsporidian and *N. bombycis* at pre cocoon stage.

DISCUSSION

Investigations on the Characterisation of the microsporidian infecting Lamerin breed of silkworm presented under the first chapter has identified specific characters that make it different from other microsporidians infecting silkworm. The spore morphology, serological affinity, features of polar filament artificial germination and electrophoretic banding pattern of spore surface protein were discussed. In this chapter results of studies on the mode and site of infection, infectivity, mode of transmission, spread of infection, virulence of Lamerin microsporidia and susceptibility of different silkworm breeds to it is compared with corresponding characters of *N. bombycis* and discussed.

Microsporidia invade insects through the three natural portals of entry *viz.*, oral, cuticular and ovarial pathways (Kramer, 1976). The Lamerin microsporidia from Lamerin breed infects silkworm through oral and ovarial portals. The oral portal is the most common route through which the microsporidians gain entry into the host. However it is the only route of infection by *Nosema apis* in honey bee (Bailey, 1963). It may be due to the contamination of mulberry leaf or through the external contamination of egg surface by microsporidia spores (tansovum). Lamerin microsporidian in Lamerin and other silkworm breeds tested is observed to infect through oral portal and cause infection in 59.69±3.3% of moths while cutaneous portal did not cause infection at all. Infection however did not cause

high percent of larval and pupal mortality. It was only 9.8 ± 0.83 and $3.2 \pm 0.83\%$ mortality in larval and pupal stages. It is also observed that $63.93 \pm 3.24\%$ progeny from the laying of the infected moths were infected (Table 1). Lamerin microsporidia infects all the breeds of silkworm screened for the infectivity of the microsporidian. It confirms that the microsporidia infectivity is not confined to Lamerin breed alone but infects other multivoltine and bivoltine breeds. However the infectivity of Lamerin microsporidian is much lower than *N. bombycis*. It ranged from 4.20 ± 3.49 - $48.00 \pm 1.58\%$ by Lamerin microsporidia as against 87.5 ± 4.19 - $91.69 \pm 3.30\%$ by *N. bombycis*.

Investigations on the mode of transmission in three breeds indicate that the Lamerin microsporidian transmit infection to the progeny. The transmission may be through the infected ovary (transovarial) or veneral transmission as is observed in some microsporidia such as *Nosema kingi* in drosophilids (Armstrong, 1976) and *Thelohania* species in mosquitoes (Kellen *et al.*, 1965). In silkworm *B. mori*, *N. bombycis* is reported to transmit infection by transovum (Masera, 1938) and by tranovarial tranmission (Han and Watanabe, 1988). The transovarial transmission of *N. bombycis* is also demonstrated in *Diaphania pulverulentalis* by Ramagowda and Geethabai (2005). The studies on the mode of transmission of Lamerin microsporidia in three-silkworm breeds *viz.*, Lamerin, Pure Mysore and CSR2 indicate that the infection is transmitted to the progeny as does *N. bombycis* (Table 3). Lamerin microsporidian infected male moth paired with healthy female moth lay eggs whose progeny were free of infection. Lamerin microsporidia infected female moth paired with healthy male moth lay eggs whose progeny carry infection. This mean that the male moth though carry the infection do not transmit infection to the progeny. The infected female moth always transmits infection to the progeny. Lamerin microsporidian infected female moth paired with Lamerin microsporidia infected male moth always transmitted infection to the progeny. The observation also holds well with the *N. bombycis* moths and female moth transmits infection to the progeny and male moths do not transmit infection to progeny. The rate of transmission of Lamerin microsporidia infection in Lamerin, Pure Mysore and CSR2 breeds was comparatively lower than transmission rate by *N. bombycis*.

The rate of Lamerin microsporidian transmission in progeny of three tested breeds ranged from $56.80 \pm 3.92\%$ to $64.00 \pm 6.78\%$. However the rate of transmission in progeny of the three breeds by *N. bombycis* was 100%. These two microsporidians differed greatly in their rate of transmission in the silkworm. A similar result of *N. bombycis* in the silkworm was obtained by Ishihara and Fujiwara (1965); Han and Watanabe (1988); Baig, (1994) and Ananthalakshmi *et al.*, (1994). Difference in rate of transmission of different microsporidians to the progeny in silkworm is also reported. The rate of transmission of *Nosema* sp. NIK-3h and *Nosema* sp. M11 is reported to be low and transmits the infection to the progeny by only $1.80 \pm 0.4\%$ and $1.2 \pm 0.41\%$ respectively (Han and Watanabe, 1988; Ananthalakshmi *et al.*, 1994). However the standard strains *N. bombycis* and *Nosema* sp. NIK-2r transmit the infection to an extent of 100 % (Han and Watanabe, 1988; Ananthalakshmi *et al.*, 1994), where as the microsporidians such as *Vairimorpha* sp. NIS-M12, NIK-4m, *Microsporidium* sp. NIS-M25, *Pleistophora* sp. NIS-N27 and *Thelohania* sp. NIS-M32 do not transmit the infection at all to the progeny (Kawarabata, 2003; Singh and Saratchandra, 2003). Although Kellen and Lindegren (1971) reported the venereal transmission of microsporidium in the Indian meal moth, *Plodia interpunctella*, the Lamerin microsporidia or *N. bombycis* in the silkworm were negative to venereal transmission in the present study suggesting that the microsporidia might not be transmitted via sperm from the male adult into the F1 progeny. The transmission is only maternal mediated. Apart from low infectivity and low rate of transmission the Lamerin microsporidia have also low rate of spread of infection in silkworm colony after introduction of infected carriers. The Lamerin microsporidian carriers, 1-9 in number, did not spread infection in a healthy colony of silkworms of the three tested breeds to an extent to cause mortality in larval stage of the colony, but infection was observed in moth stage.

Lamerin microsporidian infects different tissues of silkworm, as does *N. bombycis* but the level of infection was very low in all susceptible tissues as compared to *N. bombycis*, only 3-4 mature spores could be observed / microscopic field. The two microsporidians infects gut, malpighian tubules,

trachea, silk gland, fat bodies, gonads and haemolymph. However unlike most low virulent microsporidians such as *Nosema* sp. NIS-M11, NIS-M14, *Microsporidium* sp. NIS-M25, *Pleistophora* sp. NIS-M27 and *Thelophania* Sp. NIS-M32, the Lamerin microsporidian infects gut tissues and gonads. Compared to *N. bombycis*, the rate of infection of Lamerin microsporidian is low. It takes nine days after *per os* inoculation for the first appearance of spores in the gut epithelium while in case of *N. bombycis* it was seen on the 6th day of post inoculation. The spore makes its appearance on the 13th day of post inoculation in trachea, gonad and haemolymph while in case of *N. bombycis*, the trachea, silk gland and gonads develop spores on the 7th day itself. The intensity of infection of different tissues by Lamerin microsporidia is also lower than the intensity of infection caused by *N. bombycis*. These observations will explain the reasons for low mortality and low rate of transmission of Lamerin microsporidia in different breeds of silkworm.

Observation on the virulence of the Lamerin microsporidia to different breeds of the silkworm further confirms that it is low in virulence compared to *N. bombycis*. It is 5117, 516 and 1750 times less virulent than *N. bombycis* to Lamerin, Pure Mysore and CSR2 breeds of silkworm respectively. The LC₅₀ value of Lamerin microsporidian for 3rd instar Lamerin, Pure Mysore and CSR2 breeds are 8.7×10^9 , 4.7×10^8 and 2.8×10^5 spores/ml while for *N. bombycis* it was 1.7×10^6 , 9.1×10^5 and 1.6×10^5 spore /ml. Different breeds of the silkworm respond differently to the two microsporidian infection (Lamerin microsporidian and *N. bombycis*). The response of different silkworm breeds to Lamerin microsporidia also appear the same. CSR2 was most susceptible to Lamerin microsporidia as well as *N. bombycis*. Difference in virulence of several microsporidia infections in silkworm is reported. *N. bombycis* N1S001, *Vairimorpha* sp. NIS-M12 and *Pleistophora* sp. NIS-N27 are most virulent. *Nosema* sp. NIS-M14 and *Microsporidium* sp. NIS-M25 are moderately virulent, *Thelohania* sp. NIS-M32 is low in virulence (Saratchanra and Singh, 2003). Lamerin breed is least susceptible to both Lamerin microsporidan as well as *N. bombycis*. It is also observed that Lamerin silkworm breed was comparatively more resistant to

microsporidia infection. Screening of the Lamerin breed along with other six breeds for their susceptibility to Lamerin microsporidian and *N. bombycis* indicated that Lamerin breed is comparatively more tolerant than other breeds to not only Lamerin microsporidian but also *N. bombycis*.

Investigations in this chapter indicate that Lamerin microsporidian unlike *N. bombycis* is low in its infectivity, multiplication, transmission and rate of spread. It is also less virulent than the *N. bombycis* not only to Lamerin breed but also to other silkworm breeds tested and the Lamerin breed itself offers more resistance to Lamerin microsporidia as well as to *N. bombycis*.