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RESEARCH ARTICLE

FIRST RECORD OF A *BOMBYX MORI NUCLEOPOLYHEDROVIRUS* (BmNPV) ISOLATE FROM CUBA

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ABSTRACT

Grasserie disease of silkworm *Bombyx mori* Linnaeus (Lepidoptera: Bombycidae) caused by *Bombyx mori nucleopolyhedrovirus* (BmNPV) is one of the most serious viral disease in tropical countries and occurs throughout the year, causing considerable damage to the silkworm cocoon breeding. This report highlights the results obtained in the detection of the BmNPV for the first time in Cuba as the pathogen associated to the symptoms observed in silkworm rearing. In this context, there is an intense need to search for alternative remedial measures for control and potential restoration of ill insect breeding colonies in order to decrease economical losses in sericulture.

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INTRODUCTION

The Mulberry silkworm (*Bombyx mori*) is affected by a number of diseases caused by viruses, bacteria, fungi and microsporidia (Jiang and Xia, 2014, Ramesh-Babu *et al*, 2009). These diseases are known to occur in almost all the silkworm rearing areas of the world causing considerable damage to the silkworm cocoon breeding. The *Bombyx mori* nucleopolyhedrovirus (BmNPV) is the most harmful virus in the sericulture industry, often causing severe economic losses (Khurad *et al*, 2004, Ponnuvel *et al.,* 2003, Ramesh Babu *et al*, 2009). Baculoviruses are viruses/pathogens that produce typically fatal infections in insects and other arthropods. They are members of the *Baculoviridae* family constituted by a large double-stranded covalently closed circular DNA genome (Herniou *et al*, 2011). More than 700 baculoviruses have been isolated from invertebrates and reported in the literature.

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These viruses have been widely used in agricultural and forest pest control (Moscardi, 1999; Moscardi *et al*, 2011). Baculoviruses have also proven to be extremely valuable tools in Biotechnology. The baculovirus-insect cell expression system has become one of the most widely used methods of recombinant proteins routine production including vaccines. Expression of foreign genes on the silkworm larvae based on BmNPV vector has also been exploited (Maeda, 1989, Kost *et al*, 2005, Motohashi*et al*, 2005). The *Baculoviridae* family was previously divided in two genus: *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV) based in occlusion bodies (OB) morphology and its nucleocapsids envelopment (Blissard *et al*. 2000)). Recently, a new classification was presented to *Baculoviridae* family based manly in specificity of the virus to insect host. The family was divided in four genus: *Alphabaculovirus Betabaculovirus*,, *Gammabaculovirus* and *Deltabaculovirus* (Herniou *et al*, 2011). *Bombyx mori nucleopolyhedrovirus* (BmNPV) belongs to the *Alphabaculovirus* genus. The aim of this study was to identify the pathogen associated with the symptoms of grasserie

observed in the production areas of the Mulberry silkworm in Cuba. At first, a survey in a rearing colony of *Bombyx mori* Linnaeus (Lepidoptera: Bombycidae) was made. Larvae were then collected at different periods with the following symptoms: exudation, swollen segments, necrotic tissues, delayed growth, gut juices vomit, excretion of semi-solid feces and death. In order to detect and to confirm the presence of baculovirus as the infectious agent causing the disease, molecular techniques such PCR and viral DNA transfection, in addition to electron microscopy and *in vivo* assays were carried out.

MATERIAL AND METHODS

Polyhedra purification

Viral particles were partially purified according to Maruniak (1986) modified protocol. Infected caterpillars were macerated in homogenization buffer (1% ascorbic acid; 2% SDS; 0,01M Tris, pH 7.8; 0,001M EDTA, PH 8.0), filtered through cheesecloth and centrifuged at 10,000xg for 15 min. at 4°C. The pellet was suspended in 10 ml of TE buffer (0,01M Tris, pH 8.0 and 0,001M EDTA, pH 8.0), submitted to another centrifugation at 12,000xg and resuspended in TE buffer. Polyhedra were visualized and counted under an Olympus CK2 Microscope.

Viral DNA extraction

Initially, in order to solubilize the polyhedrin matrix and release virions, 1.5 ml of polyhedra ($5x10^7$ PIBs/ml) were dissolved by addition of 100 ul of sodium carbonate solution (Na_2CO_3 1M) and incubation at 37ºC, for 30 min. (O'Reilly *et al*, 1992). Following, to free the DNA, 50 ul of SDS 20% and 50 ul of proteinase K was added to the sample. Viral DNA purification was then carried out by extraction cycles of phenol; phenol:chlorophorm: isoamyl alcohol and chlorophorm:isoamyl alcohol, according to Sambrook *et al* (1989). The DNA was precipitated with absolute ethanol and sodium acetate, pelleted and washed with 70% ethanol. After air drying, the DNA was suspended in TE buffer and kept at 4°C.

Primers and PCR reactions

Viral DNA was used as template for amplification by Polymerase Chain Reaction (PCR) technique according Szewczyk *et al,* 2008. PCR analysis was based on the polyhedrin gene (*polh*), using universal primers designed from a variety of nucleopolyhedrovirus (NPV). The DNA sequences for this set of primers were as follows:

Forward (polhefor): 5'
CGTGTACGACAACAAGTACTACA3'

Reverse (polherev):
5'AAAGTGAGTTTTTGGTTTTTGCC3'

The reaction was carried out using the following program:

- 94°C for 5min., 49°C for 1min., 72°C for 1 min. – 3 cycles
- 94°C for 1min., 49°C for 30 s, 72°C for 1 min. – 30 cycles
- 94°C for 1min., 48°C for 30 s, 72°C for 5 min. – 1 cycle

PCR products were electrophoresed in 0.8% agarose gel using 1x TAE buffer (40 mM Tris, 20 mM glacial acetic acid, 1 mM EDTA) with ethidium bromide (Sambrook *et al*, 1989). The expected size of the amplified product was about 170 base pairs.

Transfection

The BM-5 cell line was transfected with the DNA from the isolates using Cellfectin II reagent (Invitrogen). Initially, cells were seeded at a density of $8x10^5$ per $60mm^2$ with supplemented TNMFH Medium (10% fetal bovine serum). The Cellfectin II reagent (8ul) and BmNPV DNA (1ug) were individually diluted with 100ul unsupplemented TNMFH Medium and then combined. After the incubation for 30 minutes at room temperature, the mixture was added to the cells. The plates were incubated at 27°C for five hours and the transfection mixture was then replaced with 2 mL TNMFH complete medium. BM-5 mock infected cells were used as Control. Infected cells were observed for hypertrophy, polyhedra formation and other cytopathic effects. They were photographed by phase contrast microscopy on a Nikon Eclipse TS100 Inverted Microscope.

Transmission Electron Microscopy

Standard procedures in electron microscopy were carried out according to Bozzola and Russell (1992). Polyhedra were immersed in a fixating solution (0.1M sodium cacodylate and 2.5% glutaraldehyde) and kept at 4°C for 24 hours. Then samples were washed with 0.05M sodium cacodylate, and treated with 2.0% osmium tetroxide. After complete dehydration in a degree 10% 10 100% ethanol, samples were embedded in Spurr resin. Finally, ultrathin sections (60 um) were contrasted with 2% uranyl acetate and photographed at Jeol 1011 transmission electronic microscope.

RESULTS

Initially the larval crude extract was observed for the presence of polyhedral occlusion bodies under optical microscopy. Once viral particles was confirmed in the sample, polyhedra were purified and viral DNA extracted and used as amplification template for Polymerase Chain Reaction (PCR).The PCR analysis was based on the polyhedrin gene (*polh*), using universal primers designed from a variety of nucleopolyhedrovirus (Szewczyk *et al,* 2008). As expected, a fragment around 170bp was amplified (Fig 1) strongly indicating the presence of a baculovirus. The viral DNA was then transfected in cell culture to investigate the morphological alterations typical of baculovirus infection. The *in vitro* assay was done in *Bombyx mori* BM-5 cells (Grace, 1967) based on transfection of viral DNA following the Cellfectin Reagent (Invitrogen) instructions. The induced cytopathic effects were monitored daily by light microscopy and showed infected cells

presenting successful viral replication, with nuclei hypertrophy and abundant polyhedra formation (Fig. 2).

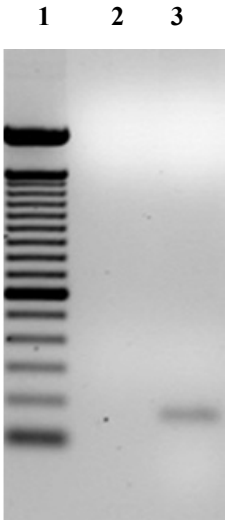

Fig. 1. Analysis of the PCR product by 0.8% agarose gel electrophoresis: 1) Thermo Fisher 50 bp DNA Ladder molecular weight marker; 2) PCR negative control (without template); 3) PCR fragment obtained by amplification using internal primers for the *polh* gene

Ultrastructural analysis of the viral particles was carried out by visualization on Transmission Electron Microscopy (Fig 3). The purified polyhedra sample presented the traditionally described occlusion bodies features with several single-occluded virions immersed in the crystalline matrix.

Virions with more than one nucleocapsid per envelope were seldom observed. The relative diameters of polyhedra were determined by measurement of the diameter of polyhedra cross sections in electron micrographs. The polyhedral occlusion bodies (OB) presented a regular shape with size ranging from 2.2 to 4.0μm, with an average size of 2.8μm. These peculiarities allow the virus to be identified as a *Bombyx mori single nucleopolyhedrovirus* (BmSNPV).

Finally, *in vivo* virulence assays was carried out using first or second day of the 5thinstar *B. mori* larvae. They were fed with semi-purified suspension of polyhedral bodies ($1.3x10^9$ PIBs/ml) and reared in quarantine at 28^{o}C until advanced stages of infection. Larvae with no addition of the inoculum as negative control, were reared under the same conditioıs. The results showed that the BmNPV isolate is very infective, causing 95% mortality at 7th day after ingestion. Larvae susceptibility was also confirmed in the fourth instar.

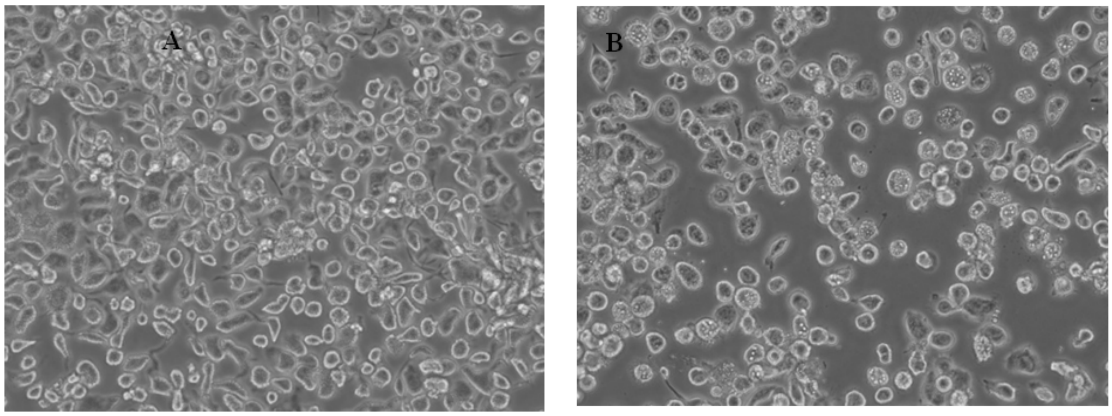

Fig. 2. Phase contrast micrographs of BM-5 cells after DNA transfection: A) mock infected cells, B) cells transfected with BmNPV DNA showing nuclear hypertrophy and occlusion bodies production, at 5 d.p.i.

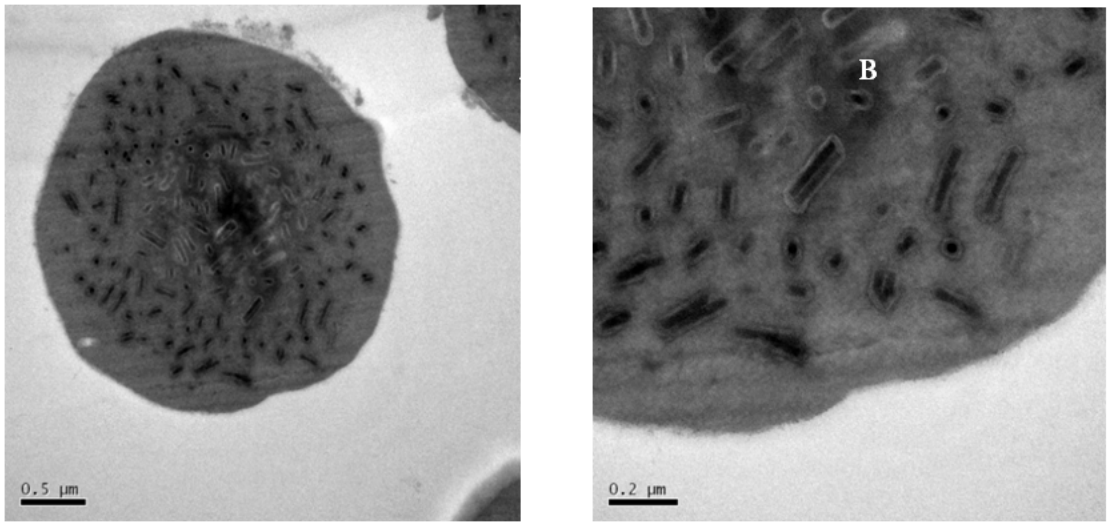

Fig 3. Electron micrographs showing the polyhedra ultrastructure: A) polyhedral body purified from *Bombyx mori* larvae with the viral disease symptoms; B) occlusion body detail showing virions with the predominance of one nucleocapsid per envelope (Single NPV)

DISCUSSION

Bombyx mori nucleopolyhedrovirus (BmNPV) selectively infects the domestic silkworm and causes great losses to sericulture. The identification of the BmNPV affecting *Bombyx mori* larvae is essential to prevent grasserie disease. In Cuba weather conditions are suitable for the development of silkworm diseases, when breeding silkworms were exposed to stress conditions, such as temperature variations, humidity, poor ventilation and nutritional deficiencies, coincides with an increase, up to 10%, in the incidence of symptoms associated to grasserie diseases. The PCR analysis initially carried out in the present work permitted detection of a 170bp DNA fragment and strongly indicated the presence of a baculovirus affecting silkworm rearing in Cuba. It was based on the polyhedrin gene (*polh*), using universal primers designed from a variety of nucleopolyhedrovirus by Szewczyk *et al* 2008. The PCR technique can be a valuable addition to the diagnostic and surveillance method in silkworm rearing and also a useful tool for vertical transmission analyses, as reported by Khurad *et al*, 2004, Khumnoi *et al*, 2008 and Saez *et al*, 2014. Multiplex PCR has also been developed for simultaneous detection of different pathogens in sericulture programs (Ravikumar *et al*, 2011).

Transfection of the viral DNA in cell culture induced the cytopathic effects peculiar to baculovirus infection, in which BM-5 cells became totally infected, confirming the cause of the disease. Such available *in vitro* system might be useful to improve molecular characterization of the Cuban viral isolate. Subsequently, visualization of the occlusion bodies by transmission electron microscopy allowed identifying the virus as a *Bombyx mori single nucleopolyhedrovirus* (BmSNPV). Multiple-enveloped nucleocapsids were seldom observed as previously reported by Ardisson-Araújo *et al.* (2015). Although the SNPV is the main form described in the literature for *Bombyx mori* NPV, the MNPV form has also been reported (Brancalhão *at al*. 2009, Maeda and Majima, 1990, Watanabe, 1975). The polyhedra size ranging from 2.2 to 4.0μm is consistent with other *Bombyx mori* NPV isolates. The virulence assays results showed that the Cuban isolate was very effective against *Bombyx mori* larvae which assure its potential for insect colony damage. The assays validated the efficacious of the virus infection which reinforces the care that must to be taken in sericulture practices. The methods used at this point confirmed the presence, for first time, of a nucleopolyhedrovirus affecting production areas of sericulture in Cuba. It was also shown that the PCR process described by Szewczyk and collaborators can be useful for the detection of BmNPV in silkworm breeding. The current study might have a significant impact for the pest management of silkworm rearing in order to prevent the transmission of the disease which leads to high economic losses.

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REFERENCES

Ardisson-Araújo, D.M.P, Lucas Melo, F.L, Andrade, M.S., Brancalhão, R.M.C, Báo, S.N., Ribeiro, B.M. 2015. Complete genome sequence of the first non-Asian isolate of *Bombyx mori nucleopolyhedrovirus* Virus Genes 49:477–484.

Blissard, G, Black, B, Crook, N, Keddie, BA, Possee, R, Rohrmann, G, Theilmann, DA, Volkman, L 2000. Family *Baculoviridae*, in *Virus Taxonomy, Seventh Report of the International Committee on Taxonomy of Viruses*, van Regenmoertel MHV, Fauquet CM, Bishop DHL, Carstens EB, Estes MK, Lemon SM, Maniloff J, Mayo MA, McGeoch DJ, Pringle CR, Wickner RB, Editors, Elsevier Academic Press 195–202.

Bozzola, J.J. and Russell, L.D. 1992. *In* Electron Microscopy: Principles and Techniques for Biologists. Jones and Bartlett Publishers, Boston, USA, 542p.

Brancalhão, R.M.C., Torquato, E.B., Fernandez, M.A. 2009. Cytopathology of *Bombyx mori* (Lepidoptera: Bombycidae) silk gland caused by multiple nucleopolyhedrovirus, Genetics and Molecular Research 8 (1): 162-172.

Grace, T.D. 1967. Establishment of a line of cells from the silkworm *Bombyx mori*. Nature 216: 613.

Herniou, E.A, Arif, B.M., Becnel, J.J., Blissard, G.W., Bonning, B, Harrison, R, Jehle, J.A., Theilmann, D.A. and Vlak, J.M. 2011. Family*Baculoviridae,* in *Virus Taxonomy, Classification and Nomenclature of Viruses, Ninth Report of the International Committee on Taxonomy of Viruses,* King, AMQ, Adams, MJ, Carstens, EB, Lefkowitz, EJ Editors, Elsevier Academic Press 163-173.

Jiang L, Xia, QY, 2014. The progress and future of enhancing antiviral capacity by transgenic technology in the silkworm Bombyx mori. Insect Biochemistry and Molecular Biology 48:1-7.

Khumnoi, S., Reanwarakoun, K., Amomsak, W., Attathom, T. 2008. Vertical Transmission of Nucleopolyhedrovirus in Thai Mulberry Silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae) Agricultural Sci. J. 39(1):45-54.

Khurad, A. M., Mahulikar, A., Rathod, M. K, Rai M. M., Kanginakudru, S, Nagaraju J, 2004. Vertical transmission of nucleopolyhedrovirus in the silkworm, *Bombyx mori* L. *Journal of Invertebrate Pathology* 87 (1) 8-15.

Kost, T.A, Condreay, J.P., and Jarvis, D.L. 2005 Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nature Biotechnology*. *23(5)*:567–575.

Maeda, S and Majima, K, 1990. Molecular cloning and physical mapping of the genome of *Bombyx mori* nuclear polyhedrosis virus. *Journal of General Virology* 71: 1851-1855.

Maeda, S. 1989. Expression of foreign genes in insects using baculovirus vectors. *Annual Review of Entomology* 34:351-72

Maruniak, J.E. 1986. Baculovirus structural proteins and protein synthesis". *In* The Biology of Baculovirus. Volume I - Eds. Granados, R. R. and Federici, B. A. CRC Press, Florida, p. 129-146.

Moscardi F, 1999. Assessment of the applications of baculoviruses for control of Lepidoptera. Annual Review of Entomology 44: 257–289.

Moscardi, F, Souza, ML, Castro, MEB, Moscardi, ML, Szewczyk, B, 2011. *Baculovirus Pesticides – Present State and Future Perspectives, In Microbes and Microbial Technology,* SpringerLink, p. 415-445.

Motohashj, T., Shimojima, T., Fukagawa, T., Maenaka, K., Park, E.Y. 2005. Efficient large-scale protein production of larvae and pupae of silkworm by *Bombyx mori* nuclear polyhedrosis virus bacmid system. *Biochemical and Biophysical Research Communications* 326 (3): 564-569.

O'Reilly, D.R., Miller, L. K., Luckow, V.A. 1992. Baculovirus Expression Vectors: A Laboratory Manual. W.H. Freeman and Company, New York. 347p. *Open Journal of Genetics*, 4: 370-377.

Ponnuvel, K.M., Nakazawa, H., Furukawa, S, Asaoka, A., Ishibashi, J., Tanaka, H., Yamakawa, M. 2003. A lipase isolated from the silkworm Bombyx mori shows antiviral activity against nucleopolyhedrovirus. *Journal of Virology* 77: 10725-10729.

Ramesh Babu, K, Ramakrishna, S, Harish Kumar Reddy, Y., Lakshmi, G., Naidu, N.V., Sadak Basha, S., Bhaskar, M., 2009. Metabolic alterations and molecular mechanism in silkworm larvae during viral infection: A review. *African Journal of Biotechnology* 8(6):899-907.

Ravikumar, G, Raje, Urs S, Vijaya Prakash, N.B., Rao, C.G.P., Vardhana, K.V. 2011. Development of a multiplex polymerase chain reaction for the simultaneous detection of microsporidians, nucleopolyhedrovirus, and densovirus infecting silkworms. *Journal of Invertebrate Pathology* 107: 193-193.

Saez, C.R.N., Munhoz, R.E.F., Pereira, N.C., Thaís Souto Bignotto, T.S., Fassina, V.A., Pessini, G.M., Laura Garay L.B., Ribeiro, L.F.C., Brancalhão, R.M.C., Fernandez, M.A., 2014. Detection of Contamination and Analysis of Vertical Transmission of BmNPV in Eggs and Moths of *Bombyx mori.*

Sambrook, J., Fritsch, E.F., Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, 2ª ed. 3 Volumes.

Szewczyk, B., Barski, P., Sihler, W., Rabalski, L., Skrzecz, I, Hoyos-Carvajal, L., Souza, M.L., 2008. Detection and identification of baculovirus pesticides by multitemperature single-strand conformational polymorphism. Journal of Environmental Science and Health Part B43:539-545.

Watanabe, H., 1975. Variation in the numbers of nucleocapsid within the envelope of nuclear polyhedrosis virus multiplied in tissues of the silkworm, *Bombyx mori. Journal of Sericultural Science, Japan* 43: 29-34.

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