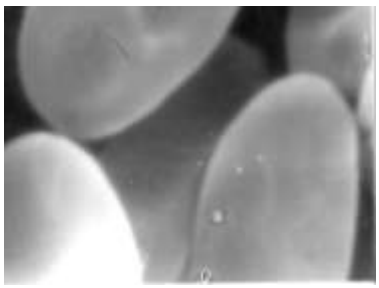
Chapter 1



ISOLATION AND CHARACTERISATION OF MICROSPORIDIAN INFECTING LAMERIN BREED OF THE SILKWORM, BOMBYX MORI L.

Microsporidia are among the 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.

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). 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).

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).

There was no published report on the microsporidian infecting the northeastern silkworm breed - Lamerin. Unlike other microsporidia infecting the silkworm breeds, the microsporidia infecting the Lamerin breed is associated with the breed for past several generations without causing much harm. The Characterisation of the microsporidian with regards to its morphology and pathology will be of immense importance in the management of the disease in Lamerin. It also provides useful information to manage different microsporidians *viz., N. bombycis*, *Vairimorpha* sp. *etc.* infection in silkworm. Isolation of the pathogen and the knowledge of its morphological and biochemical aspects are the basic requirement for further understanding of the pathogenicity and the disease. It is also essential to know the serological affinity of the microsporidia, its infectivity and the damage caused to the host. In this chapter, studies on different aspects of isolation and Characterisation of Lamerin microsporidia are presented and the results discussed.

MATERIALS AND METHODS

Collection of Lamerin breed of the silkworm, *Bombyx mori* L.: Silkworm eggs of Lamerin breed infected with microsporidia and healthy eggs were received from Regional Tasar Research Station (RTRS), Central Silk Board Imphal, Manipur, India and used in the present study.

Rearing of healthy Lamerin silkworm breed: Healthy silkworm eggs of Lamerin breed were incubated at 25±1^{o}C temperature and 80±5% RH. The larvae were brushed and reared under hygienic conditions in disinfected rearing trays and rearing room following the standard method of silkworm rearing (Datta, 1992) in Silkworm Pathology Laboratory, Central Sericulture Research and Training Institute Mysore, India. Randomly picked third instar silkworm larvae were examined for the presence/absence of microsporidian spore at 600 x magnification under Nikon (Type – 104) phase contrast microscope to confirm the healthiness of the silkworm colony. The larvae were reared further till cocooning and the moths were examined for healthiness of eggs laid by the moth.

Observations were recorded on the growth and morphometrics of larva, pupa and moth.

Isolation and purification of microsporidian infecting Lamerin breed of the silkworm: The Lamerin breed silkworm larvae hatched from the eggs marked infected with microsporidian were reared on mulberry following the standard procedure (Datta, 1992). Randomly picked third instar silkworm larvae were homogenized and examined for microsporidian spore at 600 x magnifications under Nikon (Type-104) phase contrast microscope to confirm the infection in the colony. The larvae were 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 the 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 centrifuged at 3000rpm for 15 min. The sediment obtained was suspended in minimal volume of distilled water and subjected it 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 2h. 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 isolated and purified microsporidia is the cause of the disease in Lamerin breed, the pathogen was subjected to tests for its conformity to Koch's postulates. To conduct the test, inoculum of purified spores of concentration 1×10^7 spores / ml was prepared from the stock inoculum. The stock inoculum was diluted and quantified to estimate the spore concentration following standard haemocytometer count (Cantwell, 1974) and diluted suitably to obtain inoculum of concentration 1×10^7 spores/ml. One ml of inoculum was smeared on mulberry leaves and fed to 100 third instar silkworms of Lamerin, Pure Mysore and CSR2 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 24h, the larvae were fed on uncontaminated mulberry and reared 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 sign or symptoms. The spores in the homogenate of larvae and moths were isolated and purified. These spores were fed to healthy set of silkworm to confirm its infectivity and to establish the pathogens conformity to the principles of Koch's postulates.

Morphological Characterisation of Lamerin 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 microsporidia infecting Lamerin 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 as well as photographed under phase contrast microscope Nikon (Type - 104) at 600 x magnifications. To understand the behaviour 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.

Electron microscopic studies of Lamerin microsporidian spores

Morphology and internal ultrastructure are important characteristic features of microsporidia for Characterisation. The shape, size of microsporidia, arrangement of polar filament and the number of coils of the polar filament varies in different strains of microsporidians. Electron microscopic features of Lamerin microsporidia was studied using JEOL-100 CX-11 electron microscope fitted with a (ASID-4D) scanning attachment (Tokyo, Japan 20 KV) and compared with that of *N. bombycis*.

Scanning Electron microscopic study (SEM): Spores of each microsporodia (Lamerin 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 (Lamerin microsporidia 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 ultrastructure of *Nosema* spores with that of Lamerin 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), enbloc 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^{o}C for 48hr. Semithin sections (1u) were cut with glass knives of microtome (Leica EMUC -6) placed on a hot plate at about 80^{o}C 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 - 80nm (700 –800^{o}A) 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 Characterisation of the microsporidians as the length and number of the coils of the polar filament varies among different microsporidians.

Biochemical Characterisation of Lamerin microsporidian spores

Isolation of spore surface protein: The spore surface proteins of both microsporodians (Lamerin microsporidia and *N. bombycis*) were isolated by following the method described by Kawarabata and Hayasaka (1987).The purified spores of Lamerin breed and *N. bombycis* were suspended in 0.1N K_2CO_3 (1×10^{8} spores / ml) for 30min 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 Lamerin 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, 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 Lamerin breed with different microsporidia *viz.*, *N. bombycis*, *Nosema* sp. M11 and M12 were determined following monoclonal antibody based Latex agglutination kit (Yakult, Japan) and polyclonal antibody raised against the microsporidia isolated from the Lamerin breed of silkworm.

Monoclonal antibody based affinity test: Monoclonal antibody based Latex agglutination kit (Yakult, Japan) developed for the identification of *N. bombycis*, Nosema sp. M11 and M12 was used in the present study. A drop of purified spores from Lamerin breed of silkworm was mixed with equal amount of monoclonal antibody based spore specific Latex agglutination reagent on a clean glass slide with glass rod and observed under phase-contrast microscope at 600 x magnification after 5 min of incubation at 37°C, for spore - latex bead agglutination. Five samples were observed in each category. The observation was recorded as agglutination positive (+) for positive affinity and (-) as negative indicating negative affinity of spore from Lamerin breed with the respective specific spores.

Polyclonal antibody based agglutination test: Polyclonal antibody against the purified spores of microsporidia isolated from Lamerin breed of silkworm was raised in White New Zealand breed Rabbit by following standard immunization

procedure. In brief an emulsion of the purified spores (1×10^8 spore /ml) from the Lamerin breed was prepared in complete freunds adjuvant (F5881, Sigma, USA) and injected to rabbit sub-cutaneously on either side of vertebral column at several sites. Three more inoculations of emulsion of spore and incomplete Freund's adjuvant (F5506, Sigma, USA) were given intramuscularly at an interval of 7 days from each other. After 10 days of final inoculation, the blood was collected by slitting the marginal vein of ear pinna and incubated at 37°C for 2hr and then at 4°C overnight. The serum was decanted and centrifuged at 6000 rpm for 15 min. The supernatant was used as antiserum. The antibodies present in the serum were precipitated by the addition of Amonium sulphate to obtain a concentration of 40% Ammonium sulphate in serum. The precipitate formed was sedimented, dissolved in PBS pH 7.2, dialyzed against PBS pH 7.2 and gel filtered using Sephadex – DEAE cellulose. The fragment containing the antibodies was quantified and lyophilized. The antibody was reconstituted in PBS pH 7.2 (1mg/ml) and used for affinity test. A drop of purified spores of microsporidia from Lamerin and *N. bombycis* (1×10^6) was taken separately on clean glass microslide and tested for affinity. To test, a drop of antibody was mixed with the drop of spore thoroughly using tooth pick and rotated for 2 - 3 minutes and observed for agglutination of spores under phase contrast microscope. The observation with regards to microsporidia from Lamerin and *N. bombycis* was recorded as agglutination positive (+) and agglutination negative (-).

Germination of Lamerin microsporidian spore: Hatchability or germination of spore of Lamerin microsporidia was tested following the standard method (Fujiwara, 1993) and compared with the hatchability of spores of *N. bombycis*. The spores were treated with potassium hydroxide (KOH) and neutralized. The fresh purified spores from the Lamerin breed of silkworm and the spores of *N. bombycis* (1×10^7) were incubated in different concentration (0.1, 0.3, 0.5, 0.7, 0.9 and 1%) of Potassium hydroxide (KOH) solution for 10 20 and 30min. The treated spores after the incubation period were neutralized with Phosphate buffered saline, pH 7.00 and observed for germination under phase contrast microscope at 600 x

magnifications. The germination percent was estimated by counting the hatched and unhatched spores following the standard procedure using haemocytometer. The observations were recorded. 1-40% hatching was recorded as +, 41-60% as ++, 61 – 80% as +++ and 81 -100% as ++++.

RESULTS

Rearing of healthy Lamerin breed: Lamerin breed of silkworm, *Bombyx mori* L was brushed and reared successfully following the standard method (Datta, 1992). The results are presented in table 2 and Fig 1. The eggs were light whitish yellow when laid and turned into brown after diapause (Fig.1a and b**)**. The larvae were plain without larval marking, slender and bluish white (Fig. 1c). The mature larvae measured 47.60± 2.32 mm in length and 4.82±0.30 mm in width. The average larval period was 23 days and average weight of mature larva is 2.59±0.1g. The larvae were healthy and did not develop any disease. The pupae were slender brown (Fig. 1d) and the pupal period was 11.50±0.53 days. The average pupation rate was 91±2.21% and the moth eclusion was 89.3±2.41%. The cocoons were spindle shaped orange yellow with high percentage of floss (Fig. 1e). The average cocoon and shell weight and silk ratio was 1.474±0.02g, 0.192±0.01g and 13.06±0.57% respectively.The moths were active and creamish yellow in color and were free of microsporidian infection (Fig. 1f). The average fecundity was 301±22.26.

Isolation and purification of Lamerin microsporidian spores: The percoll gradient centrifugation resulted in appearance of three clear bands (Fig. 2). The top band (B_1) lying $1/4^{th}$ 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 $1/80^{th}$ 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 Lamerin silkworm breed developed infection in healthy silkworm of Lamerin, Pure Mysore and CSR2 breeds. 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 ranging from 5.80% (Lamerin) to 11.20% (CSR2). In Pure Mysore it was 8.60%. At pupal stage the mortality ranged from 5.20% in Lamerin breed to 8.80% in CSR2 breed. In Pure Mysore the pupal mortality was 5.80%. At moth stage the infection ranged from 53.12% (Pure Mysore) to 56.95% (CSR2). In Lamerin, it was 54.29% (Table 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 57.20% (CSR2) to 59.20% (Lamerin). The isolation and purification of spores from the larva, pupa and moth and inoculation of the isolated spores to the three 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 or moth of the three tested silkworm breeds. Infection in progeny larva ranged from 12.40% (Lamerin) to 17.00 (CSR2), pupa from 6.00% (Lamerin) to 9.40% (CSR2). These observations were in conformity with the principles of Koch's postulates.

Morphological Characterisation of spores of Lamerin microsporidia

Light microscopy and micrometry: The observation on the morphological Characterisation of the spores of Lamerin microsporidia is presented in table 4 and Fig. 3 and 4.. The purified microsporidian spores from the Lamerin breed of silkworm were ovo-cylindrical measuring 4.36±0.06 µm in length and 2.14±0.01 µm in width. The spore length-width ratio was 2.03:1. The spores differ distinctly from the microsporidian spores of *Nosema bombycis* which are oval or ovoidal which measuring 3.08±0.21 µm and 2.01±0.05 µm. in length and width respectively. The length and width ratio was 1.53:1.

The wet mount of the Lamerin microsporidian spore exhibited the characteristic brownian's movement and high refractive index as is exhibited by *N.bombycis* and other microsporidian spores.

Scanning and transmission electron microscopy: The Scanning electron microscopy (SEM) of spores of Lamerin breed has indicated that the surface of the spore of Lamerin microsporidia is smooth with a depression at $1/4^{th}$ of length of the spore. It distinctly differs from the spores of *N. bombycis*. (Fig. 5 and 6). The internal ultrastructure of spore of Lamerin microsporidia shows that the spore has polar tube with 11 coils, 2 nuclei and a posterior vacuole (Table 5, Fig. 7 and 8). The average single coil length is 0.066µm and width 0.0076µm.The coil length-width ratio was 1:1.15. 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 0.078µm and 0.073µm. The coil length-width ration was 1:0.93. The spore wall of Lamerin microsporidia consists of a thick and uniform endospore coat but the exospore has ornamentation like projections. The exospore was thinner than endospore. The spore consists of a posterior vacuole, two nuclei and a mushroom shaped anchoring disc.

Biochemical Characterisation of Lamerin microsporidian

Sodium dodecyle sulphate (SDS) - polyacrylamide gel electrophoresis (PAGE): Lamerin microsporidian or *N. bombycis* spore surface protein 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 Lamerin microsporidian spore and spores of *N. bombycis* are shown in Fig. 9. The protein profile of the solubilised spore surface protein showed five major bands in the electrophoresis analysis of Lamerin microsporidia (Fig. 9, lane 3). The major peptide bands were of molecular weight 66, 50, 31, 28 and 20 kDa. In case of *N. bombycis*, six bands of molecular weight 64, 59, 31, 29, 27 and 20 kDa were observed (Fig. 9, lane 2). In case of spore of Lamerin microsporidian, 31kDa band is of high intensity than the corresponding band in *N. bombycis*.The 28kDa band is of diffused type in Lamerin microsporidia. In case of *N. bombycis* the 20 kDa band is comparatively more intense than Lamerin microsporidia.

Serological Affinity: The results of affinity test are presented in table 6**.** The spore of microsporidia from Lamerin did not react positively with monoclonal antibody based agglutination kit of *Nosema bombycis*, *Nosema* strains *viz.,* M11 and M12 indicating that Lamerin microsporidia is serologically different from them.

The polyclonal antibody was raised in New Zealand white Rabbit successfully against the spore from Lamerin microsporidia. The antibody did not cause agglutination of spores of *N. bombycis,* indicating that the Lamerin microsporidia is serologically different from the standard strain *N. bombycis*.

Germination of Lamerin microsporidian spore: The microsporidian spores from Lamerin on incubation in different concentration of potassium hydroxide (KOH) for different time durations and its rapid neutralization (pH. 7.00) by addition of phosphate buffer saline resulted in germination of spores by the extrusion of polar tube. The spores from Lamerin microsporidia and the *N.*

bombycis appeared black due to the extrusion of polar tube. The spores which did not germinate remained bluish in color and exhibited high refractive index. The polar tube could be seen as thread like structure attached to the spore (Fig. 10 and 11). One hundred percent germination of spores of Lamerin microsporidia was obtained in treatment of 1% KOH for 30min whereas 100% germination of spores of *N. bombycis* was obtained at concentration ranging from 0.1-1%. The germination of spores of Lamerin microsporidia was significantly poor in KOH of concentrations of 0.1-0.5% (1-40%) and average at 0.7 – 0.9% (41 – 80%) of KOH (Table 7).

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; Selvakumar *et al.,* 2005). But till recently Lamerin breed of silkworm, a native of state of Manipur had not received the desired attention. The Lamerin breed is often credited with possession of the charecterstic of resistance to microsporidiosis. The breed has survived for generations in spite of microsporidiosis. There were no published reports, except a preliminary unconfirmed and unpublished study, either on the breed or on the microsporidiosis in the breed. Hence the present study was of much importance to understand and manage microsporidiosis in silkworm caused by *N. bombycis* or any other microsporidians. In the present study, the microsporidia infecting Lamerin breed of the silkworm was isolated, purified following the standard menthod (Sato and Watanabe, 1980) and established its conformity to Koch's postulates in causing the infection in Lamerin and other silkworm breeds. The Lamerin microsporidia infects and causes infection to an extent of 65.29%. It also infects Pure Mysore and CSR2 breeds of silkworm and cause infection to an extent of 67.52 and 76.95% respectively. This confirms that the Lamerin microsporidia is not specific to Lamerin breed but could infect other silkworm breeds. The present study notes that the Lamerin breed is not of great economic importance except for its high tolerance to microsporidiosis. The cocoons weighs only 1400mg with 190 mg of shell and 13% of silk. The cocoons are open ended, flimsy having high percent of floss. However, the breed has high rate of survival with 91.00% of hatched larvae metamorphosing into pupae. It may be possible to improve the breed economic characters but the problem is that the breed could pose serious threat to sericulture in Manipur and other neighbouring states. The breed harbours the microsporidia and form the carrier of microsporidian infection. It form a major source for microsporidian infection to any productive and economically important silkworm breed introduced in the area where it may turn out to be more virulent and cause significant loss to cocoon crop. The problem could be viewed with much more seriousness as the farmers themselves prepare eggs of the Lamerin breed in the area and rear the infected progeny.

In the present, study the microsporidian which is endemic in the Lamerin breed of silkworm has been characterized. The spores are the characteristic feature of microsporidia. They are most distinct, unique and complicated structure of taxonomic importance. The result confirms that the microsporidia isolated from Lamerin breed posses all the characteristic features of a typical microsporidia. The spore of Lamerin 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 Lamerin microsporidia exhibits features of typical microsporidiosis, there are significant differences.

The microsporidian spores from Lamerin breed is ovo-cylindrical having a length of 4.36µm and width of 2.14µm while the spore of *N. bombycis* is oval with a length of 3.08µm and 2.01µm width. Similarly other microsporidians isolated from silkworm differ in their spore size and shape. They differ from 2.5 to 5.1µm 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 Lamerin breed indicates presence of a distinct depression at $1/4^{th}$ of length of the spore. Such a depression was not observed on the surface of *N. bombycis*.

The internal structure of spore of Lamerin 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 11-coils. The average single coil length is 0.066µm and width is 0.076. The coil length width ratio is 1:1.15. 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*, 12 coils were observed. The average single coil length and width is 0.078 and 0.073µm respectively. The coil length width ratio is 1:0.93. 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).

Lamerin 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 Lamerin microsporidian shows 5 distinct bands while of *N. bombysis* spore showed 6 protein bands. The band at 31kDa was the unique band for Lamerin microsporidia. It was of high intense than the corresponding band in of *N. bombycis*. The 28 kDa band is of diffused type in Lamerin microsporidian spores. In addition a high intensity 20kDa band is observed in spore surface protein of *N. bombycis*, which is thinner in case of Lamerin microsporidia.

Apart from the differences exhibited by Lamerin microsporidia in the form of morphology, ultra structure and electrophoretic banding patteren of spore surface protein, the Lamerin 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 Lamerin microsporidian. The polyclonal antibody based agglutination test employing antibody raised against Lamerin microspoiridian spore also confirmed that the Lamerin microsporidian is different from *N. bombycis*.These results indicates that the Lamerin microsporidian is serologically different from *N. bombycis*, *Nosema* sp. M11 and *Vairimorpha* sp M12.

The Lamerin microsporidian spores also differ in their physiological ability to germinate. Spores of invertebrates and vertebrate microsporidians can be induced to germinate artificially by various chemical treatments (Keohan and Weiss, 1999). Spore germination beings with an environmental trigger that varies for different species depending on their habitat (Undeen and Epsky, 1990; Keeling and Fast, 2002) but is largely poor understood (Keohan and Weiss, 1999; Keeling and Fast, 2002). The spores germinate by extrusion of the polar filament on incubation in potassium hydroxide and its neutralization as does many microsporidians (Oshima, 1964; Petri, 1969; Ishihara, 1968b; Weidner, 1972; Kawarabata, 2003). Lamerin microsporidia germinate to an extent of 81-100% in KOH of 1% concentration where as *N. bombyci*s spores hatch to an extent of 81-100% in 0.1% KOH itself. The germination of Lamerin microsporidian is poor in 01-0.5% of KOH, average in 0.7-0.9% of KOH. These observations confirmed that the germination of different microsporidians in KOH differs. The poor germination ability in Lamerin microsporidian may also lower the infectivity of the microsporidia to the silkworm. This may also be the reason for low virulence of the Lamein microsporidian.

These observations indicate that the microsporidia infecting Lamerin 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 and artificial germination. However, it is essential to characterize the microsporidia for mode and site of infection, infectivity, virulence, transmission *etc.* for better understanding of the host pathogen relations.