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Viral diseases of Mulberry Silkworm, *Bombyx mori* L. - A Review

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Abstract
The mulberry silkworm, *Bombyx mori* (Linnaeus) (Lepidoptera: Bombycidae) is an economically important insect domesticated for silk production. One of the main reasons for decreased cocoon production is the loss due to diseases which account for about 30 *per cent*. It is affected by a number of diseases caused by viruses, bacteria, fungi and protozoa. Among the silkworm diseases, which cause economic damages, the highest crop loss is attributed to viral diseases, accounting for 70 *per cent* of total loss of the crop every year. Viruses are obligate intracellular pathogens which need host cells to replicate. Viral diseases of silkworm comprise of inclusion and non-inclusion types. The inclusion virus diseases form typical inclusion bodies. They are nuclear polyhedrosis and cytoplasmic polyhedrosis which can be more easily identified through ordinary microscopy. The non-inclusion type consists of infectious flacherie and densonucleosis which can be detected only through electron/fluorescent microscopy and serological tests. The collection of information on investigations related to viral diseases in silkworm is highly helpful to monitor, prevent and control them. However, such collection of data on various diseases of silkworm in general and viral infection in particular in worldwide is very limited. Pathological status of the diseased animal can be evaluated in terms of symptoms resulting from the interactions between host and pathogen which in turn depends on the degree of attack, progress of diseases, metabolic modulations, physiological adjustments, molecular mechanisms, and the defence immune system of the silkworm. An attempt has been made in the present study to provide the relevant information related to the viral diseases of silkworm larvae.

Keywords: mulberry, silkworm, viral diseases, pathogens, host

Introduction
Viruses are obligate intracellular pathogens which need host cells to replicate. Viral genetic material (viral genome) is made up of different forms of DNA (DNA-virus) or RNA (RNA-virus) and is enclosed in a protein coat (capsid) and sometimes a lipoprotein envelope [16]. The genetic material and the surrounded protein are called nucleocapsid and the entire structure is called a virion or virus particle. Virions of the different virus families have different forms and shapes and can only be seen via phase-contrast or electron microscopy. A virion represents the smallest infectious entity and is sometimes vulnerable but often persistent outside its natural host. After entry of a virion into a 'permissive' host cell (a susceptible one, where the virus is 'permitted' to replicate) the viral genetic material takes control of the cell's machinery, and begins to produce viral proteins, which will be used to generate new viral particles for systemic infection. Currently there are 2,284 virus species recognised by the International Committee on Taxonomy of Viruses [1, 28] which are accommodated into 73 virus families.

The Silkworm, *Bombyx mori* L. is an important economic insect and also a tool to convert mulberry leaf protein into commercially valuable silk protein. Silk is called the "Queen of textiles" due to its glittering lustre, softness, elegance, durability, and tensile properties. Silkworm disease is a phenomenon of physiological disorders caused by invasion of pathogenic microorganism and parasites, or influence of physical and chemical factors and other adverse environmental factors, showing a variety of abnormal conditions or death of the silkworm. Mulberry silkworm, *B. mori* L. is affected by a number of diseases caused by viruses, bacteria, fungi and protozoa. Among the silkworm diseases, which cause economic damages, the highest crop loss is attributed to viral diseases, accounting for 70 *per cent* of total loss of the crop every year [7]. Viral diseases of silkworm comprise of inclusion and non-inclusion types. The inclusion virus diseases form typical inclusion bodies. They are nuclear polyhedrosis and cytoplasmic polyhedrosis which can be more easily identified through ordinary microscopy. The non-inclusion type consists of infectious flacherie and densonucleosis which can be detected only through electron/fluorescent microscopy and serological tests [58].

The nuclear polyhedrosis virus (NPV) infects various tissues, and multiplies in the nucleus forming inclusion bodies called polyhedra, which occlude virus particles. The virus is rod in shape and contains double-stranded DNA. The cytoplasmic polyhedrosis virus (CPV) infects the midgut epithelium and multiplies in the cytoplasm of columnar cell forming inclusion bodies which occlude virus particles. The virus is an icosahedral particle 60 nm in diameter and contains double-stranded RNA. The infectious flacherie virus (IFV) infects the midgut epithelium and multiplies in the cytoplasm of goblet cell without forming inclusion bodies. The virus is spherical, 28 nm in diameter, and contains single stranded RNA. The densonucleosis virus (DNV) infects the midgut epithelium and multiplies in the nucleus of columnar cell. The virus is a spherical particle, 22-24 nm in diameter and contains single-stranded DNA. Two strains of *Bombyx mori* DNV are detected serologically: DNV type-1 (DNV1) and DNV type-2 (DNV-2).

These diseases are known to occur in almost all the silkworm rearing areas of the world causing considerable damage to the silkworm cocoon crop. A number of measures have been suggested for the prevention and control of these diseases, care is also needed to be taken to see that they are not exposed to stress conditions like temperature, humidity, bad ventilation and nutritional deficiency which may make them easily susceptible to viral diseases [29]. The development of diseases in silkworm is influenced by environmental and nutritional factors such as temperature, humidity and mulberry leaf content.

Nuclear Polyhedrosis Disease of Silkworm

It is one of the most dominant and serious viral diseases in tropical countries and occurs throughout the year. The *Bombyx mori* nucleopolyhedrovirus (*Bm*NPV) (Family: Baculoviridae; Genus: Alphabaculovirus) causing grasserie disease in silkworm is the most harmful virus in the sericulture industry, often causing severe economic losses [47, 7]. In India, >50 *per cent* of silk cocoon crop losses are attributed to *Bm*NPV infection [27] and in Kashmir valley the silkworm crop losses due to *Bm*NPV are about 28-32 *per cent* [19]. The nuclear polyhedrosis of silkworm is commonly known as Grasserie disease (French name), milky disease, hanging disease, fatty degeneration disease, or Jaundice (American name). In Italy it is known as "Giallume", in Japan "Nobyo" and in Germany "Gelbsucht". The names indicate either the yellowish colour of diseased insect or its swollen fat like appearance. In India, it is also known by various local names i.e. "Halu-hula" in Karnataka, "Rasa" in West Bengal, "Pola purugu" in Andhra Pradesh, "Paala poochi" in Tamilnadu and Aab-Baimair in Kashmir.

The grasserie disease of silkworm was first reported by Nysten. Earlier *Bm*NPV the causative agent of grasserie was referred as *Borriina bombycis* [45].

Causative agent

The *Bombyx mori* nucleopolyhedrovirus (*Bm*NPV) is the most harmful virus in the sericulture industry, often causing severe economic losses [47]. The nuclear polyhedrosis viruses belong to the sub group A of the family Baculoviridae. The family Baculoviridae is classified by the basic characters as an envelope, rod-shaped virion (approximately 50×250 micron) containing a circular double-stranded DNA genome ranging from 50 to 100 million Daltons. As the name implies, this virus multiplies and forms polyhedra in the nucleus of the tracheal epithelial cells, adipose tissue cells, dermal cells and blood cells. Occasionally the nucleus of the middle and posterior portion of silk gland cells are also affected. A characteristic feature of *Bm*NPV infection is the production of crystalline proteinaceous structures called occlusion bodies or polyhedra, in which several virions are embedded and thus protected from UV rays, desiccations, proteases and nucleases. Baculoviruses have only been found in over 600 species of arthropod hosts. The majority of baculovirus hosts are within the order Lepidoptera. They have also been isolated from the insects in orders Diptera, Hymenoptera, Coleoptera, Neuroptera, Thysanura and Trichoptera and the crustacean in order Decapoda (shrimp) [17]. The baculo portion of the name refers to the rod-shaped capsids of the virus particles. The virus particle is composed of a protein shell that surrounds the nucleic acid. Within the capsid, the DNA is condensed into a nucleoprotein structure known as the core. The capsid plus the core are collectively referred to as the nucleocapsid[44]. Nucleocapsids contain a single molecule of circular super coiled double- stranded DNA. The length of baculoviral DNA is between 80 and 200 kb [60]. Nucleocapsids are made in the nucleus of infected cells.

The viral particles are rod shaped and the size is around 330×80 nm. The size of the polyhedra varies from 3-6 µ. The shape is usually octadecahedral or hexahedral and sometimes tetragon or trigon. Infection mostly takes place through feeding of polyhedra contaminated mulberry leaf, rarely through wounds. Factors inducing the outbreak of this disease are high temperature and humidity, their sudden fluctuations, bad ventilation in the rearing room, ineffective disinfection of rearing room and equipments and feeding of tender leaves during late instars. Inadequate larval spacing, starvation and excessive moisture in the rearing bed have also been known to contribute towards the outbreak and spread of the disease.

Occurrence and Incidence

The nuclear polyhedrosis prevails all through the year especially during summer and rainy seasons. This disease is having a direct correlation with climate, its highest rate in *Bombyx mori* being at 100 *per cent* relative humidity. During April and May months, NPV infection caused most adverse effect, with silkworm mortality recorded up to 52 *per cent* in Bangladesh. Nuclear polyhedrosis prevails high in summer (55.35%), followed by winter 41.66 *per cent* and in rainy season 32.97 *per cent* of Karnataka [43]. Highest incidence of the diseases 7.35 and 7.42 *per cent* was recorded during summer of 1984 and 1985, in Karnataka, India [50].

Infection and Transmission

The nuclear polyhedrosis virus multiplies in the nucleus of the infected cells of various tissues and gets crystallized on maturation into a proteinaceous material forming the polyhedra [5]. The mode of infection of *Bm*NPV to the silkworm is mainly per oral, i.e. ingestion of food contaminated with the viral polyhedral [42]. After being swallowed, the polyhedra reach the digestive tract where they are dissolved by alkaline digestive fluid and the virions are released [3]. These virions pass through the peritrophic membrane and invade susceptible columnar epithelial cells in the midgut causing primary infection [42]. The name "Propolyhedra" was proposed for such granules (0.2 to 0.4 m in diameter). These granules are the early stage in the development of polyhedral which grow around a dense central mass. Nuclei of the cell, which are filled with polyhedral finally are destroyed by the fully grown polyhedra, then which get suspended in large numbers in the hemolymph [2].

Nuclear polyhedrosis in silkworm is highly contagious. The body fluid of the infected larvae and dead infected larvae spread the infection through contamination of diet [42]. 1 to 10 nuclear polyhedrosis diseased larvae introduced during 2nd instar in to a healthy population of 100 larvae are reported to spread 42-56 *per cent* infection during rearing [24]. Only one infected larvae can transmit 42% of disease in a silkworm population. *Bm*NPV can undergo 3-5 cycles of multiplications during a silkworm rearing in larval stage to produce 10^{7-9} polyhedra /cycle and 10^{28-36} polyhedra from four cycles [42]. Due to stable character of the pathogen it can persist for a long period. *Bm*NPV polyhedra are highly stable in nature and can persist for over 100 days in soil and 3-15 years under silkworm rearing house conditions [42]. The infectivity of the silkworm nuclear polyhedral was shown to be retained well for at least twenty years, mostly at 4 °C. Dried infected silkworm hemolymph still keeps its infectivity after exposer to temperature of 100 to 120 °C for one hour. The most common sources of pathogens for infection and stress over the diseases during the rearing are the contaminated rearing trays and seat papers [21, 41].

Symptomatology of Nuclear polyhedrosis

During early part of the disease no symptoms are noticed except the worms being slightly sluggish. Initially the skin shows oily and shining appearance. The external symptoms of the *Bm*NPV disease of silkworm in the fourth and fifth instar larva, which are visible about a week after infection, include the following:

- Swelling of inter segmental regions
- Larvae become lethargic and loose appetite
- Shining and yellowing body
- Hyperactivity
- Crawling around trays and hanging
- Rupturing of the skin
- White ooze filled with polyhedra
- Crawling around the periphery of the rearing tray
- Death of worms
- Diseased larvae loose the clasping power of abdominal legs, except the caudal legs by which it hangs with the head downwards.
- Rotting and secondary infections
- Nuclei of cells of various tissue contain polyhedral bodies
- The larva becomes restless and impatient causes fatigue [31].

Epidermal cells and cells of all infected tissue become abnormal. If infection occurs in the early instars, the worms fail to spin the cocoons and die, whereas if the infection occurs at later stages, the worms could spin the cocoons but subsequently die inside producing melted cocoons, and the affected cocoons become unfit for reeling [9].

Nuclear Polyhedrosis Detection

The *Bm*NPV detection is needed to stop spread of the disease in rearing units, to take suitable preventive steps, to initiate appropriate control measures and for certification in National and International trade. The level at which the disease becomes apparent depends to a large extent on the ability of the observer to recognise the disease. Many times the disease remains undetected (latent, sub-lethal) and is only recognised once the disease had reached the epidemic levels (lethal). Once the symptoms of disease appear, it is too late to apply control measures. The present most common methodologies for *Bm*NPV detection include microscopy, *in vivo* assays and nucleic acid based analysis [22]. Other techniques that have been developed to detect *Bm*NPV include Enzyme-Link Immunosorbent Assay (ELISA) [59], DNA hybridization [6], colloidal textile dye-based dipstick immunoassay [42], protein-A linked monoclonal antibody latex agglutination test (PALMAL) [54] and viral DNA transfection [40]. All these methods stated above require isolation of the pathogen, nucleic acid extraction, skilled person and equipment, costly and are difficult to perform as routine assays. The possibilities of variations induced by the personnel, transportation of samples, processing and the testing conditions and the lack of uniform analytical platforms further complicate the process making the data unreliable. In addition, these assays lack the convenience of "on-site" testing and require a complex work flow starting from sample collection, sample labelling, sample storage and transport to appropriate facility, followed by further sample processing, after which the samples are assayed and the results are interpreted. Hence, developing suitable detection methods which permit accurate, rapid and sensitive analysis is essential for silkworm disease management. The best emerging technology is the use of biosensors and Lateral Flow Assay (LFA) that provide us with a tool to rapidly detect the presence and amount of pathogen in any given environment [13]. A biosensor is actually a compact analytical device with a ligand–specific biorecognition element (antibody, enzyme, receptor, nucleic acid, aptamers, peptide/protein, lectin, cells, and tissue. The physiological interaction between the ligand and the biorecognition element is translated, by the transducer, into a measurable electric signal, which is further deciphered by a computer–aided readout system for the user [4]. The Lateral Flow Assay (LFA) is a paper-based platform for the detection and quantification of analytes in complex mixtures, where the sample is placed on a test device and the results are displayed within 5-30 min. LFAs are very good candidates for disease detection as they are cheap to produce, easy to use and widely accepted by users [30]. Moreover, because of the long shelf life and the fact that refrigeration is not required for their storage, LFAs are very well suited for field use. LFAs are categorized into 'Lateral Flow Immunoassays' (LFIAs), in which antibodies are exclusively used as recognition elements and Nucleic Acid LFA (NALFA) which are used for the detection of amplicons formed during the polymerase chain reaction (PCR) [10]. Both these techniques can be effectively performed outside the laboratory and are well suited for development of "on-site" diagnostics. They are easy to use and do not require trained personnel, laboratory equipment or reagents, thus, capable of testing and yielding results on site, cutting short the lengthy process. As of yet, there is no report of the application of antibody-based biosensor or lateral flow assays in *Bombyx mori* NPV detection or any other silkworm pathogen.

Cytoplasmic Polyhedrosis Disease of Silkworm

The cytoplasmic polyhedrosis virus of silkworm *Bombyx mori* (*Bm*CPV) is a major viral pathogen of silkworm that causes extensive damage to the sericultural industry. Its incidence has been reported to be 27.76 *per cent* in Karnataka, out of the total loss of cocoon crop in India, 71 *per cent* was due to flacherie [9]. The CPV disease is caused by an occluded double stranded RNA virus which multiplies in cytoplasm of columnar cells of midgut epithelium. The disease is characterized by the manifestation of symptoms like, body flaccidity, paleness of skin, vomiting and semisolid whitish-

green faeces. The severity of disease is mainly dependent upon the quality of mulberry leaf, silkworm breed and rearing conditions.

Causative agent

The virus is *Bombyx mori* Cytoplasmic Polyhedrosis virus (*Bm*CPV), which belong to the CPV subfamily, which consists of 19 distinct species (electropherotyes) within the genus Cypovirus, family Reoviridae [53, 14]. The genome of *Bm*CPV is composed of 10 discrete double stranded RNA segments [12, 48]. The virus particle is globular, 60-70 nm in size, icosahedral, hexahedron, each capscmer extends outwards to form a four-joined projection, the tip of which carries a globular body concealing the two distal joints. The capsid consists of two icosahedral coats which are linked together by a tubular structure at the supposing apices. At the centre of the capsid lies the core. *Bm*CPV infects epithelial and columnar cells of the midgut of susceptible silkworm. The infected silkworms are characterized by hypogenesis, emaciation and sluggishness. As the infection advances, white wrinkles can be observed in the posterior part of the midgut, which is the typical symptom of CPV caused disease [37].

Occurrence and Infection

Occurrence is throughout the year and severity is more during summer and rainy seasons. The incidence of diseases differs from region to region, crop to crop, and even farmer to farmer in the same crop [32]. It has been found that crop losses generally more in tropics than in the temperate region which showed an annual loss due to diseases at around 30 *per cent* and another 5-10 *per cent* due to pests [52]. The weather conditions prevailing at the time of rearing greatly favours the outbreak of *Bm*CPV disease.

Infection and Transmission

Infection is mainly through oral route, the virus and polyhedra enter the digestive tract with the mulberry leaves that are swallowed. The formation of polyhedral proteins also takes place in the cytoplasm, after which the virions are enclosed to form polyhedra, which line up in rows in the cytoplasm.

After gaining entrance into the body, the *Bm*CPV first parasitizes the tubular cells at the junction of the mid and hind gut, causing a milk-white fold to form at this site. As the disease develops, the milk-white portion advances rostrally until the entire midgut becomes a milk-white annular prominence, giving the body of the worm a milk white colour. After infection of the silkworm by the *Bm*CPV, the digestive and absorptive functions of the midgut are impaired, which together with the expenditure of large quantities of host proteins to form viral and polyhedral proteins, cause disturbances in the nucleic acid and protein metabolism. As a result, the free amino-acid content of the haemolymph, the digestive fluids, the tissues and the cells decrease, and there even occurs an absence of some amino acids. All this leads to physiological disfunction and disease. Progression of the disease sees the tubular cells becoming packed with polyhedra, causing the cells to swell and finally burst. The polyhedra and viral particles released into the cavity of the intestine are excreted in the faeces, which assume a milk white colour.

Symptomatology of Cytoplasmic polyhedrosis

Slow development of symptoms and extended courses are characteristics of the disease. Small worms exposed to minute quantities of the virus may not show sickness until the fifth instar. Generally, if infection occur at the first instar, the onset of the disease would be seen in the second and third instar, if at the second instar, than the onset would be in the third and fourth instar, if at the third or fourth instar, then symptoms appear in the fifth instar and it at the fifth instar, no symptoms are seen at all.

- Larvae show symptoms of sluggishness, loss appetite, cease feeding and sometimes develops diarrhoea and vomit gut juice.
- Larvae lag behind in their growth and development.
- Head becomes disproportionately large and translucent and body shrunken.
- Frequent expulsion of semi-solid and whitish feacal matter.
- In infected larvae midgut becomes chalky white and opaque.
- Infected pupae are generally smaller than normal and the infected moth lays fewer eggs and as short lived.
- On dissection of infected larvae midgut is seen as whitish and opaque compared to greenish and transparent midgut of healthy larvae.
- Anal region is solid and occasionally rectal protrusion is noticed.
- If infection taken place at the adult larva stage, the thorax becomes slightly transparent, the body atrophies, and vomiting and diarrhoea occur.

The course of the disease is influenced by temperature, humidity, and quantity and virulence of the virus. In the fields, exposure usually occurs at the third and fourth instar, and the onset of disease at the fifth instar. This disease occurs mainly during the summer and autumn rearing seasons.

Disease Detection

Definite detection of this disease can be made by tearing open the body wall rostrally to the caudal horn and finding a milk white circular prominence in the posterior part of the midgut. If there is any doubt, tissue from the caudal part of the midgut can be dissected out, and pressed specimens should be prepared and observed under a 400-600x microscope. The presence of polyhedra would constitute a definite diagnosis of this disease.

Infectious Flacherie Disease of Silkworm

Flacherie (literally: "flaccidness") is a disease of silkworms, caused by silkworms eating infected or contaminated mulberry leaves. Louis Pasteur, who began his studies on silkworm diseases in 1865, was the first one able to recognize that mortality due to viral flacherie was caused by infection. Bacteria is an etiological agent of silkworm flacherie. The flacherie in silkworms is by virus or by bacteria alone, or it can also cause by the combined infection of viruses and bacteria.

The major diseases affecting silkworm are flacherie, grasserie, muscardine and pebrine. Among these four diseases, flacherie is more prevalent causing cocoon crop loss to the tune of 33.88 *per cent* in India [57]. *Bombyx mori* Infectious flacherie virus (*Bm*IFV) is a single stranded RNA virus that causes flacherie-like symptoms, is the most frequent virus in *Bombyx mori*, and is known to cause crop losses of up to 40 *per cent* [51]. The infected larvae show gradual reduction in size, retarded growth, reduction in body weight and flaccid condition of body followed by vomiting and death [39]. Adverse environmental conditions such as high temperature and humidity [20, 41], polluted air [23] and starvation [49] are

considered important predisposing factors for flacherie and cocoon crop loss. Temperature higher or lower than 25 °C acts as a stress factor and increases the susceptibility of silkworm to viral infections [55]. If temperature and humidity are extremely high, the susceptibility of silkworm larvae increases to viral infections [61].

Causative agent

This disease is caused by the *Bombyx mori* Infectious Flacherie Virus (*Bm*IFV), belonging to the family Picornaviridae. The virus particle is globular, about 30nm in diameter. The nucleic acid is single stranded RNA [25], no polyhedra are found in the host's cell.

Occurrence and Incidence

Infectious flacherie is found in all the silkworm rearing areas of the world. It is a highly contagious and exceedingly disastrous condition, occurring mainly in the summer, autumn and rainy seasons. The virus exhibits high virulence, and in the body of the dead worm it may retain its pathogenicity even after 2-3 years in the rearing room. Virus in the faeces can withstand 100 ˚C for 30 minutes without being destroyed.

Infection and Transmission

Infection is mainly by the oral route. After invasion of the midgut, the virus first infects the goblet cells, starting at the rostrum end and spreading upwards, without forming polyhedra. *Bm*IFV chiefly infects the goblet cells of the midgut, which perform the function of secreting digestive fluids, which in turn not only digest mulberry leaves but also have bacteriostatic and virucidal properties. Degeneration of the goblet cells affects the digestive and bacteriocidal functions and this permits rapid proliferation of enteric bacteria. Under the concerted invasion of virus and bacteria, death of the silkworm is accelerated. After infection, the goblet cell swells and later atrophy. The cells eventually disintegrate; the virus is then dispersed in the lumen of the digestive tract and is excreted with the faeces. Thus, the faeces, which carry large quantities of the virus, are an important source of infection within the rearing tray.

Symptomatology of Infectious flacherie disease

The symptoms and course of the disease vary, depending on the kind and quantity of concurrent bacterial proliferation in the digestive tract. The course of the disease is uncomplicated infectious flacherie is relatively protracted, lasting about 5-12 days. The symptoms of this disease and those of cytoplasmic polyhedrosis are rather similar.

- At the early stage of infection, symptoms are not clear and difficult to identify.
- At the initial stages of the disease consumption of mulberry leaves declines, the time needed to finish moulting is not uniform, and individual disparity in size is great.
- The larvae become soft and flaccid, the growth of affected larvae retards, become inactive and vomit gut juice.
- The faeces become soft with high moisture content. Sometimes chain type of excreta is observed. Often, rectal protrusion is also observed.
- Cephalothoracic region becomes translucent.
- The main symptoms are shrinkage, empty foregut, diarrhoea and vomiting.
- When infected with *Bacillus thuringiensis*, symptoms of toxicity such as paralysis and sudden death are observed. After death larvae turn black in colour and give foul smell.
- Sometimes the dead larvae turn red when infected with *Serratia marcescence* during injury.

Disease Detection

The conventional method of *Bm*IFV detection is the pyronine staining method, in which, a small piece of the midgut wall should be removed and a fresh specimen prepared on a slide. After fixing in carnoys fixative solution for one minute, it is washed gently and then stained with pyroninmethyl green for 5-10 minutes. After washing in water to remove unreacted stain, the specimen is observed under 400-600x microscope, detects A and B type bodies in histological observation, but this method is time consuming. Another technique is polymerase chain reaction (PCR). The applications of PCR are various, manifold, and well-documented [35]. While the PCR technique is basically a primer extension reaction used for amplifying specific nucleic acids *in vitro*, reverse transcriptase (RT) PCR is a sensitive technique used for mRNA detection, is semi-quantitative, and is employed for converting mRNA to complementary DNA (cDNA) via reverse transcriptase [38]. This technique is sensitive enough to enable quantization of RNA from a single cell [11, 34]. The nucleic acid of *Bm*IFV is an ssRNA virus [25], and since RNA cannot serve as a template for PCR, reverse transcription combined with PCR is used to convert RNA into its complementary DNA, which is suitable for PCR. The combination of both techniques is colloquially referred to as RTPCR. The process of RT-PCR has proved valuable for detecting gene expressions, amplifying DNA sequences prior to sub cloning, and analysis and diagnosis of infectious agents or genetic diseases [46].

Densonucleosis viral disease of silkworm

Viral diseases of silkworm cause a major problem to sericulture as they account for almost 70 *per cent* of total crop loss. The two major viral diseases include grasserie caused by *Bombyx mori* Nuclear polyhedrosis virus (*Bm*NPV) causes 42.60 *per cent* crop loss and viral flacherie caused by *Bombyx mori* Infectious flacherie virus (*Bm*IFV) causes 47.90 *per cent* crop loss, *Bombyx mori* Densonucleosis virus type 1 (*Bm*DNV-1) and *Bombyx mori* Densonucleosis virus type 2 (*Bm*DNV-2) causes 9.50 *per cent* crop loss [34].

Densoviruses (DVs) belong to the family Parvoviridae. Viruses belonging to the Parvoviridae family are known for having a wide host range. Hence this family has been divided into two subfamilies namely, Densovirinae and Parvovirinae. Viruses falling under the category of Densovirinae infect invertebrates while those under Parvovirinae infect vertebrates. *Bm*DNV-2 is a non-enveloped spherical DNA virus 20-24 nm in diameter and the genome contains two non-homologous single-stranded linear DNA molecules VD1 (6,543 bp) and VD2 (6,022 bp), which are encapsulated into separate virions [34].

In managing viral diseases, it is difficult to identify the pathogens causing the diseases and symptoms at early stages of the disease. Modern biotechnological tools like molecular and serological diagnosis for early detection of the virus in at early stages of silkworm growth play a critical role in management of virus.

Viral diseases of silkworm cause a major problem to sericulture industry. Grasserie and viral flacherie are the two major viral diseases. Grasserie is caused by *Bm*NPV and viral flacherie is caused by *Bm*IFV, *Bm*DNV, *Bm*KV or *Bm*DNV-2

[34]. Kenchu is a type of viral flacherie prevalent in Karnataka, India. The characterization of *Bm*DNV-2 revealed that it was a single stranded (ss) DNA virus and its bipartite genome consists of two linear ssDNA segments, which are packed into separate capsids.

Causative agent

Kenchu virus is a small, non-enveloped, linear single stranded DNA virus, which was earlier classified as *Bombyx mori* Densovirus type 2 (*Bm*DNV-2) of the subfamily called Densovirinae which comes under Parvoviridae family. The genome of *Bm*DNA-2 consists of two DNA segments of VD1 (6.5 kb) and VD2 (6 kb). Unlike any other known ssDNA virus, instead of the typical rolling circle replication initiation endonucleases (RCRE), *Bm*DNV-2 encodes a type B DNA polymerase and has been suggested to replicate its genome via the protein-primed mechanism and is the only virus that carries a single stranded DNA that encodes a DNA polymerase. According to these unique features, in 2012, the International Committee of Taxonomy of Viruses (ICTV) voted to agree with the proposal to create a new genus, Bidensovirus, as well as a new family, Bidnaviridae, and designated *Bombyx mori* Densonucleosis-2 (*Bm*DNV-2) as *Bombyx mori* Bidensovirus (*Bm*BDV), the type species of the Bidensovirus genus.

Occurrence and Incidence

This virus was isolated by Hadimani (1980) in Karnataka, India as the causal organism of Kenchu or sappe or flacherie disease with a size of 20-24 nm and it was later characterized as an ssDNA virus, multiplying in the nucleus of columnar cells of the midgut of the silkworm, then expanding to the goblet cells at the late stage, causing flacherie.

Infection and Transmission

Bombyx mori Densonucleosis-2 (*Bm*DNV-2) infect the columnar cells of midgut epithelium and cause the fatal flacherie disease and replicates predominantly in the nuclei of columnar cells of the larval midgut epithelium. Evidence showed that a gene nsd-2 encoding a 12-pass transmembrane protein only expressed in midgut was responsible for the binding of *Bm*DNV-2 to silkworm midgut.

In diseased larva, color of the postero-midgut changed to pale yellow. The main lesions occurred in the nuclei of infected cells. At the late stage of infection, the nucleus of infected cell was 2.5 times larger than that of normal cell and a voluminous dense homogenous structure appeared in each of the infected nuclei. At the late stage of infection, infected columnar cells degenerated and were discharged into gut lumen, and then the mature virions in the degenerate infected cells were egested along with faeces.

The ultra structural changes in cells infected by *Bm*DNV-2 were observed both in the cytoplasm and the nucleus. In the cytoplasm, accompanying a number of free ribosomes, vesicles or cisternae appeared near the granular endoplasmic reticulum, and small spherical particles accumulated inside of these microbody like structure. This step could represent the accumulation and transportation of viral protein to nucleus. Following the multiplication of virus, cellular organelle such as mitochondrion and endoplasmic reticulum became vacuolization and degeneration. Mitochondrion became hypertrophic and lost its cristae.

At the end of infection, the greatly enlarged nuclei were filled with thousands of small spherical virions with 20-24 nm in diameter, replacing completely the whole nuclear materials. The virions entered the cytoplasm through the enlarged nucleopores or by destruction of the nuclear membrane. Then, the complete virions were liberated into the gut lumen along with the degenerated cells.

Symptomatology of Densonucleosis disease

The symptoms of this chronic disease affecting the early instars are such that farmers tend to continue rearing diseased worms for long periods of time without favourable returns as the worms will have to be discarded, this disease causes a loss of 9.50 *per cent* out of a total crop loss of 20-40 *per cent* in India. The recent discovery of resistant gene nsd-2 in some of the *Bombyx mori* races has led to the initiation of screening of resistant parental races for the development of *Bm*DNV-2 resistant transgenic breeds and these breeds were used as a parental breed in breeding programmes for developing *Bm*DNV-2 resistant silkworm races. The symptoms of *Bm*DNV-2 infection look apparently normal just after infection but slightly dull and redness of skin will be observed after two hours. Sometimes thoracic region looks swollen. In later stage of infection, vomiting gut juice and faeces with high moisture content is noticed. Early in stars are more susceptible to the disease and among different levels. Epizootiological and serological studies have found that the virus persist in the soil, gut of chicks and rearing trays.

Disease detection

The early detection of an infection is the most effective solution for control of viral diseases to stop disease spread in rearing houses. Therefore, a technique that permits such detection of diseases at the earliest stage of infection is strongly needed [22]. There are several diagnostic methods invented for detection of some microbial diseases, and their infection in silkworm namely agarose gel double diffusion, a dot-blot hybridization assay, DNA hybridization, enzyme linked immunosorbent assay (ELISA) [59], monoclonal antibody-based sandwich ELISA [54], colloidal textile dye-based dipstick immunoassay [42] and polymerase chain reaction (PCR) based analysis [22].

Management of Viral Diseases of Silkworm

Viral diseases of silkworm are very difficult to manage due to a very short life cycle of silkworm. Several prophylactic measures are recommended to prevent the spread of the disease in the silkworm colony by the application of bed disinfectants such as Resham Keet Oushad (RKO), Vijeta, and Ankush etc. Although the disinfection of silkworm rearing environment and application of bed disinfectant is expected to maintain the pathogen load to the significant tolerable level, it may not be always adequate to prevent the diseases occurrence. Prevention is better than cure is the correct approach that should be adopted in silkworm disease management. Care is also needed to be taken to see that the silkworms are not exposed to stress conditions like temperature, humidity, bad ventilation, feeding arrest, parasitism and nutritional deficiency which may make them easily susceptible to grasserie disease [29]. Disinfection before, during and after each rearing should be carried out effectively and strictly.

For effective prevention of this disease, the silkworm rearing rooms, mulberry storage rooms, mounting rooms, equipments and rearing premises should be thoroughly disinfected before brushing. The eggs should be essentially surface disinfected. Silkworms should be reared under strict hygienic conditions. During rearing the diseased and dead larvae form the major

source of infection with the largest quantity of fresh polyhedra available. Hence, the diseased larvae should be removed carefully without breaking the skin and disposed suitably by putting them in lime vats or by burning. Depending upon the suitable temperature and humidity should be provided. Fresh air circulation should be ensured by providing cross ventilation. The silkworms should be fed with nutritive rich mulberry leaf and during later stages feeding of tender leaf should be avoided. Do not provide over matured/over stored /dirty leaf to the silkworms. Avoid starvation, overcrowding and accumulation of faeces in the rearing bed. Depending upon the stage of larvae, spacing and required quantum of leaf should be given. Proper bed drying each feed to avoid accumulation of moisture in the bed. Apply recommended bed disinfectant as per schedule and quantity. Feed Amruth as per schedule to control flacherie disease.

The use of disease resistant silkworm breeds or the utilization of inherent resistance in silkworm would be the most economical and effective way to prevent the occurrence of viral disease. The Indian silkworm germplasm bank has rich indigenous and exotic breeds and some of them are known for strong resistance to viral pathogens [33]. The productive breeds are comparatively more susceptible to diseases in general and particularly to the BmNPV. However, it is possible to exploit the germplasm to develop *Bm*NPV resistant and productive breeds thereby enhancing overall cocoon productivity.

Table 1: Some useful characteristics of viral diseases of silkworm

Disease	Causative agent	General symptoms	Some Distinct Characteristics		
			Haemolymph	Midgut	Integument
Nuclear polyhedrosis	*Bombyx mori* nuclear polyhedrosis virus (*Bm*NPV)	Swelling on inter-segmental region; shining and fragile skin; milky white fluid; cadaver's grayish black and flaccid.	Turbid and white, occlusion bodies are present.	Not distinct	Shiny just before moulting; fragile; exuding fluids; in late instars, spiracles black and cuticle with black spots.
Cytoplasmic polyhedrosis	*Bombyx mori* cytoplasmic polyhedrosis virus (*Bm*CPV)	Translucent cephalothoraxic region; diarrhoea; retarded growth; milky white midgut; whitish faeces; larvae pale, thin and small; growth slow and asynchronous; faeces remain attached to the anus; cadavers flaccid.	Clear, no occlusion bodies present.	Posterior midgut ivory in colour, shrunken, and striated, occlusion bodies are present.	Not distinct
Infectious flacherie	*Bombyx mori* infectious flacherie virus (*Bm*IFV)	Larvae vomit; head raised; growth and moulting asynchronous; larvae discoloured, flaccid; faeces watery; cadavers have a flattened shape.	Clear, without occlusion bodies.	Contains yellow-brown fluids; pink viral spherical bodies present; stains with pyroxin methyl green.	Not fragile.
Densonucleosis	*Bombyx mori* densonucleosis virus (*Bm*DNV)	Thorax mostly translucent; like flacherie or bacterial flacherie anorexia; late moulting; bodies shrunk; integument not fragile.	Clear, without occlusion bodies.	Contains yellowish green fluids; cocci and diplococcic very abundant but no occlusion bodies.	Not distinct.

Conclusion

The mulberry silkworm, domesticated and mass reared for several centuries, presumably has weakened immune system which has made the insect highly vulnerable to viral infections. Silkworm viral diseases are the major cause for cocoon crop loss. The course of infection by these viruses is largely influenced by environmental, nutritional and rearing managerial practices. Information on the aspects like causative agents, incidence and occurrence, infection and transmission, symptoms and detection, and management, in the silkworm with reference to four major viral infections in particular is scanty. Prevention of viral infections during silkworm rearing helps to increase the silk productivity, because if the diseases are controlled below the economic threshold level then there will be an increase in silk production.

Another most effective solution is timely detection of viral infections in silkworm rearing to stop further spread of the disease. The present information may prove vital in identifying the suitable symptoms in the silkworm larvae during viral infections and to protect the commercial characteristics of cocoon yield in addition to suggest suitable measures in regulating the disease. This will help in the restoration of sericulture output and will safe guard the interest of the farmers involved in sericultural practices.

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