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Review Article

Microsporidiosis in the Silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae)

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ABSTRACT

The mulberry silkworm, *Bombyx mori* L., is prone to infection of various pathogenic organisms. Microsporidiosis of the silkworm, caused by highly virulent parasitic microsporidian or *Nosema bombycis* (Nageli), is one of the most serious maladies, which determines the success or failure of sericulture industry in any country. Infections of the disease ranging from chronic to highly virulent can result in heavy loss to the sericulture industry. Several strains and species of microsporidia have since been isolated from the infected silkworms, and the disease is becoming increasingly more and more complex. Epizootiology, development of immunodiagnostic kit, fluorescent antibody technique and use of ideal disinfectant, chemotherapy and thermo-therapy techniques and management strategies have been addressed for identification, destruction, prevention and control of disease causing micro-organisms. Techniques of forced eclosion test and delayed mother moth examination have also been stated to play important roles in the detection of the disease. An attempt has also been made in this review article to briefly elucidate the various aspects of the pebrine disease and to help the researchers to develop efficient model(s) for the prevention, control and management of microsporidia infecting mulberry silkworm, *Bombyx mori* L.

Keywords: Bombyx mori L., Microsporidiosis, Pathogenesis

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INTRODUCTION

The microsporidia are spore forming, small, obligate, intracellular living eukaryote infecting both beneficial and non-beneficial insects (Nataraju *et al*., 2005). More

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than 140 genera and 1200 species of microsporidia have been recorded from insects and fish (Canning, 1993; Samson *et al.,* 1999a). Among these, at least 200 belong to the genus *Nosema* (Sprague, 1982) and most *Nosema* species are parasitic to invertebrates. A majority of these, including *N. bombycis* and *N. tyriae* (Canning *et al*., 1999), *N. mesnili* (Cheung & Wang, 1995), *N. algerae* (Muller *et al*., 2000), *N. aphis* and *N. trichoplusiae* (Malone *et al*., 1994) are pathogenic to various insects. The microsporidian infection remains a major threat to the sericulture industry with its recurrent occurrence. More than twenty wild insect species have been found to have microsporidian spores that can cross-infect silkworm. Pebrine, i.e. the spores of microsporidian (*Nosema bombycis*), is one of the most dreaded diseases of the silkworm, *Bombyx mori.* Pebrine, which determines success or failure of the sericulture industry of a nation, infects almost all ages, stages, breeds and hybrids of the silkworm by both transovarial and peroral infections. It is highly infectious and difficult to eradicate after the occurrence of infection. This is evidenced from the historical fact that the rise and fall of pebrine disease correspond with the ups and downs of the sericulture industry in the silk producing countries of the world (Tatsuke, 1971). The earliest research on pebrine was confined especially with the epizootiology and prevention of the disease (Fujiwara, 1979; Ishihara, 1963; Weiser, 1969). Meanwhile, the microscopical method of mother moth examination, although widely practiced mainly due to its simplicity, does not assure a foolproof detection of the microsporidian.

To circumvent this particular problem, efforts have been made to evolve simple, precise and more accurate method to detect the disease (Baig *et al.,* 1992; Fujiwara, 1993; Geethabai *et al.,* 1985; Shi & Jin, 1997), identify alternate host (Fujiwara, 1993; Samson, 2000), use chemotherapy and thermo-therapy for the prevention and control of disease (Hayasaka, 1990), apart from the identification of intermediary stages (Santha *et al*., 2001) but with little success. Even though research and fight against the pebrine have been continuously done for more than a century, loss due to the disease has not been completely eliminated (Singh *et al*., 2010). However, historical evidences suggest a significant relationship between the success of sericulture industry and the control of the disease. Therefore, to improve the sericulture industry and to save it from crop losses due to this chronic disease, it is essential to have a foolproof diagnostic and preventive technique.

To briefly review and discuss the recent advances achieved so far on the various aspects of the pebrine disease, an attempt has been made in this article to present annotated information on the causative organism, pathogenesis, manifestation, diagnosis and management of microsporidia infecting mulberry silkworm, *Bombyx mori,* in order to develop efficient model(s) for the prevention and control of this particular chronic disease in the days to come.

Disease History

Several historical evidences in various countries of the world showed that the outbreak of pebrine disease had greatly influenced the decline of the sericulture industry in the past. The damage of crops in Europe in the middle of the 19th century was so great and extensive which became a worldwide scale that the cocoon production sharply declined and the sericulture industry of the world suffered heavily (Tatsuke, 1971). The history of research on the pebrine disease progressed with the advancement of microbiology in the 19th century. The disease-causing microorganism was first observed in the haemolymph of the silkworm and was given the name 'Hematozoid' (Guerin-Menevillae, 1849). Quadrefague (1860) coined the name pebrine because of the appearance of pepper-like spots in the diseased larvae. Nageli (1857) of Germany stated that the disease is caused by a protozoan parasite and named this pathogen as *Nosema bombycis*. The noted French microbiologist, Pasteur (1870), in his book entitled *"Etudes sur la maladie des vers a Soie"*, called the disease 'corpuscle disease' and made a detailed study on its growth and transmission, and discovered that the disease is transmitted through transovarian transmission within the body of the mother moth and suggested methods of preventing the disease.

In India, the first record of the spread of incidence of disease was made at the end of the 19th century in the Kashmir valley (Sahaf, 2002). In 1890-1900, the disease swept through Mysore and Madras provinces. Thereafter, the disease reappeared during 1925-1930 in an epizootic form (Chitra *et al*., 1975). Disease epidemics were again observed during 1991-1992 in the southern part of the country, which resulted in considerable crop losses and revenue (Nataraju & Dandin, 2006). Since then, the incidence of the disease has been observed intermittently in silkworm crops in the different parts of India. The pebrine incidence also caused a considerable loss of silkworm seed during 1997-1999 in the seed production area of Uttar Pradesh (Quadri & Khatri, 2005).

Life cycle of Nosema bombycis

On the basis of morphological and molecular features, Undeen and Cockburn (1989) and Vossbrinck *et al*. (1987) stated that *N. bombycis* is one of the earliest known primitive eukaryotes because of the primitive type of nuclear division, but it lacks some typical organelles mainly mitochondria, stacked golgi, prokaryotic sized ribosomes and ribosomal RNAs. Several workers have studied the developmental cycle of *N. bombycis* and presented a comprehensive account of the life history of the disease (Iwano & Ishihara, 1981; Kawarabata & Ishihara, 1984). The life cycle of *N. bombycis* includes three stages, namely, spore, planont and meront. The mature spore is oval or ovo-cylindrical and measures approximately 3.4 -3.8 µm in length and 2.0 - 2.3 µm in width, with three-layered membrane (inner, middle and outer). The spores are highly refractive, and shine bluish white under microscope exhibiting

'Brownian movement'. The outline is smooth and the spores are heavier than water. The resistant form of the disease is spore and it remains either in an infected tissue of the body or discharged through excreta by leaving infected host tissue. The spore, when swallowed by the silkworm through contaminated food, germinates under alkaline conditions inside the gut of host with the help of digestive juice and produces a long polar filament measuring 500 µm in length and 0.5 µm in width, and it is more than 30 times longer than that of the lengthwise dimension of the spore, on the end of which grows a sporoplasm (Peter *et al.,* 1999). The sporoplasm has one or two nuclei and other cell organs and possesses limited membrane. The sporoplasm multiplies through fission, comes out of haemolymph through intracellular spaces, spreads to every part of the body, lives in various systems (particularly in body fat and muscular tissue) and becomes nucleus to form spore after multiplication through fission (Abe & Fujiwara, 1979). Formation of spore is apansporoblastic, disporous and dimorphic. One type of the sporoblast of the long polar tube types turns into a single spore with many coils of polar tube. The other type of sporoblast, with short polar tube, turns into a single spore with a few coils of the polar tube. Spore with short polar tube hatches directly in the host cell. Secondary sporoplasm reaches the other cells of the host. The spore completes its life cycle within 4 days. Complete developmental stages of the pathogen have been studied and elucidated in detail (Takizawa *et al.,* 1975). The mature spore is unicellular endo-membranous differentiation of its sporoblast (Vavra & Maddox, 1976). These authors designated the sporoblasts as Phase-I sporoblasts and Phase-II sporoblasts. The Phase-I sporoblasts are characterized by the presence of a dark staining spherical body (Singh *et al*., 2007).

Characteristics of the Disease

The disease infects all ages, stages, breeds and hybrids of the silkworm. Larvae suffering from pebrine do not show any external symptoms until the disease is far advanced. At advanced stage, larvae become sluggish and show symptoms like poor appetite, retarded growth and development and irregular moulting. As the disease progresses, the larvae appear pale, dull and translucent with wrinkled skin, shrink in size and become flaccid (Jolly, 1986; Singh & Saratchandra, 2003). Due to the chronic nature of the disease, the infected larvae do not die immediately and continue to survive for some time. The infected gut becomes opaque and white pustules appear on the silk glands. Infected pupae are flabby and swollen with lusterless, blackish and softened abdomen and black spots occasionally appear on this region (Ishihara, 1963). Highly infected pupae fail to metamorphose into adult. Irregular moth emergence, clubbed wings, distorted antennae, improper mating, low fecundity, and sometimes clumpy egg laying, as well as high percentage of unfertilized and dead eggs, apart from eggs with less gluey substance leading to their detachment from the egg sheets, lack of uniformity in egg

shape, and easily coming off scales from the wings and abdominal area are some of the symptoms of the disease at the moth stage. The accessory glands of pebrinized moths are also infected and this results in production of loose eggs which easily role-off the egg sheets.

Source and Stage of Contamination

Transovarially infected seeds are the primary source of contamination. Contaminated rearing and grainage buildings, appliances, silkworm litter, and mulberry leaf fed to the silkworm harboured by infected insects, *etc.* also contribute to the spread of the disease. The incidence of pebrine varies with the variety of silkworms, the developmental stage and the rearing environment. Meanwhile, resistance to pebrine is greater in the Chinese breeds, but less in the Japanese and the least in European breeds (Govindan *et al.,* 1998). The multivoltine breeds are relatively more resistant than bivoltines (Patil & Geethabai, 1989). Young silkworms, newly moulted and starving larvae are susceptible and show high mortality. In India, Nistari and C. Nichi are more resistant silkworm breeds as compared to the others. Patil and Geethabai (1989) reported that among the bivoltines breeds, NB7 is the most susceptible, and this is followed by NB4D2, KA and NB18. Although the disease resistance appears to depend on the genetic constituents of a particular breed, factors such as pathogen load, inadequate nutrition, and the environment in which the insects are reared may also affect their resistance. In addition, the physical and physiological characteristics of the hosts may make the invasion of microsporidians possible (Weiser, 1969, 1977). The larvae infected during the 1st and 2nd instars show a normal growth up to the 3rd instars. Meanwhile, disease symptoms appear during the later half of the 4th instars to the first half of the 5th instars and die before spinning. If the contamination takes place in the 3rd instars, the larvae will show symptoms of the disease in the late 5th instars and die on the mountage before cocooning. It is important to note that these larvae discharge spores through faecal matter during the 4th and 5th instars. If these larvae are reared with healthy larvae, the spore discharge by the infected larvae provides the source of contamination and digestion of spores by healthy silkworms will result in a spread of the disease. This stage of contamination is known as the 'second stage of contamination.' Larvae infected during the 4th and 5th instars pupate and on emergence lay contaminated eggs. This phenomenon is referred to as 'transovarian transmission.' Most of the larvae infected through transovarian transmission show irregular moulting and growth, become tiny or under grown and die before the 3rd moult, after discharging spores. The contamination occurring from the transovarially-infected larvae is termed as the 'first stage of contamination.' The minimum number of the spores required for contamination through per oral infection varies with each instar. Among other, Iwano and Ishihara (1981) stated that 1-10 spores are sufficient enough to cause disease in

the 2nd instars larvae, while approximately 100 of such spores are required in the 5th instars for the same symptoms to occur. Transovarian transmission is 100% in the case of *N. bombycis* and only 1.2% with *Nosema* sp. M11 (Han & Watanabe, 1988).

The spores of different microsporidia infecting silkworms differ in their morphological characters; some are larger than mature spore and some are long, thin and pear-shaped with different sizes, shapes and lusters. Sometimes, the conidia of green muscardine and red muscardine bear a striking resemblance to the spore of the pebrine disease. Horizontal transmission of the pebrine spore is possible through contaminated rearing bed, mulberry leaf and layings (Govindan *et al.,* 1998). Baig *et al.* (1988a) reported that the spread of disease in rearing trays is also dependent on the density of diseased silkworms. Growth and multiplication of pathogen are influenced by the growth of its host. When egg enters into diapause, the growth and multiplication of pathogen stops simultaneously and when egg starts to grow, the pathogen will also start to grow and multiply.

Physiological Stability

Generally, a large number of factors, *viz.,* temperature, humidity and abiotic components of the substrate influence the survival of microsporidians (Kramer, 1976). The spores belonging to the dormant stage of pathogen and possessing great resistance can remain infective after 3 years in the dried body of the female moth, and become active after being submerged in water for 5 months (Li, 1985). When kept in the dark, the spores are reported to remain viable for as long as seven years, but when the spores are directly exposed to sunshine, they remain viable for 6-7 hrs and when treated with hot water, they survive for just 5 minutes. Studies conducted on the viability of the pebrine spores in soil and compost under tropical conditions have shown the survival of spores for a maximum period of 225 days in wet soil and a minimum of 135 days in wet compost (Patil, 1993). Srikanta (1986) observed that spores remained infective even after 150 days of refrigeration and after 90 days in moist soil and faeces. He further stated that the viability of spores is lost in 60 days in dry soil and in 5 days when they are stored at room temperature. The resistance of spores to different disinfectants indicates that they can remain viable for 10-30 minutes in the solution of corrosive sublimate, for about 5 hrs in formalin and 10 hrs in chlorinated lime solution (diluted 10,000 times). Bleaching powder containing 1% and 3% active chlorine can render spore inactive in 30 minutes and 10 minutes, respectively. When the degree of infection is relatively high, the egg often becomes sterile or dead, but when the contamination is of low degree, the egg hatches and the disease develops at the larval stage and causes death of larvae at later stages of development.

Alternate Host

Most microsporidians prefer having alternate hosts because these offer many advantages to them, *viz.*, dispersal, transmission and

survival. The perpetual incidence of microsporidian infection in silkworms may be due to various sources of secondary contaminations which include alternate hosts in and around mulberry garden. In addition to *N. bombycis,* seven other microsporidia belonging to the genera *Nosema, Pleistophora, Thelohania, Vairomorpha* and *Leptomonas* spp. have been isolated from the silk moth (Govindan *et al.,* 1998). They differ in their spore morphology, target tissues and virulence, and have been designated as M11, M12 and M14 (*Nosema* sp.), M24, M25, M27 *(Pleistophora* sp.) (Fujiwara, 1984a and b) and M32 (*Thelohania* sp.) (Fujiwara, 1985), as shown in Table 1. Three microsporidia designated as NIK-2r, NIK-3h and NIK-4M have been isolated from silkworms in Karnataka (India) and these are immunologically dissimilar to *N. bombycis* (Ananthalakshmi *et al*., 1994).

N. bombycis has also been reported to infect *Samia cynthia ricini* and Indian tropical tasar, muga and Chinese tasar silkworms (Talukdar, 1980). *N. bombycis* has also been found to infect several other lepidopteron like *Spodoptera exigua*, *S. litura*, *Diaphania pulvurentalis*, *Pieris rapae, P. brassicae, etc.* Veber (1958) reported 32 species of lepidopteron which are known to develop infection to the peroral inoculation of *N. bombycis* spores. These include *Chilo suppressalis, Pieris rapae, P. brassicae, Spodoptera exigua*, *S. litura, S. maurilia, Balataea funeralis, Cruptophlebia illepida, Exartema mori, E. morivirum, Diaphania pyloalis, Mycalesis gotoma, Abracus miranda, Descorba simplex, Boarmia selenlia, Menophra atrilinecta, Elydna nonagrica, Otosema odera, Perigea illecta, Plusia chalcites, Pseudaletia unipuncta, Stilpnotia lubricipeda, S. imparilis, Callimorpha quadripunctata, Thaumetopoea processionea, Malacosoma neustria, Gastropacha quercifolia, Lasiocampa quercus, Bombyx mandarina, Antheraea pernyi, A. yamamai, Sphinx ligustris, Agrotis ipsilon, Agrius cinagulatus, Pholera assimilis, Acronicta major,*

TABLE 1
Different types of microsporidian spores

Types of microsporidian	Spore size (µm) (L x W)	Site of infection	Virulence
Nosema bombycis	3.8 x 2.2	Systemic	High
Nosema sp. (M11)	3.9 x 1.9	Various tissues	Low
Nosema sp. (M12)	4.2 x 2.7	Various tissues	Low
Nosema sp. (M14)	5.1 x 2.0	Various tissues	High
Pleistophora sp. (M24)	2.7 x 1.6	Mid gut	Low
Pleistophora sp. (M25)	3.2 x 1.8	Mid gut	Low
Pleistophora sp. (M27)	5.4 x 3.0	Various tissues	Low
Thelohania sp. (M32)	3.4 x 1.7	Muscle	Low

Source: Fujiwara (1985)

Acrotomycis aceris and *Achaea janata* (Kawarabata, 2003; Samson *et al.,* 1999b; Singh *et al*., 2007, 2010). The lawn grass cut worn, *Spodoptera depravata,* serves as a natural reservoir for the pathogen (Ishihara & Iwano, 1991) which shares the surface specific antigens with *N. bombycis and* results in transovarial transmission with less virulence.

Cross Infectivity

Different species of insects, known to carry microsporidians causing cross-infectivity to silkworms, were found harbouring in and around mulberry garden. Enormous quantity of microsporidian spores was observed in *Catopsilia* sp., an inhabitant of mulberry garden (Kishore *et al*., 1994) and found infective to silkworms. Butterflies causing microsporidian infections to silkworms were also reported (Samson *et al*., 1999a, b). Singh *et al.* (2007) reported that butterflies, i.e. *Eurena hecabae* and *Zizina otis*, carry microsporidian spores infective to silkworms. These insects are potential source of contamination as spores of pathogen are excreted along with litter on the mulberry leaves in the garden, and when these leaves are fed to silkworms, they cause the disease to appear. The different microporidia isolated relatively recently from India, their spore morphology, target tissues, as well as the infection rate and rate of transovarian transmission in progeny of silkworm are presented in Table 2, which reveals the transmission to the extent of 100% in NIK-3r, whereby only 1.8% is found in NIK-3h.

Spore Isolation and Purification

Isolation, purification and identification of spores from the host are the first steps involved in the study of pebrine disease and its management. To isolate the spores, diseased larvae/pupae/moths are homogenized in sterile water for 1-2

TABLE 2
Microsporidian spores isolated from India

Characters	Microsporidian spores		
	Nosema sp. NIK-2r	*Nosema* sp. NIK-3h	*Nosema* sp NIK-4m
Spore size (µm) (L x w)	3.6 x 2.8	3.8 x 1.8	5.0 x 2.1
Spore shape	~ round	Oval	Ovocylindrical
Site of infection	Gut epithelium, malpighian tube, muscle, fat body, silk gland, gonad	Malpighian tube, muscle, fat body, silk gland, gonad	Gut epithelium
Infection rate	High	Medium	High
Mortality rate	High	Low	High
Rate of transovarian transmission of spores in progeny	100%	1.8%	No report

Source: Nataraju *et al.* (2005)

minute(s) using a mixer. The homogenate is filtered through cotton or fine muslin cloth. The filtrate obtained is transferred into a centrifuge tube and is centrifuged at 3,000 rpm for 5 minutes. The supernatant is discarded and the sediments obtained consist mostly of spores, which can be confirmed with microscopical examination. However, serological and biochemical studies of microsporidians require high degree of purity. Gochnaner and Margetts (1980) described a rapid method for concentrating *Nosema* spores based on continuous flow centrifugation method. Another method based on 'Brownian movement' was also reported. Sato and Watanabe (1980) purified spores using sucrose and percol gradient centrifugation and reported that centrifugation using percol at 73,000g for 30 minutes resulted in 3 bands viz., a sharp band consisting of tissues of silkworms, mulberry leaves, bacteria, etc., a dim band consisting of mature but inactive spores and sharp band consisting of only mature and active spores.

Sporulation Rate

Sporulation rate is a significant step in the area of pebrine disease detection through microscopic test. In this method, the mother moths are collected in groups after oviposition and in perforated cardboard boxes/covers and preserved alive. Alternatively, they can be left on dummy sheets in the oviposition trays itself. The boxes/oviposition trays are properly numbered as per egg sheets and preserved in well-ventilated room at ambient room temperature (25-30^{0}C) for a period of 3-4 days prior to the microscopic test. This enhances sporulation of the pathogen in older moths facilitating an easy and more accurate detection of the disease. After the stipulated period, moth testing was carried out as per recommended procedure in-vogue. Through this method, easy and effective detection of pebrine disease is possible due to enhanced sporulation in older moths. Even under moderately low infection levels, pebrine can be detected using this method. This technique is very useful during basic seed multiplication and production of P1 seeds. It has also been reported that the rate of multiplication of *N. bombycis* increases substantially with the age of moths and the cephalothoraxic region has the highest spore concentration, especially around the wing and wing muscles (Sashidharan *et al*., 1994) (Table 3), and therefore, testing of silk moths 3-4 days after oviposition would be a more effective method to detect pebrine with better accuracy.

Approaches for Prevention and Management of Pebrine

Pebrine has been a threat to the sericulture industry since time immemorial. The disease has become more complex now because of the occurrence of the different types of microsporidians infecting the silkworm. Some of them belong to other genera like *Vairomorpha* and *Thelohania* and exhibit differences in their patterns of infection (Samson, 2000). Apparently, the biology of the pathogen has been used

TABLE 3
Sporulation rate of *Nosema bombycis* in different tissues after emergence of moths of silkworm

Body parts	Breeds	Quantity of spores on different hours after emergence ($x10^7$/gm wt of tissue)				
		0 h	24 h	48 h	72 h	96 h
Whole moth	PM	4.39	4.50	5.67	21.90	25.50
	NB18	5.92	6.34	12.40	22.00	28.70
Cephalothorax	PM	8.20	10.50	9.40	35.80	44.00
	NB18	7.10	10.20	14.70	38.40	40.10
Abdomen	PM	1.49	2.60	5.50	14.90	21.60
	NB18	5.02	3.80	7.94	14.00	20.30
Wing	PM	6.30	8.50	11.00	25.00	31.30
	NB18	8.61	12.80	24.45	28.60	34.60
Gut	PM	9.62	10.81	10.60	24.60	22.60
	NB18	8.11	8.94	12.40	20.00	21.20
Fat body	PM	0.19	0.15	0.10	0.20	0.20
	NB18	1.34	2.17	2.10	1.77	2.41

Source: Sashidharan *et al*. (1994)

as a basis in disease control. The disease is transmitted horizontally by ingestion of spore and vertically by transovarian transmission. This unique characteristic of the disease makes it difficult to be completely eliminated from the silkworm crops. The earliest method suggested by Pasteur, based on the selection of pathogen free eggs through a careful systematic examination of mother moths for pathogens after laying eggs, is one of the most effective methods to avoid the disease in the silkworm crops.

Meanwhile, proper monitoring and testing of the seed crops at every successive stage of progress of the crop are done to ensure the production of pebrine free seed cocoons for commercial seed production. Quadri and Khatri (2005) stated a three-tier examination approach (namely, larval, pupal and moth) to detect the incidence of pebrine disease in the multiplication of silkworm seed and suggested destruction of infected crops as soon as identification of infection as an important step towards pebrine disease management. Since the disease is seed borne, the surface sterilization of eggs immediately after egg laying and also during the pin-head stage of incubation should be followed to prevent the occurrence of the disease from surface contamination (Singh *et al.,* 1992). Several reports have documented the efficiency of the thermal treatment of silkworm eggs in minimizing pebrine infection (Bedniakova & Vereiskava, 1958; Fujiwara & Kagawa, 1984; Hayasaka, 1990). The maximum lowering of infection rate was reported in eggs incubated during the first two days of their development to 44°C. Singh and Saratchandra (2003)

stated that the incubation of eggs at higher temperature within 3 days of laying would result in significant reduction in pebrine disease. Meanwhile, thermal treatment, in combination with hydrochlorization to achieve dual objectives of elimination of pebrine and termination of diapause, has also been reported (Austrurov *et al.,* 1969). Liu *et al.* (1971) reported a remarkable success in reducing pebrine infection after a treatment at 47°C for 10-20 minutes. Chowdhary (1967) suggested exposure of cocoons to high temperature (33.8°C) at the time of pupation for 16 hrs a day, whereas 55 - 65% of humidity tends to reduce infection in the resulting eggs. Sheeba *et al* (1999) reported that a thermo-therapy of 7 days old pebrinized cocoons at 36°C for 16 h tended to significantly reduce pebrine infection without affecting the growth and development of the larvae.

Certain insect hosts tolerate high temperature than their microsporidian parasites and the hosts can be freed of the disease by rearing the infected individuals at higher temperatures until the disease is cured. Meanwhile, attempts have been made by several authors/researchers to control pebrine infection in silkworm eggs through temperature treatment. Among other, Ovanesyan and Lobzhanidze (1960) and Austrurov *et al.* (1969) attempted hot water treatment of pebrinized eggs and reported a sharp decrease in the degree of infection. Similarly, Smyk (1959) expressed varying successes with hot water treatment. Fujiwara and Kagawa (1984) reported that the parasites in non-diapausing eggs were more sensitive to hot water (46°C for 4 minutes) treatment and there was no harmful effect of the treatment on the normal development of silkworm embryos. The treatment of silkworm eggs, with HCL of 1.03 - 1.09 specific gravity at 47^0C for 10-20 minutes, has been known to reduce the disease incidence by 97.4 - 100% (Liu & Zhong, 1988). In the same manner, a hot air treatment (48-50^0C) of 12-18 h old silkworm eggs also inhibited the development of microsporidians. Silkworm eggs of 36-60 h old treated with hot water at 46^0C for 90-150 minutes, 48^0C for 50-70 minutes, and 52^0C for 4 minutes, also inhibited the development of pebrine disease. However, these methods are not effective enough to completely eliminate the infection. Of the several therapeutic drugs, Benomyl, Nosematol, Bavistin and Thiophanate have been identified as anti-microsporidian agents to control *N. bombycis* infections (Alenkseenork, 1986; Chandra & Sahakundu, 1983). Although these fungicides have been proven to be experimentally effective in reducing the multiplication of spores, further studies clearly showed that these fungicides could not significantly eliminate transovarian transmission. *N. bombycis* is made to be inactive by hilite (Potassium dichloro isocyanurate) (Iwano & Ishihara, 1981). Baig *et al.* (1988b) studied the comparative efficacy of four disinfectants (*viz.,* hilite, sodium hypochlorite, bleaching powder and formalin) in four concentrations of 0.5%, 1.0%, 1.5% and 2% as surface sterilents against the spread of pebrine disease in

a colony of silkworms hatched from the surface contaminated laying and reported that all the tested concentrations were effective in preventing the spread of the disease and also successful in inactivating the spores of *N. bombycis* when exposed to 5, 10, 20 and 30 minutes, respectively. Kagawa (1980) studied the efficacy of formalin as a disinfectant against pebrine and reported an increased death rate of the spores with a raise in the concentration and temperature of formalin. Iwano and Ishihara (1981) tested nine chemical types as inhibitory agents against *N. bombycis*, with high degree of inhibitory effects on the spores.

However, the methods attempted to control pebrine disease by several authors have been found to yield limited success. Therefore, development of better and more reliable diagnostic methods to detect pebrine during seed production and silkworm rearing has always remained one of the most important and valid strategies to eliminate the disease from silkworm crops. Relatively recently, delayed mother moth test is recommended as a significant step in the area of pebrine disease diagnosis in microscopic test. In this method, the female moths are preserved alive at room temperature for a period of 3-4 days after oviposition and before subjecting for microscopic test. This allows improved sporulation of the pathogen to facilitate an easy and a more accurate detection of the disease (Samson, 2000). It has been reported that the multiplication rate of *N. bombycis* increases substantially with the age of moths and that the cephalothoracic region has the highest spore concentration, especially around the wing and wing muscles (Sasidharan *et al.,* 1994). Therefore, testing silk moths around 3-4 days after oviposition is a more effective way or method to detect pebrine with a much better accuracy. An improved testing method has also been recommended for better detection at egg stage. A sample of eggs was incubated at a moderately higher temperature of 32±1ºC for 48 h to enhance the sporulation of *N. bombycis.* Testing of such eggs will therefore enhance the chances of disease detection. On these lines and based on the principles of immunology, even the diagnostic techniques were also attempted in several countries, including India, for the detection of pathogen and spore identification, but with only limited success (Baig *et al.,* 1992).

N. bombycis and closely related spores were diagnosed using several techniques such as antibody-sentisized latex agglutination (Hayasaka & Ayuzawa, 1987), slide agglutination (Baig *et al.,* 1992; Li, 1985), ELISA procedures (Kawarabata & Hayasaka, 1987), fluorescent antibody (Sato *et al.,* 1981, 1982), serological (Grobov & Rodionova, 1985) and SPA coagglutination (Mei & Jin, 1998), etc. The development of the monoclonal antibody technique, which has very high specificity and stability, has played a great role in the studies of the classification and identification of specific microsporidians (e.g., Carlos *et al.,* 1996; Chen *et al.,* 1989). Meanwhile, Ke *et al.* (1990) raised monoclonal antibodies against *N. bombycis* spores and applied

them to identify pebrine and other closely related microsporidian spores infecting silkworms using the ELISA procedure. Shi and Jin (1997) reported that agglutination test using N5 McAb (hybridoma cell lines secreting monoclonal antibody) sensitized latex particle was a very practical technique for the diagnosis of the pebrine disease. Nonetheless, a simple dipstick immunoassay method tried later for the diagnosis of pebrine was also unsuccessful in the field. A simple negative staining procedure (Geethabai *et al.,* 1985) and an immunoperoxidase staining procedure (Han & Watanabe, 1987; Kawarabata & Hayasaka, 1987) were developed for the clarity during the examination of spores. Sironmani (1997) developed the Western blot method to identify the microsporidian infection and observed that immunological reaction with *N. bombycis* infected silkworm larvae and eggs showed the presence of 17-kDa polypeptide, which is specific to infection. The researcher further reported that 17-kDa polypeptide could be used as a virulent marker for the identification of microsporidian infection. DNA based probes have also been developed to identify *N. bombycis* (Malone & Mclvor, 1995).

Based on the amplification of rRNA gene fragments, several PCR methods are available for the diagnosis and species identification of insect microsporidia (Kawakami *et al*., 1995, 2001). The molecular techniques developed were found to have more sensitivity and specificity in the detection of the disease (Hatakeyama & Hayasaka, 2001). Nageswararao *et al*. (2004) studied the pathogenecity, mode of transmission, tissue specificity of infection and SSU-rRNA gene sequences for the microsporidian isolates from the silkworm, *Bombyx mori*. Using inter-simple sequence repeat PCR (ISSR-PCR) analysis, the genetic characterization and relationship between different microsporidia infecting mulberry silkworms have been reported (Nageswararao *et al*., 2005). The researchers further differentiated six different microsporidians through molecular DNA using ISSR-PCR and stated that the ISSR-PCR analysis might emerge as a powerful tool to detect, diagnose and identify microsporidians using inter simple sequence repeat PCR (ISSR-PCR) analysis, as it is difficult to study with microscope because of their extremely small size. A new technique based on the identification of intermediary stages has also been suggested for diagnosing pebrine (Santha *et al.,* 2001).

Although these tests are simple and sensitive, they still cannot create any impact on the pebrine disease diagnosis in the field, unless standard methods are evolved for their effective field applicability. To maintain the quality of silkworm eggs, several attempts have been made from time to time to improve the sampling procedure (Fujiwara, 1993; Kurisu, 1986; Kurisu *et al.,* 1985). Moreover, procedures have also been developed for the detection of pebrine spores in soil/dust, rearing and grainage houses, on mulberry leaves, eggshells/unhatched eggs, litter, etc. (Singh & Saratchandra, 2004). The sample size for the examination of faecal matter to

detect the presence of pebrine has also been described by Patil *et al.* (2001). As it is not possible to examine all the emerging moths in the commercial grainages, Fujiwara (1993) suggested a 20% sampling method and reported the probability of detection of the pebrine disease, as shown in Table 4.

Destruction of disease-causing microorganisms at various levels is a general method used in preventing and controlling the disease. Surface sterilization of disease free laying, maintenance of strict sanitation, hygienic rearing, frequent and careful examination of stock, disinfections of rearing rooms and appliances, removal of dead and infected larvae are to be strictly adopted to get rid of the disease. Meanwhile, exposing all the contaminated materials and equipments to direct sunlight, and disinfections with 2% formalin solution or 5% bleaching powder solution are the most effective and simple eradication methods for the disease. However, the pathogen killing action of the disinfectants is influenced by several factors such as temperature, humidity, concentration of disinfectants and duration of treatment (Kagawa, 1980). Recently, a new disinfectant chlorine dioxide (serichlor) is considered as an ideal disinfectant for all types of rearing/ grainage houses. In combination with slaked lime, it is 2.5 times stronger than chlorine and 2 times stronger than sodium-hypochloride. Furthermore, it is the least corrosive and also non-hazardous. When no single technique is sufficient enough to be used in checking the disease in field, it becomes obligatory to choose a multi-pronged approach. Unfortunately, the technique can only assist in detecting the

TABLE 4
Probability of detection of pebrine in 20% sampling method (Index)

No. of egg cards (20 layings on each card)	Population (No. of layings)	Pebrine	Samples	Probability	
				Non Detectable	Detectable
20	400	2	80	0.6400	0.3600
30	600	3	120	0.5120	0.4880
40	800	4	160	0.4096	0.5904
50	1000	5	200	0.3277	0.6723
60	1200	6	240	0.2621	0.7379
80	1600	8	320	0.1678	0.8322
100	2000	10	400	0.1074	0.8926
150	3000	15	600	0.0352	0.9648
200	4000	20	800	0.0115	0.9885
250	5000	25	1000	0.0380	0.9620
300	6000	30	1200	0.0012	0.9988
500	10000	50	2000	0.0000	1 .0000

Rate of pebrine infection = 0.05% in female moths

(Source: Fujiwara, 1993)

disease and the only way out is to destroy the diseased silkworm crops which cause loss, apart from making efforts to prevent the said disease at all levels.

A burning problem in the field of microsporidiosis is the increasing number of different microsporidians that are being encountered in silkworm crops (Fujiwara, 1980, 1993). These microsporidians have been shown to exhibit varying degrees of virulence and many of them have demonstrated low multiplication rate in silkworm although they are infective and pathogenic. Some of them have not shown vertical transmission in the host. As of today, however, there has been no specific testing procedure to discriminate these microsporidians in the field to take appropriate action, while preparing disease free silkworm seed. If pebrine is to be controlled effectively, a system has to be evolved, where either a seed cocoon grower or a seed producer is not put in hardship due to the reoccurrence of the disease.

Future Research Strategies

Application of the molecular techniques for diagnosis, species differentiation, identification of intermediary stages of development, multiprimer PCR techniques will lead to enormously increased knowledge of the microsporidians infecting silkworms (*Bombyx mori*) in the near future. In addition, there is also a need to develop better, rapid, systematic and feasible techniques for early detection of the disease, to evolve pebrine resistant region and season specific breeds/ hybrids of silkworm for commercial use, and to identify the potential target organs of both the parasites and host for control through chemical agents, apart from elaborating serological and epidemiological studies in natural epizootics involving biology, host parasite interactions, taxonomy, etc. and developing effective and efficient model(s) for forecasting of the disease outbreaks.

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