Chapter 1

CHARACTERIZATION OF THE NEWLY ISOLATED MICROSPORIDIAN NIK-5hm INFECTING SILKWORM, *BOMBYX MORI* L.

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The Microsporidia are a diverse group of spore forming obligate intracellular parasites belonging to the phylum Microspora. Microsporidia are smallest and most primitive of eukaryotic cells, representing one of the earliest branches of the eukaryotic phylogenetic tree (Vossbrinck *et al.,* 1987; 1993; Baker, 1994). The spore is the most visible sign of infection by microsporidia and its structure, texture and nature are the characteristic feature of microsporidia. The spore of microsporidians are small, usually ranging between 3.0-5.0 µm in length and 2.0-3.0 µm in width, oval, ovoidal or ovo-cylindrical, shining having high refractive index and exhibiting the characteristic brownian movement. They are also characterized by having unique organelle, the polar capsule involved in the invasion of a host. Some species of microsporidia have complex life cycle, involving sexual reproduction, vertical and horizontal transmission, two obligate hosts, and three or more morphologically distinct spores (Hazard and Weiser, 1968; Sweeney *et al.,* 1985; Andreadis, 1985a and b; Avery and Undeen, 1990; Becnel, 1992). Other microsporidia have simple life cycle with only one kind of spore, formed during the course of a simple asexual reproduction.

Microsporidia are extremely widespread. They infect nearly every organism on earth from honey-bees and silkworm to mammals and birds. Microbiological studies of microsporidiosis in silkworm started after the outbreak of Pebrine disease in France in 1845. Pasteur (1870) established that the certain corpuscles are the cause of pebrine disease. The corpuscles were later identified as the spore stage of the microsporidia - *Nosema bombycis* in silkworm. By the end of 19th century, only *N. bombycis* was known as causative agent of pebrine in silkworm. At present several microsporidians are known to cause the disease in silkworm (Ananthalakshmi *et al.,* 1994; Kishore *et al.,* 1994; Samson *et al.,* 1999a,b; Sharma *et al.,* 2003; Singh and Saratchandra, 2003; Nagewara Rao *et al.,* 2004; Selvakumar *et al.,* 2005). They differ in their morphology, serology, site of infection and virulence (Table 1.1). Among them, the most common one are the strain of *Nosema* (NIS-001, NIS-M11, NIS-M14, and NIK-2r,

and NIK-3h.), Species of *Vairimorpha* (NIS-M12 and NIK-4m), and *Microsporidium* (NIS-M25), *Pleistophora* (NIS-M27), *Thelohania* (NIS-M32) and *Leptomonas*. The environmental spore of these microsporidians is oval, cylindrical or ovocylindrcal, either uninucleate or binucleate (Kawarabata, 2003; Singh and Saratchandra, 2003). However, the *Nosema* sp. NIS-11, *Vairimorpha* sp. NIS-12 and *Microsporidium* sp. NIS-M14 also form uninucleate octospores in muscle and adipose tissue but all the environmental spores are binucleate. The environmental spores of *N. bombycis* NIS-001 are oval in shape and medium in size while *Nosema* sp. NIS-M14 produces larger environmental spores than NIS-001. In *Pleistophora,* NIS-M27, *Microsporidium* NIS-25 and *Thelohania* NIS-M32 species, the spores are oval and uninucleate. The spores measure 3.6 to 5.0 µm in length and 1.8-2.8µm in breadth. The spores of *Vairimorpha* sp. are longer measuring 5.0µm in length to 2.1 µm in breadth (Table 1.1).

Microsporidian spores are ubiquitous and are capable of infecting any animal cell including those of insects, fish, mammals and even other parasites. The spores tide over the adverse environmental factors with a resilient spore wall that consists of a proteinaceous exospore and chitinous endospore. It is backed by a plasmalemma that probably mediates the diffusion of ions and other small molecules between the cytoplasm of the spore and the external environment. A unique extrusion apparatus – polar capsule is present occupying most of the spore and consists of polar filament with its anchoring apparatus and polaroplast. At the anterior end of the spore is the anchoring disc to which the basal end of the polar filament is attached. The polar filament is enclosed in the polar sac or polaroplast. The disc functions as a hinge when filament is everted. The polaroplast, a system of sac-like or lamellar components occupies 25-35% of the mature spore volume. The polar filament serves as an inoculating needle which is a thread like tubular structure through which the sporoplasm extrudes (Peter *et al.,* 1999). Its presence in spore places the organism in the phylum Microspora (Issi, 1986). It is anchored at the anterior portion (Polaroplast complex) of the spore wall by an anchoring disc. The attached filament extends posterior and forms several number of coils. The number of coils and their arrangement relaed to one another and even the angle of tilt of coil is of taxonomic importance (Sprague *et al.,* 1992; Keeling and Fast, 2002). The straight basal portion of the filament near the attachment site is thicker than its other parts. The polar filament is elastic, stretching to three times

the length of the filament in coiled state when extruded from the spore. It is also elastic transversally to increase its diameter (Lom and Vavra, 1963). The filament consists of concentric layers and the proportion of individual layer varies along the longitudinal axis (Vavra, 1976). The coils of the polar filament are frequently seen in electron micrographs as a series of cross sections lying in rows, just inside of the plasmalemma (Keeling and Fast, 2002).

Genetic material is carried in either as a single nucleus or a diplokaryon (two nuclei in close association). The cytoplasm is rich in ribosomes and endoplasmic reticulum. At the end of the spore, opposite to the anchoring disc is posterior vacuole containing a posteriosome, amorphous material, or a clear fluid. The chromosome number and genome size of microsporidians infecting silkworm is determined by pulsed field gel electrophoresis. The chromosome number varies from 13-18 and their genome size 4.4 -16 kb (Keeling *et al.,* 2005). *N. bombycis* has 18 chromosome bands ranging in size from 380-1,150 kb. *Nosema* sp. NIS-M11 has 13 bands of 610-1,240 kb and *Vairimorpha* sp. NIS-M12 has 13 bands of 960-1,900 kb (Kawakami *et al.,* 1994).

Mono- and polyclonal antibodies are useful tool for species differentiation of microspridia infecting the silkworm, *B. mori* (Shi and Jin, 1997). Serological affinity of different microsporidians has been studied with the polyclonal anti- environmental spore antibody. It demonstrates the occurrence of specific antigens on the surface of *N. bombycis* NIS-001, *Nosema* sp. NIS-M11 and *Vairimorpha* sp. NIS-M12. The monoclonal antibody raised against environmental spores of *N. bombycis* NIS-001, cross-reacts with *Nosema* sp. M14 (Fujiwara *et al.*, 1966; Sato *et al.,* 1981; Kobayashi and Yamazaki, 1987; Abe and Kawarabata, 1988). The polyclonal anti-environmental spore antibody of *N. bombycis* reacts positively with the environmental spore of *Nosema* sp. NIK-1s and *Nosema* NIK-2r. However, the polyclonal anti-environmental spore antibody sensitized latex beads of *Nosema* sp. NIS-M11 and *Vairimorpha* sp. NIS-M12 did not react positively with *N. bombycis* spore. *Nosema* sp. NIK-3h spore polyclonal antibody reacted positively with *Nosema* sp. M11, *Vairimorpha* sp. NIK-4m and *Vairimorpha* sp. NIS-M12 (Ananthalakshmi *et al.,* 1994).

The disease has become increasingly complex today because of different strains of microsporidia being isolated from silkworm and some mulberry insect pests. In

recent years, different strains of microsporidia *viz*., NIK-2r, NIK-4m, NIK-3h, NIK-4m and NIK-5hm has been isolated from silkworm in India (Ananthalakshmi *et al*., 1994; Nageswara Rao *et al*., 2004)

As per the available literature spore formation occur in midgut epithelium, malpighian tubules, silk gland, fat bodies, gonads and trachea. Recently Selvakumar *et al*. (2005) isolated a new microsporidia NIK-5hm from the silkworm larval sample received from Kempayanahundi, Karnataka. The microsporidian sp. was found to be highly pathogenic to the silkworm, *Bombyx mori.* The microsporidian sp. was also reported to infect the haemocytes of silkworm haemolymph.

In the present study, a newly isolated microsporidian sp. NIK-5hm infecting silkworm *Bombyx mori* L*.,* was investigated for their morphology, pathogenecity along with hematological, histo-pathological and biochemical changes caused by the new microsporidian infection in silkworm. The study also provides useful information on the pathogenicity of the newly isolated microsporidia in silkworm and its impact on the economic characters of the silkworm. The management aspects of the new microsporidians *vis-à-vis, N. bombycis* will be studied

MATERIALS AND METHODS

Isolation and Purification of NIK-5hm microsporidian: For multiplication, isolation and purification of NIK-5hm, the susceptible silkworm breed CSR2 was selected. The layings of CSR2 breed received from Germplasm bank of Central Sericultural Research and Training Institute, Mysore, Karnataka, India. Randomly picked third instar silkworm larvae were homogenized and examined for the presence of microsporidian spore at 600 x magnifications under Nikon (Type-104) phase contrast microscope to confirm the infection in the colony. The inoculated batch of larvae was reared till cocooning. The cocoons were stored 25±1^{o}C temperature and 80±5% RH. The pupae were allowed to emerge as moths. The moths were homogenized in-group of 20 moths in 80 ml 0.6 % K_2CO_3 solution using homogenizer at 3000 rpm for 3min. The homogenate was allowed to stand for 5 min. and filtered through double-layered muslin cloth to remove the tissue debris. The filtrate was centrifuged at 5000 rpm for 15 min to sediment the spores. Finally the sediment was suspended in distilled water and once again centrifuged at 3000rpm for 15 min. The sediment obtained was suspended in minimal volume of

distilled water and subjected to discontinuous neutralized Percoll (Sigma) gradient centrifugation (Sato and Watanabe, 1980) using Hitachi Ultra centrifuge CPO56G11 and Swingout rotar P56ST. The percoll gradient was constructed by sequential layering in equal volume of 100, 75, 50 and 25% percoll in distilled water. 1ml spore suspension was layered on the gradient and centrifuged at 10,000 rpm for 2 hours. The band formed along the vertical length of the tube was collected separately using Pasteur pipette, diluted with distilled water by 5 times in the original volume. The suspension was centrifuged at 5000 rpm for 20 min. and the supernatant was discarded. The sediment was suspended in 1 ml of distilled water and washed thrice in distilled water by repeated centrifugation. The final sediment was suspended in physiological saline (0.85% NaCl) and stored for further experimentation.

Conformity to Koch's postulate: To confirm that the microsporidia isolated as cause of the disease in the silkworm, *Bombyx mori* L., the isolated microsporidia were subjected to tests for their infectivity in silkworm following the Koch's postulates. To conduct the test, inoculum of purified spores of 1×10^6 spores / ml spore concentration was prepared from the stock inoculum following Cantwell, 1974. One ml of inoculum of NIK-5hm was smeared on mulberry leaves and fed to 100 third instar silkworm larvae of CSR2 and PM breeds immediately after 2^{nd} moult. The larvae were allowed to feed on the treated leaves for 24h to ensure complete consumption of the treated leaves. After 24 h, the larvae were fed with normal mulberry leaves and rearing was conducted and till cocooning. The pupae were preserved to metamorphose into moth. The dead larvae, pupae and the moths were examined for the microsporidian infection. The live moths were allowed to lay eggs and moths were examined for infection. The eggs were treated in hydrochloric acid (HCl) of specific gravity of 1.075, 47.1ºC for 5 min. to break the diapause and the hatched larvae were examined for microsporidian infection. During the progressive infection, the larva, pupa and moths were examined for morphological disease symptoms. Which were similar to the original batch of infected larvae there by establishing the pathogens conformity to the principles of Koch's postulates.

Morphological Characterization of NIK-5hm microsporidian spores: Morphological Characterisation of the spore stage is one of the important criteria used to identify the microsporidians.

Light microscopy and micrometry: Purified spores of newly isolated microsporidia NIK-5hm infecting CSR2 breed of silkworm were subjected to morphological Characterisation following standard method (Fujiwara, 1980) and compared with the spore of *N. bombycis.* Observations were recorded on spore shape, size, texture and behaviour. To determine the spore shape and size, the spores were immobilized on microslide coated with mineral oil droplet and observed and photographed under phase contrast microscope Nikon (Type-104) at 600x magnifications. To understand the behavior of the spore, a wet mount of spores was observed under phase contrast microscope and photographed.

One hundred spores were measured for their length and width following the standard micrometry method (Fujiwara, 1980). The device used for measuring objects is an ocular micrometer in a microscopic field. It consists of a simple ruled grid placed in one of the oculars of the microscope and calibrated with a stage micrometer. The stage micrometer has marking divisions and each division represents 10 μm. The calibration factor with 10x and 40x eyepieces for the particular microscope and its objective lens was calculated. The unit of each division for 10X and 40X in ocular micrometer was calculated using the formula S / O x 10 where "S" is mean value of the coinciding divisions of stage micrometer and "O" is mean value of the coinciding divisions of ocular micrometer. The spores were first immobilized using a drop of mineral oil. A drop of mineral oil was placed on a slide and a cover slip with a small drop (< 5 μl) of dense spore suspension was applied on top of the oil. Water, having a better affinity for glass, spreads out on the surface of the cover slip, leaving spores individually trapped in "holes" in the oil for measurement. One hundred spores were measured and the calibration value is applied to the spore measurement to transform dial readings to μm.

Scanning Electron microscopic study (SEM): Electron microscopic features of the isolated microsporidia were studied using JEOL-100 CX-II electron microscope fitted with an ASID-4D scanning attachment (Tokyo, Japan 20 KV) and compared with that of the Electron microscopic features of *N. bombycis*. Spores of each microsporidia (NIK-5hm microsporidia and *N. bombycis*) were purified by the method described by Sato and Watanabe (1980) and were scanned following standard scanning electron microscopy method. Samples of purified spores were air dried at room temperature for electron

microscope studies at Central Sericulture Research and Training Institute, Mysore, India. The microsporidian spores were transferred into double stick cellophane tape pasted on copper stubs used for mounting specimen for scanning electron microscope. The mounted stubs were coated with about 20 nm gold in sputter coater (EMS-550) and viewed under JEOL-100 CX-11 electron microscope fitted with a (ASID-4D) scanning attachment (Tokyo, Japan 20 KV). The spores were observed for the shape and size and photographed at 20,000 x - magnification and compared with the spores of *N. bombycis.*

Transmission electron microscopic study (TEM): The purified spores of each microsporodia (NIK-5hm and *N. bombycis*) were processed for transmission electron microscopy at National Institute of Mental Health and Neuro Sciences (NIMHANS), Bangalore, India. Transmission electron microscopy of *N. bombycis* spores were carried out for comparison of ultra structure of *Nosema* spores with that of NIK-5hm microsporidia. The spores were fixed in 3% (v/v) glutaraldehyde ($C_5H_8O_2$) in phosphate buffer saline (PBS, pH. 7.4) kept at 4^oC for 24h, washed several times with buffer (pH. 7.2) till the odour of the fixative was completely removed. The samples were post fixed in 1% (w / v) Osmium tetraoxide (OsO_4) for 2h, washed, dehydrated in an ascending series of alcohol of 70, 80, and 90% (1h in each change), e Nosema bombycisloc stained with 2% uranyle acetate and dehydrated in absolute alcohol (100%) for 1h. The samples were again passed through propyle oxide (2-changes of 15min each for clearing) and were infiltrated with Araldite and propyle oxide in ration of 1:1 ratio for 12h. The samples were centrifuged and sediment ware infiltrated again with fresh araldite (3-changes of 4h each) embedded in Araldite and kept at 60^oC for 48hr. Semithin sections (1u) were cut with glass knives of microtome (Leica EMUC -6) placed on a hot plate at about 80^oC and dried. The sections were stained with 1% toludine blue dried on hot plate, washed under running water, dried and observed under light microscope (Motic). Ultra thin sections 70-80 nm (700–800^oA) were double stained with Uranyl acetate and lead citrate, observed and photographed under 60KVA (JEOL 100CX) electron microscope at different x-magnification, to study the coiling pattern of the polar filament. The number of the coils of the polar filament is reported to be one of the important criteria for the Characterization of the microsporidians as the length and number of the coils of the polar filament varies among different microsporidians.

Virulence: To determine the virulence of the NIK-5hm microsporidian isolated from CSR2 breed of silkworm, two silkworm breeds, CSR2 and PM were selected. The eggs of these breeds received from germplasm bank of CSRTI, Mysore were surface disinfected and incubated at 25±1ºC and 80±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 NIK-5hm 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 three replications of 100 larvae. One set of larvae of each breed were inoculated with different concentration inoculum of NIK-5hm 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 NIK-5hm microsporidian and *N. bombycis* in respective treatments till cocooning. The dead larvae were homogenized and examined for the micropsoridian infection under phase contrast microscope. The virulence of the microporidia was expressed, as the dosage required for killing 50% of the larvae *i.e..,* 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 CSR2 breed. The virulence of NIK-5hm microsporidia were compared with *N. bombycis* and discussed.

Screening of selected silkworm breeds for their susceptibility to new microsporidian by bioassay method

To determine the susceptibility status of different silkworm breeds to NIK-5hm microsporidia and *N. bombycis*, 13 multivoltine breeds and 16 bivoltine breeds were selected. The layings of CSR2 breed received from Germplasm bank of Central Sericultural Research and Training Institute, Mysore, Karnataka, India. and incubated at 25±1ºC and 80±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 of microsporidian 1 × 10^6 spores / ml of NIK-5hm and *N. bombycis* and were reared till cocooning. The inoculum was prepared from the purified stock. The breed formed the treatment and each breed had three replications of 100 larvae. The observation was made on the mortality due to the microsporidiosis and recorded. The dead larvae and pupae were homogenized and the cause of the mortality was determined 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 NIK-5hm microsporidia was compared with the susceptibility of these breeds to *N. bombycis*.

Mode of transmission: To determine the mode of transmission of the newly isolated microsporidia, popular multivoltine (Pure Mysore) and bivoltine silkworm (CSR2) breeds were selected and on day zero of fourth instar, larvae of the said breeds were per orally inoculated separately with the spores of the isolated microsporidia at a dosage of 1×10^6 spores/ml. To compare the results, one set of larvae was inoculated with spores of *Nosema bombycis*. The inoculum containing 1×10^6 spores/ml of the isolated microsporidia or *N. bombycis* was prepared from the stock inoculums by proper quantification using Neubar haemocytometer (Cantwell, 1970). One ml of inoculum (1×10^6 spores/ml) of each microsporidia was smeared separately on 100 sq. cms surface area of mulberry leaf disc and fed to 100 larvae immediately after 3rd moult. The larvae were allowed to feed on the contaminated leaves for 24 hours. The second normal feeding was given after 24 hours and the rearing on uncontaminated mulberry leaves was continued till cocooning. After cocoon formation, the cocoons from each treated batch were cut open for sex separation of the pupae. The male and female pupae were kept in separate trays for moth emergence. Yet another set of larvae was 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 which formed the treatments was as follows,

In PM with NIK-5hm

T1: Healthy male (HM) × Infected female (IF) moth.

T2: Infected male (IM) × Healthy Female (HF) moth.

T3: Infected male (IM) × Infected female (IF)

In PM with *Nosema bombycis*

T4: Healthy male (HM) × Infected female (IF) moth..

T5: Infected male (IM) × Healthy Female (HF) moth.

T6: Infected male (IM) × Infected female (IF)

T7: Healthy male (HM) × Healthy female (HF) (Control)

In CSR2 with NIK-5hm

T11: Healthy male (HM) × Infected female (IF) moth.

T12: Infected male (IM) × Healthy female (HF) moth.

T13: Infected male (IM) ×Infected female (IF)

In CSR2 with *Nosema bombycis*

T14: Healthy male (HM) × Infected female (IF) moth.

T15: Infected male (IM) × Healthy female (HF) moth.

T16: Infected male (IM) × Infected female (IF) moth.

T17: Healthy male (HM) × Healthy female (HM) (Control).

After mating, female moths were allowed to lay eggs on egg sheets. The layings were prepared from individual mother moths. The male and female moths were homogenized 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 progeny larvae were reared as per standard methods (Datta, 1992). After I moult, 100 larvae/batch were collected randomly and homogenized individually and the smear was observed under phase contrast microscope for the presence of microsporidian spores and thus, the transmission rate was calculated by the standard formula (Han and Watanabe, 1988) which is as follows;

$$\text{Transmission rate} = \frac{(A \times B) + (C \times D)}{A + C}$$

Where, A- Number of dead eggs

B- % of dead eggs infected

C- Number of larvae hatched

D- % of larvae infected

Isolation of spore surface protein: The spore surface proteins of both microsporodians (NIK-5hm microsporidia and *N. bombycis*) were isolated by following the method described by Kawarabata and Hayasaka (1987). The purified spores of NIK-5hm breed and *N. bombycis* were suspended in 0.1N K_2CO_3 (1×10^8 spores / ml) for 30 min. at room temperature. The suspension was gently stirred at interval of 5 min. and the pH of the spore suspension was adjusted to 7.0 using 0.1N HCl. The spore suspension was centrifuged at 5000 rpm for 30min. and the supernatant containing spore surface protein was collected. An equal volume of chilled acetone (80%) was added to the supernatant and kept at 4^{o}C over night. The suspension was centrifuged at 3000 rpm for 30 min and the sediment was air dried to collect the spore surface protein in powder form. It was suspended in PBS, pH 7.4 and stored at –20^o C till use.

Sodium dodecycle sulphate - polyacrylamide gel electrophorosis (SDS-PAGE): The spore surface protein of microsporidia from NIK-5hm breed and *N. bombycis* was separated using 10% SDS-PAGE discontinuous system of Laemmli (1970). The commercial standard marker protein of molecular weight of range 14.3-97.4 kDa, received from Bangalore Genei, Pvt. Ltd. Bangalore was used for comparing polypeptide mobility in SDS- PAGE. The silver staining was used for gel staining as it is sensitive at nanograms (ng) level. The observation was compared with that of spore of *N. bombycis* surface protein.

Serological affinity: Serological affinity of microsporidian spore from NIK-5hm breed with *N. bombycis*, *Nosema* sp. were determined following monoclonal antibody based Latex agglutination kit (Yakult& co, Japan) against the microsporidia isolated from the CSR2 breed of silkworm.

Effects of different temperature regimes on the infection and sporulation of the newly isolated microsporidian NIK-5hm and the standard strain *Nosema bombycis*:

To determine the effect of different temperatures on the sporulation of the isolated microsporidian NIK-5hm, disease free layings (dfls) of the CSR2 breed of silkworm were surface disinfected and incubated following the standard procedure at 25±2ºC

and 80±5% relative humidity. After the stipulated period of incubation, the hatched larvae were brushed and reared under hygienic conditions as per standard rearing methods (Datta, 1992) up to second moult. Inoculum of each microsporidian *viz.*, NIK-5hm and *Nosema bombycis* was prepared from purified stock of each microsporidian and quantified by standard method using Neubar haemocytometer (Cantwell, 1970). One ml of inoculum of each microsporidian strain containing 1×10^6 spores/ml was smeared separately on the mulberry leaf disc (100 cm^2 surface area) and fed to 100 larvae separately immediately after II moult. The larvae were allowed to feed on the microsporidia smeared mulberry leaf till the complete consumption of the leaf and then the larvae from each treatment were divided into groups of four, each group containing 25 larvae. The larvae from each different treatment were then shifted to BOD incubators (Sanyo) that had been preset with temperatures of 15ºC, 30ºC and 33ºC separately. The treatment, 25±2ºC (room temperature) served as control for comparison purpose. The rearing of the treated larvae was continued for ten days at the selected temperatures. On tenth day, the larvae from each treatment were homogenized individually in one ml of distilled water and the spores obtained from the homogenate of each individual larva were quantified using Neubar haemocytometer.

RESULTS

Rearing of healthy CSR2 silkworm breed: CSR2 breed of silkworm, *Bombyx mori* L. was brushed and reared successfully following the standard method (Datta, 1992). The results are presented in Table 1.2. The eggs were light whitish yellow when laid and turned into brown after diapause (Fig. 1.1a and b**)**. The larvae were plain without larval marking, plumpy and bluish white (Fig. 1.1c). The average larval period was 23-24 days and average weight of mature larva is 4.59±0.1g. The larvae were healthy and did not develop any disease. The pupae were brown in colour with oval in shape (Fig. 1.1d) and the pupal period was 12.50±0.53 days. The average pupation rate was 85-90%. The cocoons were oval shaped and white in color with less percentage of floss (Fig. 1.1e). The average cocoon and shell weight and silk ratio was 1.80-1.95g, 0.45-5.00g and 23.80-25.4 respectively. The moths were active and creamish yellow in color and were free of microsporidian infection (Fig. 1.1f). The average fecundity was 500-550.

Isolation and purification of NIK-5hm microsporidian spores: The percoll gradient centrifugation resulted in appearance of three clear bands (Fig. 1.2). The top band (B_1) lying 1/4th distance from the top of the tube consisted of host tissue. The second band (B_2) about the middle distance from the top of the tube consisted of dead and immature spores. The third band (B_3) lying 1/80th distance from the top of the tube consisted of mature spores. The repeated washing of the spores collected from the band B_3 with distilled water by repeated centrifugation yielded final sediment of pure live spores. The spores were suspended in physiological saline (0.85% NaCl) and stored at 4°C.

Conformity of the pathogen to Koch's postulates: The *per os* inoculation of the microsporidian spores from NIK-5hm developed infection in healthy silkworm of CSR2 breed. The larvae inoculated on the zero day of 3rd instar developed specific infection and symptoms by inoculated pathogen and it followed the principles as indicated in Koch's postulate**.** The microsporidia caused larval mortality 26.33% and 53.67 % in PM and CSR2 breeds respectively. At pupal stage the mortality was 15.67% and 18.00% in PM and CSR2 breeds respectively. At moth stage the infection was ranged from 13.33% (Pure Mysore) to 26.67 (CSR2) (Table 1.3). The microscopic examination of homogenate of dead larva, pupa and moth indicated the presence of microsporidia inoculated. The progeny from the infected moth also had the infection indicating that the infection is transmitted to progeny from parent and it ranged from 84.00% (PM) to87.33% (CSR2). The isolation and purification of spores from the larva, pupa and moth and inoculation of the isolated spores to the two breeds during the first instar also resulted in infection. The microsporidian infection was observed in all stages of silkworm life cycle and the spores were recovered from the homogenate of dead larva, pupa and moth of the two tested silkworm breeds. Infection in progeny larva ranged from 31.67% (PM) to 59.33 (CSR2), pupa from 19.33% (PM) to 22.67% (CSR2). These observations were in conformity with the principles of Koch's postulates.

Morphological Characterisation of spores of NIK-5hm microsporidia

Light microscopy and micrometry: The observation on the morphological Characterisation of the spores of NIK-5hm microsporidia is presented in Table 1.4 and Fig. 1.3 and 1.4. The purified microsporidian spores isolated from the CSR2 breed of silkworm were ovo-cylindrical measuring 4.58±0.009 μm in length and 2.10± 0.01μm

in width. The spore length-width ratio was 2.18:1. The spores differ distinctly from the microsporidian spores of *Nosema bombycis* which are oval or ovoidal, measuring 3.10± 0.008μm and 2.05±0.007 μm. in length and width respectively. The length and width ratio was 1.51:1. The wet mount of the NIK-5hm microsporidian spore exhibited the characteristic brownian's movement and high refractive index as exhibited by *N.bombycis* and other microsporidian spores.

Scanning and transmission electron microscopy: The Scanning electron microscopy (SEM) of spores of NIK-5hm microsporidia has indicated that the surface of the spore is smooth with a slight depression at 2/3th of length of the spore. It is differs from the spores of *N. bombycis* (Fig. 1.5 and 1.6). The internal ultrastructure of spore of NIK-5hm microsporidia shows that the spore has polar tube with 12 coils, 2 nuclei and a posterior vacuole (Table 1.5, Fig. 1.7 and 1.8). The average single coil length is 0.112μm and width 0.131μm.The coil length-width ratio was 1:0.75. In the case of *N. bombycis* the spore polar tube has 12 coils with an angle of tilt. The average single coil length and width is 0.105μm and 0.130μm. The coil length-width ration was 1:1.23. The spore wall of NIK-5hm microsporidia consists of a thick and uniform endospore coat. The exospore was thinner than endospore. The spore consists of a posterior vacuole, two nuclei and a mushroom shaped anchoring disc.

Determination of Median Lethal concentrations (LC_{50})

For finding Median Lethal concentrations (LC_{50}) of NIK-5hm and *Nosema bombycis* five different inoculum doses were used i.e., 1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7 and 1×10^8 to test 3rd instar silkworms of PM and CSR2 breeds. The LC_{50} values were calculated for 12 DPI. The larvae were *per orally* inoculated with NIK-5hm and *Nosema bombycis* inoculum of above mentioned concentration @ 1ml of inoculum/ 100 sq.cm leaf /100 larvae. Three replications for each concentration were maintained. A normal control batch fed on mulberry leaves smeared with distilled water was also maintained for comparison. The larvae were observed everyday for macroscopic symptoms of pebrine symptoms for a period of 12 days and recorded the larval mortality due to the pathogens. The observations were recorded and analyzed to determine the LC_{50} value. The percent mortality was calculated as per formula given below.

$$\text{Percent mortality} = \frac{\text{Number of dead larvae}}{\text{Number of dead larvae}} \times 100$$

Total number of larvae

The LC_{50} and fiducial limits were calculated following the Probit analysis method (Finney, 1971) by utilizing Indostat software package. The results are presented in Table 1.6 and 1.7.

Virulence: The result of studies on the virulence of NIK-5hm microsporidian and *N. bombycis* to PM and CSR2 silkworm breeds is presented in Table 1.8. It is observed that the NIK-5hm microsporidia caused larval and pupal mortality at a concentration of 1×10^3 spores /ml but relatively higher from 1×10^6spores /ml both in Pure Mysore and CSR2 breeds. At the concentration 1×10^6 spores / ml the larval mortality was 26.67 % in PM breeds while in CSR2 breed it was 54.00 %. In CSR2 and PM larval mortality was recorded at concentration of 1×10^7 spores/ml was 54.33 % in PM and 100.00 % in CSR2. 1×10^8 spores / ml caused 100.00 % mortality in PM and CSR2. The total infection (inclusive of mortality in larva and pupa) ranged from 17.33% (1×10^3 spores / ml) to 100.00% (1×10^8 spores / ml) in PM where in CSR2 it was 88.83% (1×10^3 spores / ml) to 100.00% (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 total infection (inclusive of mortality in larva and pupa) ranged from 15.33% (1×10^3 spores / ml) to 100.00% (1×10^8 spores / ml) in PM where in CSR2 it was 85.33% (1×10^3 spores / ml) to 100.00% (1×10^8 spores / ml).

The LC_{50} and fiducial limits for NIK-5hm microsporidia and *N. bombycis* were calculated for larval mortality with regards to PM and CSR2 silkworm breeds following the Probit method and is presented in Table 1.8. The LC_{50} value estimated for 12 days PI for NIK-5hm microsporidia to PM breed was $1\times10^{6.7}$ spores/ml with the $10^{6.529}$ and $10^{6.932}$ as Upper and lower fiducial limits. The LC_{50} value of the NIK-5hm microsporidian to CSR2 breed was $1\times10^{5.6}$ spores with $10^{5.526}$ and $10^{5.856}$ as upper and lower fiducial limits. The LC_{50} value estimate for 12 DPI for *N. bombycis* to PM breed was $1\times10^{7.5.}$ spores/ml with $10^{7.275}$ and $10^{7.781}$ as Upper and lower fiducial limits. The LC_{50} value of *N. bombycis* to CSR2 breed was $1\times10^{6.1}$ spores/ml with $10^{5.998}$ and $10^{6.359}$ as upper and lower fiducial limits. From the above result, it is observed that the NIK-

5hm microsporidian is more in virulence as compared to *N. bombycis.*

Susceptibility: The results of screening of sixteen bivoltine breeds for susceptibility to NIK-5hm and *N. bombycis* are presented in Table 1.8. It is observed that the breeds inoculated with NIK-5hm and *N. bombycis* were shows retarded growth. The larvae of CSR2 silkworm breed is most susceptible toNIK-5hm and *Nosema bombycis* microsporidia with total mortality of 54.00±1.73% followed by CSR2(SL) (52.33±1.15%), CSR5 (50.00 ±3.00%), CSR4 (49.33±1.52), CSR18 (49.33±1.52), CSR19 (47.33±2.51), IBF (45.00±2.00), CSR50 (44.33±2.08), CSR51 (42.67±2.51), NOSEMA BOMBYCIS4D2 (40.67±1.52%), 34T (39.00±1.00), SD7 (37.33±1.52), SD12(36.00±1.00), 5N (34.00±1.00%), Boropolo (32.67±1.52%) and Diazo breed was least susceptible with mortality of (31.00. ± 1.73).

Similar trend was observed in *N. bombycis* also, the results were presented in Table 1.9. The larvae of CSR2 silkworm breed shows less mortality compare to NIK-5hm was 51.00±1.00% followed by CSR2(SL) (51.00±0.00%), CSR5 (50.00±3.00%), CSR4 (48.67± 0.57), CSR18 (48.33±0.57), CSR19 46.67± 2.88), IBF (44.33±1.52), CSR50(43.00±1.73), CSR51 (42.00±1.73), NOSEMA BOMBYCIS4D2 (40.00±2.00%), 34T (37.33± 1.15), SD7 (36.67±1.52), SD12(35.33±11), 5N 32.67±2.51%), Boropolo (31.67±1.15%) and here also and Diazo breed was least susceptible with mortality of (29.00 ±2.00).

The results of screening of thirteen multivoltine breeds for susceptibility to NIK-5hm and *N. bombycis* are presented in Table 1.10. It is observed that the breeds inoculated with NIK-5hm and *N. bombycis* showed retarded growth. The larvae of SBNP9 silkworm breed is most susceptible to NIK-5hm with total mortality of 61.33±3.60% followed by SBNP12 (60.66±4.04%), BL37 (58.67±1.15%), 96A (50.00±3.60) BL34 (50.67±0.57), TX (45.33±2.88), Madagaskar (42.00±1.00), BL69 (39.00±1.73), ND5 (37.33±1.52), NP1 (36.66±5.58%), Sarupath (34.67±3.05), Mysore Prince (27.00±2.00) Where PM breed least susceptible to NIK-5hm infection with mortality of 26.00± 1.00.

Similar trend was observed in *N. bombycis* also, the result were presented in Table 1.11. The larvae of SBNP9 and BL37 silkworm breed shows highest mortality was 56.33±1.15% followed by SBNP12 (53.00±1.00%), BL34 (49.67±2.08%), 96A (48.67± 1.52) TX (43.67±2.08), Madagaskar (40.33± 2.08), BL69(38.00± 5.29), NP1 (37.67± 4.16), ND5 (36.00± 2.64) Sarupath (33.33±0.52), Mysore Prince (26.00± 2.00), where as PM breed was least susceptible to *Nosema bombycis* infection with mortality of 24.33±0.57.

Mode of transmission: The result of studies on the vertical transmission of NIK-5hm microsporidian in two silkworm breeds *viz.,* PM and CSR2 is presented in Fig. 1.9 and 1.10. The results were compared with rate of transmission of *N. bombycis* for both the breeds. It is observed that larvae hatched from the eggs obtained from infected male × healthy female (IM×HF) in respect of both NIK-5hm microsporidian and *N. bombycis*, in both silkworm breeds (T2, T5, T12 and T15) and healthy male × healthy female (HM × HF) *viz.,* T7 and T17 did not reveal infection in the progeny of both the breeds, while in progeny larvae hatched from eggs obtained from healthy male × infected female (HM × IF) *viz.,* T1, T4, T11 and T14 as well as infected male × infected female (IM × IF) *viz.,* T3, T6, T13 and T16 revealed infection.

The F1 progeny larval examination of NIK-5hm microsporidian infected male x infected female (IM×IF) *viz.,* T3, and T13 revealed infection in all the breeds. The rate of transmission in PM and CSR2 breeds were ranged from 79.33±0.00 to 81.33±1.53 and 84.00±4.58 to 86.33±3.79 respectively. The F1 progeny larval examination of *N. bombycis* infected male × infected female (IM × IF) *viz.,* T6, and T16 revealed infection and the rate of transmission was 100% in both the breeds. The progeny larval examination of NIK-5hm microsporidian and *N. bombycis* infected male × healthy female (IM×HF) *viz.,* T2, T5, T12, and T15 did not reveal infection in the progeny and the rate of transmission was nil with respect to both the breeds tested. The progeny of the control group (HM × HF) *viz.,* T7 and T17 of all the two breeds with no inoculation with any of the microsporidians (NIK-5hm microsporidian and *N. bombycis*) did not reveal infection and the rate of transmission was nil. No transmission of either NIK-5hm microsporidian or *N. bombycis* to the progeny occurred in the healthy female × infected male (HF×IM) crosses, indicating that there was no venereal pathway for either

microsporidian. Only the female moth transmits the infection and male moth does not transmit to next progeny.

Biochemical Characterisation of NIK-5hm microsporidian

Sodium dodecyle sulphate (SDS) - polyacrylamide gel electrophoresis (PAGE): NIK-5hm microsporidian or *N. bombycis* spore surface proteins were successfully isolated, purified and quantified. The spore surface protein were collected and subjected to SDS-PAGE electorphoretic study with 10 % SDS and the banding pattern obtained from NIK-5hm microsporidian spore and spores of *N. bombycis* are shown in Fig. 1.11. The protein profile of the solubilised spore surface protein showed five major bands in the electrophoresis analysis of NIK-5hm microsporidia (Fig. 1.11, lane 3). The major peptide bands were of molecular weight 64, 59, 31, and 20 kDa. In case of *N. bombycis,* six bands of molecular weight 64, 59, 31, 29, 27 and 20 kDa were observed (Fig. 1.11, lane 2). In case of spore of NIK-5hm microsporidian, 31kDa band is of high intensity than the corresponding band in *N. bombycis*. The 28kDa band is of diffused type in NIK-5hm microsporidia. In case of *N. bombycis* the 20 kDa band is comparatively more intense than NIK-5hm microsporidia.

Serological Affinity: The results of affinity test are presented in Table 1.13**.** The spore of microsporidia from NIK-5hm did not react positively with monoclonal antibody based agglutination kit of *Nosema bombycis*, *Nosema* strains *viz.,* M11 and M12 indicating that NIK-5hm microsporidia is serologically different from them. The antibody did not cause agglutination of spores of *N. bombycis,* indicating that the NIK-5hm microsporidia is serologically different from the standard strain *N. bombycis*.

Effect of different temperature regimes on the infection and sporulation of the isolated microsporidia and the standard strain, *Nosema bombycis*:

The results of the investigation with regard to the impact of different temperature regimes on the sporulation of the isolated microsporidia are presented in Table 1.9 and graphically represented in Fig. 1.19. The data shows that at 15^{o}C, there was no sporulation in the NIK-5hm microsporidian tested as well as in the standard strain

Nosema bombycis. With respect to different temperatures, the highest spore production (33.3×10^6 spores/ml spores/ml) in NIK-5hm microsporidian under study was observed at 33ºC and followed by 30ºC in NIK-5hm (32.7×10^6 spores/ml). At 25±2ºC, the sporulation was relatively low (28.3×10^6, spores/ml) when compared to *Nosema bombycis*, the sporulation was much higher at 33ºC (39.7×10^6) followed by 30ºC (29.3×10^6) and 25ºC (24.3×10^6) spores/ml respectively. From this study it is observed that NIK-5hm shows high rate of multiplication between 25ºC-30ºC as the temperature increases the above 30ºC the rate of multiplication very low when compared to *Nosema bombycis* which clearly indicate that the NIK-5hm microsporidian is temperature sensitive than *Nosema bombycis.*

DISCUSSION

The literature of recent period on microsporidiosis in silkworm indicates that the silkworm microsporidiosis is caused by *N. bombycis,* different species/strains of *Nosema* and by several other microsporidians (Kawarabatta, 2003). The microsporidians infecting silkworm have been isolated from different breeds of silkworm from different locations (Tanaka *et al.,* 1972; Abe, 1979; Lim *et al.,* 1982; Fujiwara, 1980; 1984a; 1985; Fang *et al.,* 1991; Iwano and Ishihara, 1991; Baig, 1994; Ananthalakshmi *et al.,* 1994; Hatakeyama *et al.,* 2000; Hayasaka *et al.,* 2002; Dash and Nayak, 2003; Sasidharan *et al.,* 2003; Canning *et al.,* 2004; Wang *et al.,* 2005; Selvakumar *et al.,* 2005; Mohanan *et al.,* 2005) and many of them have been characterized (Nageswara Rao *et al.,* 2005). Greater emphasis on understanding the microsporidiosis is often made by researchers as the disease caused by microsporidia in silkworm is most destructive. The history of sericulture speaks of microsporidiosis in silkworm that resulted in wiping off of sericulture in several European countries (Tatsuke, 1971). The destructive potential of the microsporidiosis in sericulture is attributed to their virulence and efficient mode of spread and transmission of infection to the progeny (Singh and Sartchandra, 2003; Kawarabata, 2003).

In India, a few microsporidians have been isolated from silkworm and charectarised (Ananthalakshmi *et al.,* 1994; Sasidaharan *et al.,* 2003; Nageswara Rao *et al.,* 2004; 2005). But the genesis of the present study is a chance while testing the larval sample in silkworm pathology laboratory, CSRTI, Mysore. Initial observation revealed

the presence of large number of spores in the haemolymph of the infected larval sample, Subsequent survey, sample collection and preliminary study revealed that the newly isolated pathogen was highly virulent and has potential of causing serious damage to the industry. Keeping view the importance of the microsporidiosis, the newly isolated microsporidia which was tentatively designated as NIK-5hm was made the subject of present study.

The present study was of much importance to understand and manage microsporidiosis in silkworm caused by NIK-5hm or any other microsporidians. In the present study, the NIK-5hm microsporidia infecting breed of the silkworm was isolated, purified following the standard method (Sato and Watanabe, 1980) and established its conformity to Koch's postulates in causing the infection in CSR2 and other silkworm breeds. The NIK-5hm microsporidia infects and causes infection both Pure Mysore and CSR2 breeds of silkworm and cause infection to an extent of 55.33 and 98.34% respectively. This confirms that the NIK-5hm microsporidia is not specific to PM and CSR2 breeds but could infect other silkworm breeds. The present study notes that the CSR2 breed is of great economic importance except for its low tolerance to microsporidiosis. The cocoons weighs 1.800 to 1.900 g with 0.45 g to 0.50 g of shell and 23.80 to 25.40 % of silk content. The cocoons are oval shaped, white in color having low percent of floss. However, the breed has high rate of survival with 85-90 %. Nik-5hm infection form a major source for microsporidian infection to any productive and economically important silkworm breeds introduced in the area where it may turn out to be more virulent and cause significant loss to cocoon crop.

In the present, study the microsporidian NIK-5hm which is endemic in the breed of silkworm has been characterized. The spores are the characteristic feature of microsporidia. They are most distinct, unique. The result confirms that the microsporidia isolated from CSR2 breed posses all the characteristic features of a typical microsporidia. The spore of NIK-5hm microsporidia exhibits characteristic Brownian's movement and posseses unique extrusion apparatus which are of taxonomic value (Sprague, 1977; 1982; Issi, 1986; Canning, 1990; Sprague *et al.,* 1992; Canning and Vavra, 2000). Though the NIK-5hm microsporidia exhibits features of typical microsporidiosis, there are significant differences.

The microsporidian spores NIK-5hm is ovo-cylindrical having a length of 4.58µm and width of 2.10µm while the spore of *N. bombycis* is oval with a length of 3.10µm and 2.05µm width. Similarly other microsporidians isolated from silkworm differ in their spore size and shape. They differ from 2.5 to 5.1 µm in length and 1.3 to 2.8 µm in width and from oval to almost round, cylindrical to ovo-cylindrical in shape (Keeling and Fast, 2002; Kawarabatta, 2003; Sasidharan, *et al.,* 2003; Singh and Saratchandra, 2003). The Scanning electron microscopy (SEM) of the microsporidian spores of NIK-5hm breed indicates presence of a distinct depression at 3/4th of length of the spore. Such a depression was not observed on the surface of *N. bombycis.*

The internal structure of spore of NIK-5hm microsporidian and *N. bombycis* has several similarities. It indicates the presence of extrusion apparatus which is unique for microsporidians (Issi, 1986; Canning, 1990). It occupies most of the spore and consists of the polar filament with its anchoring apparatus and the polaroplast. There is also posterior vacuole and nucleus. The polar filament has 12-coils. The average single coil length is 0.082µm and width is 0.072. The coil length width ratio is 1:1.14. The lumen of the filament is electron dense. The coils also exhibit certain degree of tilt. The number of coils, their arrangement is related to one another and the angle of tilt is of taxonomic value for a particular species (Burges *et al.,* 1974; Sprague *et al.,* 1992; Keeling and Fast, 2002). In *N. bombycis*, also 12 coils were observed. The average single coil length and width is 0.079 and 0.071µm respectively. The coil length width ratio is 1:1.11. In microsporidia, the number of coil varies from 3-5 in *Encephalitozoon cuniculi* (Petri and Shiodth, 1966) to 44 in *Nosema apis* (Scholtyseck and Danneel, 1962).The number of coils were 7-9 in *N. galerucellae*, 8-10 in *N. couilloudi*, 15-18 in *N. nisotrae,* 12-14 in *N. birgi.* (Toguebaye and Marchand, 1984, 1986, 1989; Toguebaye and Bouix, 1989; Yaman and Radek, 2003).

NIK-5hm microsporidian also differ from *N. bombycis* spore surface protein banding pattern obtained through Sodium dodecyle sulphate (SDS) - polyacrylamide gel electrophoresis (PAGE). The banding pattern of NIK-5hm microsporidian shows 5 distinct bands while of *N. bombysis* spore showed 6 protein bands. The band at 31kDa was the unique band for NIK-5hm microsporidia. It was of high intense than the corresponding band in of *N. bombycis*. The 28 kDa band is of diffused type in NIK-5hm microsporidian spores. In addition a high intensity 20kDa band is observed in spore

surface protein of *N. bombycis*, which is thinner in case of NIK-5hm microsporidia.

Investigations on the mode of transmission in two breeds indicate that the NIK-5hm 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 NIK-5hm microsporidia in two-silkworm breeds *viz.,* Pure Mysore and CSR2 indicate that the infection is transmitted to the progeny as does *N. bombycis* (Table 1.12). NIK-5hm microsporidian infected male moth paired with healthy female moth lay eggs whose progeny were free of infection. NIK-5hm 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. NIK-5hm microsporidian infected female moth paired with NIK-5hm 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 NIK-5hm microsporidia infection in Pure Mysore and CSR2 breeds was comparatively lower than transmission rate by *N. bombycis*. The rate of NIK-5hm microsporidian transmission in progeny of two tested breeds ranged from 79.33±0.00% to 86.33±3.79%. However the rate of transmission in progeny of the two 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 Ananthalakhsmi *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±0.4% and 1.2±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 NIK-5hm microsporidia or *N. bombycis* in the silkworm were negative to venearl 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.

Observation on the susceptibility of the NIK-5hm microsporidia to different breeds of the silkworm further confirms that CSR2 was most susceptible to NIK-5hm microsporidia as well as *N. bombycis*. Difference in susceptibility level 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). PM is least susceptible to both NIK-5hm microsporidan as well as *N. bombycis*. It is also observed that PM was comparatively more resistant to microsporidia infection. Screening of the PM along with other twelve breeds for their susceptibility to NIK-5hm microsporidian and *N. bombycis* indicated that PM is comparatively more tolerant than other breeds to not only NIK-5hm microsporidian but also *N. bombycis*.

Apart from the differences exhibited by NIK-5hm microsporidia in the form of morphology, ultra structure and electrophoretic banding patteren of spore surface protein, the NIK-5hm microsporidia are also serologically different from *N. bombycis*, *Nosema* sp. M11, *Vairiomorpha* M12 *etc.* The monoclonal antibodies of *N. bombycis*, *Nosema* sp. M11 *Vairimorpha* sp. M12 did not recact positively with spore surface of NIK-5hm microsporidian. These results indicate that the NIK-5hm microsporidian is serologically different from *N. bombycis*, *Nosema* sp. M11 and *Vairimorpha* sp M12.

These observations indicate that the microsporidia NIK-5hm infecting CSR2 breed of the silkworm have distinct characteristic features of general microsporidia and

differ from *N. bombycis*, the most common strain of microsporidia on several counts such as spore morphology and its internal structure, electrophoretic banding pattern of spore surface protein, serological affinity. Susceptibility and transmission. However, it is essential to study the histopathological, haematological and Biochemical changes during the course of progressive infection of NIK-5hm *etc.* for better understanding of the host pathogen relations.

Table 1.1: Characteristic features of microsporidians infecting silkworm

Microsporidian isolates	Spore shape	Spore size (μm)		Virulence
		Length	Width	
Nosema bombycis	Oval	3.8	2.6	High
Nosema sp. (NIS-M11)	Oval	3.9	1.9	Low
Nosema sp. (NIS-M14)	Oval	4.1	2.3	High
Nosema sp. (NIK-2r)	Ovidal	3.6	2.8	High
Nosema sp. (NIK-3h)	Ovo cylindrical	3.8	1.8	Low
Vairimorpha sp. (NIS-M12)	Ovo cylindrical	4.5	2.0	Low
Vairimorpha sp. (NIK-4m)	Ovo cylindrical	5.0	2.1	High
Microsporidium sp. (NIS-25)	Oval	4.9	2.8	Low
Pleistophora sp.(NIS-M24)	Oval	2.7	1.6	Low
Pleistophora sp.(NIS-M27)	Ova	5.4	3.0	Low
Thelohania sp.(NIS-M32)	Oval	3.4	1.7	Low
Lamerin(Lbm)	Oval	4.4	2.1	Low

Table 1.2: Characteristic features of the CSR2 breed of silkworm

Metamorphic stage	Characteristic features
Egg	Light whitish yellow at the begining and turns brownish on hibernation.
Larva	Plain with bluish white body colour Weight: 4.59±0.1g Larval period: 23-24 days.
Cocoon	Cocoons were oval shaped, white in color Single cocoon weight: 1.80-1.95g, Single shell weight: 0.45-0.50g Silk content is about 23.80-25.4%
Pupa	Relatively big and brown in color. Pupation rate is 85-90 % Pupal period: 12.50±0.53 days.
Moth	Relatively big, creamish yellow. Fecundity: 566±10.26.

Table 1.3: Infectivity of NIK-5hm microsporidian in PM and CSR2 breeds of silkworm

Silkworm breeds	Mortality (%)		% Infe-ctivity in moth stage	Total % infection and mortality	% infection in progeny	% Mortality in F1 progeny by microsporidiosis	
	Larva	Pupa				Larva	Pupa
PM	26.33	15.67	13.33	55.33	84.00	31.67	19.33
CSR2	53.67	18.00	26.67	98.34	87.33	59.33	22.67
CD@5%	1.53	1.00	2.52	-	4.04	2.08	1.53

Table 1.4: Morphlogical features of NIK-5hm and *N.bombycis* microsporidians

Microsporidian isolates	Form	Spore size (µm)		Length/width ratio
		Length±S.D	Width±S.D	
NIK-5hm	Ovo-cylindrical	4.58±0.01	2.10±0.01	2.18:1
N.bombycis	Oval	3.10±0.01	2.05±0.01	1.51:1

Table 1.5: Spore polar filament characteristics of NIK-5hm and *N. bombycis* microsporidians

Microsporidian isolates	No. of coils of polar tube	Coil size (µm)		Coil length / width
		Length	Width	
NIK-5hm microsporidian	12	0.08	0.07	1:1.14
N. bombycis	12	0.07	0.07	1:1.11

Table 1.6: Comparative virulence of NIK-5hm and *N. bombycis* to different silkworm breeds

Microsporidian isolate	Doses Spores/ml	Pure Mysore				CSR2			
		% larva dead	% Pupa dead	% Moth infected	Total % infection	% larva dead	% Pupa dead	% Moth dead	Total % infection
NIK-5hm	1×10^3	8.00	5.33	4.00	17.33	9.00	7.67	71.67	88.33
	1×10^4	11.33	8.67	7.67	27.67	14.67	13.33	64.67	92.67
	1×10^5	18.00	12.00	11.67	41.67	25.00	16.67	55.00	96.67
	1×10^6	26.67	16.67	13.33	56.67	54.00	18.67	27.33	100.00
	1×10^7	54.33	23.00	22.67	100.00	100.00	0.00	0.00	0.00
	1×10^8	100.00	0.00	0.00	100.00	100.00	0.00	0.00	0.00
	CD@5%	2.52	1.62	2.75	2.33	1.33	2.10	2.62	1.03
N. bombycis	1×10^3	6.67	4.67	4.00	15.33	7.67	6.33	71.33	85.33
	1×10^4	9.67	7.67	7.67	25.00	12.33	11.33	67.33	91.00
	1×10^5	15.67	11.67	11.67	39.00	22.00	13.33	58.00	93.33
	1×10^6	24.33	16.33	13.00	53.67	51.33	15.67	33.00	100.00
	1×10^7	53.00	22.67	24.33	100.00	100.00	0.00	0.00	0.00
	1×10^8	100.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00
	CD@5%	1.51	2.14	2.62	1.18	1.03	2.75	2.69	1.58

Table 1.7: LC_{50} and fiducial limits of NIk-5hm microsporidia and *N. bombycis* to different silkworm breeds

Treatment	X mean	Y mean	Probit Equation	Chi square	S.E-b	Fiducial limit	LC_{50}
Pure Mysore							
NIk-5hm	6.3482	4.7562	Y=0.71+0.63X	3.793NS	0.0911	$10^{6.529}$-$10^{6.932}$	$1\times10^{6.7}$
N. bombycis	6.7540	4.4824	Y=-0.04+0.67X	2.602NS	0.1227	$10^{7.275}$-$10^{7.781}$	$1\times10^{7.5}$
CSR2							
NIk-5hm	5.6593	4.9740	Y=0.36+0.82X	6.401NS	0.1068	$10^{5.526}$-$10^{5.856}$	$1\times10^{5.6}$
N. bombycis	6.0098	4.8790	Y=0.58+0.72X	7.819NS	0.0976	$10^{5.998}$-$10^{6.359}$	$1\times10^{6.1}$

NS: Non-significant

Table 1.8: Susceptibility of different bivoltine silkworm breeds to the NIK-5hm microsporidia.

Silkworm breed	% larval mortality	% pupal mortality	% moth emerged	% moths infected	% of total infection
CSR2	54.00±1.73	18.67±1.53	0.00	27.33±3.06	100±0.00
CSR2 (SL)	52.33±1.16	19.33±0.58	0.00	28.33±0.58	100±0.00
CSR5	50.00±3.00	20.67±1.16	0.00	29.33±3.22	100±0.00
CSR4	49.33±1.53	19.67±1.53	0.00	31.00±1.00	100±0.00
CSR18	49.33±1.53	20.67±1.53	0.00	30.00±2.00	100±0.00
CSR19	47.33±2.52	22.33±1.53	0.00	30.33±1.16	100±0.00
IBF	45.00±2.00	27.00±1.00	0.00	28.00±1.00	100±0.00
CSR50	44.33±2.08	29.00±2.00	0.00	26.67±0.58	100±0.00
CSR51	42.67±2.52	28.67±0.58	0.00	28.67±2.08	100±0.00
NB4D2	40.67±1.53	25.33±2.08	16.33±3.22	17.67±0.58	83.67±3.22
34T	39.00±1.00	18.33±2.52	26.67±2.16	16.00±1.00	73.33±2.52
SD7	37.33±1.53	17.33±1.528	29.67±1.16	15.67±1.16	70.33±1.16
SD12	36.00±1.00	18.67±0.53	30.33±0.58	15.00±1.00	69.67±0.58
5N	34.00±1.00	16.67±1.53	34.00±2.00	15.33±1.16	66.00±2.00
Boropolo	32.67±1.53	15.33±0.577	35.33±2.31	16.67±1.16	64.67±2.31
Diazo	31.00±1.73	15.33±0.58	38.00±2.65	15.67±1.16	62.00±2.65
CD at 5%	2.998	2.376	2.448	2.585	2.448

Table 1.9: Susceptibility of different bivoltine silkworm breeds to the *Nosema bombycis* microsporidia.

Silkworm breeds	% larval mortality	% pupal mortality	% moth Emerged	% moths infected	% of total infection
CSR2	51.00±1.00	16.00±1.00	0.00	33.00±1.00	100±0.00
CSR2 (SL)	51.00±0.00	18.33±0.58	0.00	30.67±0.58	100±0.00
CSR5	50.00±3.00	19.67±0.58	0.00	30.33±3.06	100±0.00
CSR4	48.67±0.58	18.67±1.16	0.00	32.67±1.53	100±0.00
CSR18	48.33±0.58	19.67±0.58	0.00	32.00±1.00	100±0.00
CSR19	46.67±2.89	20.67±0.58	0.00	32.67±3.22	100±0.00
IBF	44.33±1.53	27.33±1.16	0.00	28.33±0.58	100±0.00
CSR50	43.00±1.73	29.33±2.08	27.00±2.00	27.67±0.58	100±0.00
CSR51	42.00±1.73	29.33±0.58	0.00±0.00	28.67±1.53	100±0.00
NB4D2	40.00±2.00	26.00±1.73	16.67±3.06	17.33±0.58	83.33±3.06
34T	37.33±1.16	19.00±1.73	29.00±3.61	14.67±1.16	71.00±3.61
SD7	36.67±1.53	17.00±1.00	30.67±2.08	15.67±0.58	69.33±2.08
SD12	35.33±1.16	19.67±1.16	30.00±1.73	15.00±1.00	70.00±1.73
5N	32.67±2.52	15.67±0.58	36.00±1.73	15.67±0.58	64.00±1.73
Boropolo	31.67±1.16	14.67±0.577	37.00±1.73	16.67±1.16	63.00±1.73
Diazo	29.00±2.00	14.33±1.16	41.33±0.58	15.33±0.58	58.67±0.58
CD at 5%	2.880	1.859	2.629	2.364	2.494

Table 1.10: Susceptibility of different multivoltine silkworm breeds to the NIK-5hm microsporidia.

Silkworm breed	% larval mortality	% pupal mortality	% moth emerged	% moths infected	% of total infection
PM	26.00±1.00	17.33±1.53	43.00±2.65	13.67±0.58	57.00±2.65
M.prince	27.00±2.00	17.67±0.58	39.67±2.08	15.67±0.58	60.33±2.08
Sarupat	34.67±3.05	17.00±1.00	27.67±2.31	20.67±0.58	72.33±2.04
Madagaskar	42.00±1.00	17.00±1.00	16.33±2.52	24.67±1.53	83.67±2.52
BL69	39.00±1.73	16.66±2.08	19.00±2.65	25.33±0.58	81.00±2.65
NP1	36.667±5.86	16.33±1.16	20.00±2.65	25.33±±1.53	80.00±2.65
ND5	37.33±1.53	14.33±1.16	22.67±1.53	24.67±0.58	77.33±1.53
SBNP9	61.33±3.61	34.66±1.53	0.00	4.33±2.08	100.00
SBNP12	60.667±4.04	32.67±1.53	0.00	6.67±3.06	100.00
BL34	50.67±0.58	23.00±1.00	11.67±3.06	14.67±1.528	88.33±3.06
BL37	58.67±1.16	27.00±2.65	2.00±1.73	12.33±1.155	98.00±1.73
96A	50.00±3.606	20.33±1.16	12.00±5.57	17.67±3.055	88.00±5.57
TX	45.33±2.89	22.33±0.58	15.66±3.79	16.67±0.577	84.33±3.79
CD at 5%	4.822	2.373	4.584	2.687	4.658

Table 1.11: Susceptibility of different multivoltine silkworm breeds to the *Nosema bombycis* microsporidia.

Silkworm breed	% larval mortality	% pupal mortality	% moth emerged	% moths infected	% of total infection
PM	24.33±0.577	16.33±0.58	46.33±0.58	13.00±1.00	53.67±0.58
M.prince	26.00±2.00	17.33±0.58	42.33±±2.08	14.33±0.58	57.67±2.08
Sarupat	33.33±0.53	16.33±1.16	29.67±1.16	20.67±0.58	70.33±1.16
Madagaskar	40.33±2.08	16.67±1.16	19.00±1.73	24.00±1.00	81.00±1.73
BL69	38.00±5.30	16.00±1.00	18.67±1.53	24.67±0.58	81.33±1.53
NP1	37.67±4.16	15.67±0.58	21.67±3.51	25.00±1.73	78.33±3.51
ND5	36.00±2.65	15.00±1.00	23.67±2.89	25.33±0.58	76.33±2.89
SBNP9	56.33±1.16	32.67±2.08	3.33±3.215	7.67±1.16	96.67±3.26
SBNP12	53.00±1.00	26.67±3.22	6.00±3.00	14.33±1.53	94.00±3.00
BL34	49.67±2.08	22.67±1.16	13.67±2.08	13.00±1.00	86.33±2.08
BL37	56.33±1.16	26.33±2.89	3.67±2.89	13.67±1.155	96.33±2.89
96A	48.67±1.53	21.00±2.00	15.33±3.79	15.00±1.00	84.67±3.79
TX	43.67±2.082	21.67±1.16	18.33±3.79	16.33±0.58	81.67±3.79
CD at 5%	4.111	2.776	4.481	1.721	4.481

Table 1.12: Mode of transmission of NIK-5hm microsporidia and *N.bombycis* in different silkworm breeds.

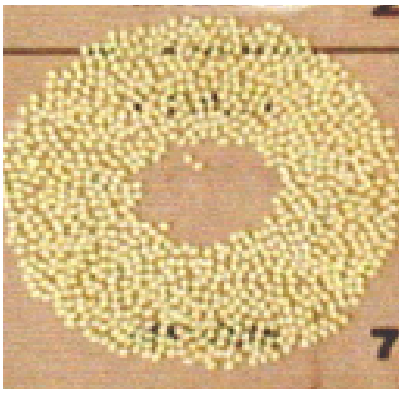
Microsporidian Isolate	Treatment	Eggs/ laying	Hatching %	Transmission percent
PM				
NIK-5hm	T21(HM× IF)	288.33±3.51	68.00±2.64	81.33±1.53
	T22(IM× HF)	402.33±4.16	91.67±1.00	0.00
	T23(IM×IF)	288.00±3.00	58.00±3.00	79.33±0.00
N.bombycis	T24(HM×IF)	284.00±4.00	65.33±2.08	100.00±0.00
	T25(IM×HF)	410.67±1.53	88.67±2.52	0.00
	T26(IM×IF)	291.00±3.61	64.33±1.53	100.00±0.00
Control	T27(HM× HF)	412.67±3.51	94.33±0.58	0.00
	CD at 5%	6.73	4.07	1.44
CSR2				
NIK-5hm	T31(HM× IF)	283±6.00	63.00±3.00	86.33±3.79
	T32(IM× HF)	520±1.53	86.33±2.52	0.00
	T33(IM×IF)	279.67±2.31	55.67±2.08	84.00±4.58
N.bombycis	T34(HM×IF)	282.00±7.81	61.00±2.00	100.00±0.00
	T35(IM×HF)	413.33±3.78	90.33±1.53	0.00
	T36(IM×IF)	285.00±8.54	60.00±1.00	100.00±0.00
Control	T37(HM×HF)	559.00±4.36	91.67±1.15	0.00
	CD at 5%	7.46	4.32	5.61

NIK-5hm microsporidian; Values are mean±SD;

Table.1.13: Serological affinity of NIK-5hm microsporidian and *N. bombycis*

Microsporidian isolates	Serological affinity of NIK-5hm Monoclonal Antibodies of		
	N.b.	M11	M12
NIK-5hm	-	-	-
N.bombycis	+	-	-

Positive reaction +; Negative reaction -



a. Non-diapausing eggs

b. Diapausing eggs

c. Fifth instar larvae

d. Pupae

e. Cocoons

f. Moth.

Fig. 1.1: Life cycle of CSR2 breed of the silkworm

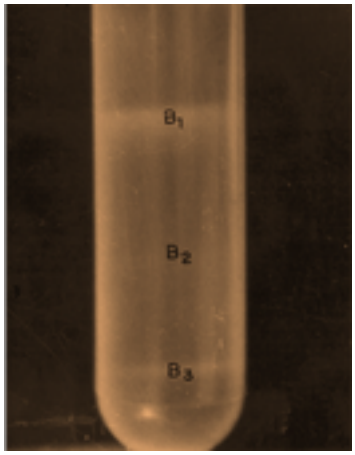


Fig. 1.2: Purification of the spores of microsporidian from NIK-5hm. Percoll gradient centrifugation showing three bands. B_1: A sharp band consisting of tissues of host, mulberry leaves, bacteria *etc*.; B_2: Light band consisting of immature and dead spores and B_3: Sharp band consisting of mature spores.

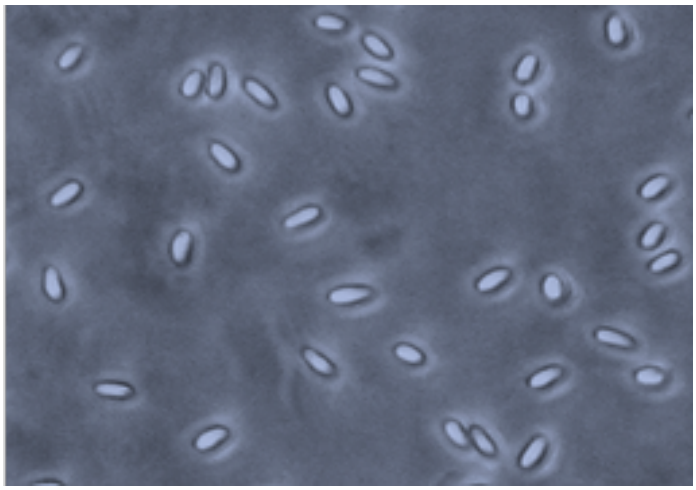


Fig. 1.3: Microphotograph of purified NIK-5hm spores (600X)

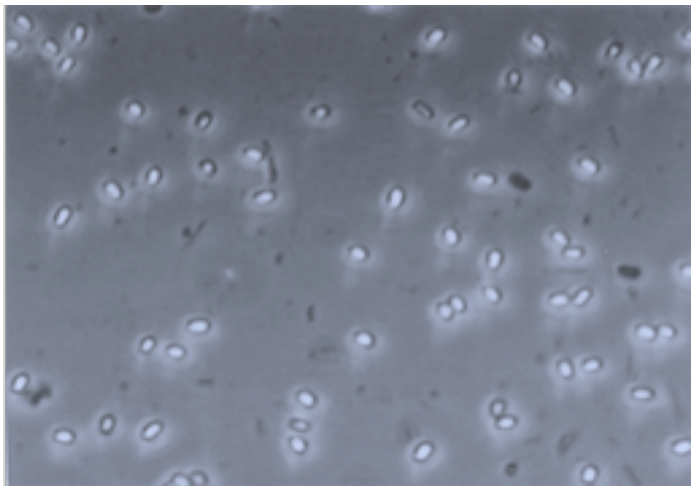


Fig. 1.4: Microphotograph of purified *Nosema bomycis* spores (600X)

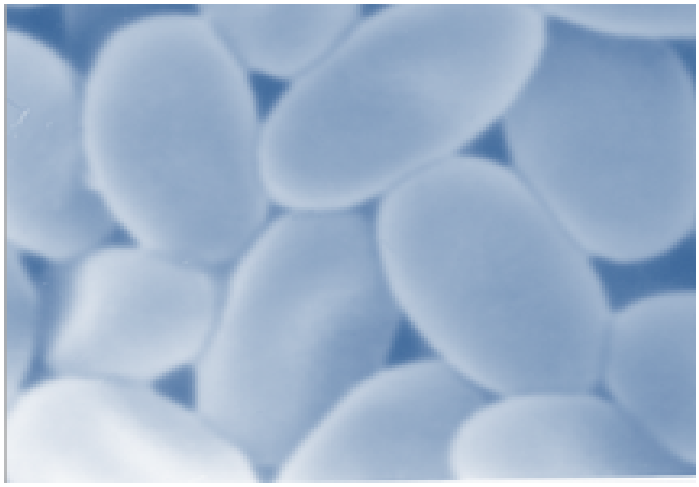


Fig. 1.5: Scanning electron microphotograph of purified spores of NIK-5hm microsporidia (20,000x) (Arrowhead showing the depression in spore)

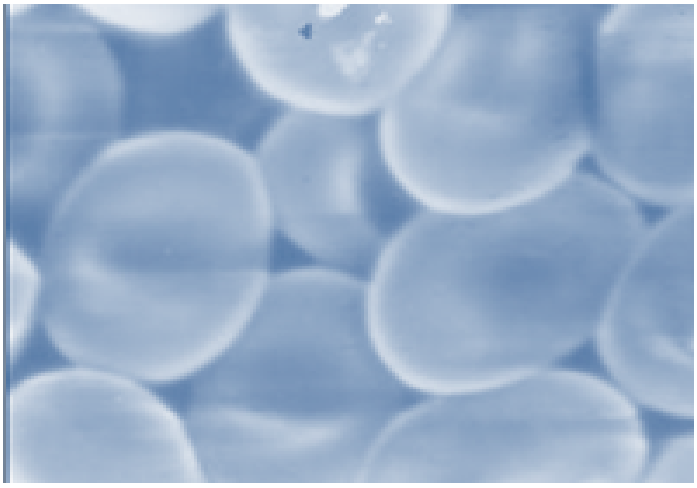


Fig. 1.6: Scanning electron microphotograph of purified spores of *Nosema bombycis* (20,000x) (Arrowhead showing the depression in spore)

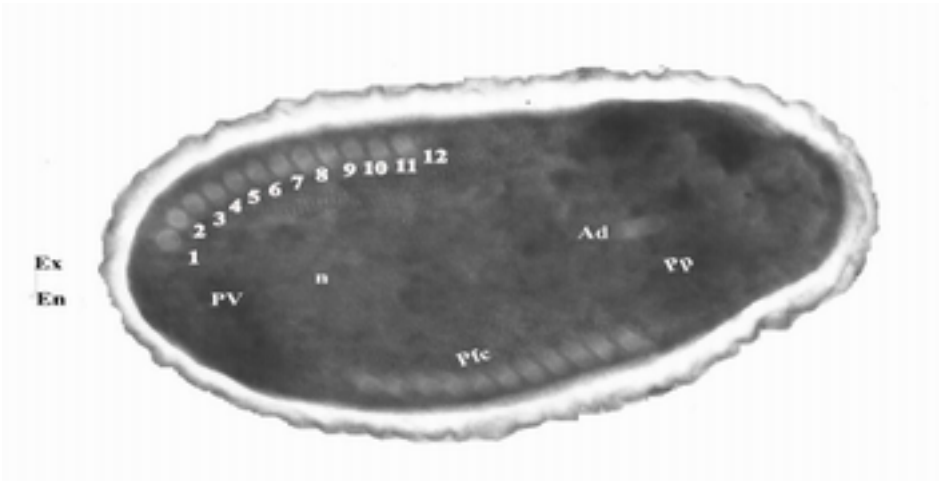


Fig. 1.7: Longitudinal section of NIK-5hm spore showing 12 coils of polar tube: pt – polar tube; Ex- exospore; En – endospore; Ad – anchoring disc (mushroom shaped); Pv – posterior vacuole; n- nucleus; (53307x)

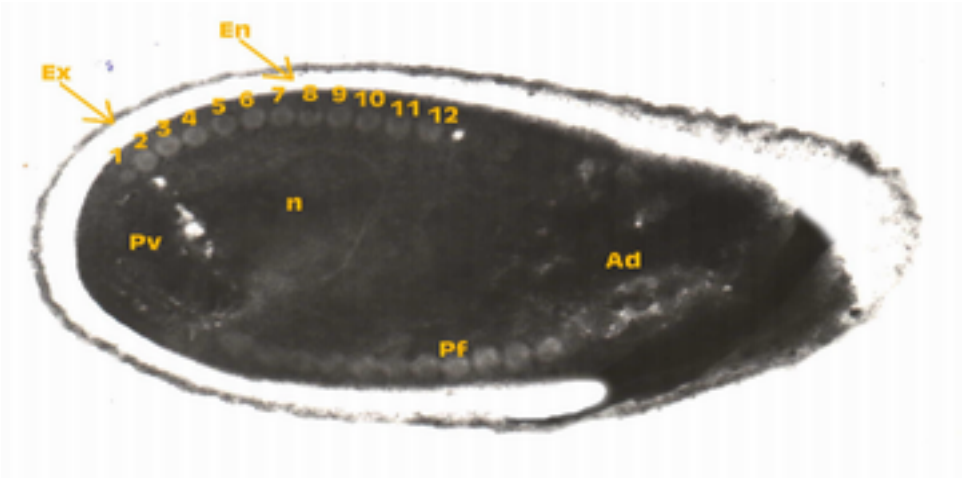


Fig. 1.8: Longitudinal section of *N. bombycis* spore showing 12 coils of polar tube: pt – polar tube; Ex- exospore; En – endospore; Ad – anchoring disc (mushroom shaped); Pv – posterior vacuole; n- nucleus; (53307x)

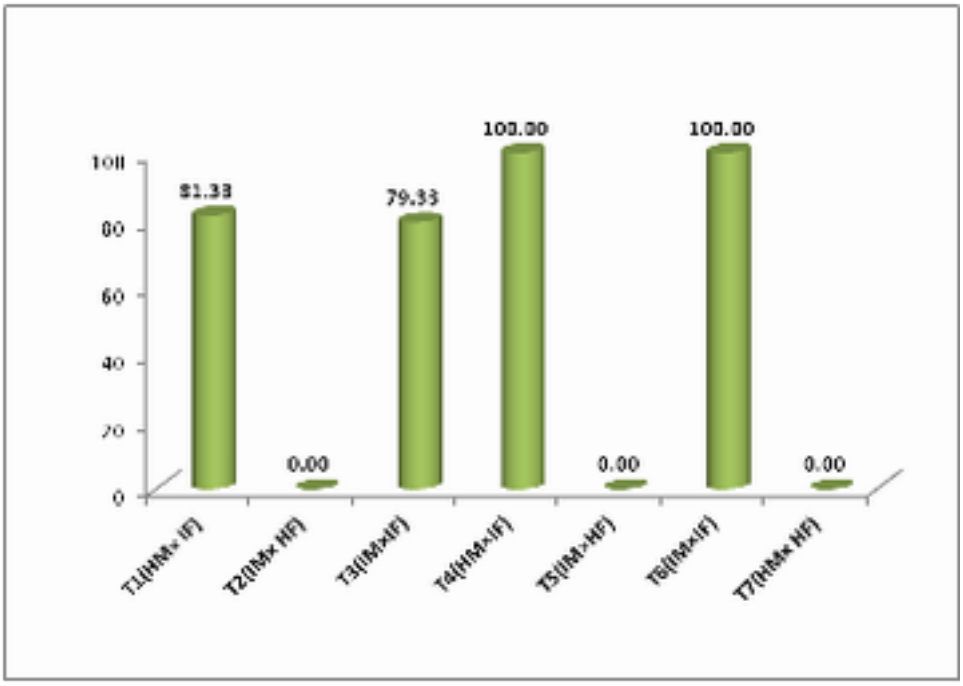


Fig. 1.9: Mode of transmission in PM

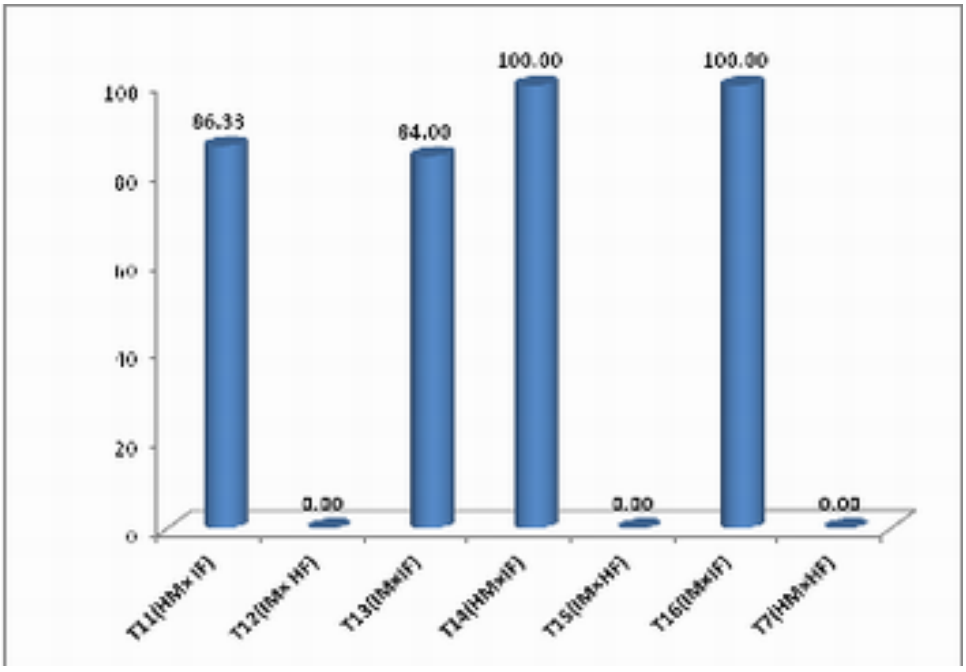


Fig. 1.10: Mode of transmission in CSR2

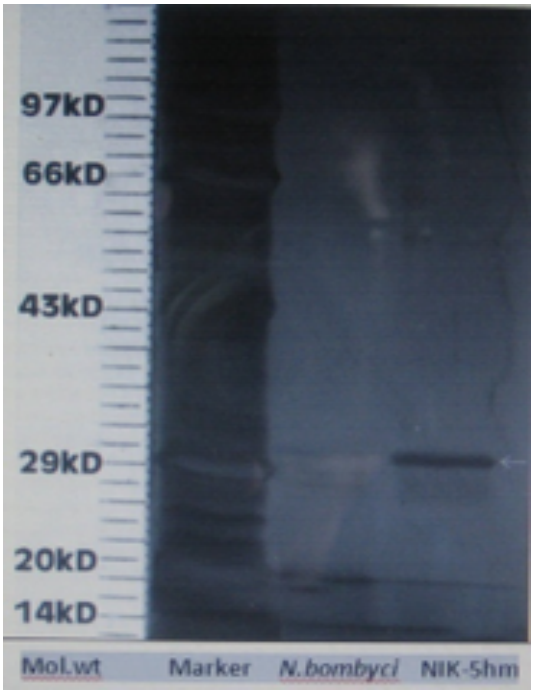


Fig. 1.11: Electrophoretic banding of spore surface protein of NIK-5hm microsporidian spore and *N. bombycis* distinguishes from *N. bombycis* (Lane 2) on the basis of thick 31 kDa band Molecular weight is given in KDa on the left side. Arrow heads shows the unique band of NIK-5hm microsporidium