SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

Nucleopolyhedrovirus: Scanning Electron Microscopy Technique

Ednéia F.B. Torquato[1] Marcílio H. de Miranda Neto[2] Rose M.C. Brancalhão[1] and Valdeni S. Franco[3]

[1]*Lab. Biologia Celular, Univ. Estadual do Oeste do Paraná, Campus de Cascavel, Rua Universitária, 2069, Jardim Universitário, 85814-110, Cascavel, PR*
[2]*Lab. Anatomia Humana;* [3]*Depto. Matemática. Univ. Estadual de Maringá, Avenida Colombo, 5790, Jardim Universitário, 87020-900, Maringá, PR*

Neotropical Entomology 35(6):787-790 (2006)

Nucleopolyhedrovirus: Técnica de Microscopia Eletrônica de Varredura

RESUMO - Um método simples foi desenvolvido para se estudar a forma geométrica do Bombyx mori *Nucleopolyhedrovirus* múltiplo, BmMNPV, para microscopia eletrônica de varredura. O vírus, pertencente à Família *Baculoviridae*, foi obtido do filtrado, do inóculo e da hemolinfa de lagartas de *Bombyx mori* (L.) (Lepidoptera: Bombycidae) infectadas. O material foi colocado diretamente sobre o suporte de varredura, onde previamente foi aderida uma fita de cobre. Após secagem e metalização com carbono e ouro, o vírus foi analisado ao microscópio de varredura. Os poliedros apresentaram-se com as faces lisas, perfeitamente definidas, possibilitando a análise matemática que o identificou como um octaedro truncado. A forma do poliedro pode apresentar valor taxonômico, uma vez que ela é específica para cada linhagem viral.

PALAVRAS-CHAVE: Baculovírus, *Bombyx mori*, BmMNPV, poliedro, octaedro truncado

ABSTRACT - A simplified methodology was developed to study the geometric form of multiple Bombyx mori *Nucleopolyhedrovirus* by scanning electron microscopy. The virus belongs to *Baculoviridae* family and was isolated from the silkworm *Bombyx mori* (L.) (Lepidoptera: Bombycidae). The polyhedra of *Nucleopolyhedrovirus* were obtained from the filtrate, inoculum and hemolymph of the silkworm experimentally infected with nuclear polyhedra. This material was placed on stubs, where a copper tape was previously adhered. After dry at room temperature the virus was covered with carbon and gold. Scanning electron microscopy analysis reveled a well defined morphology for the polyhedra of multiple Bombyx mori *Nucleopolyhedrovirus*, making possible the mathematical study that identified it as a truncated octahedron. The form of the polyhedron can present taxonomic value, once it is specific for each viral lineage.

KEY WORDS: Baculovirus, *Bombyx mori*, BmMNPV, polyhedra, truncated octahedron

Nucleopolyhedrovirus (NPV) is an important entomopathogenic virus belonging to *Baculoviridae* family. It is made up of double stranded DNA molecule, associated with proteins of the capsid, forming the nucleocapsid. Two different subgroups are recognized for NPV: the occlusion derived virions and the budded virus. These virion subgroups have different functions in the infection cycle. The occluded subgroups could be divided into two subgroups: those containing one nucleocapsid per virion are referred to as single-capsid NPVs (SNPVs), whereas those containing multiple nucleocapsids per virion are known as multiple NPVs (MNPVs) (Murphy et al. 1995, Blissard 1996).

Nucleocapsid and envelope constitutes the virion or infectious particle. In NPV several virions are contained in a protein structure, named occlusion body or polyhedron (Adams & McClintock 1991, Murphy *et al.* 1995, Blissard 1996).

Polyhedra are stable structures that can persist for long time in the environment, where they are commonly found on the surface of the plants or in the soil. In the soil they are resistant to deterioration and sufficiently stable to persist from a season to another (David 1975). This way, the occlusion of the virus is to protect the virions of the adverse conditions of the environment (Blissard & Rohrmann 1990, Adams & Mcclintock 1991, Blissard 1996). This characteristic, allied to the fact of NPV could infect several orders of insects, particularly Lepidoptera (Murphy *et al*. 1995), make these organisms particularly efficient to be used in biological control programs, aiding in the elimination of insects that affect agriculture, such as the caterpillar of the soybean *Anticarsia gemmatalis* (Hübner) and the caterpillar of the corn *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae). However, the NPV also infect useful insects, such as the silkworm, *Bombyx*

mori (L.) (Lepidoptera: Bombycidae), used in the production of silk threads and cloth (Brancalhão 2002).

In this paper, we report the development of a scanning electron microscopy technique to analyze the structure of the *B. mori* viral polyhedra, BmMNPV. This virus was isolated from *B. mori* silkworm in Parana State, Brazil (Brancalhão 2002, Brancalhão & Ribeiro 2003). The polyhedra with the preserved structure were submitted to mathematical analysis to define their geometric form.

Material and Methods

Viral suspension of BmMNPV was obtained from previously infected silkworm, *B. mori*. The virus was identified in studies of light and transmission electron microscopy, and symptomatologic analyses (Brancalhão 2002). The experiments were conducted in the cell biology laboratory from State University of Western Parana and electron microscope laboratory of São Paulo University, Brazil.

The viral suspension was prepared from 5th instar silkworms died by BmMNPV and frozen. Infected silkworms were homogenized in 50 ml 0.001M tris-HCl buffer at pH 7.5 or 50 ml of distilled water (where the same results were obtained). The homogenate was filtered in hydrophilic cotton and was centrifuged three times at 2,000 rpm in a room temperature micro centrifuge, where the supernatant was discarded and the precipitate resuspended in distilled water for each centrifugation. The resulting pellet containing the polyhedra, identified by the whitish coloration, was resuspended in distilled water, put in sterile flasks and stored at -10°C until used. The material resulting from this semi-purification process was denominated inoculum. We also obtained inoculum using Granados & Lawler (1981) technique, however the amount of virus obtained was small and, for this inoculum we used the above technique, with satisfactory results. A similar method to this is mentioned by Smith (1967).

The BmMNPV inoculum was processed for scanning electron microscopy (SEM) by three different techniques:

Technique 1. BmMNPV inoculum was fixed in 5% glutaraldehyde (aqueous solution) + 0.2% tannic acid for 2h, postfixed in 1% osmium tetroxide (aqueous solution) for 1h, dehydrated in a standard aqueous acetone series (50%, 70%, 80%, 90% and twice in 100%) about five seconds each bath. Samples of that inoculum were then seeded onto stubs, previously covered with copper tape, and were allowed to air dry. Then stubs were coated with carbon and gold and particles were viewed on SEM.

Technique 2. BmMNPV inoculum was fixed in 5% glutaraldehyde (aqueous solution) + 0.2% tannic acid for 2h, dehydrated in a standard aqueous acetone series (50%, 70%, 80%, 90% and twice in 100%) about five seconds each bath. Samples of that inoculum were then seeded onto stubs, previously covered with copper tape, and were allowed to air dry. Then, stubs were coated with carbon and gold, and particles were viewed on SEM.

Technique 3. BmMNPV inoculum was seeded directly onto stubs, previously covered with copper tape, and were allowed to air dry. Then, stubs were coated with carbon and gold, and particles were viewed on SEM.

BmMNPV polyhedra were also obtained directly from hemolymph of infected caterpillars. In this case, drops of hemolymph were collected directly from the silkworm legs, with needle puncture and then processed for SEM. Also the viral filtrate could be used; however, in these cases, a great amount of cellular and tissue fragments remain.

The geometric form of the polyhedra was defined by using Pedagoguery Software (1988).

Results and Discussion

The BmMNPV viewed with the SEM using techniques one and two revealed a little clear polyhedra with granulations in all surfaces. However, a great number of polyhedra were damaged with cracks in their surface (Fig. 1 A). This damage did not happen with the application of the third technique, coincidentally the simplest (Fig. 1 B). In this case, the BmMNPV polyhedra were clear, with the faces smooth and perfectly defined. These characteristics made possible his morphologic identification, of a truncated octahedron (Fig. 2). In the geometric analysis the truncated octahedron is a solid of Archimedean with 24 vertices and 36 edges forming six square faces and eight hexagonal ones (Pedagoguery Software 1988).

Techniques for viral polyhedra processing for MEV are described in several publications (Adams and Wilcox 1982, Zhou *et al.* 1998, Bravo-Patiño & Ibarra 2000), where usually the material is fixed in glutaraldehyde and postfixed in osmium tetroxide, in several concentrations. However, the images obtained by these authors do not differ from that obtained with the use of the technique three, described in this work, where the faces of the polyhedron appeared smooth, facilitating the morphologic descriptions. Similar methodology is described by Cheng *et al.* (1998), Cheng & Carner (2000) and Cheng *et al.* (2005), used to determine the morphology of SNPV and MNPV of *Thysanoplusia orichalcea* (L.) (Lepidoptera: Noctuidae).

The polyhedral structure of different NPV of Lepidoptera is formed basically by the same constituents: virions, protein matrix of poliedrin, and viral envelope (Tanada & Kaya 1993, Blissard 1996, Brancalhão 2002). So, the technique 3 applied for BmMNPV polyhedron of *B. mori*, besides being simpler and fast, makes possible a morphologic identification reliable, and can be applied for other NPVs, needing new tests to verify his applicability. Although we have observed the form of an octahedron truncated in our isolated geographical viral, Sengupta *et al.* (1990) mentioned tetrahedral, hexahedral forms and also octahedral without specification, for polyhedra of *B. mori.* The meaning of the variations in the form of the polyhedra of NPVs is little discussed (Adams & McClintock 1991, Bilimoria 1991). However, according to Cunningham (1970) Apud Watanabe *et al*. (1975), a genetic variation seems to exist in the form of the body of occlusion of *B. mori* NPV, specific for each viral lineage.

The polyhedra obtained from the filtrate and the haemolymph of the infected caterpillars, and processed by the same technique, also showed much defined images.

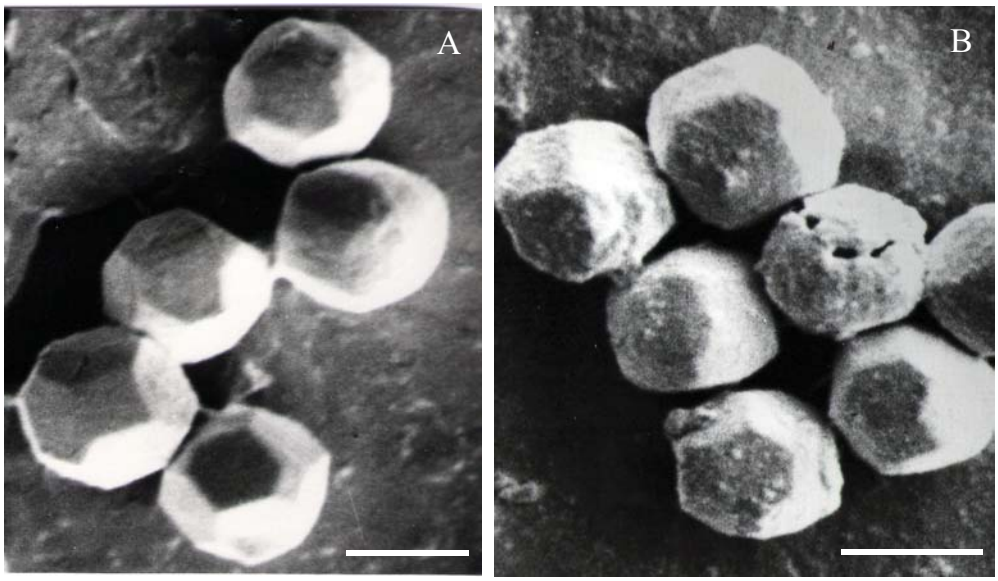

Fig. 1. Scanning electromicrograph of viral polyhedra of *B. mori*, BmMNPV. In A, technique 3 presenting the clear polyhedra. In B, technique 2, polyhedra with granulations and cracks in the surface; scale 1 μm.

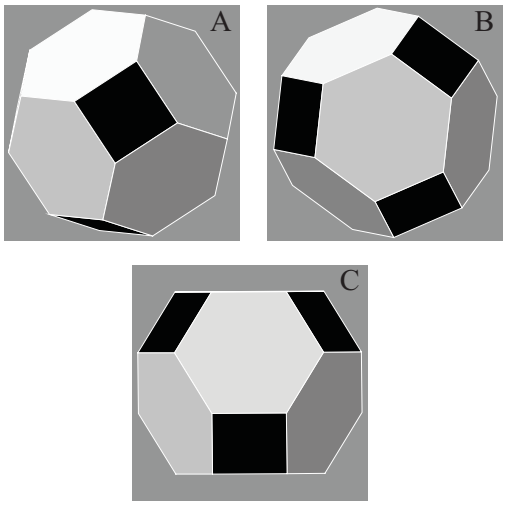

Fig. 2. Three-dimensional positioning of the geometric forms of truncated octahedron (A, B and C) obtained starting from scanning electromicrograph of viral polyhedron of BmMNPV of B. *mori*.

Acknowledgments

I thank the Núcleo de Apoio à Pesquisa em Microscopia Eletrônica Aplicada à Pesquisa Agropecuária (NAP/MEPA), Escola Superior de Agricultura "Luiz de Queiroz"/ Universidade de São Paulo (ESALQ/USP) and Centro de Microscopia Eletrônica da Universidade Federal do Paraná for providing equipment and facilities during virus preparation and silk companies in the State of Paraná (BRATAC, COCAMAR e KANEBO), for providing the *B. mori* caterpillars.

References

Adams, J.R. & J.T. McClintock. 1991. Baculoviridae. Nuclear polyhedrosis viruses. Part 1. Nuclear polyhedrosis viruses of insects, p.89-180. In J.R Adams & J.R. Bonami (eds.), Atlas of invertebrate viruses. Florida, CRC Press, 694p.

Adams, J.R. & T.A. Wilcox. 1982. Scanning electron microscopical comparisons of insect virus occlusion bodies prepared by several techniques. J. Invertebr. Pathol. 40:12-20.

Bilimoria, S.L. 1991. The biology of nuclear polyhedrosis viruses, p.1-72. In E. Kurstak, Viruses of invertebrates. New York, Marcel Dekker, 360p.

Blissard G.W. 1996. Baculovirus-insect cell interactions. Cytotechnology 20: 73-93.

Blissard, G.W. & G.F. Rohrmann. 1990. Baculovirus diversity and molecular biology. Annu. Rev. Entomol. 35:127-155.

Brancalhão, R.M.C. 2002. Vírus entomopatogênico no bicho-da-seda: Taxonomia e citopatologia causada por *Nucleopolyhedrovirus* em células de *Bombyx mori*. Biotecnologia 24: 54-58.

Brancalhão, R.M.C. & L.F.C. Ribeiro. 2003. Citopatologia da infecção causada por BmNPV no tegumento de *Bombyx mori* L., 1758 (Lepidoptera:Bombycidae). Arq. Ciên. Vet. Zool. 6: 15-20.

Bravo-Patiño, A. & J.E. Ibarra. 2000. Site-directed mutagenesis of Autographa californica nucleopolyhedrovirus (AcNPV) polyhedron: Effect on polyhedron structure. Arc. Virolog. 145: 827-834.

Cheng, X.-W. & G.R. Carner. 2000. Characterization of single-nucleocapsid nucleopolyhedrovirus of *Thysanoplusia orichalcea* L. (Lepidoptera: Noctuidae) from Indonesia. J. Inverteb. Pathol. 75: 279-287.

Cheng, X.-W., G.R. Carner & H.W. Fescemyer. 1998. Polyedrin sequence determines the tetrahedral shape of occlusion bodies in *Thysanoplusia orichalcea* single-nucleocapsid nucleopolyhedrovirus. J. Gener. Virolog. 79: 2549-2556.

Cheng, X.-W., G.R. Carner, M. Lange, J.A. Jehle & B.M. Arif. 2005. Biological and molecular characterization of multicapsid nucleopolyhedrovirus from *Thysanoplusia orichalcea* L. (Lepidoptera: Noctuidae). J. Inverteb Pathol. 88: 126-135.

Watanabe, H., Y. Aratake & T. Kayamura. 1975. Serial passage of a nuclear polyhedrosis virus of the silkworm, *Bombyx mori*, in larvae of rice stem borer, *Chilo suppressalis*. J. Invertebr. Pathol. 25: 11-17.

David, W.A.L. 1975. The status of viruses pathogenic for insects and mites. Annu. Rev. Entomol. 20: 97-117.

Granados, R.R. & K.A. Lawler. 1981. *In vivo* pathway of *Autographa californica* baculovirus invasion and infection. Virology 108: 297-308.

Murphy, F.A., C.M. Fauquet, D.H.L. Bishop, S.A. Ghabrial, A.W. Jarvis, G.P. Martelli, M.A. Mayo & M.D. Summers. 1995. Virus taxonomy: Classification and nomenclature of viruses. Sixth Report of the International Committee on Taxonomy of Viruses. New York, Springer-Verlag, Wien, 586 p.

Pedagoguery Software. 1988. Solids Archimedean in version 1.11, 2005. Available in the site http://www.peda/poly.

Sengupta, K., P. Kumar, M. Baig & M. Govindaiah. 1990. Handbook on pest and disease control of mulberry and silkworm. Bangkok, UNESCAP - United Nations Economic and Social Commission for Asia and the Pacific, 88p.

Smith, K.M. 1967. Insect virology. New York, Academic Press, 256p.

Tanada, Y. & H.K. Kaya. 1993. Insect pathology. DNA-viral infections: Baculoviridae. p.171-244. Academic Press. San Diego, 666p.

Zhou, C.E., R. Ko & S. Maeda. 1998. Polyhedron-like inclusion body formation by a mutant nucleopolyhedrovirus expressing the granulin gene from a granulovirus. Virology 240: 282-294.

Received 21/IX/05. Accepted 29/VI/06.