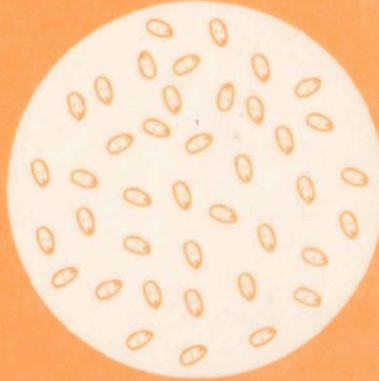


PEBRINE DISEASE OF SILKWORM



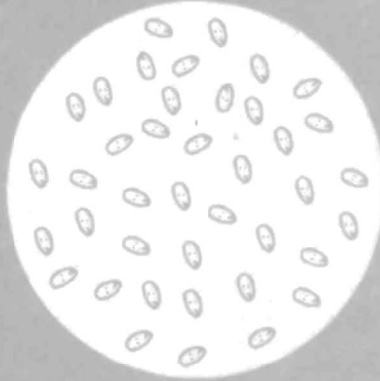
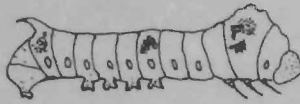
R. GOVINDAN
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M. C. DEVAIAH



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**PEBRINE DISEASE
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SILKWORM**

R. GOVINDAN

Professor of Sericulture

T. K. NARAYANASWAMY

Associate Professor of Sericulture

M. C. DEVAIAH

Dean, UAS

UNIVERSITY OF AGRICULTURAL SCIENCES

GKVK, BANGALORE-560 065

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FOREWORD

Sericulture is an important agro-based enterprise generating more employment potential and fetching remunerative returns to the silkworm rearers, silk reelers, twistors and weavers. Silk also earns considerable foreign exchange to the country. India ranks second in global raw silk production next only to China and Karnataka alone contributes the bulk of Indian raw silk. The research advancement made in mulberry leaf production technology and silkworm breeding though has contributed for considerable increase in productivity, the silkworm diseases and pests have been playing a pivotal role in reducing the cocoon yields as well as the quality of raw silk produced. Diseases are responsible for 20-40 per cent of cocoon crop loss. Silkworm diseases are caused by Protozoa, fungi, bacteria and viruses. Pebrine is a protozoan disease and this is a dreadliest melody in all sericultural nations since the time of Louis Pasteur. This is a transovarially transmitted disease and was responsible for the collapse of sericultural industry in Europe. This disease has been appearing in epizootic form cyclically in Karnataka too. This necessitates for possessing comprehensive knowledge on various aspects of pebrine including its management. The authors Dr. R. Govindan, T. K. Narayanaswamy and Dr. M. C. Devaiah have attempted to pool the scattered literature on pebrine disease and the same has been brought out in this series under appropriate headings.

This compilation contains information on bio-diversity of Protozoa infectious to silkworm and historical aspects, etiology, epizootiology, symptoms and pathologies, detection and prevention and control of pebrine disease. It is useful to teachers, research workers and students of Sericultural Sciences.

Bangalore
September, 1977

G. K. VEERESH
Vice-Chancellor



INTRODUCTION

Pebrine is a protozoan disease of the silkworm, *Bombyx mori* L. caused by *Nosema bombycis* Naegeli (Protozoa; Microsporidia). It was first recorded in France in 1845 and later spread to Italy, Spain, Syria and Romania. It was responsible for the collapse of the French and Italian silkworm industry in 1865. The name pebrine was coined by De Quadrefagues (1860) because of pepper-like spots seen on the diseased larvae in advanced stage of infection. The disease is known as KATA or MATHA KATA in Bengali and as GANTU or GANTU ROGA in Kannada.

The most frequent source of infection is through ingestion of the food (mulberry leaves) contaminated with spores of the parasite by silkworm larvae. The second common method and route of infection is through the mother moth (transovarial transmission) known since Pasteur's time. Also, the disease is to some extent transmitted from one generation to the next through the external contamination of eggs by spores (Transovum transmission).

Infected worms lose appetite, become sluggish, opaque and ultimately die. Diseased worms may spin cocoons which are flimsy and poor in silk content. In coloured cocoon races, the intensity of the colour comes down. The fast spreading nature of the disease through secondary contamination has been established, the extent of contamination being in proportion to the initial density of infected larvae in the batch (Baig *et al.*, 1988 a).

Life history of the pathogen is known (Stempell, 1909). Pioneering work on various aspects of this disease has been done in India by Mukherji (1912), Jameson (1922; 1924) and Ghosh (1949). Later, the works of Neriki and Ito (1896), Ishiwata (1897), Ishikawa (1935) and Weiser (1957) mainly have concern to the epizootiology and prevention of the disease.

Eventhough the fight against pebrine has continued for more than a century, the loss due to the disease has not been completely eliminated.

In fact, there appears to be a close relationship between the control of pebrine and the success of the sericulture industry. Therefore, it is essential to acquire indepth knowledge of this disease for the improvement of the sericulture industry.

Pebrine being a transovarially transmitted disease, the young silkworms infected in 1st and 2nd instars usually die in 3rd instar but rarely in 4th instar so that the cocoon production is greatly affected. If infection occurs in 4th instar, the development may proceed to the adult stage and eggs are laid but they contain infected embryos and thus constitute a menace to the egg supply. In commercial cocoon production pebrine infection reduces the quantity and quality of the cocoons produced thereby rendering heavy loss to the sericulturists.

The damage of cocoon crops in Europe in the middle of the nineteenth century was so great and extensive that the cocoon production declined sharply and the sericultural industry of the world suffered heavily. In France, for example, pebrine disease brokeout in the province of de Vaucluse in 1845 and spread to three neighbouring provinces in the following years. Within 5 to 6 years the disease spread all over France and in 1851 there was not a single province in France which was not affected by the disease. The menace of the disease was so intense that the total annual cocoon production of 2,10,00,000kg during 1846-52 was reduced to 1/3 of the production (75,00,000kg) during 1856 in short span of four years.

Around 1850 France began importing silkworm eggs from Lomardie, Italy and as a result the cocoon production increased again. In 1854 the pebrine disease spread to Italy and Spain, forcing the merchant to seek silkworm eggs in Greece and Turkey. However, these two countries were also affected by the disease in 1859-1860 and silkworm eggs had to be sought in Syria, Caucasus, Moldavie and Valachie. These regions, however, were also affected by the disease gradually and finally in 1864, attention of the world began to focus on the silkworm eggs produced in Asia, particularly those in Japan, for healthy silkworm eggs and France began importing silkworm eggs from

Japan. However, the damage to crops by pebrine disease in France could not be prevented by imports of silkworm eggs and far exceeded the supply of new silkworm eggs. As a result, the cocoon production decreased to one sixth of that in the best time. At last in 1856 the disease was so rampant that sericulture in France was on the verge of total collapse. Silkworm inspection in France in 1866 revealed that approximately two third of the silkworm eggs imported from Japan had been infected and all sericultural countries of the world were exposed to the menace of pebrine disease. Prior to this explosive outbreak of pebrine disease in the middle of the 19th century, the damage to crops believed to have been caused by pebrine disease was also seen in a cycle of several decades. However, confirmation of the pathogen through microscopic observation was not in vogue at that time and therefore, it cannot be claimed that pebrine disease was the lone and the unequivocal cause for crop damage. It is believed, however that the disease which had existed unnoticed for a long time spread widely again following the revival of silk industry.

For about a century since the fight against pebrine disease began, the threat from the disease has not yet been eliminated to the present. Even in Japan where laws and regulations are relatively complete for the prevention of the disease, there is every possibility of whole enterprise facing bankruptcy if the regulations are not strictly adhered to even in a comparatively small region. An analogy may be drawn from the outbreak of epidemics like gastroenterites and small pox that are frequently reported inspite of strict quarantine laws. Prevention of such disease can not be effectively implemented through preventive measures of one region or one country alone but there must be constant and strict surveillance through International co-operation. This also holds true for the prevention of pebrine disease. All sericultural nations should realise their International obligations for the prevention and control of this disease.

The history of sericulture industry speaks volumes on the downfall of many sericulturally advanced countries in the middle and later part of 19th century due to the rampant outbreak of pebrine. Cocoon production declined sharply, resulting in heavy loss to sericulture industry. During

19th century the incidence was to an extent of 20 per cent in the major sericultural countries and presently this has come down to about 1 per cent due to its systematic control and management. Pebrine is commonly prevalent in China, Japan, Korea, India, Thailand, Cambodia, Vietnam etc. The incidence is very high in the countries where the farmers produce their own silkworm seed.

Unlike in the temperate or sub-tropical countries, silkworm rearing is practised throughout the year in tropical countries, which enhances the possibility of secondary contamination from the rearing rooms, equipments and mulberry gardens. The fact that the symptoms of pebrine are often mistaken for flachérie disease by the farmers makes the disease unnoticed and thus becomes a further source of contamination. The condition of the rearing houses in tropical countries is not ideal to take up thorough disinfection. Seed production is a continuous process and the time available between batches in a grainage is too short to take up suitable precautionary measures in an effective way.

In temperate countries the incidence is reported to be more during summer followed by autumn and spring, which may be due to continuous silkworm rearing helping in the persistence of the pathogen. Contrary to this it is observed in tropical countries like India that the incidence is low during summer and increases by winter. Low incidence during summer may be due to the higher temperature and lower humidity which reduce the pathogenicity and reduction in rearing intensity may also contribute. Though the reasons for the increased incidence during cooler months are not clear, it may be possibly due to the increase in the lepidopteran pests during rainy and winter seasons (De, 1919 ; Jameson, 1922 ; Dasgupta, 1950 ; Noamani *et al.*, 1971).

The incidence of pebrine under Mysore conditions was seen during 1969. It varies with seasons. Temperature and humidity appear to play some role on the incidence (Devaiah and Krishnaswami, 1975). The disease prevails throughout the year. The percentage of infection is more during July to March (73.6 to 78.9° F) at Mysore and less during April to June (78.4 to 83.3° F). Similar is the situation under West Bengal climatic

conditions (Noamani *et al.*, 1971). In Karnataka pebrine incidence is 2.32 per cent of the total disease occurrence (Samson *et al.*, 1990).

2. BIODIVERSITY OF PROTOZOA

Protozoa injurious to mulberry silkworm, *Bombyx mori* L. exhibit considerable biodiversity and this group includes Sporozoa but the most disastrous of all is *Nosema bombycis* which causes pebrine disease. Though a sarcodinan, *Entamoeba blattae* and a mastigophoran, *Herpetomonas* sp. have been reported in silkworm from Japan as back as 1924, further work has not been there.

A flagellate, *Leptomonas* sp. is found infectious to silkworm (Aratake and Kayamura, 1977 ; Abe, 1978) and its ultrastructure is known (Abe and Iwashita, 1989). It multiplies by binary fission in silkworm pupae intracellularly as leishmanial forms (amastigote) and extracellularly as leptomonad form (promastigote) (Abe, 1978c). When leptomonad forms increase in number to more than 10^7 per ml in haemolymph, they aggregate to make rosette and encyst. When cysts are inoculated orally to silkworm, encystation also occurs in midgut lumen and the cysts are expelled into faeces with the peritrophic membrane. As per Abe (1979), acridine orange and sodium azide stop its multiplication in vitro but not so in vivo. When exposed to more than 35°C for 24 h, it dies both in vivo and in vitro. It appears to be eliminated from haemolymph by phagocytic action of host insect since degenerated protozoans are detected in blood cells.

In addition to *N. bombycis* seven other microsporidians belonging to the genera *Nosema*, *Pleistophora* and *Thelohania* have been isolated from the silkworm (Fujiwara, 1984 a, b.). These microsporidians differ in their spore morphology, target tissues and virulence and have been tentatively designated as M11, M12 and M14 (*Nosema* spp.), M24, M25, M27 (*Pleistophora* spp.) and M32 (*Thelohania* sp.). Spores of *Thelohania* sp. measure 3 to $3.7\ \mu$ and 1.5 to $2.0\ \mu$ in length and width, respectively with filament length of $65\ \mu$ (45 to $82\ \mu$).

Pleistophora sp. has been isolated from female silk moths in Japan (Fujiwara, 1984a). Its incidence is extremely low in spring and slightly increases in fall. Spores are oval ($5.06 \times 2.97 \mu$) and polar filament is 140μ in length. The parasite invades muscles, fat bodies, malpighian tubules and silk glands of larvae. Pansporoblasts are observed in infected cells and later 16 or more spores are formed in a cyst (Watanabe, 1984). It is infective to *Antheraea eucalypti* cells (Sato and Watanabe, 1986b). Indirect fluorescent antibody technique seems to be one of the most reliable methods for species discrimination of microsporidians infecting silkworm. The morphology of spores, site of infection and virulence to silkworms are shown in Table I.

Table I. Microsporidians isolated from silk moth *Bombyx mori* L. (Fujiwara, 1980 ; 1984a)

Microsporidian	Spore size (μ)	Site of infection	Virulence
<i>Nosema bombycis</i>	3.8 x 2.2	Systemic	High
<i>Nosema</i> sp. M11	3.9 x 1.9	Various tissues	Low
<i>Nosema</i> sp. M12	4.2 x 2.7	Various tissues	Low
<i>Nosema</i> sp. M14	5.1 x 2.0	Various tissues	High
<i>Pleistophora</i> sp. M24	2.7 x 1.6	Midgut	Low
<i>Pleistophora</i> sp. M25	3.2 x 1.8	Midgut	Low
<i>Pleistophora</i> sp. M27	3.4 x 3.0	Various tissues	Low
<i>Thelohania</i> sp. M32	3.4 x 1.7	Muscle	Low

Recently, three microsporidians designated as NIK-2r (*Nosema* sp., Mysore), NIK-3h (*Nosema* sp. M11, Hassan) and NIK-4m (*Nosema* sp. M12, Mysore) have been isolated from Karnataka and they are immunologically dissimilar with *Nosema bombycis* (Ananthalakshmi *et al.*, 1994a). Even they differ in spore size ($3.6 - 5.00 \times 1.8 - 2.8 \mu$) and shape of *N. bombycis* ($3.8 \times 2.6 \mu$). *Vairimorpha* sp. NIS M12 isolated from *B. mori* infects the cell line of *Spodoptera frugiperda* (Inoue *et al.*, 1995). Short coiled spores occur 72 hr pi and long coiled mature spores

at 10 days pi. Syncytia are not formed. The microsporidian spores isolated from the progeny of infected moths are serologically identified as the same species that is fed to the larvae. No transmission of either *N. bombycis* or *Nosema* sp. M11 progeny occurs in cross between healthy female and infected male, indicating that there is no veneral pathway for either microsporidians. The progenies that are transovarially infected with the two Microsporidia show differential mortality. In the progeny population infected with *N. bombycis* the infections are acute, killing 66 per cent of the progeny in the egg stage, and an additional 33.1 per cent by the end of the 3rd stadium. In the progeny population that has been infected with *Nosema* sp. M11 however, only 0.4 per cent die in the egg stage, and larval death does not occur until the end of fifth instar, where mortality increases to 1 per cent to eclosion. The resulting adults in three batches have infection rates of 2.2, 65.9 and 97.9. Further *N. bombycis* and *Nosema* sp. M11 differ greatly in their virulence to the silkworm. *N. bombycis* is highly virulent and produces acute disease, while *Nosema* sp. M11 is comparatively less virulent and produces a chronic disease.

Nosema sp. infects tropical tasar silkworm, *Antheraea mylitta* (Jolly and Sen, 1972). *N. antheraea* has been isolated from Chinese tasar *A. pernyi* in USSR (Simchuk *et al.*, 1979). *Nosema* sp. also infects muga silkworm, *A. assamensis* in India with high incidence during October (Talukdar, 1974). Infection results in delayed moth emergence and reduction in fecundity (Talukdar, 1995). Transovarian transmission reveals the rate of infection to increase with each successive hatching date resulting in 100 per cent infection among larvae hatching on last day (Talukdar, 1980).

3. HISTORICAL ASPECTS OF PEBRINE

The historical developments on pebrine disease are arranged chronologically as follows :

1750-1780 : Extermination of sericulture industry in France due to outbreaks of pebrine

- 1763-1818 : Boissier and Dandolo gave description of various diseases in which spots on the skin are cited as symptoms
- 1845 : Existence of pebrine disease was known in France. Then its occurrence was observed in Italy, Spain, Turkey, Syria, Rumania etc.,
- 1849 : Guerin - Meneville of France named the pebrine pathogen as "Hematozoid" *ie.*, a microorganism living in haemolymph of silkworm
- 1850-1870 : Leybig and Balbiani showed for the first time that Microsporidia are characterised by production of minute oval spores. In Europe the epidemic of pebrine swept the silkworm rearing districts
- 1856 : Lebert and Fray made observations on pathological significance of the corpuscles
- 1857 : Naegeli briefly described the corpuscles as representing some form of yeast-like fungus and gave the present name, *Nosema bombycis*
- 1858 : Lebert studying the pathological significance of pebrine corpuscles, named the organism as *Panhistophyton oyatam*
- 1859 : Hutton described the pebrinised laying and also black spots appearing at the junction of the annulations of the body
- 1860 : De Quadrefagues gave a popular name pebrine to this disease
- 1865-1870 : Pasteur innovated transovarial transmission of pathogen and suggested mother moth examination for diagnosis of pebrine disease. He wrote a voluminous work entitled "EIUDES SUR LA MALADIE DES VERS A SOIE". In this book the

disease has been called "MALADI CORPUSCULAIRE" (CORPUSCLE DISEASE) (Pasteur, 1870)

- 1866 : A disease in Mysore described as a sort of atrophy was also doubtlessly pebrine
- 1878 : Pebrine epidemic swept through Kashmir
- 1882 : Balbiani of France wrote a book "Lecons Surles Sporozoaires" and made known that the pathogen belongs to Microsporidia under Sporozoa of Protozoa
- 1888 : Mukerji stated that pebrine had appeared only 10 or 12 years back and becoming more destructive in each year
- 1890-1900 : Epizootic outbreak of pebrine in the then Mysore and Madras provinces
- 1899 : Hutchinson wrote in all probability that pebrine had played a role in depressing the successful rearing of silkworm in India
- 1909 : Stempell from Germany published well known paper describing the various stages in the life cycle of *N. bombycis*
- 1913 : Paillot brought out that deadly epidemics were caused by *N. bombycis* among silkworms in France
- 1915 : Basu reported that pebrine disease on eri silkworm *Philosamia ricini* was not much important
- 1916 : Lefroy's Report on an enquiry into the silk industry in India stated this disease to have appeared

Bengal by about 1895 and the probability in Mysore was between 1890 and 1900

- 1916-1920 : Hutchinson (1918) opined that pebrine disease in India showed its appearance only towards the close of the last century. Infectivity, pathogenecity, transmission and viability of *N. bombycis* were studied
- 1917 : Fauchere stated that silkworms in Europe and Madagascar were infected seriously by pebrine rendering more serious loss in tropics
- 1926 : According to Deboudeau, pebrine appeared in Persia at about the same time as in Europe with the same disastrous results
- 1929 : Flass working with tasar silkworm, *Antheraea pernyi* observed a disease, "Nosematose" caused by *Nosema* sp. closely resembling *N. bombycis*
- 1931 : Condura working with one microsporidiosis of silkworm *B. mori* found that pebrine was responsible for serious losses in Italy
- 1955 : Pebrine started as mildly epidemic in a large seed campaign during 1940-47 in Mysore
- 1960 : Janakiraman stated that pebrine was not known until 1895 but he believed it must have existed during 1850-1870
- 1963 : As per Yokoyama, pebrine was destructive to sericulture before Pasteur's principle was known and at present the actual damage caused by this disease is very slight in Japan.

4. ETIOLOGY

A. Causative Agent

The disease causing microorganism was first observed in the haemolymph of silkworms and was given the name "Hematozoid" (Guerin Meneville, 1849). Naegeli (1857) named the pathogen scientifically as *Nosema bombycis*. Later, Balbiani (1882) reiterated its position to Sporozoa of the Phylum : Protozoa. Recently, Thomson (1960) and Sprague (1977) while listing microsporidians infectious to insects presented the following classification :

Phylum	:	Protozoa
Order	:	Microsporidia Balbiani (1882)
Family	:	Nosematidae Labbe (1899)
Genus	:	<i>Nosema</i> Naegeli (1857)
Species	:	<i>Nosema bombycis</i> Naegeli (1857)

Based on the microsporidian's unusual cytological and molecular characteristics such as primitive type of nuclear division and absence of mitochondria they have been phylogenetically considered as one of the earliest known eukaryotes (Vossbrink *et al.*, 1987 ; Undeen and Cockburn, 1989). The work on pebrine has been reviewed (Patil, 1993).

Purification of Spores : Purification of the pathogen will have to be achieved for enumerating various aspects *viz.*, morphology, ultra structure, serology, infectivity etc. of the pathogen. Bonisha (1959) and Cole (1970) used centrifugation and triangulation method, respectively to purify *Nosema* spores. The theoretical and actual distribution of spores at various levels after centrifugation were studied.

In partial modification of the above methods the usual method is that pebrinised larvae collected based on the external symptoms at fifth instar are triturated in a sterilized mortar and pestle using sterile distilled water. The filtered crude spore suspension is centrifuged at 3500 rpm/ 15 min. The sediment is suspended in distilled water and centrifuged

repeatedly till a clear white pellet of pebrine spores is obtained. The stock spore suspension is stored in refrigerator ($3\pm 1^{\circ}\text{C}$) for further use and a rapid method for concentrating *Nosema* spores based on continuous flow centrifugation has been described (Gochnauer and Margetts, 1980). Hostounsky (1981) described a method based on brownian movement for concentration of spores. Sato and Watanabe (1980) purified pebrine spores using sucrose and percoll gradient centrifugation. In the sucrose gradient of 50-60 per cent (W/W) centrifugation the mature spores did not clearly separate from immature spores and cell debris, while a band consisting of only mature spores with a recovery rate of 90 per cent was possible in percoll gradient. Centrifugation using percoll at 73000 g for 30 minutes resulted in 3 bands viz.,

- 1) a sharp band consisting of tissue debris of silkworms, cell debris of mulberry leaf, bacteria etc.,
- 2) a dim band consisting of mature but inactive spores
- 3) another sharp band consisting of only mature and active spores.

B. Morphology and Chemistry

Three unidentified *Nosema* spp. and a *Pleistophora* sp. were known to infect the silkworm. The *Nosema* differed in pathogenecity *ie.*, target tissues, spore shape and pathology in the infected tissues (Fujiwara, 1980). Indirect fluorescent antibody tests showed that the spores of twenty of the *Nosema* spp., *Pleistophora* sp. and *Nosema bombycis* had no antigenic cross reactivity (Sato *et al.*, 1982a). The ultrastructure of the spores of four sporozoans was elucidated (Sato *et al.*, 1982b).

Nosema bombycis : The polar filament has 12 coils with an angle of tilt of atleast 49° . Filament has central core surrounded by four concentric layers. The core is surrounded by a relatively electron lucent layer with a substructure consisting of 16 small granules. The internal and the outermost layer are translucent like the membrane of endoplasmic reticulum or nucleus and between the two layers is an electron dense layer.

Spores, under light microscope are oval in shape, approximately $3.4 \times 1.5 - 2.5 \mu$ with a three layered membrane (inner, middle and outer). A micropyle is located at the anterior end and in the centre is a belt-like sporoplasm containing two nuclei. The polarplast has two parts. At each end there is a vacuole. A polar capsule and polar filament are present. The polar filament is tubular, coiled, whip-like structure lodged within polar capsule. The polar filament is 30 times the length of spore.

The spores are highly refractile appearing light green under the microscope. The outline is smooth and the spores are heavier than water. The spores are the dormant stage of the pathogen and possess great resistance. For example they can remain infective even after three years in the dried body of the female moth and remain active after being submerged in water for five months.

Nosema sp. M11 : Polar filament has about 11 coils with an angle of tilt of at least 39° . Filament is composed of a core surrounded by three concentric layers. Ribosome arrangement is similar to that of *N. bombycis*.

Spore has two nuclei placed close together. Nuclei are slightly elongate in the direction of the major axis of the spore. The polarplast consists of two parts as in the case of *N. bombycis*.

Vairimorpha sp. (*Nosema* sp. M12) : Polar filament has about 15 coils with an angle of tilt of at least 43° . Structure of polar filament and polarplast are similar to *Nosema* sp. M11 but the polarplast is more slender than that of *N. bombycis* and *Nosema* M11. The arrangement of nuclei and ribosomes is as in *Nosema* sp. M11. The polarplast consists of two morphologically different components: an anterior part composed of laminated membranes, which may be tightly flattened sacs of loose shoe shape in a longitudinal section of the spore and a posterior part made up of loosely flattened sacs of lozenge shape.

Spore possesses two nuclei in the posterior half that are slightly elongate in the direction of the minor axis of the spore. The posterior

vacuole is enclosed by double or more membranes and occurs near the posterior pole.

Occasionally the vacuole has vesicles along its side. These vesicles have been observed separating from the vacuole in a spore. Spores fix well with osmium tetroxide, show an arrangement of ribosomes in the cytoplasm, just internal to the coiled polar filament.

Pleistophora sp. : The polar filament has eight coils with an angle of tilt of at least 31° . The filament has a central core surrounded by concentric layers. The polarplast is composed of three parts. The spore contains single nucleus in the posterior half. A small vacuole enclosed by many fold membranes occurs near the posterior pole and sometimes it has vesicles along its side. Around the coil of polar filament long endoplasmic reticulum is often seen.

Serological techniques have been widely used to determine taxonomic relationships among parasitic Protozoa. The application of these techniques to Microsporidia has been hampered by the difficulty in producing sufficient quantities of spores and by the lack of a suitable method for disrupting them. Fowler and Reeves (1974 a & b) have successfully employed the MSK Braun cell homonizer to disrupt spores and extract water soluble proteins for use as antigens.

Cali (1970) studied the development of *N. bombycis*, *N. apis* and *N. curiculi* with the electron microscope. It was suggested that the genus *Nosema* be restricted to those Microsporidia in which each sporont gives rise to two spores and in which the diplokaryon nuclear arrangement is present throughout the life cycle.

Ishihara (1968) described the fine structure of *N. bombycis* spores which had been treated to discharge the polar filaments.

Vaura and Barker (1978) innovated a preparatory technique for scanning electron microscopic studies of *N. bombycis* and *N. plodiae*.

Earlier, the identification of pathogens was mainly based on morphological characters observed with light and electron microscopy, but the dimorphism exhibited by microsporidians causes difficulties in accurate identification (Hazard and Weiser, 1968). This gave an impetus to the study of biochemical characters. The sedimentation value of monoribosomes from the sporoplasm of *N. bombycis* (typical of lower Protista) as determined by sucrose density centrifugation (Ishihara and Hayashiya 1968). Later, the nature of metachromatic granules, distribution of ribosomes within the spores, their sedimentation coefficients and chemical composition of the spore wall of *Nosema* sp. have been studied (Erickson and Sprague, 1970). Further identification of various microsporidian species is achieved using polyacrylamide gel electrophoresis (Fowler and Reeves, 1974a; 1974b) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (Street and Briggs, 1982). The polypeptides separated with the above methods provide reproducible electrophoretic profiles.

The spore polypeptides of *N. bombycis* and *Vairimorpha necatrix* were separated by SDS PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis). Page electrophoresis provided unique reproducible electrophoretic profiles which were not influenced by the host species. The host proteins were not detected in the polypeptide profiles. Mixed spore samples of *V. necatrix* and *N. bombycis* could be separated in the presence of the profiles of the individual species. A total of 31 polypeptide bands were detected on electrophoretic profiles of spore mixtures, whereas individual profiles of *V. necatrix* and *N. bombycis* had 24 and 26 polypeptide bands, respectively (Street *et al.*, 1978). The SDS PAGE systems could detect spore mixtures of these two species.

Assays such as Fluorescent antibody test (Fujiwara *et al.*, 1966; Sato *et al.*, 1981) and indirect enzyme linked immunosorbent assay (Kawarabata and Hayasaka, 1987) have been suggested for detection of *N. bombycis* infection. The simple latex agglutination test for microsporidian infections of silkworm has been developed (Hayasaka and Ayuzawa, 1987). Baig *et al.* (1992a) described a rapid, accurate and economical latex agglutination test for detection of pebrine infection on simple glass slide.

Nosema bombycis was studied cytochemically using light and electron microscopy. DNA and RNA were detected in all the stages. Polysaccharides and acid phosphatase were found in the spore but not in the schizogonic stages. Polysaccharides were detected in the polar cap, polar filament and the limiting membrane of the spore cytoplasm. Acid phosphatase was associated with polar cap, posterior vacuole and polar filament (Hirani *et al.*, 1975).

DNAs from reference strains of *N. bombycis* are amplified specifically by polymerase chain reaction (PCR) using primers from a putative pseudogene together with those derived from conserved regions of a small subunit RNA gene of *N. bombycis* and when DNA templates of six microsporidians *viz.* two reference strains (SES-NLI, NIS-do1) and one from *Spodoptera depravata* under *N. bombycis*, *Nosema* sp., *Vairimorpha* sp. and *Pleistophora* sp. and PCR was conducted, the PCR products are obtained only from the first two reference strains of *N. bombycis* (Kawakami *et al.*, 1995). This is useful in pebrine inspection.

C. Life Cycle

The transmission channel of *N. bombycis* is quite interesting in that the pathogen gets passage to the next generation through transovarial transmission thus getting access into the eggs and also through wide contamination of mulberry leaf. The pathways of oral infection in the second instar larva of *B. mori* with *N. bombycis*, *Nosema* sp. M11, *Vairimorpha* sp. M12 and *Pleistophora* sp. have been reinvestigated (Sato and Watanabe, 1986).

Kawarabata and Ishihara (1984) studied the infection and development of *N. bombycis* in a cell line of *Antheraea eucalypti*. The first increase in the number of infected *A. eucalypti* cells was observed 48 hour subsequent to inoculation and it was caused by the secondary infective forms of *N. bombycis*. The secondary infective forms were produced during the course of sporoblast differentiation. The parasites in cell cultures divided synchronously until 36 hours post inoculation (pi). Mature spores were observed initially 6 days after inoculation at 27° C. An actual increase in the number of infected cells was detected 48

hour pi and this increase in the rate of infection was caused by the secondary infective forms. At 72 hours pi rapid increase in parasitic cells was observed. The rate of infection reached 80 per cent or more by 10 days pi.

Regarding growth, the number of the parasites per infected cell started to increase 24 hour pi. and increased rapidly upto 60 hour. The multiplication of the first microsporidian generation reached a stationary state at 72 hour. The proliferation growth of *N. bombycis* in *A. eucalypti* cells can be divided into 3 successive stages. 1st stage is additive growth stage (6 hour-12 hour) during which the sporoplasms grew into schizonts, 2nd stage being vegetative stage (19 hours to 24 hours) during which there was multiplication by binary fission of the schizonts and 3rd stage being differential stage (36 hours to 39 hours) during which the sporonts differentiated into spores and the production of secondary infective forms occurred. The Phase I sporoblasts grew into Phase II sporoblasts within 6 hour and the production of the secondary infective forms began at the same time (Kawarabata and Ishihara, 1984).

The dimorphic development of *N. bombycis* spores in midgut epithelium of larvae of *B. mori* has been studied (Iwano and Ishihara, 1991a). When spores were inoculated to second instar larvae, two types of spores viz., short polar tube type spores and long polar tube type spores, identical with the ones revealed in cultured cell, developed. One type corresponded to the spores with few coils and the other type to the spores with many coils in the cultured cells, respectively. The significance of the dimorphism has been discussed. The long polar tube spores were thrownout to the midgut lumen while the short polar tube type spores helped for further infection within the host.

Sato and Watanabe (1986) studied the pathways of oral infection in the four Microsporidia in silkworm. The spores of *N. bombycis* extruded polar filaments in every area of the midgut lumen. Sporoplasms and intermediate forms between sporoplasms and schizonts were found in the cytoplasm of the midgut epithelium and the circular muscle of the middle part of the midgut between 5 and 24 hour pi. Two days later colonies of schizonts appeared in the cytoplasm of the cells of midgut

epithelium, circular muscles, longitudinal muscles and adjacent tissues of midgut. Sporoplasms of *Nosema* sp. M11 were found in the cytoplasm of midgut epithelium 6 hour pi. Four days later schizonts of M11 proliferated in the midgut epithelium, posterior midgut muscle and adjacent tissues.

The spores of *Vairimorpha* sp. M12 germinated in every area of midgut lumen and their sporoplasms were observed in the cytoplasm of the midgut epithelium and middle part of the midgut. Two days later schizonts of M12 were seen in the same tissue and sometimes in the adjacent tissues. Spores of *Pleistophora* sp. extruded polar filaments in every area of the midgut lumen (Sato and Watanabe, 1986a).

Kudo (1916) succeeded in extruding the polar filament of *N. bombycis* by treating the freshly extracted spores with hydrogen peroxide solution. The spores when suspended in a mixture of Hydrogen peroxide and potassium chloride at 40°C are known to get primed and hatch (Iwano and Ishihara, 1979).

The presence of Juvenile hormone (JH) activity in microsporidian spores has been reported (Fisher and Sanborn, 1964; Erickson and Sprague, 1970). The possible explanation suggested was that the microsporidian parasite either produces a chemical with an activity similar to that of JH or the parasite absorbs JH from the host haemolymph. It has been reported that Microsporidia - infected larvae undergo supernumerary moults (Fowler and Reeves, 1975; Milner, 1972), a condition which is characteristic of abnormally high or persistent levels of JH. This would indicate that infection of *N. bombycis* may have some effect on the endocrine system of silk worms which eventually may lead to the extension of the larval period which would enable the parasite to continue multiplication for its complete development.

D. Infection Cycle

The basic sources of infection for pebrine pathogen are transovarial transmission and contamination of food i.e., mulberry leaf through various means.

When spores of *N. bombycis* were ingested by silkworm larvae, the inner part of the spore lost its affinity and small sporoplasm discharged from the spores developed into large ovoid cells with two nuclei and a vacuole. The sporoplasms were observed in the peritrophic membrane and microvilli of the midgut (Abe, 1978a). Histological observations on silkworm larvae orally infected in the 2nd instar with *N. bombycis* spores revealed that after 24 hour *Nosema* cells occurred only in the granulocytes (Abe, 1978a). The parasites developed into round acidophilic cells (APC) in vesicles in the granulocytes and were released into the blood stream which entered the muscle layer of the midgut in 48 hour. Cytoplasm of midgut epithelial cells, silk gland cells and malpighian tubule cells were found to contain APC and empty vesicles at the distal end near the basement membrane and young and mature sporoblasts had developed into spores in 96 hour. At this time large parasitic cells appeared in granulocytes and were liberated into the haemocoel. The cells became amoeboid cells with two nuclei and invaded other host cells. APS appeared to be responsible for earlier propagation while amoeboid cells engage in later spread. Intranuclear development of *N. bombycis* in the cells of experimentally infected *B. mori* studied by Takizawa *et al.* (1975) revealed that 7 days after the initial infection (per os) all developmental stages of *N. bombycis* were present demonstrating thereby that the host nuclei satisfy all the nutritive requirements of the pathogen. The vegetative forms showed abundant microvilli at the periphery of the parasitized.

Change of relation between infectivity and filament evagination of debilitated spores of *N. bombycis* revealed that per os infection with *Nosema* spores was normally performed by filament evagination (Oshima, 1973). However, when spores were weakened by preservation for several years or by disinfectants, the relationship between filament evagination and infectivity did not necessarily exist. The degree of discordance changes not only with the kind of disinfectant used but also with the duration the spores were treated with formalin. Hydrogen peroxide (H₂O₂) evaginated the filaments well but the sporoplasm was completely destroyed.

Though the microsporidian restricts to the cytoplasm of the target cell, occasionally it invades the nucleus too (Takizawa *et al.*, 1973).

Spores treated with Potassium hydroxide (KOH) solution on ingestion by silkworm larvae changed their stainability in that the inner part of spore lost its affinity for basic stain and two granules stained with acid dye. Small sporoplasms with poor cytoplasm discharged from spores developed to large ovoid cells with two nuclei and a vacuole in the cytoplasm. The sporoplasms were observed in peritrophic membrane so that they seemed to be protected by the membrane. Some sporoplasms were also seen in the area of microvilli (Abe, 1978a).

Ananthalakshmi *et al.* (1992) studied the intermediary stages in the life cycle of *N. bombycis* by Giemsa stained smears and progressive infection by paraffin sections. Midgut formed the primary site of infection and binucleate round dividing schizonts were observed at 48 h and spore formation was at 96 h pi. Malpighian tubules formed the second site of infection followed by fat bodies, muscle fibres, silk glands and gonads. Hayasaka and Kawarabata (1990) cloned *N. bombycis* in cell culture of *Antheraea eucalypti* by a limiting dilution method.

The developmental stages of *N. bombycis* observed with fresh Giemsa stained smears of the midgut revealed two and four nucleated schizonts at 48 h pi (Ananthalakshmi *et al.*, 1994b). Schizonts ready to undergo division and two binucleate (diplokaryon) stage at the end of division were observed. Sporonts were elongated, binucleated with lightly stained, granulated nuclei and cytoplasm. Young and mature sporoblasts, with two nuclei were observed at 96 h pi. Mature sporoblasts were spindle shaped. The secondary infective form was observed at 96 h pi.

5. SYMPTOMS AND PATHOLOGIES

The symptoms of pebrine vary depending on the stage of *B. mori* viz., egg, larva, pupa and moth and form important criteria for identifying the disease.

Egg : As per the process of transovarian transmission, the pathogen parasitizes the ovary first when the female larva infected by the pebrine disease at the 4th – 5th instar becomes a moth, moves into the egg and

after the deposition of the egg undergoes multiplication and develops the disease in the embryo or in the body of the silkworm.

Growth and multiplication of the pathogen in the egg are influenced by the growth of host egg. When the egg diapauses, the growth and multiplication of the pathogen stop simultaneously and when the egg starts growing by incubation and other reasons, the pathogen also starts growing and multiplying. When the degree of infection of the egg is relatively high, the egg often becomes a non-fertilized egg or dead egg, but when the contamination is of light degree, the egg hatches and the disease develops at larval stage with the resultant death of the larva later.

Lack of uniformity in egg shape, reduction in egg size and weight, eggs being laid in clumps, poor egg attachment, high percentage of unfertilized and dead eggs are the symptoms. There is disparity in the time required for pigmentation of head and body of the embryos. Severely diseased eggs cannot hatch or if they do hatch, the worms soon die. Slightly infected eggs do not show any special symptoms. Often pebrinised eggs are paler in colour, which is more evident in breeds like Nistari which lays deep yellowish eggs. Veber and Jasic (1961) observed a great reduction in fecundity of pebrinised moths (209 to 341) as against 543 eggs laid by the healthy females.

Larva : First instar larvae that have been infected embryonically show no spacing of body hairs (ie., no growth). Two days after hatching the worms become darker, thin and exhibit slow growth. Those heavily infected may linger onto the second or third instar. If infection takes place at the beginning of first instar, the symptoms are generally similar, but normally the worms moult later or in some cases, not at all. Infection at the second and third instar may, in the grown larval stage, reveal such symptoms as wrinkled silkworms, ie., after feeding, larvae of the different instars show wrinkled skin with rusty colour; spotted silkworm ie., black pepper-like irregular spots appearing on the body mostly on the caudal horn and lateral to the prolegs usually in European breeds, incomplete moulting, difficulty in moulting, delayed moulting, non-pupating larvae (ie., mounted worms keep moving around without spinning, or fall onto the ground after extruding a small amount of silk). Unequal size of the

worms in the same tray is the striking symptom. The affected gut becomes opaque and the silk gland shows white pustules or spots in different places along its length.

Though there is variation depending on the intensity of contamination, most of the hatched larvae infected through transovarial transmission show irregular moulting and irregular growth before they reach the 2nd – 3rd instar. These undergrown or tiny larvae die subsequently. The progeny from eggs infected with *N. bombycis* develop an acute disease and all larvae die by the end of third instar (Ishihara and Fujiwara, 1965).

If these larvae infected through transovarial transmission are reared together with healthy larvae, the spores discharged by infected larvae serve as source of infection and the ingestion of the spores by healthy silkworms results in the spread of the disease. This process takes place during the 1st and 2nd instars and the contamination in these instars is called the first stage of contamination.

The silkworms infected in the first stage of contamination show comparatively normal growth until about the third instar but show symptoms from the later half of the 4th instar to the first half of the 5th instar and succumb to death by late 5th instar. The spores discharged through the excreta at this stage cause per oral infection in the larvae reared in the same trays. Infection at this stage is called the second stage of contamination. The majority of silkworms infected in the 4th–5th instar undergo eclosion and the deposited eggs are contaminated through transovarial transmission.

The increase in the density of infected silkworms mixed artificially with healthy silkworms increases the rate of contamination of moths. In Japan the rate of contamination of moths is high during summer and autumn rearing seasons. The intensity of prevalence of the disease varies with the rearing conditions. It is needless to say that any spores that have remained in the rearing room or on the rearing tools after being discharged by the pebrine infected silkworms will get into the rearing bed and become the source of the disease.

The minimum number of spores required for moth contamination through per os infection varies with each instar. The progress of the disease is slow paced and it takes 2 to 3 weeks for an infected worm to die.

Pupa : Cocoons spun by pebrine infected worms may become flimsy, malformed and less coloured with thin ends and less raw silk content.

Diseased pupa becomes lustreless and relatively less reactive to tactile stimulus. The abdomen softens and irregular black spots appear on the body wall. Highly infected pupae may fail to metamorphose into adults.

Moth : Delayed moth emergence, complete inability to emerge in severely diseased moths and inability to eclose through the pupal skin after wing development are some of the symptoms. Sick moths may develop clubbed wings, developed wings not spreading, moths with vesicles or black spots on wings, being dull, naked moths without abdominal scales, moths not showing mating instinct, discolouration of abdominal scales and shedding of scales from wings and body are also observed. In pebrinised female moths the accessory glands being infected, result in laying loose eggs which easily fall off from the egg cards. There is also a tendency of the moths to lay eggs in clusters.

Pathologies

Lesions : The pebrine protozoan passes from the digestive tract and invades various organs and tissues and multiplies with the exception of the chitinous epidermis, tracheal spiral filaments and the wall of the fore and hindgut. It can multiply in the cells of the digestive tract, fat bodies, malpighian tubules, silk glands, gonads and body wall. The parasitized cells turn milky white, but different tissues exhibit different lesions as follows :

Silk glands : The lesions of the silk glands are the most prominent, being visible to the naked eye. The parasitized silk glands show milky white abscesses, which can be used as a basis for definite diagnosis of this disease. The affected glands lose the ability to secrete silk.

Musculature : The greater part of the muscular tissues is dissolved to form cavities and the neighbouring tissues are also affected. Thus the diseased worms are sluggish in movement and appear shrunken.

Haemocytes : The granulocytes, leucocytes and plasma cells are those chiefly affected. The infected cells stain weakly and appear enlarged. The haemolymph becomes somewhat turbid due to disintegration of the haemocytes.

Body wall : Invasion of dermal cells of the body wall by the pathogen causes vacuolation and enlargement of the cells, during which process encirclement by granulocytes produces brown spots, which are often covered by newly formed dermal cells and then outwardly the body of the silkworm displays numerous pepper - like small black spots.

Histopathology : Histopathological studies of *N. bombycis* infecting the silkworm revealed that malpighian tubules were more susceptible to the attack than any other tissue. In various tissues the damage may be due to infection of muscles and nerves causing disturbance of their normal function rather than direct contact (Wafa and Kotby, 1971). When the pathogen invades the muscle, the tissues may be softened and liquified (Chitra *et al.*, 1975). The parasite can also multiply within the layers of the integument. As the protozoan multiplies, the cytoplasm within the host cell may disappear slowly. The nucleus is rarely invaded. Severe infection of fat results in poor energy turnover and death of larvae or pupae may result. Infection of silk glands causes very poor synthesis of silk fibroin.

The effects of *N. bombycis* on *B. mori* are more severe than those caused by other microsporidians. Tissue damage to different organs of silkworm has been studied in detail (Mitani, 1920; Hassanein and Ibrahim, 1959; Wafa and Kotby, 1971; Abe and Fujiwara, 1979 and Fujiwara, 1979). Localization of pathogens in the muscular and nervous systems causes the disturbance to functioning of organs which leads to disorganisation (Wafa and Kotby, 1971). On the otherhand, Issi and Onatksi (1984) showed the pre-sporulation stages not to damage the tissues.

6. EPIZOOTIOLOGY

There are several factors which influence the development and incidence of the disease. These factors may be biotic including those inbuilt in silkworms and abiotic.

A. Routes of infection

Transovarial transmission and per os infection are the two routes of pebrine infection in silkworm.

a. *Transovarial/Embryonic/Vertical Transmission* : Embryonic infection occurs when the pathogen infects the fourth or fifth instar larvae in which it invades the epithelial cells of the ovaries and then the parasites are transferred to the oogonia, oocytes and nutritive cells. Parasitism of the oocytes may result in death of the resultant eggs after being laid. If non-infected oocytes absorb the nutrient material from infected nutritive cells, the Protozoa will pass over to the oocytes and may cause infection of the embryos. The result of embryonic infection, however differs according to the stage of the embryos during invasion. If infection occurs during the formation of the embryo, then no embryonic development follows and the eggs die. It is only when the embryo has reached the stage of reversion or blastokinesis, the pebrine protozoan enters the digestive tract of the embryos with the absorbed yolk nutrients so that the first instar larva that hatches out is embryonically infected,

Han and Watanabe (1988) studied the transovarial transmission of two Microsporidia in *B. mori*. The examination of dead eggs and newly hatched larvae shows that *N. bombycis* is transmitted transovarially by all infected females. On the other hand, only 15 to 20 egg batches produced by female moths infected with *Nosema* sp. M11 are themselves found to be infected: Maternal mediated transovarial transmission of *N. bombycis* to the progeny eggs takes place at a frequency of 100 per cent while that of *Nosema* sp. M11 averages to only 1.2 ± 0.4 per cent.

The rate of transovarian transmission was 100 per cent in case of *N. bombycis* and it was only 1.2 per cent in the silkworm batches infected

with *Nosema* sp. M11 (Han and Watanabe, 1988) thus suggesting that the former is a more virulent one than the latter.

The entry of the pathogen to the egg through micropyle cannot occur as the size of the infective stage is larger than the former. Also the egg cannot be infected after the formation of the follicular epithelium. The early laid eggs of a moth may escape infection if the disease has not advanced but the ones laid later show high infection rate as most of the yolk cells are affected towards the end of the oviposition. In the infected hibernated egg the development of the pathogen is arrested temporarily and becomes activated when the eggs are incubated. Further, the pathogen cannot affect the sperms of the diseased male moth as the sperm is small in size.

b. **Contamination (Horizontal Transmission)** : Per oral infection occurs when silkworms eat mulberry leaf contaminated with spores or just hatching out worms eating the egg shell contaminated with the pathogen. Transmission within the rearing tray is caused by silkworms in which large quantities of pebrine spores are formed, excreted with the faeces or attached to surface of integument thus contaminating the mulberry leaf in the rearing bed and thereby transmitting the disease to the healthy worms. Infection inside the tray is of two stages. Primary infection refers to that occurring in the first and second instars and excretion of spores through faeces by these infected ones in third or fourth instars. Ingestion of these spores by other healthy worms constitutes secondary infection. Secondarily infected silkworms are able to feed normally and become moths but they lay eggs with infected embryos.

The extent of rearing tray transmission depends on the number of diseased worms present initially (Baig et al., 1988a). If even a few sick worms are mixed with healthy ones at early stage, there are many opportunities for transmission since the period of contact is protracted. The consequent losses incurred will be correspondingly greater. At the beginning of the first instar when 3 per cent diseased first instar larvae were added the mortality rate could reach 50-60 per cent in the mature silkworms. All the resultant moths were found infected with pebrine.

Contamination takes place usually from rearing rooms, appliances, mulberry garden and surface contaminated layings.

i) **Contamination in rearing bed** : Secondary contamination takes place in the rearing bed through the faecal matter of infected larvae, contaminated tray, seat paper, dust from the contaminated room and dead larvae infected with disease. The spores liberated from the above sources settle along with dust on mulberry leaf, forming the source of secondary contamination in the rearing bed. Among the rearing equipments, rearing trays are known to be the most frequent source of secondary contamination (Jameson, 1922).

ii) **Through mulberry leaf** : Improper disposal of silkworm litter and infected larvae and use of incompletely decomposed silkworm bed refuse in mulberry fields as manure may form sources of contamination along with the mulberry leaves contaminated by the spores of pathogen from the faeces of infected lepidopterous pests of mulberry in the field.

iii) **Through layings** : Incubation of layings in contaminated room, without proper disinfection, result in surface contamination of eggs and larvae get infected. The healthy larvae get infected during the process of hatching. Surface contamination is also possible through the moth scales and urine of infected moths. If layings are not surface disinfected, the secondary contamination can occur.

The percentage of secondary contamination resulting from transovarially infected larvae is positively correlated with the larval deaths due to infection as well as infection rate in moths. The alarming rate of secondary contamination poses serious threat to successive generations of silkworm crop.

As per Jafri *et al.* (1978) also pebrine is transmitted per orally and transovarial transmission and it causes losses to sericulture and the cocoons of infected larvae are of very inferior quality.

B) Virulence and Infectivity

Nosema bombycis was more pathogenic than *Nosema* sp.M11, the former causing acute disease and more mortality compared to the

latter (Han and Watanabe, 1988). Similarly, *Pleistophora* sp. caused chronic disease with less mortality than *N. bombycis* (Fujiwara, 1984a). Eventhough all the three regions of midgut are preferred, in distal end of midgut the percentage spore germination was more in *N. bombycis* followed by *Vairimorpha* sp. M12 and *Pleistophora* sp. (Sato *et al.*, 1986). Pathogenecity of *Nosema* sp. to *B. mori* popular bivoltine breed, NB4D2 due to secondary contamination has been compared with that of *N. bombycis* infection. *Nosema* sp. was found to cause considerable damage (Geethabai and Mahadevappa, 1995).

C) Alternate Hosts

The relation between silkworm pebrine pathogen and other insects is not fully understood. *N. bombycis* is reported to infect 26 different lepidopteran insects in addition to *B. mori*. A study undertaken in and around silk farms in Hungary has shown 20 different Lepidoptera as hosts (Machay, 1957). However, transovarial transmission has not been reported.

N. bombycis was found infecting eri silkworm, *Samia cynthia ricini* in Assam (Basu, 1915; Jameson, 1922).

Indian tropical tasar, muga silkworm and Chinese tasar silkworm are also known to be infected by an unidentified species of *Nosema* sp. (Jolly and Sen, 1972; Talukdar, 1980 and Vander Flass, 1929).

N. bombycis is known to infect other insects viz., *Arctia caja* (Malenotti, 1925); *Chilo simplex* (Oshima, 1935); *Rodoptia menciana* (Ku, 1936); *Pieris brassicae* (Blunk, 1952), *Pieris rapae* and *Pyrausta nubilalis* (Zimmack *et al.*, 1954); *Malacosoma neustria*, *Stilpnotia salicis*, *Thaumetopoea processionea* and *Hyphaniria cunea* (Veber, 1956; Weiser, 1957); *Brachyrhinus lingustici* (Weiser, 1958) and *Choristoneura fumiferana* (Thomson, 1958), *Bombyx mandarina*, *Glyphodes pyloalis* and *C. suppressalis* (Yokoyama, 1962).

The lawn grass cutworm, *Spodoptera depravata* serves as natural reservoir of *N. bombycis* which shares the surface specific antigens with *N. bombycis* from *B. mori* (Ishihara and Iwano, 1991). Its infection to

B. mori is systemic and results in transovarial transmission but it is less virulent. As per Iwano and Ishihara (1991c), it produces two types of spores in the cultured cells of *Antheraea eucalypti*.

A strain of *N. bombycis* causing high larval mortality of beet army worm *Spodoptera exigua* was detected (Yasunaga *et al.*, 1992). Kumar and Ahsan (1993) isolated *Nosema* sp. infecting *Theophila religiosa* and found it to be infectious to *B. mori*. Microsporidian infection was observed in *Catopsilia pyranthe* (MCpyB1), *C. pomionae* (MCpoB1) and *C. crocale* (MCcnB1) in and around mulberry fields (Kishore *et al.*, 1994). With latex antibody test reagents of *Nosema* sp. (M11 and M12) and *N. bombycis* (Yakult, Japan), MCpyB1 and MCcrB1 reacted with *Nosema* sp. M11 and MCpoB1 reacted with both *N. bombycis* and *Nosema* sp. M11. These microsporidians were infective to silkworm, *B. mori*.

D) Cross infectivity

Pebrine pathogen is known to infect other insects when artificially inoculated. Picard (1913) while studying biology of *Pthorimoea operculella* succeeded in infecting the larva with *N. bombycis* obtained from silkworm *B. mori*.

Veber and Jasic (1961) infected *N. bombycis* collected from *B. mori* to *Hyphantria cunea*. Infection resulted in decline in the fecundity (443 eggs) compared to healthy female (607 eggs).

Kudo and De-coursey (1940) experimentally infected the larvae of *H. cunea* with *N. bombycis*.

The *N. bombycis* was found to be able to cause infection in *Agrotis segetum*, *A. exclamatus*, *Heliothis armigera*, *Autographa gamma*, *Malacosoma neustria* and *Lymantria dispar* (Kashkarova, 1981). When inoculated to 1st instar *Spilosoma obliqua*, all the individuals died before reaching the second instar (Singh, 1978). Kashkarova and Khakhanov (1980) artificially inoculated some lepidopterans viz., *A. segetum*, *H. armigera*, *Spodoptera exigua*, *Laphygma exigua*, *A. gamma* and

Lymantria dispar with *N. bombycis* and all were successfully infected but *L. dispar* was not susceptible. Cross infection of *Nosema* from the saturniid *Antheraea mylitta* to *B. mori* in the laboratory resulted in variation of rates of infection and disease development between larvae of *B. mori* of different ages, third and fourth instar larvae being most susceptible (Patil, 1989).

E) Survival of the pathogen

The survival of microsporidians in the environment is influenced by a large number of factors like temperature, humidity and biotic components of the substrate in which they are found (Kramer, 1976). Survival of *N. bombycis* for less than 2 months in moist fermenting materials has been reported (Kudo, 1931). Pebrine spores will remain viable for a maximum period of 225 days in wet soil and a minimum of 135 days in wet compost under tropical conditions (Patil, 1993). Apparently, spores coated with tissues and spores present under humid conditions have been found to be viable for several years (Anonymous, 1975). On the other hand naked spores are susceptible to desiccation and thus lose infectivity very quickly. Maddox (1973) is of the opinion that survival of microsporidian spores depends on the kind of material (substrate) in which they are found, in addition to the moisture content of the material.

The adverse effects of environmental conditions on microsporidian spores, especially sunlight, have been well documented (Kaya, 1977; Sikorowski and Lashomb, 1977; Wilson, 1974). Sunlight contains ultra violet light (UV) which has a sporicidal effect (Nilova, 1974). The amount of actual UV content of sunlight reaching the earth surface depends on the prevailing atmospheric conditions (Coulson, 1975) and solar angle (Withrow and Withrow, 1956). In addition, the intensity and amount of bright sunlight (high UV content) available to spores depends on the seasons of the year and duration of time for which spores are exposed (Patil, 1993). The overall effect of the above parameters is directly related to the rate of inactivation of spores.

Reduction in spore size occurs with increased storage duration and also in faecal matter, moist soil and at 15°C after 60 days. Spores

stored in refrigerator, dry soil and room temperature under dry condition show reduction after 90 days (Srikanta, 1986).

F. Instar and breed susceptibility

Pebrine incidence varies with the silkworm developmental stages, breeds and rearing environment. Resistance to pebrine is greatest among Chinese breeds, less in Japanese breeds and least in European breeds. Generally, the multivoltine (Polyvoltine) breeds are relatively resistant followed by bivoltine breeds. Univoltine (Monovoltine) breeds show the poorest resistance.

Early instar silkworms are more susceptible compared to late instars and newly moulted worms are also highly susceptible compared to the aged worms in the same instar. As per Devaiah (1973), the total larval mortality was highest (86.67 %) when first instar larvae were infected by *N. bombycis* followed by 2nd (77.00%), 3rd (55.00%), 4th (51.00%) and 5th instar infection (4.00%). This was even reflected by the rate of pupation when these instars were infected in that maximum and minimum rate of pupation were encountered with 5th and 1st instar infection, respectively.

Noamani *et al.*, (1971) studied the breed susceptibility to pebrine infection in six different seasons with Nistari and other improved breeds viz., MBD IV, MBD V and D3C. Significant difference seldom existed between pebrinised and healthy cocoons in all the breeds, in respect of single cocoon weight and filament length. Filament weight and denier were significantly lower in case of cocoons spun by diseased worms compared to those spun by healthy larvae, while no significant differences were found regarding single cocoon weight, filament length, number of breaks in filament and renditta (except in case of MBD IV where renditta was significantly lower) in respect of the two categories of cocoons.

The disease incidence appeared in all the breeds and during all the rearing seasons. But the percentage of pebrine infection varied in different breeds. In general Nistari showed lowest incidence and C122 showed highest (Devaiah and Krishnaswami, 1975). Devaiah *et al.*,

(1975b) recorded highest pebrine incidence on Chinese Farmer (13.05%) followed by Belakokonaya (7.52%), Pure Mysore (3.36%), C. Nichi (3.15%) and Nistari (2.08%). In general the percentage of infection was more during July to March and less during April to June.

The breed susceptibility of *B. mori* to *N. bombycis* in third instar based on larval mortality revealed C. Nichi to be less susceptible (49.67%) and Pure Mysore and HS6 to be highly susceptible (100%). Nistari was less susceptible (8.92%) compared to other breeds in fourth instar (Devaiah, 1973).

The relative susceptibility was studied in twenty nine silkworm breeds in IV instar (Anonymous, 1988). The rate of infection in Nistari (94.75%) was followed by CPP-1 (94.33), G-race (91.84%), NB4B2 x CPP-1 (84.67%), Tamil Nadu White (82.18%) and JSV-2 (64%). In others 100 per cent infection was recorded. The order of resistance of silkworm breeds to pebrine disease as revealed by rate of mortality was C. Nichi > PM > NB18 > KA > NB4 D2 > NB7 (Patil and Geethabai, 1989). The probable reasons attributed for differential susceptibility and resistance of insects to microsporidian disease were supposed to be (1) Failure of spores to germinate in the digestive tract because of unfavourable conditions (2) Non-susceptibility of host tissue (3) Elimination of infected cells by the host.

Pure Mysore was comparatively less susceptible (16.6%) to *N. bombycis* infection than AP11, RD1, Nistari and Sarupat (20.3 to 31.2%) while KA (52.3%) was more susceptible and the per cent infection among NB7, NB18, P2D1 and My₁ was ranging from 45 to 51 per cent (Baig and Nageshwara Rao, 1991).

As research on disease resistant breeds has been paid much attention in recent years, there has been evidence showing resistance in silkworms against microsporidian infections (Tanada, 1963). Although disease resistance appears to depend on the genetic constituents of a particular breed, nevertheless factors such as inoculum size, nutritional status and the environment in which the insects are reared also affect

resistance. In addition, the physical and physiological characteristics of hosts may make the invasion of microsporidians possible (Weiser, 1969; Brooks, 1971). However, the studies directed towards the factors responsible for differential host resistance for *N. bombycis* are very limited.

G. Rearing Practices

Rearing silkworm by employing new technologies may have a prominent role in reducing the incidence of pebrine. Providing highly nutritious mulberry leaf, optimum ecological requirements and optimum quantity of leaf and proper spacing, timely bed cleaning and practising of hygienic and disease preventive measures may curtail the disease. Use of moist trays, cowdung smeared bamboo trays, may increase the possibility of ingesting the contaminated mulberry leaf by silkworms.

Refrigeration of aged multivoltine eggs may act as a predisposing factor in increasing the susceptibility of *B. mori* to pebrine infection (Devaiah, 1973).

The frequency of feeding as well as the quantum of food provided to the silkworm are known to influence the disease development. When 3rd to 5th instar silkworms were partially fed with mulberry leaf, the pathogen took 6 days from spore to spore. While under normal feeding conditions it was eight days. Starvation of 4th instar silkworms for period of 24, 36 and 48 hours and with pebrine infection the total mortality was 39.50, 73.20 and 100.00 per cent compared to 3.7, 11.8 and 27.8 per cent in their respective uninoculated starved controls. Further, starvation along with infection also resulted in reduction of larval weight and cocoon parameters (Devaiah, 1973).

7. DETECTION

For successful prevention and timely curtailing of dreadliest infectious diseases such as pebrine all the possible detective methods should be employed effectively. The presence of the pathogen in the silkworm rearing habitat, mulberry ecosystem and seed material should

be examined thoroughly to prevent carryover of the pathogen. Though some detective methods are in practice, they should be carried out without ambiguity. Also modern methods of detection may be practised.

A. Existing Techniques

i) **Predictive Test** : The suspected silkworms in early instars and the faeces will be subjected for detection of spores. First sampling must be done during 1st and 2nd instars. Another sampling must be made from the fourth instar upto the mounting period to identify second stage of contamination.

ii) **Pupal Test** : When the seed cocoon lots arrive to the market, random sampling of cocoons suspected to be pebrinised is made and the presence or otherwise of the pathogen in pupae is confirmed before the disposal of seed cocoons to the granearns.

iii) **Forced Eclosion Test** : The confirmity of the presence of the pathogen is made even before the moth emergence so that if the lot contains more pebrine, atleast those cocoons can be used for reeling silk. In this test, a small number of seed cocoons from each batch is preserved at temperature of 32°C which facilitates early emergence by 1-2 days. The early ecloded moths are examined and if found to be pebrinised, the whole batch of cocoons is sent for reeling after proper stifling. This saves labour in seed preparation and prevents subsequent spread of the disease (Jolly, 1986).

iv) **Mother Moth Examination** : Production of pebrine free layings is ensured by conducting mother moth examination on scientific lines. There are different types of moth examination. It can be conducted by using either green (live) or dry moths.

If the eggs are multivoltine and also if bivoltine eggs are required for immediate use and are to be acid treated, mother moth examination is conducted on the following day after oviposition. When the eggs are hibernated, there is sufficient time for testing and can be done at leisure by drying and preserving the moths. Dry moth examination is better

than fresh moth examination as the testing is made easier and spores are killed rendering them non-infective. The moths are dried at 60-70°C for a duration of 5 hours and preserved under dry condition. Sufficient care should be taken while drying the moths as higher temperature of 80°C and above results in deformity of spores and insufficient drying leads to multiplication of saprophytic organisms. Before the moths are crushed, the heads and wings are clipped off to facilitate better clarity.

Pasteur's method/Individual mother moth examination :

Individual mother moth examination is practised in reproductive egg production (P3, P2, P1) and also when the disease is in epizootic form. This method is aimed at complete elimination of pebrine.

For microscopic examination of mother moth to determine the existence of pebrine spores, the green or dry moth must be crushed prior to the examination. For this purpose, the individual mother moth and the mass mother moth inspection methods are available. With the individual moth inspection method, the dried moths are soaked in about 2 ml of 2 per cent KOH so that the body of the moth may be swollen sufficiently and the tissues and cells are completely crushed with the use of mortar and pestle to provide good separation of the pathogen.

Preparation of Labarraque solution : The first solution is prepared by dissolving 20 g of bleaching powder in 500 ml of water and making the volume to 500 ml. The second solution is prepared by dissolving 32.5 g of NaOH in 500 ml. of water. The first and second solutions are mixed in equal quantities and the supernatant is taken as the base fluid and preserved in a sealed container in darkness.

When a close examination is required, the spore collection method is most effective. To collect spores, the triturate is poured into the sedimentation tube, about 7 cm long and about 6 ml in volume with rubber plug on the top and the bottom through a coarse filter paper, and increased to about 5 ml with the addition of water. When undried moths are used, the tube is left undisturbed for 48 hours. When the temperature is high and there is a possibility of the multiplication of bacteria, a few drops of 10 per cent formalin or 5 per cent cresol must be added. After

the lapse of 48 hours, the tube is turned upside down and the plug, which has so far been at the bottom side of the tube, is removed and the sediment deposited on the plug is transferred to the glass slide.

Then the test sample is dropped on a microscope slide over which a cover glass is placed for microscopic examination. A method for easy detection for pebrine spores during mother moth examination has been developed (Geethabai *et al.*, 1985). To a drop of smear placed on glass slide a pin head drop of Indian Ink is added from the edge. After a few minutes the microscopic field becomes black and the spores become shiny thus enabling their easy detection. This is based on negative staining principle where the background is stained.

The required magnification of microscope is 600 x or above for satisfactory observation. For each mother moth two smears will have to be prepared and in each smear 5 microscopic fields will have to be viewed at 600 x for confirmation. If oval, translucent shining spores are encountered in any of the fields, the eggs laid by that particular moth will be destroyed.

In phase contrast microscope, the spore shows itself with a glowing pale colour and with a distinct contour. The immature spore and schizont appear as lustreless, dark brown images against a bright background.

Though the pebrine inspection is generally conducted by the examination of mature spores, the presence of other developmental stages of the pathogen such as schizonts provides additional evidence for the occurrence of the disease.

Though several modified methods of moth examination viz., Italian method, Industrial method, Bengal method, Hutchinson's method, Mukerji's method etc., had been in use, the individual mother moth examination developed by Louis Pasteur and the mass mother moth examination are widely practised in reproductive and industrial egg production, respectively.

Mass mother moth examination : The method of mass pebrine inspection of moths was adopted in Japan for the first time in 1968. In mass pebrine inspection 14-30 moths are crushed together and spores are separated from this mass. However, with this method, infection of a single moth among the mass results in the rejection of all eggs laid by the group of moths. Therefore, this method can be used only in the country where there is less possibility of the incidence of pebrine disease.

In mass pebrine inspection a large number of moths (20-30) are crushed together in a cutter type mixer with 80-90 ml of 0.5 per cent K_2CO_3 solution. The moths are ground and centrifuged at 9000-10000 rpm for 2 min for spore liberation from tissues. The crushed material is allowed to stand for 2 min and filtered. The filtrate thus collected is centrifuged at 260 rpm for 3 min. The sediment is suspended in 2 ml of 2 per cent KOH solution if dry moths are used and in case of green moths a small quantity of 0.15 per cent KOH is added to the sediment which is put on a microscopic slide for microscopic examination. Using an automatic equipment called Hirano moth testing machine that has a grinding, a washing and pouring unit and a centrifuge, has been developed at Japan using which in a day of 8 hours about 500 samples can be tested. 0.5 per cent pebrine contamination in commercial egg preparation is allowed whereas in reproductive egg production per cent freedom of pebrine is expected.

B. Techniques to be Followed

i) **Corrective Test** : Eggs, empty chorions and newly hatched out larvae are subjected for inspection in this test. Whether the eggs infected through transovarial transmission have been completely eliminated by the pebrine inspection of mother moth or not may be determined by supplemental sample inspection of silkworm eggs including unfertilised and dead eggs.

Portions of eggs, empty chorions and newly hatched out larvae are ground with a few drops of 2 per cent KOH and centrifuged at 1000 rpm for 2 min., allowed to settle and the sediment is examined. If pebrine is detected, the lot has to be destroyed by burning.

ii) **Rearing room Dust Examination** : Examination of dust from silkworm rearing venue and equipments immediately after disinfection process reveals the effectiveness of the disinfection process and subjecting the dust from the mulberry leaves for inspection aids in finding out whether the leaf carries the inoculum from the infected mulberry defoliators in the garden.

Dust collected from floor and walls of rearing house, rearing trays, equipments and harvested mulberry leaf is collected and soaked in 0.1–0.2 per cent KOH solution or 0.5 per cent K₂CO₃, mixed thoroughly and the sample is then filtered through absorbant cotton. It is then precipitated by centrifugation (1500g for 3 min) and the sediment is examined microscopically.

iii) **Immunological Techniques** : Recently, immunological techniques have been applied for detection of pathogens and identification of species. *N. bombycis* and closely related species were diagnosed with the use of double immunodiffusion technique (Knell and Zam, 1978), slide agglutination test (Hayasaka, 1983), antibody sensitized latex (Grobov and Rodionova, 1985; Hayasaka and Ayuzawa, 1987; Li, 1985; Baig *et al.*, 1992b), ELISA procedure (Greenstone, 1983; Kawarabata and Hayasaka, 1987; Watanabe, 1987) and fluorescent antibody technique (Sato *et al.*, 1981; Huang *et al.*, 1983). These tests could be mainly used for early detection thereby enabling prompt adoption of adequate preventive and control measures. Of late development of monoclonal antibody technique (Kohler and Milston, 1975; Ke *et al.*, 1990) has provided a means for the rapid diagnosis of microsporidians in silkworms. In addition monoclonal antibodies have also been used for identification of specific microsporidians. Ke *et al.* (1990) raised monoclonal antibodies against *N. bombycis* spores and used the same to identify this pathogen and other closely related microsporidians infecting silkworms using the ELISA. Immunoblot analysis and Protein–A linked antisera test have been employed recently for the detection of *N. bombycis* (Irby *et al.*, 1986; Baig *et al.*, 1992a). The agar gel diffusion test was negative for immunodiagnosis of pebrine while positive result was obtained from the agglutination test which was later confirmed by indirect fluorescent antibody test and ELISA (Sengupta *et al.*, 1993).

In view of the above, it necessitates for the development of an immunodiagnostic kit for early detection of pebrine in chawki rearing centres as well as in seed cocoon rearings.

8. PREVENTION AND CONTROL

The protection of cocoon crops from the severe menace of pebrine is of paramount importance as the pathogen is characterised by transovarial transmission and per oral transmission through contaminated food, persists in rearing room and on appliances, survives in mulberry lepidopterous defoliators resulting in apparent epizootic and serious-crop losses. Individual mother moth examination innovated by Pasteur successfully eliminates the disease. The adoption of mass mother moth examination allows certain flow of pathogen in industrial eggs. Also the pathogen may survive without losing viability in the rearing ecosystem and rearing appliances in view of ineffective disinfection process. As well the pathogen may gain entry to the rearing through mulberry leaf from the mulberry field ecosystem. Thus certain proportions of the pathogen get access to rearing through means of transovarian transmission and the contaminated mulberry leaf. It is essential that basic source of inoculum responsible for contaminating the mulberry leaf should be destroyed adopting several preventive and hygienic measures.

Seed area is the back bone of sericulture industry. The success of commercial sericulture depends upon the quality and pebrine free seed cocoon production in seed areas. The importance of commercial F1 hybrids needs to be much stressed in view of the elevation of both qualitative and quantitative aspects of silk cocoon production for which the pebrine free seed cocoons of parental breeds particularly of the female parent are an essential component since the pathogen is transovarially transmitted.

Otherwise the disease enters even the commercial cocoon producing areas through eggs and the crops will fail. If the problem of pebrine in seed areas gets aggravated, then the seed cocoon lots will be unfit for production of disease free layings (DFLs) and thus the

requirement of DFLs in commercial cocoon producing areas cannot be met with to the fullest extent and this obviously results in less cocoon production inturn affecting the quantity of raw silk produced. Furthermore, the secondary contamination of silkworm with pebrine pathogen results in deterioration of both qualitative and quantitative cocoon parameters considerably. The floss content of cocoon increases, both filament length and denier decrease and the resultant raw silk will be of inferior quality. The aforesaid factors are enough to infer that the ultimate net profits of silkworm rearers of both seed area and commercial cocoon producing area are brought down due to the menace of pebrine disease in seed area.

It is essential that one should have a thorough knowledge of the available methods of detection and also make sincere efforts to inculcate the modern methods of detection of pebrine pathogen along with the existing methods already brought out so that the pebrine pathogen can be detected timely which further helps to plan for successful prevention of the same.

1. Two rounds of effective disinfection of rearing house and appliances may have to be ensured once immediately after cocoon harvest with formalin 3 per cent and the other just prior to the next brushing with 5 per cent bleaching powder.

Use of substandard formalin will result in the application of the chemical in sublethal doses to the pathogen which inturn may induce resistance to formalin in this pathogen and thus the chemical becomes ineffective even at lethal dose. Hence, the supply of standard formalin may have to be well regulated by establishing quality control laboratories which will monitor the active ingredient of chemical particularly disinfectants used in sericulture industry.

2. Prompt surface disinfection of incubated eggs two days before hatching with 2 per cent formalin for 5 min. to overcome the contamination during transit and incubation.

As per Kagawa (1980), the efficacy of formalin as disinfectant of spores of *N. bombycis* is more with increase in concentration, rise of

temperature and the extension of period of exposure. Iwano and Ishihara (1981) observed 0.001 per cent solution of Hilite (Potassium dichloro isocyanurate) to inhibit the hatching of spores of *N. bombycis* most effectively and immediately after treatment. Feeding silkworm with such treated spores did not result in infection. 0.1 per cent Hilite treated mulberry leaves when fed did not retard the growth of the larvae. Immersing *B. mori* eggs in HCl (1.05 sp. g) at 47° C for 20 min reduces the incidence on infection with *N. bombycis* to 0.92 per cent. In field experiments with such treatment the incidence is 1.21 to 7.41 per cent (Liu *et al.*, 1984). *N. bombycis* parasitizing diapause eggs of silkworm was effectively controlled when 48 h old eggs were stored at 5° C for 30 days and then treated with 20 per cent HCl at 48° C for 5 min (Fujiwara and Kagawa, 1984). The parasites in the non-diapause eggs were also sensitive to similar treatment when 48 h old eggs were stored at 5° C for 30 days. Hot water treatment at 46° C for 4 min was an alternative to acid treatment. Li Ai Qun (1962) also suggested previously the hot water treatment for controlling pebrine.

Baig *et al.* (1988b) while investigating the surface disinfecting ability of hilite, sodium hypochlorite, bleaching powder and formalin at 1.5 and 2.0 per cent against pebrine contamination on the eggs found all the chemicals to be effective in killing the pebrine spores on the eggs as revealed by no larval mortality due to pebrine. Further, the pebrine contamination in moth stage was significantly low in all the chemicals (1.34 – 2.54 %) compared to bleaching powder at 1.5 – 2.0 per cent (2.75 – 2.85 %). The lowest moth contamination was with 2 per cent formalin.

Silkworms inoculated with pebrine spores exposed to disinfection by Asiphor, an iodine compound at 0.5 to 2.0 per cent concentration resulted in an incidence of 50.67 – 67.67 per cent compared to 80.0 per cent in silkworms inoculated with untreated pathogen (Reddy *et al.*, 1990) indicating Asiphor as an ineffective nosematidicide.

Bleaching powder in different concentrations (1–5 %) was tested against *N. bombycis* and compared with those of formalin (Balavenkata-subbaiah *et al.*, 1994). All the concentrations were significantly effective

in inactivating the spores. Bleaching powder 4 and 5 per cent was more effective than 2 per cent formalin.

3. Chemotherapeutic measures though are very less attempted, Liu (1988) proved that Bavistin and Benomyl each at 4 per cent were effective in controlling the pebrine disease in silkworm to 0 per cent compared to 94.5 per cent in control when larvae were fed with 10^{3-5} spores per ml.

The treatment of pebrinised eggs in hot water for the control of the disease when *N. bombycis* infected silkworms were treated with aerosols of Nosematol I and Nosematol II, the resulting moths were infected 2.5 to 3.0 times lower than the untreated group. The preparations were non-toxic to silkworms. The treatment of infected fourth and fifth instar larvae protected with Nosematol revealed an infection of only 5 to 10 per cent of the control batch (Aleseenok and Pavelenko, 1984; Aleseenok, 1986). Administration of the drug pantelmin (Mabandazole) to silkworm immediately after second stadium with an aqueous suspension of 2,000 ppm concentration thoroughly mixed with mulberry leaves has been found effective in controlling the microsporidian infection in *B. mori* (Chandra and Kundu, 1983).

Trinidazole, Metronidazole and Benomyl at 5 to 200 ppm when administered to third to fifth instar larvae of silkworm against pebrine infection are known to result in disease control and higher cocoon production (Radha and Muthukrishnan, 1981).

4. Providing incentives like offering more price for pebrine free seed cocoons and subsidised supply of standard formalin and other disinfectants as well as disinfectant application equipments, particularly during epizootics.

5. Allowing a non-rearing period between two rearings during epizootics, though appears to be non-practicable, may help to some extent.

6. Prompt destruction of pebrine infected rearings by burying or burning.

7. Timely elimination of lepidopterous defoliators of mulberry in the mulberry gardens may help to a great extent in checking the possible spread of pebrine pathogen from mulberry gardens to the rearing houses.

8. Practising improved rearing technology for young and late age silkworms.

9. Timely feeding of worms with optimum quantity of suitable quality leaves.

10. Bed cleaning at regular intervals.

11. Proper composting of silkworm litter and using the manure for cultivation of other crops.

12. Providing optimum spacing, temperature, ventilation and relative humidity to the worms during rearing.

13. Timely picking of pebrinised worms and their destruction by dipping in 3 per cent formalin.

14. Carrying out mass disinfection of farmers' rearing houses to get pebrine free rearing houses.

15. Avoiding borrowing of rearing trays and mountages from other silkworm rearers.

16. Establishment of central pebrine monitoring laboratory equipped with required equipments and technically competent personal is useful. The squad of this laboratory may visit and monitor the pebrine incidence in the field and issue pebrine free certificates to be accompanied with cocoon lots arriving to the seed cocoon market. Establishment of a mini pebrine laboratory with technical staff at each seed cocoon market to verify the pebrine freeness of cocoon lots by adopting the technique of pupal test. The seed cocoon purchaser may be issued a certificate by the concerned seed cocoon market pebrine laboratory as to the pebrine freeness of the cocoon lot procured.

17. Thermotherapy to seed cocoons after 8 to 9 days of mass spinning may be done at a temperature of 40°C and relative humidity of 55 to 60 per cent for 12 to 16 hour at grainage. The heat treatment is given when pupae are most resistant and the phagocytes engulf the pebrine pathogen and thus prevent the passage of pebrine spores into the eggs.

18. Forced eclosion may be achieved in grainage by exposing some seed cocoons of each lot to a temperature of 30 – 32°C which enables the moths to emerge 1 to 2 days earlier compared to the normally preserved seed cocoons. The early emerged moths are used for pebrine spore detection. This avoids further spread of the disease.

19. Individual mother moth examination should only be practised in seed area. Use of Indian ink is known to help for easy detection of pebrine spores in moth body fluid smears (Geethabai *et al.*, 1985). Trained moth testers should be employed. Atleast five fields may have to be viewed from each mother moth's smear. Counter checking of already tested smears by another person ensures complete elimination of the pathogen. Moth testers should be sincere and honest and should possess lot of patience in moth examination.

20. Prompt burning of pebrinised layings, moth residues after mother moth examination and used male moths to prevent further contamination.

21. The layings are surface washed with 2 per cent formalin for 5 to 10 min and cold storing of eggs is done for only required period in effective cold storage.

22. Prompt disinfection of various grainage rooms and appliances with formalin (3 to 4 %) and bleaching powder (5%) immediately after the preparation of eggs from a cocoon lot so as to prevent the possible surface contamination of washed DFLs.

23. The seed cocoons that are 100 per cent free from pebrine infection only are to be used for laying preparation.

PROSPECT

A thorough understanding of the various factors governing the epizootics of silkworm pebrine disease in Karnataka appears to be an essential pre-requisite for successful combating of the disease intime. Also, silkworm is known to be infected by several sporozoan Protozoa viz., *Nosema bombycis*, *Nosema M11*, *Nosema M12*, *Vairimorpha* sp., *Pleistophora* sp. and *Thelohania* sp. in Japan and the former is more infective. It is speculated that these may also be existing in Karnataka and a detailed systematic search in this regard is necessary as the spores of these look more or less alike. The possible associative effect of *N. bombycis* with other sporozoan parasites as well as other pathogens of silkworm may have to be investigated with relevance to pebrine epizootics. Many a times silkworms exhibiting flacherie symptoms reveal *N. bombycis* along with bacteria or and virus. This knowledge may be highly useful to the field workers in detection.

Stress may have to be given on the research concerning to the detailed epizootiological aspects of the disease. The survival of the pathogen under mulberry field ecosystem thus probably getting access to rearing through mulberry leaf is interesting to be surveyed.

Rearing of silkworm breeds and hybrids less susceptible to pebrine will help to minimise the cocoon crop loss. Genetic engineering may also be useful to transfer the genes concerning to pebrine resistance among productive silkworm breeds.

Methods for early detection of pebrine must be invariably employed. Designing of simple and effective immuno-diagnostic kit which can be conveniently used by field workers and farmers for early detection of pebrine appears to be an urgent need so as to curtail the disease intime without giving further scope for any epizootics to occur. Practising single method can never be effective to curtail pebrine and hence it is wise to adopt various measures so that the pathogen is eliminated by preventing its entry to silkworm through contaminated mulberry leaf and transovarian transmission. More than all these, creating awareness amongst seed cocoon rearers on various measures of preventing pebrine through effective programmes greatly helps to bring down the menace of the disease.

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