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Bombyx mori pylorus infection by *Alphabaculovirus*

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ABSTRACT. *Alphabaculovirus* is an entomopathogenic virus genus that infects *Bombyx mori*, which is known as the *Bombyx mori* multiple nucleopolyedrovirus (BmMNPV). This virus is polyorganotrophic, and a series of tissues are known as targets; however, there is currently no information regarding infection in the pylorus, the segment of the hindgut that is present in the midgut transition and is responsible for food passage control. Thus, in the present study, we aimed to analyze infection of the *B. mori* pylorus by BmMNPV. To do so, hybrid *B. mori* larvae were inoculated with a viral suspension of BmMNPV, and segments of the intestine containing the pylorus and its subdivisions, the posterior interstitial ring (PIR), pyloric cone, and pyloric valve, were dissected and processed for light microscopy on different days post inoculation. The results showed that *B. mori* pylorus subdivisions respond differently to infection, and the anterior area of the PIR is susceptible with these cells being the secondary infection targets. Cytological analysis revealed the presence of viroplasm in the hypertrophic nucleus, followed by the formation and development of viral polyhedra. Cytolysis occurred at the end of the infectious cycle, thereby releasing polyhedra and enabling the spread of the disease. There was no evidence of BmMNPV infection in the posterior area of the PIR, cone, or pyloric valve. These results will

contribute to greater understanding of the virus infectious cycle, whose consequent epizootic disease can negatively impact this economically important insect that is used in silk production in Brazil.

Key words: Insect; Baculovirus; Lepidoptera; Hindgut

INTRODUCTION

Alphabaculovirus (AlphaBV) is an entomopathogenic virus genus of the Baculoviridae family that can cause disease in beneficial insects, such as *Bombyx mori* (Lepidoptera, Bombycidae) (Dourado et al., 2011). AlphaBV is a nucleopolyhedrovirus (NPV) that is specific to lepidopterans, consisting of double-stranded circular DNA that associates itself with proteins, constituting the nucleocapsid. This is surrounded by a membranous envelope and forms the enveloped nucleocapsid or virion. The several nucleocapsids of AlphaBV are grouped into an occlusion body protein or a polyhedron, which provides protection to the virions (Rohrmann, 2011; Liang et al., 2013; Ikeda et al., 2013).

AlphaBV may contain only one nucleocapsid per envelope, which is referred as a "single nucleopolyhedrovirus" (SNPV) or several, which is referred to as "multiple nucleopolyhedrovirus" (MNPV). According to the literature, MNPVs are more virulent than SNPVs, and there is evidence for geographical viral variability of NPVs to the same species, which may affect their virulence (Adams and McClintock, 1991; Hong et al., 2000; Fan et al., 2007; Liang et al., 2013). However, Hu et al. (1999) and Rohrmann (2011) stated that the genetic basis for understanding the enveloping of one or more nucleocapsids remains unknown. One of the main characteristics of AlphaBVs is their infectious cycle, which presents two phenotypically distinct viral forms: the occlusion-derived virus (ODV) and the budded virus (BV). ODVs are virions that are released from occlusion bodies and act on primary infection sites, being responsible for the horizontal transmission of the disease. BVs never become occluded, and are instead released into the hemolymph, have an envelope that is distinct from that of ODVs, and are responsible for the establishment of systemic infection or secondary infection, that is, the cell-to-cell transmission mechanism (Rohrmann, 2011; Satadal et al., 2012).

The infectious cycle of baculoviruses has been studied in numerous insects (Adams and McClintock, 1991). In *B. mori*, an analysis was performed to evaluate the infection caused by a *B. mori* NPV (BmNPV) geographic isolate in the State of Paraná, Brazil, in which the viral species was identified as *B. mori* MNPV (BmMNPV), based on the fact that its polyhedra contains both single and multiple enveloped nucleocapsids (Pereira et al., 2008).

BmMNPV is polyorganotrophic and various target organs are known including the integument, trachea, fat body, nerve ganglion, and testicles (Brancalhão et al., 2002; Brancalhão and Ribeiro, 2003; Torquato et al., 2006a; Pereira et al., 2008). However, there is currently very little information about the susceptibility and infection of the digestive tract, especially the pylorus. The pylorus is a segment of the hindgut with great morphological complexity. It is subdivided into the posterior interstitial ring (PIR), pyloric cone, and pyloric valve (Byers and Bond, 1971; Reinecke et al., 1973), and is responsible for controlling the food passage from the midgut to the hindgut, thereby assisting in the finalization of the digestive process (Judy and Gilbert, 1969; MacGown and Sikorowski, 1982; Snodgrass and Eickwort, 1993; Landim, 2009). The morphology of the pylorus reveals a simple epithelium lining that is covered on the

luminal side by a chitinous intima, which has specializations in some areas, the spicules (Judy and Gilbert, 1969; Byers and Bond, 1971; Reinecke et al., 1973; Levy et al., 2004). Underlying the epithelium is the muscle layer, which is formed by bundles of striated fibers that function in peristalsis (Judy and Gilbert, 1969; Byers and Bond, 1971; Reinecke et al., 1973; Levy et al., 2004, 2008).

In this sense, the present study aimed to analyze the susceptibility and infection of the *B. mori* pylorus against the isolated geographic BmMNPV strain. The knowledge gained from this study will contribute to a greater understanding of the infectious cycle of this important entomopathogenic virus, which is responsible for nuclear polyhedrosis disease, popularly known as grasserie or milky diseases. This is a serious worldwide problem for sericulture, for once the larvae are sick, they must be eliminated to prevent the spread and propagation of the disease in the environment (Sengupta et al., 1990; Qin et al., 2012; Satadal et al., 2012; Liang et al., 2013).

MATERIAL AND METHODS

Insects

The 5th-instar hybrid *B. mori* larvae that were used in the following experiments were obtained from silk spinning companies that produce silkworms for commercial purposes in the State of Paraná, Brazil. Larvae were bred in polyethylene boxes that were kept in a breeding room, and were fed with fresh mulberry leaves (*Morus* sp) twice a day.

Virus and inoculation

The BmMNPV (GenBank accession No. EU251694.1) was previously obtained from infected larvae (Brancalhão et al., 2002) and stored at -4°C. The viral suspension was quantified in a Neubauer chamber at a concentration of 2.4 x 10^7 polyhedral occlusion bodies/mL.

The inoculation was performed in 25 silkworms after ecdysis from the 4th to the 5th instar. The silkworms were fasted for 24 h and were then fed with mulberry leaf disks 2 cm^3 in diameter, which were previously dipped in a 10-µL viral suspension. An equal number of larvae were fed with leaf discs containing only water, constituting the control group.

During feeding, the silkworms remained individually confined until feeding on the entire leaf disc, thus ensuring ingestion of viral suspension in the case of the inoculated group. At the end of feeding, larvae were transferred to the boxes where they remained until the end of the experiment, and received mulberry leaves without BmMNPV daily. The symptoms manifested by the larvae were monitored daily (Ribeiro et al., 2009), which were used as an additional parameter for confirmation of BmMNPV infection.

Pylorus microscopy

For microscopic analysis, both the control and inoculated silkworms were anesthetized with ether, dissected, and the intestinal segment containing the pylorus was removed and fixed in Dubosq Brazil (Beçak and Paulete, 1976) for 24 h at 4°C. This procedure was performed from the 2nd to the 9th day post-inoculation (dpi) at 24-h intervals.

After fixation, the intestinal segment was embedded in paraffin following routine histological techniques (Brancalhão et al., 2002). The cuts were made on a microtome Olympus

CUT4055, in thicknesses of 5 and 7 µm, and the slides were stained for viral occlusion bodies with a modified Azan technique (Hamm, 1966) for cytopathology. Control slides were subjected to the same preparations used for the inoculated material.

RESULTS AND DISCUSSION

The pylorus subdivisions of *B. mori* larvae showed differences in susceptibility to the geographic BmMNPV isolate. In particular, the anterior area of the PIR was susceptible to the virus, and the first signs of infection were observed from the 5th dpi (Figure 1A and B). Ribeiro et al. (2009) also found BmMNPV susceptibility in the cardia, another transition region of the intestine and of the same embryonic origin as the PIR. The other subdivisions, i.e., the posterior area of the PIR, pyloric cone, and pyloric valve, did not show any evidence of infection with BmMNPV at any of the time periods analyzed (Figure 1C, D, and E).

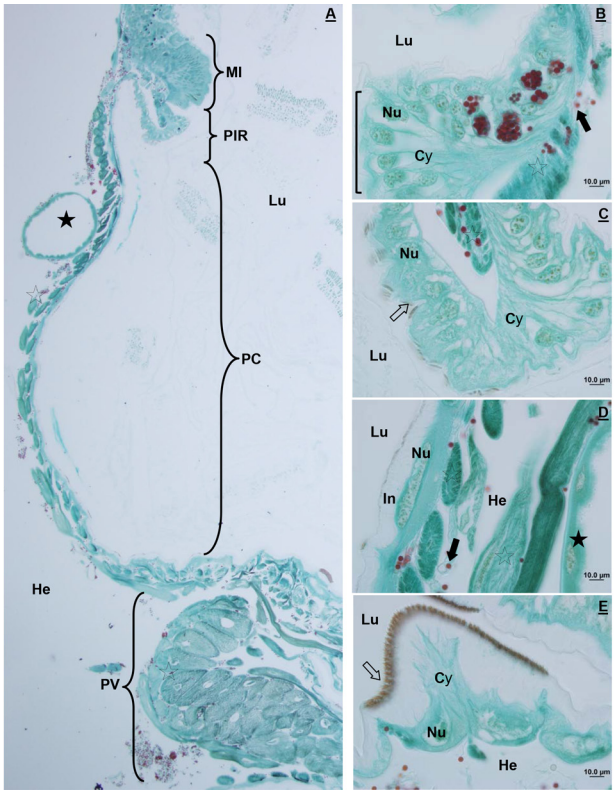

Figure 1. Photomicrographs of the pylorus of *Bombyx mori* larvae, 5th instar, infected by BmMNPV, 7th dpi, longitudinal cutting, modified Azan staining. **A.** Overview of the pylorus with its subdivisions, posterior interstitial ring (PIR), pyloric cone (PC) and the pyloric valve (PV). Part of the midgut (MI), Malpighian tubules (black star), hemocoel (He), muscles (hollow star), and intestinal lumen (Lu). **B.** Anterior area of PIR with mature polyhedra (in red) in the cell nucleus and in the extracellular environment (black arrow), part of the posterior area of PIR (bracket), no polyhedra in the nucleus (Nu), cytoplasm (Cy). **C.** Posterior area of PIR, intima spicules (hollow arrow). **D.** PC showing epithelial cell nucleus, smooth intimate (In). Polyhedra in the extracellular environment (black arrow). **E.** Anterior area of the PV showing Cy and Nu.

The anterior area of the PIR was found to be a secondary target of infection caused by the viral phenotype BV. This occurs because the cover provided by the intima, which is present on the luminal surface of the epithelium, represents a barrier to primary infection caused by ODVs (Ribeiro et al., 2009; Satadal et al., 2012; Liang et al., 2013). Moreover, the very action of digestive juice proteins, such as serine protease and fluorescent red, and the alkaline pH present in the midgut are responsible for the ODV release from the polyhedron, but can also degrade it, and they become inactivated if remaining in the lumen too long (Rohrmann, 2011; Sunagar et al., 2011; Qin et al., 2012; Liang et al., 2013). Thus, ODVs with infective potential do not reach the pylorus. Nonetheless, due to the time of infection in the anterior PIR at the 5th dpi, being greater than that of the surrounding tissues such as the fat body (4th dpi), reinforces infection by BVs from the hemolymph and tracheal systems, the organs responsible for the dispersion of infection in the insect's body (Brancalhão et al., 2009; Rohrmann, 2011).

Cytopathological analysis revealed infection in the susceptible epithelial cell nucleus, enabling the analysis of various stages of the infectious cycle. Initially, the infection induces the formation of the virogenic stroma or the viroplasm (Figure 2A), where enveloped nucleocapsids or virions are produced. These are grouped concentrically around a protein matrix, consisting of polyhedrin, which ultimately forms the viral polyhedron. At this stage of infection, the nucleus appeared hypertrophic (Figure 2A and B), which was also observed in previous studies (Brancalhão et al., 2009; Ribeiro et al., 2009; Rohrmann, 2011). The control materials are shown in Figure 2C for comparison.

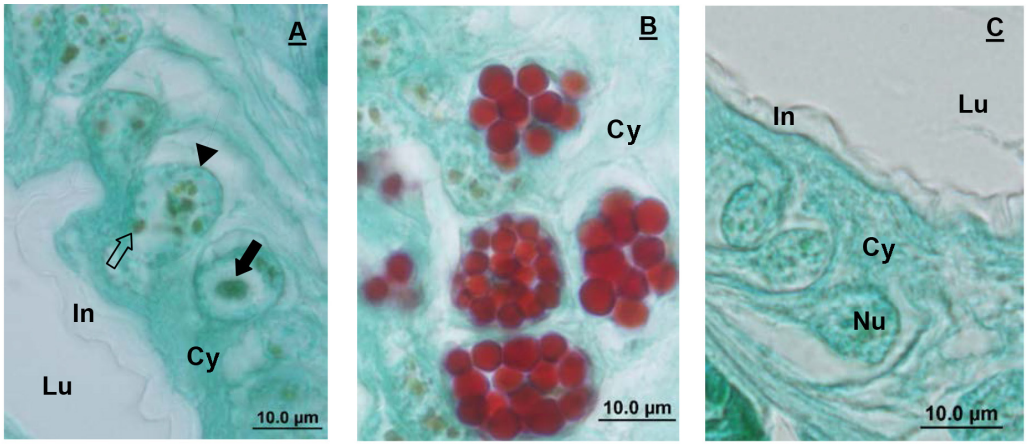

Figure 2. Photomicrographs of the anterior area of the posterior interstitial ring of *Bombyx mori* larvae, 5th instar, longitudinal cutting, modified Azan staining. **A. B.** Inoculated material at 5th and 7th dpi, respectively, and **C.** control material, for comparison. **A.** Viroplasms (black arrow) in the hypertrophied nuclei and immature polyhedra (hollow arrow). Nuclear envelope (arrowhead), cytoplasm (Cy) and intima (In) facing the lumen (Lu). **B.** Mature polyhedra (in red) in the nucleus and in the extracellular environment. **C.** Epithelial cell nucleus (Nu) and Cy.

The polyhedron develops between the viroplasm and the nuclear envelope. Initially, it is small, without a defined geometric shape, and only identifiable by its green coloration (Figure 2A). Subsequently, it becomes larger and takes on a characteristic geometric shape, being present in the nucleus and in the extracellular environment, as displayed by red color-

ation (Figures 1B and 2B) resulting from chemical interactions with the dye (Hamm, 1966). Brancalhão et al. (2009) classified these two states as immature and mature, respectively, where the immature forms are not enveloped and the mature forms contain the envelope. The geometrical shape of BmMNPVs was described by Torquato et al. (2006b) as a truncated octahedron. However, they may also present in other geometric forms, such as cuboids, hexahedral, dodecahedral, triangular, or tetrahedral, which may be the result of genetic variations specific to each viral strain, or even due to polyhedrin mutations (Katsuma et al., 1999; Hong et al., 2000; Ribeiro et al., 2009; Brancalhão et al., 2009; Liang et al., 2013).

In the nucleus of infected cells, variations in the amount and size of polyhedrons were observed (Figure 2B). According to the literature, such variations are common, and may be related to the number of occluded nucleocapsids, the developmental stage of the polyhedron, infected cell metabolism, genetic variations of the virus, and the positions occupied by polyhedra in the infected nucleus, which should be considered as they cause different angulations in histological sections (Brancalhão et al., 2009).

Cytolysis occurred at the end of the infectious cycle (Figure 2B) due to the action of a multifunctional viral p10 protein, which is involved in nuclear membrane disintegration and cell lysis, providing the release of mature polyhedra to the extracellular environment and the hemocoel, as well as the intestinal lumen (Hong et al., 2000; Rohrmann, 2011; Liang et al., 2013). This cell lysis behavior is characteristic of BmMNPV infections, having been observed in adipose tissues (Brancalhão et al., 2002), the integument (Brancalhão and Ribeiro, 2003), the male reproductive system (Pereira et al., 2008), in the silk gland (Brancalhão et al., 2009), and in the cardia (Ribeiro et al., 2009). At this stage, symptom analysis showed leakage of the hemolymph due to frequent integument injuries that were caused by the action of chitinase and cathepsin enzymes encoded by the viral genome (Brancalhão and Ribeiro, 2003; Brancalhão et al., 2009). Thus, hemolymph containing viral polyhedra reaches the environment.

Viral polyhedra are resilient structures that may persist in the environment over several seasons, where they can adhere to mulberry leaves, the main food source of *B. mori*. When taken to silkworm rearing rooms, these leaves can infect healthy silkworms, thus restarting the infectious cycle in the new host (Brancalhão et al., 2002; Liang et al., 2013). Thus, it is essential that prophylactic measures for control be adopted as soon as the presence of this virus is noticed, such as the removal of diseased silkworms, as they are a potential focus of the pathogen (Potrich et al., 2007; Brancalhão et al., 2009).

The non-infection of the posterior area of the PIR, pyloric cone, and pyloric valve may be related to defense mechanisms, whose genetic bases are not well known at present (Ponnuvel et al., 2003; Qin et al., 2012; Feng et al., 2013). Adams and McClintock (1991) suggested that the absence of infection of certain cell types might be caused by the differential expression of viral genes that are inserted into the genome of the host cell once the BmMNPV presents asynchronous replication. In a study of insect cell culture, Rohrmann (2011) showed that beta-N-acetylglucosaminidase 2 played a role in resistance, altering the binding of glycans to the fusion protein of the viral envelope, GP64, thus reducing the infective capacity of the BV.

In this sense, infection of the anterior area of the PIR is a novel addition to the other known BmMNPV targets, and will affect the functioning of the hindgut, particularly with regards to food passage into the ileum and the formation of fecal pellets. Therefore, the disease affects the metabolism of the insect, compromising the production of quality cocoons, with consequent losses to producers and to the sericultural industry.

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