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Pebrine-A Silent Threat to Indian Sericulture

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Authors' contributions

This work was carried out in collaboration among all authors. Authors MAH, SSR and Author RS designed the study and wrote the first draft of the manuscript. Authors KK and SAH managed the literature searches. Authors MAH and KK wrote the final draft of the manuscript. All authors read and approved the final manuscript.

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Review Article

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ABSTRACT

Indian culture is deeply rooted in sericulture, with different regions having a track record for supporting distinct populations of silkworms. For example, the North-Eastern region of the country is home to a diverse range of Muga (*Antheraea assamensis* Helfer), Eri (*Samia ricini* Donovan), and Oak Tasar (*Antheraea proylei*Jolly) silkworms. The eastern region, which includes Jharkhand and Odisha, is well-known for its tropical tasar (*Antheraea mylitta* Drury) while the southern region, comprising Karnataka, Tamil Nadu, and Kerala, is the primary source of India's yearly output of raw silk made from Mulberry silkworms (*Bombyx mori* Linnaeus). As an agro-based industry, sericulture is vulnerable to a number of pests and diseases, the most destructive of which is pebrine. The disease is particularly dangerous since it spreads transovarially from one generation to the next, therefore it's critical to monitor its spread constantly in order to produce disease-free layings

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(DFLs), which will support the industry's continued expansion. This article provides a thorough overview of the current state of pebrine disease, including its mode of transmission, symptoms, and advancements in diagnosis techniques. By doing so, the reader will be able to comprehend the disease more easily and develop a sense of awareness.

Keywords: Antheraea assamensis; Nosema bombycis; silkworms; Bombyx mori, Antheraea mylitta, pebrine.

1. INTRODUCTION

Pebrine, a deadly parasite illness brought on by microsporidia from several genera, continues to be one of the major threats to the global advancement of sericulture. Microsporidia are a common category of obligate intracellular parasites that are eukaryotic. They were first identified more than a century ago when Louis Pasteur in France identified *Nosema bombycis* as the cause of pebrine in mulberry silkworms, or *Bombyx mori* Linnaeus, 1758. Additionally, *Nosema bombycis*—a systemic disease that seems to be unique to the genus *Bombyx*—has been reported from a number of insects (Table 1).The French Sericulture Industry's survival was threatened in the middle of the 1800s when an epizootic (microsporidiosis) emerged. The illness quickly spread to other nations, including China, Italy, Spain, Syria, and Turkey, and it posed a serious threat to the silk industry's continued existence. The dire circumstances prompted Sir Louis Pasteur (1822–95) to look into the causes of the threat, which led to the identification of microsporidiosis in silkworms, also known as pebrine and represented by Näegeli (1857) as *Nosema bombycis* [1]. Pasteur clarified that the sickness might be passed from mother moth to offspring through the egg, through contaminated food, or by coming into touch with sick worms. This finding was the first indication of a pathogenic bacterium spreading vertically [2]. Additionally, he described a tactical method that is still frequently employed in the global sericulture business to screen mother moths for pebrine. Pasteur's groundbreaking research on pebrine was published in "*Etudes sur la Maladie des Vers a Soie*" in 1870.

The *Nosema* species that infect silkworms are members of the evolutionary group *Nosema* or *Vairimorpha*, which is the microsporidian taxon that is most commonly isolated from lepidopterans [3]. Microsporidia are eukaryotic, single-celled parasites that form spores. They are classified into two main clades: typical (also known as advanced) and atypical (also known as primitive). Atypical microsporidia are a small group of 13 genera and 42 species, while the majority of known microsporidia are of the typical type, which includes 190 genera and an estimated 1300–1500 species [4]. Numerous microsporidians can infect both vertebrate and invertebrate hosts, giving them a wide host range. According to reports, silkworm microsporidiosis is caused by microsporidia from the genera *Microsporidium*, *Nosema*, *Vairimorpha*, *Pleistophora*, and *Thelohania* [5,6,7,8,9]. According to Li *et al.* (2018), these microsporidia have evolved specialized methods to take energy and nutrients from the silkworm [10]. Typical microsporidia have lifecycles and developmental sequences that range from basic to complex.Microsporidian infections are typically identified by using light microscopy to look for distinctive spore presence or absence. Spores of the *Vairimorpha spp.* species are cylindrical (3.9 × 1.7µm) and binucleate, while those of the *Microsporidium* (4.9 × 2.8µm), *Pleistophora* (2.5 × 1.3µm), and *Thelohania* (3.4 × 1.7µm) genera are characterized as being oval and uninucleate. Owing to their light-refractive characteristics, *Nosema* spp. spores can be easily observed in aqueous preparations under a light microscope at 600× magnification without the need for contrast coloring. Since *Nosema bombycis* demonstrates transovarial transmission from parent to offspring, pebrine monitoring systems worldwide have relied on mother moth examination as their primary method of surveillance since the beginning of time [11]. Though most microsporidian spores are similar in appearance and just slightly different in width and length, light microscopy is less sensitive and dependent on the subjectivity of the observer or examiner. Additionally, the accuracy of light microscopy can be impacted by urea crystals, yeasts, diatoms, and other factors. While normal light microscopy shows distinct light reflections from yeast and *Nosema* spores, phase contrast microscopy prevents misidentification. Subjective assessment on an arbitrary infection scale can be used to gauge the *Nosema* infection level. For accurate estimations of the amount of spores, a conventional hemocytometer should be used [12].

Table 1. List of Microsporidia

Sl. No.	Microsporidia	Species	Reference
1.	*Nosema bombycis*	*Bombyx mori*	Roy *et al.,* 2017 [13]
2.	*Nosema antheraeae, Nosema pernyi*	*Antheraea pernyi*	Wang *et al.,* 2006; 2015 [14,15]
3.	*Nosema mylitta*	*Antheraea mylitta*	Roy *et al.,* 2017 [13]
4.	*Nosema spodopterae*	*Spodoptera litura*, *Helicoverpa*, *Pieris*, *Plutella xylostella*	Tsai *et al.,* 2003 [16]
5.	*Vairimorpha sp.*	*Bombyx mori*	Luo *et al.,* 2014 [17]
6.	*Vairimorpha imperfecta, Nosema plutellae*	*Plutella xylostella*	Canning *et al.,*1999 [18]
7.	*Nosema philosamiae*	*Samia cynthia ricini*	Zhu *et al.,* 2013 [19]
8.	*Nosema heliothidis*	*Helicoverpa armigera*	Gaugler and Brooks, 1975 [20]
9.	*Nosema trichoplusiae*	*Trichoplusia ni*	Tanabe and Tamashiro, 1967 [21]
10.	*Nosema portugal, Vairimorpha lymantriae*	*Lymantria dispar*	Maddox *et al.,*1999 [22]
11.	*Nosema fumiferanae*	*Choristoneura fumiferana*	Wilson, 1982 [23]
12.	*Vairimorph aephestiae*	*Galleria mellonella*	Vorontsova *et al.,* 2004 [24]
13.	*Nosema assamensis*	*Antheraea assamensis*	Chakrabarty and Manna, 2007 [25]
14.	*Nosema ricini*	*Samia ricini*	

Source: Singh et al., 2021 [26]

2. THE TRANSMISSION MODE OF PEBRINE DISEASE

Nosema bombycis can spread either vertically or horizontally to infect *Bombyx mori*. In most cases, horizontal transmission occurs when the disease enters the silkworm through the mouth when the larval stage eats contaminated mulberries. However, the infection can also spread vertically by transovarial or trans-ovum routes. Similar to this, the diseases of *Antheraea mylitta*, *Antheraea assamensis*, and *Samia ricini* are similarly passed from mother moth to offspring through transovarial transmission, in addition to secondary infection sources such transovum and oral [27,28]. Whereas transovarial transmission is characterized by the disease moving from parent to offspring through contaminated embryos, trans-ovum transmission is described by the pathogen entering freshly emerging larvae through the surface of egg shells. As a result, when the eggs hatch, contaminated offspring are produced. In silkworms, transovarial transmissions of a variety of strains from the *Nosema* genera have been observed. Nonetheless, transovarial transmission is not observed for the species belonging to the genera *Microsporidium*, *Pleistophora,Thelohania*, and *Varimorpha* [29].

3. SYMPTOMS OF PEBRINE DISEASE

Typically, *Nosema bombycis* infects every tissue in the silkworm. The distinct symptoms of infection are present in the infected egg, larva, pupa, and moth developmental stages. Pebrinized eggs are characterised by a meager number of eggs, the majority of which are unfertilised, poor adhesion to the egg sheet, lack of regularity, and erratic hatching. Larvae of *Nosema bombycis* -infected silkworms display a range of symptoms, including anorexia, anal and oral discharges, opaque stomach, and white pustules extending the length of the silk gland. They also lose their appetite. Larvae release an unpleasant stench and turn black as they die as a result of bacterial secondary invasion. The lustreless, bloated, and softened abdomens of the pebrinized pupae are flabby. Black dots that are irregular in nature are frequently seen close to the wing and abdomen rudiments. Pupae that are severely affected do not develop into moths. *Nosema bombycis* -infected moths can be identified by their clubbed wings with malformed antennae, delayed and inappropriate moth emergence, and improper mating [11]. When compared to uninfected ones, *Antheraea mylitta* larvae infected with *Nosema sp.* exhibit a longer development period, decreased size, and decreased larval weight [30]. The black pepper-like patches on the integument of infected larvae are caused by larger, vacuolated, and blackened hypodermal cells as a result of melanin production [31]. In *Antheraea assamensis*, microsporidiosis manifests as decreased growth and black patches that spread throughout the integument of the insect body during the late infection stage. These "black pepper"-like spots are thought to be hypodermal cells that expand and vacuolate when infected, then turn black from the production of melanin pigment. *Antheraea pernyi* and *Antheraea mylitta*, two other non-mulberry silkworms, have also been found to exhibit similar symptoms [15,32,33]. *Samia ricini* exhibits the disease's symptoms at different stages of development. The majority of the unfertilized and dead eggs laid by the female moth are stacked one atop the other rather than side by side. The larva stops feeding, becomes motionless, and becomes inactive. The larva experiences abnormal growth, irregular moulting, rust-coloured body throughout the instar stage, and eventual death [34].

4. ADVANCEMENT IN DIAGNOSIS OF PEBRINE DISEASE

4.1 Microscopic Approach of Diagnosis

A critical component of producing silkworm eggs for commerce is pebrine spore diagnosis. One of the main goals of the National Silkworm Seed Organisation (NSSO) of India is to produce and distribute disease-free layings (DFLs). Through microscopic analysis using light microscopy, Pasteur produced the first diagnosis of pebrine spore in sericulture in 1870 [2]. The infected mother moths were identified as pebrine sick. This procedure involves severing an adult's abdomen with scissors, putting it in a tiny mortar, adding water, and crushing it with a pestle. Under a microscope, a drop of the smear is put on a sterile surface and checked for *Nosema sp.* spores. Large grainages—facilities that produce silkworm eggs for commercial use—need this procedure, which is crucial but time-consuming. Later, Fujiwara (1979, 1984c, 1993) refined the method by crushing mother moths with 0.6% K_2CO_3 solution and centrifuging them for two minutes at 10,000 rpm. Following content settling, the filtrate is once more centrifuged for five minutes at 3000 rpm. The supernatant is then discarded, and the sediment is dissolved in two to three drops of 0.6% K_2CO_3 solution. A

small layer of the mixture is applied to a glass slide and examined under a microscope at 600–650× magnification to look for pebrine spores [35,36,37].

An enhanced technique for identifying pebrine has been proposed by Chakrabarty *et al.* (2013), which involves crushing live larvae, pupae, and moths in a sterile solution (0.6% K_2CO_3) and separating the spores. After filtering, the suspension was kept for thirty to sixty minutes at room temperature. After that, the suspension was either decanted or centrifuged, combined with 0.8% KOH, and chilled for six hours at 5±3ºC. The suspension was then combined with Transco Trichostar and Ambistryn-S powder formulation, inspected under a microscope, and stained with 10% nigrosin to identify the spores [38]. The Central Tasar Research and Training Institute in Ranchi created Pebrine Visualization Solution (PVS) by combining various chemicals at different concentrations to solve the problems associated with laborious microscopic examination. In addition to clearing debris and enhancing spore visibility on the slide, PVS facilitates the simple identification of pebrine spores during microscopic study of mother moths [39]. Vijay *et al.* (2024) utilized Chromotrope-2R, a widely used tool in microbiology and clinical research for the identification of microsporidia, in the detection of pebrine. According to their comparative analysis, pebrine spores were effectively stained among the tested concentrations by a 0.5% concentration of Chromotrope-2R, which gave the spores a noticeable red color and made them easy to distinguish from other non-pebrine artifacts and cellular debris. At lower concentrations, it was particularly difficult to distinguish unstained samples from debris. That being said, Chromotrope-2R shows promise as a staining agent for the simple detection of *Nosema bombycis* spores in grainage operations [40].

4.2 Molecular Approach of Diagnosis

The field of sericulture has undergone a revolution in the last ten years due to the recent advent of genetic tools for pathogen identification, which has reduced the tedious procedure of mother moth testing for pebrine spore detection. The application of efficient disease control strategies is made possible by the quick and precise identification of the causal agents of silkworm diseases by molecular diagnostics [41]. "Molecular diagnostics" is the term for the application of DNA/RNA or protein-based molecular biology techniques to the identification and diagnosis of infectious agents. Molecular diagnostics has several important uses in sericulture, such as early detection and identification of silkworm infections, diagnosis of genetic abnormalities in silkworms, and evaluation of genetic diversity in silkworm populations [42]. Using molecular techniques for pebrine detection and diagnosis necessitates the completion of preparatory steps like genomic DNA extraction and RNA extraction, which can be accomplished using commercially available kits or traditional protocols to effectively lyse the tissue sample and recover the DNA and RNA. The extracted DNA and RNA need to be further purified and quantified. Table 2 lists the many molecular methods that were employed to identify it in silkworms. Zhao *et al.* (2023) have successfully established a rapid visual detection technology for *Nosema bombycis* through the combination of Recombinase Polymerase Amplification (RPA) technology and CRISPR/Cas12a system. This includes CRISPR/Cas12a fluorescence detection and CRISPR/Cas12a immunochromatographic detection. The technology was achieved by optimizing the concentration of key components. In contrast to conventional nucleic acid detection methods, this technique demonstrated a detection sensitivity of up to 2 copies/μl, a detection limit of 2 fg/μl for the *Nosema bombycis* genome, and a high sensitivity of up to 100 microsporidia injected in 100 healthy silkworm eggs. They showed that the CRISPR/Cas12a detection system has good specificity by identifying silkworm tissues infected with several infections. The real sample has a 100% accuracy rate. The technology is useful for the on-site detection of *Nosema bombycis* and has the advantages of speed, accuracy, visibility, cheap cost, minimal equipment needs, and easy operation [43].

4.3 Software and Applications for Diagnosis

A collaborative research project has been carried out with C-DAC (Centre for Development of Advanced Computing, India) for the full proof pebrine identification technology. Over the course of three years, C-DAC developed an instrument known as "Pebrine-O-Scope," which is a microscope-mounted device with a software backend that analyzes the photo-micrographic image of the smear of tissue sample from female silk moths and accurately detects the presence of "Pebrine Spore" disease. The technology was presented at the International Sericulture

Table 2. List of Technique used

Sl.No.	Technique used	Microsporidia	Host	Target Gene/Protein	Reference
1.	Polymerase chain reaction (PCR)	*Nosema bombycis*	*Bombyx mori*	RNA polymerase	Roy *et al.* (2017) [13].
2.		*Nosema assamensis*	*Antheraea assamensis*	SSU rRNA	Subrahmanyam *et al.,* (2019) [28].
3.		*Nosema pernyi*	*Antheraea pernyi*	SSU rRNA	Jiang *et al.* (2011) [44].
4.	Multiplex PCR	*Nosema bombycis*	*Bombyx mori*	SSU rRNA	Ravikumar *et al.* (2011) [45]; Wu *et al.*(2017) [46].
5.	Reverse Transcription PCR (RT-PCR)	*Nosema bombycis*	*Bombyx mori*	β-tubulin	Jagadish *et al.* (2021) [47].
6.	Quantitative PCR (qPCR)	*Nosema bombycis*	*Bombyx mori*	Small-subunit rRNA gene	Fu *et al.* (2016) [48].
7.		*Nosema bombycis*, *Nosema mylitta*, *Nosema assamensis*and *N. ricini*	*Bombyx mori*, *Antheraea mylitta*, *Antheraea assamensis*, *Samia ricini*	EB1, 16S rRNA, β-tubulin, PTP1, PTP2, PTP3, SWP5 and MetAP2	Esvaran *et al.* (2020) [49].
8.	Loop-mediated Isothermal Amplification (LAMP)	*Nosema bombycis*	*Bombyx mori*	SSU rRNA	Yan *et al.* (2014) [50]; Sivaprasad *et al.* (2021) [51].
9.	Lateral Flow Assay (LFA)	*Nosema bombycis*	*Bombyx mori*	LSU rRNA	He *et al.* (2019) [52].
10.	Slide agglutination	*Nosema mylitta*	*Antheraea mylitta*	Purified *Nosema mylitta* spores	Madhusudhan *et al.* (2016) [53].
11.	Western Blot	*Nosema bombycis*	*Bombyx mori*	Endospore protein EOB13320	Li *et al.* (2015) [54].
12.				SWP26	Zheng *et al.* (2020) [55].
13.				NbHSWP11	Yang *et al.* (2014) [56].
14.	Enzyme-Linked Immunosorbent Assay (ELISA)	*Nosema bombycis*	*Bombyx mori*	SWP5	Esvaran *et al.* (2022) [57].
15.				NbPTP6	Lv *et al.* (2020) [58].
16.				SWP12	Huang, Chen, *et al.* (2018) [59].

Source: Deepika et al., 2024 [60]

Commission, which led to substantial demand for the technology. On July 27, 2016, at a function hosted by C-DAC Kolkata and attended by the Secretary of the Department of Information Technology, Government of India, TDF (Tasar Development Foundation) received the formal transfer of technology. C-DAC granted TDF the authority to manufacture and sell Pebrine-o-scope in the industry [61].

5. CONTROL AND MANAGEMENT STRATEGIES FOR PEBRINE DISEASE

In order to prevent embryonic infection, the primary strategy for managing and preventing this illness is to generate healthy ovum. Examining mother moths in a methodical manner will help accomplish this. The other approaches include maintaining stringent hygienic standards during rearing and performing efficient disinfection of the surroundings, equipment, and rearing rooms. To prevent the spread of disease from the eggs, thorough egg washing and disinfection procedures should be carried out using Depuratex, a liquid soap created by CTRTI for tasar egg cleaning and surface disinfection. It is possible to look for pebrine spores in the grainages by examining dust, dead and unhatched eggs, egg shells, and faeces. It is advised that seed cocoon lots be regularly inspected by the disease monitoring team by sample pupae examination during I, II, and III crop [62]. Prior to incubation, the space and its furnishings need to be cleaned and sanitised. It's important to raise young silkworms in sanitary circumstances. Unhatched blue eggs, dead eggs, hatched larvae, and eggshells can all be examined as a precaution. If pebrine is found, such eggs should not be brushed, and if they are, the larvae should be destroyed. For the purpose of finding pebrine spores, a similar predictive analysis might be carried out using uneven larvae, late moulters, feather debris, and exuviae. These tests may reduce the likelihood of raising transovarially infected layings while simultaneously examining cross-contamination and disease dissemination. In order to stop cross-contamination and infection, infected silkworms, faeces, and mulberry field pests should be appropriately disposed of.In addition to inspecting the mother moth during seed production, precautions should be taken to avoid external contamination. It is not advisable to utilise equipment that has been used for one lot on another until it has been properly cleaned and sanitized. Following surface cleaning, eggs need to be dried and kept in a different room from the areas used for egg production and testing. A number of compounds have been used in an attempt to control pebrine illness, and some of them have been proven to be somewhat successful. It has been shown that feeding the worms with the infection a fungicide called Benomyl at a level of 100–150 ppm is successful in treating the disease [63]. Microsporidiasis in Andhra native ecoraces of tasar silkworm is reported to be effectively controlled by administering 0.1% Bengard and 0.005% Bavistin [26].

6. CONCLUSION

In order to ensure accuracy in the diagnosis of pebrine spores, which may be lacking in the traditional method of microscopy observation due to human error, current research has paved different ways for the diagnosis of pebrine disease. These methods primarily focus on different molecular approaches, such as PCR, RT-PCR, Multiplex-PCR, qPCR, LAMP, LFA, ELISA, etc. Since, pebrine is passed on transovarially from parent to progeny, any neglect in the progeny's monitoring could have a catastrophic effect on the sericulture sector. This is especially crucial because farmers are unable to use accurate molecular methodologies to detect pebrine spores due to the lack of expertise. Therefore, the only option left for pebrine surveillance is the tried-and-true, less sensitive microscopic inspection. For all stakeholders involved, producing silkworm eggs free of pebrine is crucial. In commercial seed production centers and seed multiplication farms, pebrine can be identified or detected using the mother moth examination method, which uses an advanced protocol that incorporates light microscopy. The availability of an AI-based pebrine identification technology is critically needed in the current situation, and at the farm level, adopted seed rearers (ASRs) stand to gain the most from it as the current approach is time-consuming and labour-intensive.

7. FUTURE PROSPECTIVE

As the cause of the decline in the sericulture sector both nationally and in other European nations, pebrine disease is well known to be extremely harmful. In addition, farmers bear the burden of loss from a crop that did not succeed owing to pebrine transmission during silkworm rearing. It is therefore clear that the entire scientific community is focused on managing the pathogen, *Nosema sp*., but as of yet, no

technology has been developed that can genuinely claim to have prevented the spread of the spore, despite the fact that numerous techniques have been developed for the diagnosis and management of pebrine disease.Mother moth testing has been the main method used to diagnose pebrine up to this point. However, mass producing silkworm eggs, particularly in Vanya sericulture, where Muga, Eri, and Tasar silkworms are directly exposed to outdoor rearing activities, has proven to be a difficult operation. Thus, the ability of artificial intelligence (AI) to be applied can be a significant step forward in the diagnosis of mother moths in the large-scale manufacturing of silkworm eggs.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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