The Importance of Proactive Approach to the Microscopic Laboratory Techniques for the Prevention and Control of Major Silkworm Diseases

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Abstract
The aim of the paper was to highlight the importance and relevance of the proactive approach of microscopic laboratory techniques for prevention and control of major silkworm diseases thus contributing to the improvement of silkworm health and to augmentation of the sericulture production. The examinations were carried out by direct microscopy and laboratory diagnostic methods for specific diseases (bacterial, parasitic, mycotic and viral) according to the methodology of the OIE Manual, adapted to the conditions of the Laboratory of Genetics, Breeding and Pathology of Silkworms of S.C.S. Baneasa, Bucharest. Direct microscopical and bacterioscopic examinations were carried out to exclude the risk of the presence of pathogens in future generations selected for the conservation of the genetic background. Samples collected from the 84 breeds under conservation were subjected to direct microscopy and bacterioscopic examinations on two developmental stages of silkworms - egg and larval stage (L3). The results of the laboratory examinations required the elimination of inappropriate biological material from the reproduction, that allowed obtaining disease-free reproductive material of the best quality. In the present research paper, we demonstrated the importance of the proactive approach of microscopic laboratory techniques for prevention and control of major silkworm diseases in order to select breeds, lines and hybrids of silkworms with resistance to diseases owned by S.C.S. Baneasa, Bucharest.

Keywords: contagious and non-contagious diseases, laboratory diagnosis, silkworms

1. Introduction

Silkworm diseases are caused by contamination with viruses, fungi, microsporidians and bacteria etiological agents of some individuals that spread the disease within the population, but also the consequence of the interaction between various favourable factors (pathogen, host and environment). Depending on the factors involved, silkworm diseases are divided into two categories: infectious (viruses, bacteria, fungi, protozoa) and non-infectious (arthropods, chemicals used in agriculture) [1, 2, 3]. In warm climates there are high rates of bacterial and other diseases [4, 5]. Silkworms are susceptible to a variety of diseases, and viral diseases are no exception [6, 7]. In the management of viral diseases, it is difficult to identify the pathogens that cause the diseases and symptoms in the early stages of the disease [8]. The silkworm is susceptible to numerous diseases, and there is currently no breed with total disease resistance. Most of the diseases are chronic in nature and the curative measures are not practical. Major silkworm diseases are viral such as nuclear polyhedrosis (*Bombyx mori* nucleopolyhedrovirus; BmNPV), cytoplasmic polyhedrosis (*Bombyx mori* cytoplasmic polyhedrovirus; BmCPV), infectious flacherie (*Bombyx mori* infectious flacherie virus; BmIFV), densonucleosis (*Bombyx mori* densovirus; BmDNV); bacterial diseases (sepsis, bacterial toxicosis (*Bacillus thuringiensis*) and diseases of digestive organs (*Staphylococcus spp., Streptococcus spp.*); fungal diseases (white

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muscardine - *Beauveria bassiana*, green muscardine (*Nomuraea rileyi*) and aspergillosis (*Aspergillus flavus*); and internal parasitic diseases (pebrine or nosemosis - *Nosema bombycis*, *Vairimorpha spp., Pleistophora spp., Thelohania spp*. and *Microsporidium spp.)* [9, 10, 11].

Non-infectious diseases of silkworms are represented by poisoning (poisoning by contact, poisoning by ingestion) and pests (ants, wasps, flies, bacon weevil) [12, 13].

Pebrina or nosemosis is an internal parasitic disease with the most serious evolution in silkworms, produced by the microsporidia *Nosema bombycis*. The route of contamination is achieved by ingesting Nosema spores, and the disease is transmitted to silkworms through 3 transmission routes (oral, contact and transovarian) [14]. According to the type of source of infection, pebrina is divided into 2 types: hereditary (infection transmitted by infected butterflies, infected eggs and hatched larvae showing pathogenic spores) and acquired (healthy silkworms that have ingested infected material). As clinical signs, Nosema-infected eggs lack adhesive ability (easily detached from the egg film) and are laid on top of each other instead of next to each other, irregular hatching. The pathognomonic symptom of the disease is represented by the appearance of dark brown or black dots on the larva [15].

Grasserie or polyhedrosis, a viral disease of silkworms produced by the etiological agent Borrelina virus, it is called anaemia due to the yellow colour of the affected larvae [16]. The route of infection is represented by the ingestion of food contaminated with the virus that crosses the intestinal wall into the body cavities and invades the cells of susceptible tissues. The disease can be induced by keeping the larvae at low or high temperatures and irregular feeding on too tender or mature leaves favouring the incidence of polyhedrosis. The virus forms polyhedral bodies with 5-6 faces, two types of polyhedra are found: cytoplasmic polyhedra virus (CPV) and nuclear polyhedra virus (NPV). One week after infection, clinical signs appear for the first time, manifested by the swelling of the first segment of the infected larvae, then it gradually extends to the whole body, the swelling is more pronounced at the intersegmental level, giving the larva a bent appearance like bamboo. The cutis becomes stretched and fragile, the larva fails to move and crawls aimlessly, the cutis cracks and a whitish liquid ooze from the cracks, if the larva is infected in the terminal stage with polyhedra it splits and the silk is damaged or forms a flimsy cocoon. The larva dies inside the cocoon before pupating.

Flacherie is a disease caused by a single-stranded RNA virus - *B. mori* Infectious Flacherie Virus (BmIFV), which causes crop losses of up to 40% [17, 18]. Several other virus types believed to be BmIFV have also been reported from Karnataka, India [19]. The synergy between bacterial and viral diseases that increase crop losses due to virus infection is poorly understood [20]. The conventional method of detecting BmIFV is pyronin staining, which detects type A and B bodies in histological sections, but this method is time-consuming and not a confirmatory test. The preferred technique for confirmation is reverse transcriptase polymerase chain reaction (RT-PCR), which is a sensitive technique used for mRNA detection, is semi-quantitative, and is used to convert mRNA to complementary DNA (cDNA) by reverse transcriptase [21].

It is more advantageous than Northern blot analysis and RNase protection techniques because it can quantify mRNA levels from much smaller samples [22]. This technique is sensitive enough to allow quantification of RNA from a single cell [23]. The nucleic acid of BmIFV is an ssRNA virus [24], and since RNA cannot serve as a template for PCR, reverse transcription combined with PCR is used to convert the RNA into its complementary DNA, which is suitable for PCR. The RT-PCR test has proven valuable for detecting gene expression, amplifying DNA sequences before sub-cloning, and analysing and diagnosing infectious agents or genetic diseases [25].

The main causes that produce flacherie are represented by factors favouring the disease (overpopulation, overfeeding, feeding with dry, dirty, fermented and unsuitable leaves for the age of the larvae, high temperatures, high humidity or poor ventilation). Depending on the etiological agents and symptomatology, flacherie is classified into: typical form (super acute, acute) and atypical (slow). Infected larvae become inactive and delay growth. The cephalon-thoracic region becomes translucent, the head portion will be bulging, which is the typical symptom of BmIFV infection [25]. The larva begins to vomit intestinal juice and dysentery begins.

Muscardine is the mycotic disease of silkworms that evolves in the form of white muscardine (*Beuveria bassiana*), green muscardine (Spicaria prasine), yellow muscardine (*Isaria farinose*) and brown muscardine (*Aspergillus*). When the fungal conidia come into contact with the skin, they germinate, the fungi penetrate the skin, spread throughout the organism and the mycelium develops, which comes into contact with the body fluid on the worm and forms spores. The fungus continues its development in the body. The larvae have diarrhoea, liquid vomit, the larva's body loses its elasticity, the larva dies in 3-4 days after infection, the larva's body hardens and becomes rigid, the larva's body is covered with a white, yellow, green or brown mycelium. The conidia germinate in the haemolymph and penetrate the adipose tissue, muscles, nervous system, sericogenic glands, etc. In the advanced stage of infection, the haemolymph circulation stops and the larva dies. Dead larvae spread the infection. Affected larvae lose their appetite and become inactive. An oily secretion flows on the cutis, and the numerous conidia on the body form a powdery substance [26, 27].

2. Materials and methods

Health monitoring through morpho-clinical and laboratory examinations on samples of eggs and larvae (L1-L3) of Bombyx mori was carried out for the purpose of prophylaxis and control of diseases that may develop in silkworms. Within the Laboratory of Genetics, Breeding and Pathology of Silkworms within the S.C.S.B.B. during the 2023 silkworm season, investigations were carried out on the biological material of silkworms from the Bombyx mori species, respectively on the 84 existing breeds/lines/hybrids, which constitute the genetic background maintained in conservation [28, 29, 30, 31, 32]. An experimental lot consisting of 84 egg samples (1 cm^2 each) and 84 larval samples (L1-L3) from all breeds under conservation at SCSBB was constituted. Monitoring sheets were drawn up which included the following details: breed/line/hybrid, female characteristics, egg morphology, size and body weight of larvae at different ages (L1-L3), results of direct microscopic and bacterioscopic examination to identify pathogens involved in the occurrence 24of diseases. Microscopic examination (Nikon Eclipse CI-L Plus microscope, 400 x and 40 x resolution) was carried out based on the regulations of the International Office of Epizootics (OIE, 2016) and Insect Pathology (Steinhaus, 1967), adapted for samples of female B. mori butterflies (www.idsa.ro) (Figure 1).

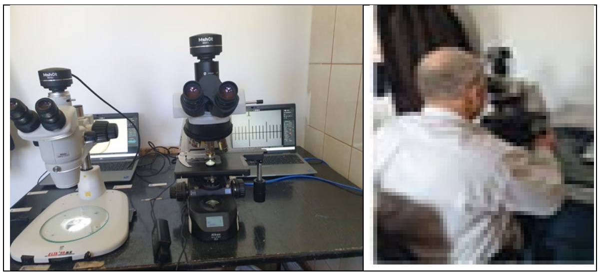


Figure 1. Microscopic examination of silkworm biological material

3. Results and discussion

The biological cycle of the silkworm shows 4 stages characteristic of a complete metamorphosis (egg, larva, chrysalis and butterfly). The particularities of occurrence and the risk of disease evolution in the 4 stages of evolution, as well as the lack of specific therapeutic means, recommend carrying out sanitary-veterinary surveillance of growing batches of silkworms, at each stage of development, the most important stages being those of the adult stages (butterfly) and the larval stages (L1-L5).

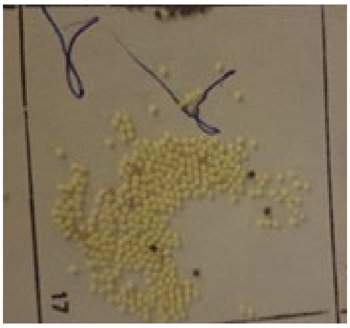
Monitoring of the state of health in the egg, young larva and adult larva stage is done by laboratory examinations (morpho-clinical, direct microscopic and bacterioscopic), and the monitoring of the adult stage is carried out by direct microscopic control of the intestines of butterflies selected for reproduction.

The obtained results indicated that each developmental stage presented specific particularities, dependent both on the influence of the external environment and on the functioning of the neuroendocrine systems that trigger and regulate these processes.

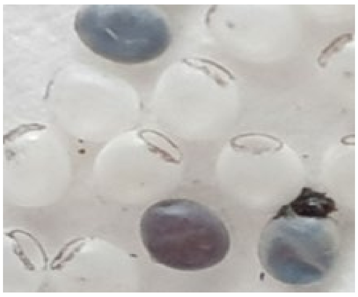
A series of environmental factors acted either directly on the health of silkworms (food, temperature, humidity, light intensity, hygiene conditions in the growing space), or indirectly (geoclimatic conditions, which are reflected especially in the nutritional value of the leaves of mulberry) [33, 34].

During the morpho-clinical and laboratory sanitary-veterinary examination, various aspects were highlighted that required the withdrawal from reproduction of eggs, larvae and females that presented different non-conforming characteristics (uniformity of hatching, eggs from butterflies and males with congenital wing defects , unfertilized eggs, overcrowded and petrified eggs, dehydrated eggs, eggs subject to heat shock, larvae with digestive symptoms from eating young leaves, hypoxic larvae, crushed larvae, etc.) (Figure 2).


Clinically healthy fertilized eggs


Unfertilized eggs


Eggs from the butterfly and the male with congenital wing defect


Unevenness of hatching


Eggs hatch unevenly


Eggs kept at elevated temperature


Dehydrated eggs


Heat-shocked eggs


Deck overcrowding


Supraaglomerarea larvelor



Larve cu simptome digestive de la consumul de frunză tânără

Crushed larvae Larvae with hypoxia

Figure 2. Specific features of non-conforming silkworm eggs and larvae

The results obtained following the morpho-clinical sanitary-veterinary examination on the different stages of silkworm evolution did not record losses in the 2023 active season that had infectious causes and that determined the appearance and evolution of infectious-contagious diseases (viral, bacterial, mycotic and specific parasites) and/or other non-infectious causes (due to non-compliance with technological parameters). It was observed that in the young larva stage there were no losses at all due to technological mistakes, and in adults the appearance and evolution of specific diseases were not recorded.

No episodes of sporadic (infectious anaemia and septicaemia) or epizootic diseases (polyhedria, flaseria, nosemosis and muscardina) were recorded, which led to obtaining generations of larvae free of infectious-contagious diseases.

All these beneficial results were due to well-designed preventive actions (periodic disinfection, changing the waste substrate on time, keeping the growing space in favourable conditions in terms of temperature, humidity, air currents and light, but also as a result of the removal of the larvae that have migrated from the breeding bed, the removal of crushed larvae from the branches with mulberry leaves administered for feeding, the provision of the protective equipment necessary for the growth and disinfection activity, the permanent change of the disinfectant solution and the refreshing of the impregnated carpets with disinfectant solution from the entrance door to the growing spaces, ensuring quality mulberry leaves (without being wet, dirty) and corresponding to the age of the larvae (finely chopped at the L1-L2 age and then more coarsely chopped at the older ages).

Sivaprasad describes that optical microscopy and histological methods are known to have low sensitivity and specificity therefore the use of immunodiagnostics is recommended which is often used to diagnose/detect silkworm diseases using antibodies produced against specific silkworm pathogens [35].

4. Conclusions

The morpho-clinical sanitary-veterinary examination did not highlight the evolution of any infectious-contagious or non-infectious diseases in the breeds/lines/hybrids of silkworms from the 84 breeds that constitute the sericulture gene pool of the Băneasa-Bucharest Sericulture Research Station.

In the young larva stage, there were no losses at all due to technological mistakes, and in adults, the appearance and evolution of specific diseases were not recorded.

During the morpho-clinical and laboratory sanitary-veterinary examination, various aspects were highlighted that required the withdrawal from reproduction of the eggs, larvae and females that presented different non-compliant characteristics.

Well-designed sanitary-veterinary prophylactic actions allowed obtaining a biological material free of pathogens, which would ensure the next generation to obtain a genetic material of the best quality.

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