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DIRECT APPLICATION OF THE NEW PCR PROTOCOL FOR EVALUATION AND MONITORING OF *BOMBYX MORI* INFECTION BY NUCLEOPOLYHEDROVIRUS

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ABSTRACT

Polymerase chain reaction (PCR) with complementary primers to the polyhedrin gene region was used to diagnose *B. mori* nucleopolyhedrovirus (BmNPV) infection. Experimental infections were performed for the standardization of the PCR assay. The PCR products were sequenced and specificity of the amplification was confirmed by comparison with BmNPV nucleotide sequences available in the GenBank. An identity level of about 99% was observed for those PCR products when compared to the known BmNPV sequences. The viral gene fragment amplification protocol proposed here is useful for early diagnosis of nucleopolyhedrosis in whole larvae, hemolymph, and infected eggs or in asymptomatic insects. Moreover, the standardized PCR can be used as a tool for surveillance and control of nucleopolyhedrosis in silkworm rearing.

KEY WORDS: *Bombyx mori*, diagnosis, nucleopolyhedrovirus, PCR, nucleotide sequence.

RESUMO

APLICAÇÃO DE NOVO PROTOCOLO DE PCR PARA AVALIAÇÃO E MONITORAMENTO DA INFECÇÃO DE *BOMBYX MORI* PELO VÍRUS DA NUCLEOPOLIEDROSE. A reação em cadeia pela polimerase (PCR) com *primers* complementares a uma região do gene da poliedrina foi usada para diagnosticar a infecção de *B. mori* pelo vírus da nucleopoliedrose (BmNPV). Infecções experimentais foram realizadas para a padronização do ensaio de PCR. Os produtos de PCR foram seqüenciados e a especificidade da amplificação foi confirmada por comparação com seqüências de nucleotídeos do BmNPV disponível no GenBank. Um grau de identidade ao redor de 99% foi observado quando tais produtos de PCR foram comparados com as seqüências de BmNPV conhecidas. O protocolo de amplificação do fragmento gênico viral proposto aqui é útil para o diagnóstico precoce da nucleopoliedrose na larva total, hemolinfa e ovos infectados ou em insetos assintomáticos. Além disso, o ensaio de PCR padronizado pode ser empregado como uma ferramenta para a vigilância e controle da nucleopoliedrose em criações de bicho-da-seda.

PALAVRAS-CHAVE: *Bombyx mori*, diagnóstico, vírus da nucleopoliedrose, reação de PCR, seqüenciamento de nucleotídeo.

INTRODUCTION

Sericulture is an important agricultural activity in Brazil; about 97% of the national production is exported. Brazil is the sixth largest silk producer and the industry has 6,000 workers organized in cooperatives. This activity is developed mainly in the States of Paraná, São Paulo, Mato Grosso do Sul, Santa Catarina, and Goiás (Brancalhão et al., 2002). Silk production in Brazil is low when compared with that of other countries and it is limited mainly by the occurrence of silkworm diseases (Watanabe et al., 2000). Silkworm is subject to a number of severe diseases during the different life stages, for example, the disease caused by nucleopolyhedroviruses (NPVs).

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Nucleopolyhedroviruses (NPVs) belonging to family *Baculoviridae* are significant arthropod pathogens, especially to insects of the order Lepidoptera, such as the silkworm *Bombyx mori* (Lepidoptera: Bombycidae). *B. mori* nucleopolyhedrovirus (BmNPV) is constituted by a large, circular, and double-stranded DNA, which causes a serious and often fatal nucleopolyhedrosis, a disease responsible for considerable economic losses in the Brazilian silk industry. Persistent infection by NPV may be an important factor in the induction, transmission, and spread of the virus in an insect population (Chou et al., 1996).

The silkworm life cycle in which the animals are susceptible to NPV infection goes through four marked stages: 1st as eggs; 2nd, as caterpillars or worms; 3rd, as chrysalides inside of cocoons; and 4th, as full-grown moths. It takes from 20 to 30 days for the eggs hatching. The caterpillar stage lasts about 25 days and before the worm is full-grown, it molts 5 times up to the next stage of the cocoon or chrysalis, which finally transforms into a moth. The period between successive molts is called instar.

Apparently, healthy insects collected from the field are often persistently infected with NPV, although symptoms may not be obvious or detectable. NPV infection is usually detected with the onset of symptoms (Gomi et al., 1999). In early instars, infected larvae may die within 1 to 2 days postinfection. The signs and symptoms of larvae infected after third instar are not apparent for several days. The larvae exhibit a loss of appetite, they change the color of the tegument, which becomes opaque and assumes a shiny and swollen appearance. The infection gives rise to systemic disease and culminates in the death of the larva. The internal tissue is in a state of disintegration and the silkworms are fragile to the touch, rupturing and releasing a whitish fluid filled with virus particles. Sometimes, black markings are observed on the body surface of the pupae on time of death. Even though death usually occurs in the larval stage, larvae infected in the last instars do not have their development blocked and may survive until reaching the adult stage where cocoons are small, misshapen and dappled (Tinoco, 2000). Susceptibility to infection decreases with insect development through the instars (Engelhard & Volkman, 1995, Washburn et al., 1995), promoted by the insect ecdysteroid molting hormones. The enzyme encoded by the virus egt gene inactivates the insect hormone favoring increase of virus particles number and infection progress (O'Reilly et al., 1998).

The epizootiology of the NPV is influenced by ecological and environmental factors (Burand et al., 1992). Although NPV is transmitted vertically via environmental contamination, the virus can be maintained in an insect population by transgenerational transmission in eggs. At present, in our country there is no routine diagnostic assay for early detection of NPV infection in a silkworm population. The diagnosis of nucleopolyhedrosis is based on the observation of disease symptoms. Here in Brazil, only health inspection and elimination of infected animals have been conducted to prevent disease dissemination. Several conventional diagnostic methods such as immunocytochemical localization (Fuxa et al., 1992), dipstick immunoassay (Nataraju et al., 1994), or latex agglutination assay (Shamim et al., 1995) are described for detection of BmNPV infection. In controlled-infection experiment, the kinetics studies revealed that a sandwich ELISA can detect virus 96h after infection and by immunofluorescence the infection can be detected as early as 72h after infection (Shamim et al., 1994). However, these methods are dependent on antibody specificity, time-consuming, and sometimes they are insensitive to detect low antigen concentrations or unsuitable for large-scale screening.

An effective surveillance of the NPV infection and health control may be implemented with the availability of a diagnostic assay that detects early on the presence of the virus genome in an insect population or in eggs.

Hemolymph from insect with nucleopolyhedrosis usually bears a large quantity of virions within the occlusion bodies (OB), constituted by a lattice of crystalline protein called polyhedrin. Polyhedrin has molecular weight of about 29 kDa. It is encoded by a highly conserved nonessential 738 bp fragment gene (Rohrmann, 1992; Wang et al., 2000), with highest expression towards the end of the infective cycle. Moreover, the insect cells can be infected also by more than one rod-shaped nucleocapsid per viral envelope, a highly virulent phenotype of multiple nucleopolyhedrovirus (BmMNPV). In this case the enveloped nucleocapsid or virions are embebed in a polyhedron lattice (Brancalhão et al., 2002). Several authors have investigated the presence of NPV in insect host, eggs, and environment by amplifying different regions of polyhedrin gene using polymerase chain reaction (PCR) (Burand et al., 1992; Burand et al., 1986; Moraes & Maruniak, 1997). For standardization of this assay, a careful design of experimental protocols is of critical importance to avoid many artifacts that inhibit the reaction, reducing the sensitivity, specificity or interfering in the reproducibility. We report herein, a polymerase chain reaction (PCR) protocol for the amplification of a polyhedrin gene fragment from BmMNPV. The amplified PCR product was sequenced and compared with BmNPV sequences in the GenBank.

MATERIALS AND METHODS

Samples

B. mori larvae used in this work were kindly provided by producers from Bauru region, São Paulo, Brazil. The experimental infection was carried out in laboratory conditions and the hemolymph was collected in different larval life phases (instar L1-L5). Before DNA extraction, they were frozen in liquid nitrogen and ground to a fine powder, according to CABALLERO et al. (1992). DNA from field sample larvae was prepared according to the same procedure. *B. mori* eggs were washed with sodium hypochloride. After that, they were crushed and ground in STE-buffer [10 mM Tris-HCl, pH 8.0, 1 mM ethylene diamine tetraacetic acid (EDTA), 50 mM NaCl, and 10 mM cysteine], and DNA was extracted from 40 mg of egg samples (approximately 11 eggs).

The virus used for experimental infection was provided by Unidade de Pesquisa e Desenvolvimento de Bauru / Polo Centro–Oeste APTA, São Paulo, Brazil, and identified as MNPV by using transmission electron microscopy.

Purification of occlusion bodies

Polyhedral OBs were collected from the hemolymph of BmMNPV-infected larvae. Hemolymph was washed and purified in a 40-63% (w / v) sucrose linear density gradient and submitted to centrifugation at 17,000*g* for 30 min at 4º C using a 60TI Beckman L15 rotor. Sucrose was removed from the preparation by diluting the suspension with PBS pH 7.2 and pelleting by centrifugation at 15,000*g* for 20 min. The pellet was resuspended in PBS pH 7.2. Polyhedral particles were estimated by counting in a Neubauer chamber. The suspension was stored at -20º C and was used to standardize the PCR reaction later on.

Detection of BmMNPV during larval infection

In order to simulate the infection, a preliminary study was performed in triplicate, in two different experiments, with L1-L5 instars larvae kept in Petri dishes. Larvae (10 individuals / instar) were infected by feeding them once with 5 g of mulberry leaves to which 1mL with 10^{-6} OBs was applied. First and second instar individuals consumed all contaminated leaves within 2h, and 3rd, 4th, and 5th instars in 2 or 3 min. Controls received leaves treated with distilled water. After 7 days, hemolymph (300mL / individual larva) from instars L4 and L5 was collected; larvae were frozen in liquid nitrogen and ground to a fine powder. PCR was performed using individual larvae and 40 mg of biomass as samples.

DNA extraction

The isolation and purification of OBs from infected insects were carried out as described by CHOU et al. (1996). Briefly, virions were released by means of alkaline treatment of 40 mg of individual larvae in each instar, except L1 and L2. For these instars, approximately 10 individuals were used to obtain the same concentration. DNA was extracted from the virions (LEVIN et al., 1997) by means of incubation with 1.0% SDS and proteinase K (10 mg / mL) followed by purification with Wizard® Plus Minipreps DNA Purification System kit (Promega®). The same extraction procedure was used for field samples, control samples and eggs.

PCR amplification of viral DNA sequences

Primers P1 (5'AATTATTCATACACCCCCACCATC 3') nucleotides 126–149 and P2 (5'GCCACCCTTTTTA GCCAGACT 3') nucleotides 627-647 of the polyhedrin gene were selected based on BmNPV polyhedrin gene nucleotide sequences (GOMI et al., 1999). PCR was performed in 25µL reaction mixture (KCl 50 mM; Tris HCl 10 mM, $MgCl_2$, 2.5 mM), containing 0.2µM of each dNTPs, 0.1 µM of each primer and 0.87 ng of sample DNA. 35 amplification cycles were carried out in a Perkin Elmer 2400 thermocycler. Cycling parameters for PCR were as follows: 2 min at 94º C, 15 sec at 94º C, 20 sec at 60º C, 45 sec at 72º C and 7 min at 72º C. PCR products were analyzed by electrophoresis on 1.5% (w / v) agarose gel (80V for 1h) using 40 mM Tris-acetate, 1 mM EDTA pH 7.5 as running buffer. DNA fragments were visualized by ethidium bromide staining.

PCR specificity

PCR specificity was evaluated by enzyme-restriction analysis and by analysis of nucleotide sequence of amplified fragments. PCR products were purified by Concert™ Rapid PCR Purification system (Gibco, BRL), according to the recommendations of the manufacturer. Samples were digested with 5U of *Alu* I restriction enzyme (Invitrogen™) with 3h incubation at 37º C. Products obtained were analyzed by electrophoresis on 1.5% agarose gels (w / v) stained by ethidium bromide.

DNA-sequencing

PCR fragments were separated from primers and nucleotides using Concert™ Rapid PCR Purification system (Gibco, BRL). Sequences of purified PCR products were determined using BigDye terminator II kit (Applied Biosystem), and analyzed using ABI

Prism 377 DNA sequencer (Applied Biosystems). The sequences were aligned and compared with registered ones in GenBank (access number L33180, X63614, U75359, NC-001962, M30925, M10043).

RESULTS

Optimization of PCR amplification conditions was performed using DNA extracted from different larval stages of experimentally infected *B. mori*. Under these experimental conditions, the use of primers P1 and P2 generated a single amplification product with a size corresponding to the predicted 522 bp (Fig.1). No amplification was observed in the absence of viral DNA (Fig 1, lanes 9, 10). In this experimental model, 7 days after ingestion of contaminated diet, the viral genome was detected in infected larvae, independent of the onset of infection signs or the larvae age, but 2 days after the onset of the administration of contaminated diet MNPV DNA was detected in animals collected by chance in all stages of life. Results of PCR of first to fifth instar were positive only when DNA was extracted from a biomass near 40 mg, corresponding to approximately one or two animals for each instar (Fig. 1B). When DNA was extracted from 20 mg or lower values of biomass from first to fifth instar animals, we observed that PCR results showed great variation between triplicate samples (Fig 1A). In the same way, commercially available eggs submitted to regular surface decontamination also showed positive PCR results using 40 mg biomass or above. Moreover, hemolymph of infected larvae from fourth and fifth instars was positive by PCR independent of disease symptom manifestation. Hemolymph from uninfected larvae used as negative control revealed no PCR product (data not shown).

The PCR with 35 amplification cycles detected BmMNPV in 100% of the infected larvae of all instars, however about 50% of later instar animals were not showing clinical signs of infection. The sensitivity of PCR assay for NPV DNA detection was correspondent to 1.8 pg/µL (equivalent to 3 OBs) and it was determined by ten-fold serial dilutions of 18 ng/µL purified viral DNA (data not shown). The selected primers did not present sequence homology when aligned with GenBank sequences of putative environmental NPVs (data not shown).

The enzyme-restriction analysis and nucleotide sequencing of viral DNA amplified fragment evidenced the specificity of amplification, yielding PCR products with identity rates higher than 99% when compared with available BmNPV sequences (accession number L33180, X63614, U75359, NC-001962, M30925, M10043) (Fig. 3).

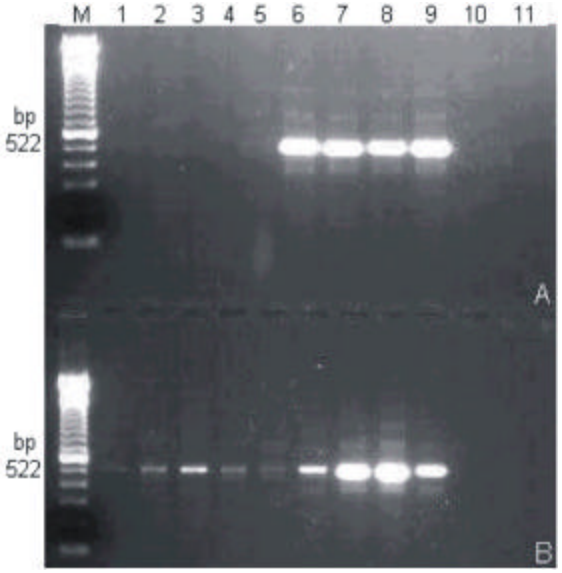

Fig. 1 - Agarose gel electrophoresis analysis of PCR-amplified DNA gene fragment from infected *B. mori* larvae and control samples. - PCR amplification of BmNPV DNA using 20 mg (A) and 40 mg (B) of each sample. Lanes: M = 100 bp DNA Ladder (Gibco, BRL); 1= eggs; 2 – 6 = BmNPV DNA from first to fifth instars, respectively; 7 - 9 = BmNPV DNA positive control; 10 = negative control sample (DNA extracted from uninfected larvae) 11 = reagent control (all the PCR reagents included, except template DNA). Electrophoresis conditions: 10 µLof PCR product, 1.5% agarose gel stained by ethidium bromide, 80V / 1h.

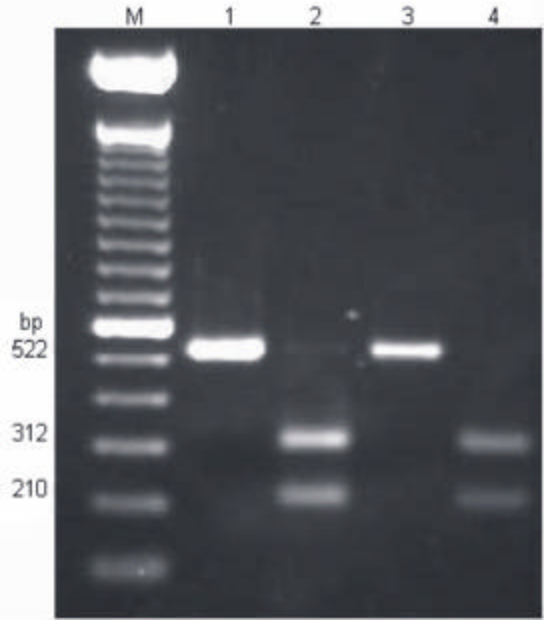

Fig. 2 - Restriction-enzyme analysis of PCR-amplified polyhedrin gene fragment of MNPV obtained from infected *B. mori* larvae. Lanes: M = 100 bp DNA Ladder (Gibco, BRL); 1,3 = BmMNPV polyhedrin gene amplified product; 2,4 = BmMNPV polyhedrin gene amplified product digested with *Alu* I (Invitrogen™). Electrophoresis conditions: 10 mL of PCR product, 1.5% agarose gel stained with ethidium bromide, 80V / 1h.

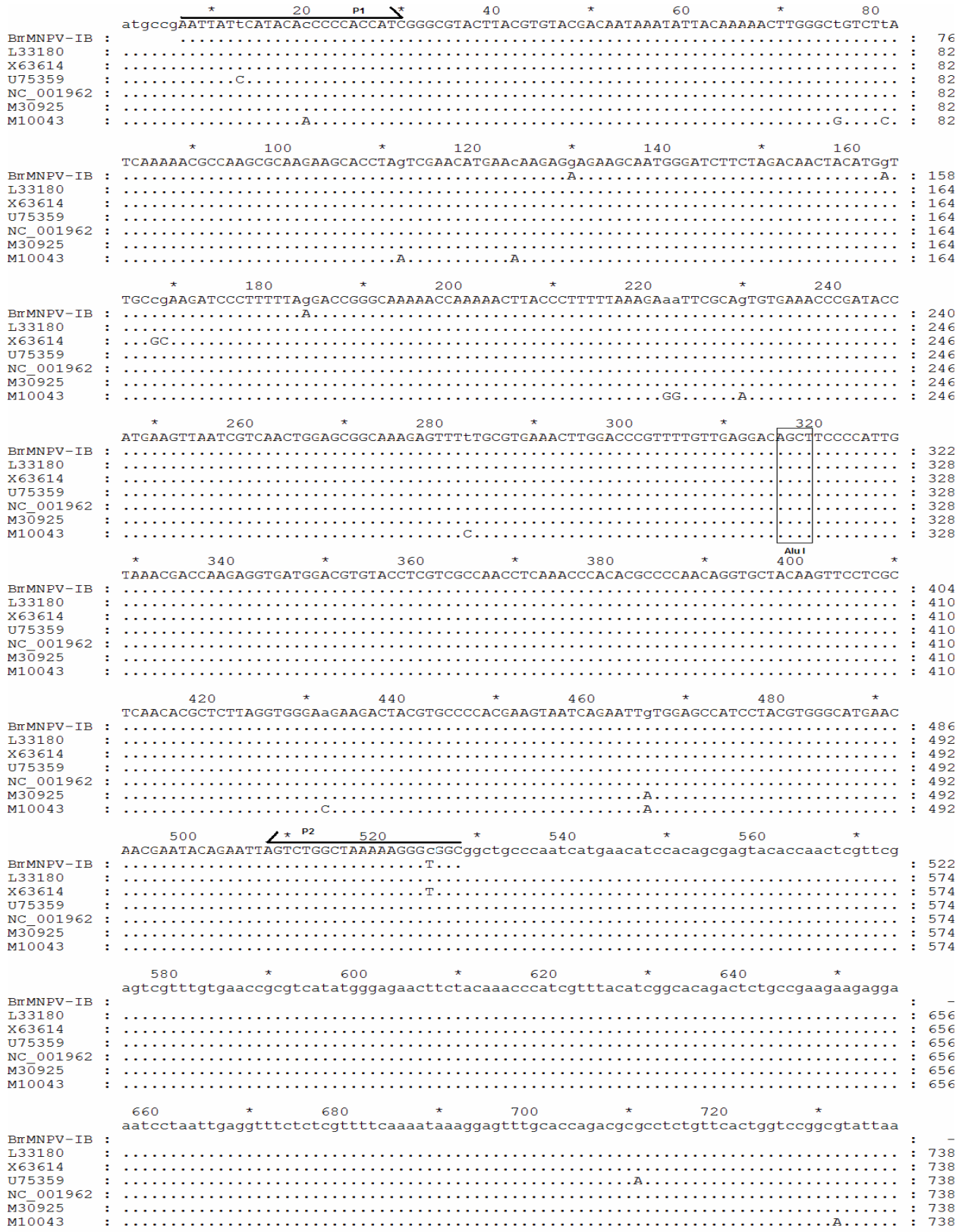

Fig. 3 - Alignment of nucleotide sequences of BmMNPV DNA polyhedrin gene fragment (BmMNPV-IB) amplified by PCR using primers P1 and P2. The *Alu* I restriction site is indicated by boxed nucleotides. The arrowhead indicates the position and direction of the 522-nucleotide amplified fragment.

Analysis of amplified polyhedrin gene sequences using Vector NTI Viewer 4.0.1 software (Invitrogen™/ InforMax) showed a single restriction site for the enzyme *Alu* I. As expected, two bands with molecular size corresponding to 312 bp and 210 bp were observed by *Alu* I digestion of the amplified product (Fig.2).

DISCUSSION

In this report, a single round PCR using BmNPV-specific primers proved to be a good alternative to detect NPV in experimentally infected insects. In the assay outlined here, the reaction was performed with DNA extracted from larvae, hemolymph or eggs and proved to be useful to analyze field-collected samples. An advantage of this assay is to detect NPV presence in samples of diverse nature with the same sensitivity. PCR assays described by several authors for detection and discrimination of NPV in different insects generally use degenerated primers and cell cultures; hemolymph or eggs as the DNA source (Burand et al., 1986; Moraes &Maruniak, 1997; Wang et al., 2000). In the experimental conditions determined by Wang et al. (2000), one-step PCR could detect about 0.57 ng of viral DNA or 10^2 to 10^3 times less when using nested PCR amplification and was affected by the NPV species or tissues used for virus replication. In our study, these protocols did not present good performance to appraise field samples, showing inconsistent results even when using the highly sensitive nested PCR. We could explain this fact either because of the use of degenerated primers or the influence of contaminants in the DNA extracted from field samples, which are common inhibitors of the PCR (Wilson, 1997). Considering that the DNA extraction procedure from field samples could be the cause of inconsistent results, we used a reliable extraction protocol employing SDS, proteinase K and a commercial kit of silica resin. In this way, a 522 bp fragment of the BmNPV polyhedrin gene was amplified, allowing detection of MNPV in animals 2 days after infection. This corresponds to a PCR detection sensitivity of 1.8 pg of viral DNA per µL. Very low levels of virus particles infecting first and second instar larvae probably explain the amount of biomass necessary to obtain detectable PCR products. Different intensities of the PCR products in agarose gel observed in different instar larvae may reflect a variable amount of viral DNA or a viral population size that can be influenced by stresses during animal handling (Tinoco, 2000). The homology between amplified products and known NPV sequences were higher than 99%, the restriction enzyme analysis reinforced the specificity of our protocol. The selected primers did not present homology with sequences of putative environmental NPVs, detecting either BmSNPV or BmMNPV populations, assuring the specificity of the PCR protocol standardized by us. This protocol showed to be a useful tool for early diagnosis of the disease, to prevent NPV transgenerational transmission and to guarantee silkworm health, avoiding possible egg-surface contamination by viruses, the main source of inoculums for newly hatched larvae.

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