

Purification and Amplification of DNA from *Penaeus monodon*-Type Baculovirus (MBV)

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The purpose of this paper was to purify and amplify the DNA fragment of *Penaeus monodon*-type baculovirus (MBV). Using 30–50% caesium chloride gradients, MBV virions and occlusion bodies with density parameters of 1.28–1.29 and 1.32–1.33 g/ml, respectively, were purified. Two oligonucleotide primers have been successfully designed and utilized for the amplification of a DNA fragment of MBV. After 35 amplification cycles of the MBV DNA fragment, a large amount of amplified product with an approximate molecular weight of 600 bp was obtained. This is the first successfully published work on the amplification of MBV using the polymerase chain reaction (PCR). Using the same primers, DNA extracted from MBV noninfected *P. monodon*, *P. japonicus*, and *P. orientalis* had a negative PCR response. However, a positive PCR response was obtained from DNA extracted from MBV-infected postlarval *P. monodon*. DIG-dot blot hybridization technique using PCR product obtained from the present study as a probe further confirmed that the product is originated from a portion of MBV polyhedrin gene. It is also suggested that PCR product may be beneficial for an accurate and early diagnosis of MBV infection in larval shrimp. © 1993 Academic Press, Inc.

KEY WORDS: *Penaeus monodon*; Baculovirus; MBV; polymerase chain reaction.

INTRODUCTION

Penaeus monodon-type baculovirus (MBV) is considered to be one of the most important pathogens for larvae of the giant tiger prawn (*P. monodon*, Fabricius) (Chen *et al.*, 1989) and frequently causes mass mortality or growth retardation in infected animals (Lightner *et al.*, 1983; Chen *et al.*, 1989). The high infection rate of MBV may result in a fluctuant production on the hatchery system of *P. monodon*. To reduce the losses caused by MBV infection, the development of early and accurate diagnostic techniques is essential. Technologies for the diagnosis of MBV infection have been reported by Lightner *et al.* (1981, 1983) and Chen *et al.* (1989) using histopathological staining methods. These techniques, however, have been limited to the detection of occlusion bodies in late larval stages and

no reliable methods have been developed for the detection of early infection of MBV. This paper therefore attempts to describe an application of polymerase chain reaction (PCR) for the amplification of MBV DNA.

PCR is a technique that utilizes flanking oligonucleotide primers and repeated DNA synthesis cycles to amplify small quantities of target DNA sequences (Mullis *et al.*, 1986; Saiki *et al.*, 1986). Modifications and applications of PCR have been widely described (Matsumoto *et al.*, 1990; Cartin *et al.*, 1991; Mack and Sninsky, 1988; Korber *et al.*, 1991). This technique has been demonstrated to be a sensitive method for the detection of microbiological infections at the picogram level. Therefore, the purpose of this study was to detect a DNA fragment of MBV in larval shrimp using PCR.

In this experiment, virions and occlusion bodies of MBV from postlarval stage of *P. monodon* were purified and digested. The procedures for the development of a diagnostic system for MBV using PCR technique were also developed.

MATERIALS AND METHODS

1. Preparation of Purified Virions and Occlusion Bodies

MBV infection in the experimental shrimps was diagnosed using wet mounts as described by Lightner *et al.* (1983). The hepatopancreata removed from postlarvae were placed on a slide with one drop of TE buffer (0.02 M Tris, 1 mM EDTA, pH 7.5) and stained with 0.1% eosin. The specimen was then observed under an Olympus (BH-2) light microscope.

Collection of MBV-infected postlarvae (PL) was performed at stages of PL 10 to 30. The larvae were immediately dissected under a dissection microscope and the hepatopancreata were collected with forceps and immediately immersed in cold TE buffer.

Approximately 100–300 pieces of hepatopancreata in 1 ml TE buffer were homogenized and then filtered through 400- μ m Nytex-meshed sieves. The filtrates were then centrifuged at 1000g for 20 min at 4°C. Both

the supernatant and pellet were collected and suspended in an appropriate volume of TE buffer and loaded on a 30–50% discontinuous caesium chloride (CsCl) density gradient and then ultracentrifuged at a speed of 200,000g for 16 hr. The band from the CsCl gradient was removed and pelleted by centrifugation at 200,000g for 1 hr at 4°C. Subsequently, the pellet was resuspended in TE buffer and then checked for the presence of viral particles using a negative stain and a HITACH 500 transmission electron microscope.

2. Preparation of Template for DNA Amplification

Following purification and confirmation of the presence of MBV virus and occlusion bodies as described above, the bands containing occlusion bodies and virions were harvested and stored at 0°C until needed for experimental use. The DNA of MBV was extracted from purified MBV virions with TE buffer containing 1 mg/ml proteinase K, 1% *N*-lauroyl sarcosine, and 0.1 M KCl at 65°C for 3 hr, followed by phenol–chloroform extractions and ethanol precipitation. Precipitated DNA was resuspended in 0.1 × TE buffer and the concentration of DNA was determined by electrophoresis using a 1% agarose gel. The DNA was stained with ethidium bromide and observed under uv light (TF-20M, VILBER LOURMAT, France).

The DNA from hepatopancreata of postlarval *P. monodon* was also prepared with proteinase K digestion, phenol–chloroform extraction, and ethanol precipitation. This crude DNA was used as the template for DNA amplification to determine the specificity of the MBV DNA amplification system designed in this experiment. The DNA obtained from muscle of *P. japonicus*, *P. orientalis*, and *P. monodon* prepared by the same procedures as described above was used as negative control.

3. Detection of MBV DNA by Amplification with PCR

According to nucleotide sequences (46–75 and 691–722) of insect polyhedrin genes described by Rohrmann (1986), two mixed primers, No. 35 (5'AC(CT)TA(CT)GTGTACGACAACAAATA(CT)TACAAA3') and No. 36 (5'GG(TC)GCGTC(TG)GG(TC)GCAAA(CT)TC(CT)TT(TA)AC(TC)TT(GA)AA3'), were designed to amplify MBV DNA. Amplification of MBV DNA was performed in a 100- μ l PCR mixture containing 300 ng template DNA, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 1.25 mM of each deoxynucleotide triphosphate, 2.5 μ g of each primer, and 2.5 units of AmpliTaq DNA polymerase (Perkin–Elmer Cetus). Subsequently, the mixture was overlaid with 50 μ l of mineral oil to prevent evaporation. The reactions were promoted by 35 cycles of denaturation, annealing, and polymerization. The treatment for each cycle included a heat denaturation temperature of 94°C for 1 min, annealing temperature of 42°C for 1

min, and polymerization temperature of 72°C for 5 min, followed by exposure to 72°C for 15 min after the last cycle. The prepared samples were electrophoresed in a 1% agarose gel followed by staining with ethidium bromide for the presence of amplified DNA of MBV.

4. DIG–Dot Blot Hybridization

To characterize the end product of PCR obtained from DNA MBV obtained in the above section, the technique of chemiluminescent detection of digoxigenin-labeled nucleic acid as described by Holtke *et al.* (1992) was used. The DNA fragment produced from PCR with molecular weight of approximately 600 bp labeled with digoxigenin-labeled deoxyuridine-triphosphate was used as probe. DNAs derived from occluded MBV, MBV-infected postlarvae, and noninfected MBV were transferred into nylon membranes via a 96-well dot-blot vacuum filtration Manifold (Schleicher & Schuell, Inc., Keene, NH) under low vacuum with a slow filtration rate. Subsequently, the materials were denatured in denatured buffered solution containing 0.5 M NaOH and 1.5 M NaCl and crosslinked in an uv crosslinker. Hybridization, stringent washes, and chemiluminescent detection were then performed according to the procedures described by Holtke *et al.* (1992).

RESULTS

Using the direct smear technique for the detection of MBV, 92% of the 50 randomly selected postlarvae of *P. monodon* were demonstrated to be positive. The majority of the postlarvae revealed heavily infected hepatopancrease with the presence of large amounts of occlusion bodies in the infected cells (Fig. 1). The infected cells showed hypertrophic nuclei containing spherical occlusion bodies. Using 0.1% aquatic eosin solution wet mounts the occlusion bodies were a dark red color in the nuclei and a light red color in hepatopancreatic cells (Fig. 1).

Two distinct bands were observed when the homogenate of MBV-infected hepatopancreata of PLs were ultracentrifuged at a speed 20,000g for 16 hr on a 30–50% CsCl density gradient (Fig. 2). Each band was then collected separately and CsCl was eradicated by centrifugation. The presence of occlusion bodies and viral particles in each band was determined by using light and transmission electron microscope (TEM). Band 1 from the CsCl gradient as presented in Fig. 3 was characterized by a specific density of 1.28–1.29 g/ml and contained free virus with or without nucleic acid. Band 2 localized at a density of 1.32–1.33 g/ml and contained only occlusion bodies of MBV (Fig. 4). Observation of band 2 under TEM after negative staining, revealed the presence of both polyhedrin and virions (Fig. 5).

The MBV DNA extracted from purified MBV virions

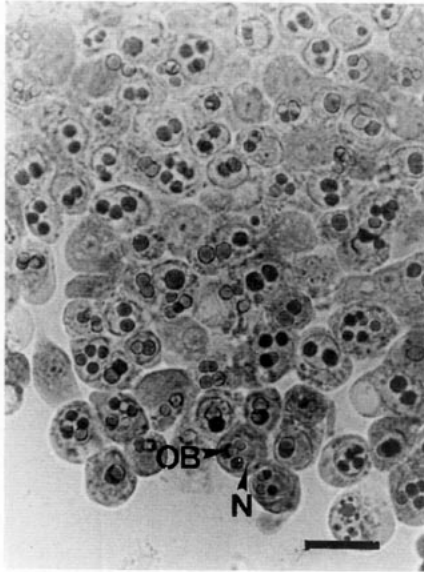


FIG. 1. Wet mounting of hepatopancreata, stained with eosin, *Penaeus monodon*-type baculovirus (MBV) occlusion bodies observed in majority of hepatopancreatocytes. Bar = 50 μ m. OB, Occlusion bodies of MBV; N, nucleus.

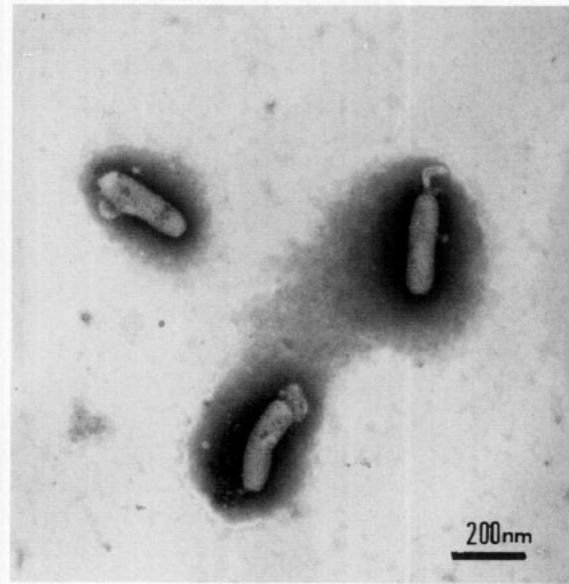


FIG. 3. Nucleocapsid of *Penaeus monodon*-type baculovirus observed under electron microscope by using negative-staining technique. Magnification 50,000 \times .

and occlusion bodies showed a single band at a high-molecular-weight position after agarose gel electrophoresis (Fig. 6).

The results of agarose gel electrophoresis performed on the product of PCR of purified MBV DNA revealed a distinct band (Fig. 7) following 35 amplification cycles. When compared to the control marker, the size of the PCR products was approximately 600 bp. In the same gel, the excess primers were present at a lower position on the gel (Fig. 7).

To test the specificity of the amplification system for

the detection of the MBV polyhedrin gene, crude DNA extracted from hepatopancreata of postlarval stages of *P. monodon* was used as template. As shown in Fig. 8, a single band of a 600-bp DNA fragment was visualized in the gel under uv light. DNA extracted from uninfected *P. japonicus*, *P. monodon*, and *P. orientalis* gave no response as indicated in Fig. 8.

The results of DIG-dot blot presented in Fig. 9 shows that positive reaction with an appearance of black dot

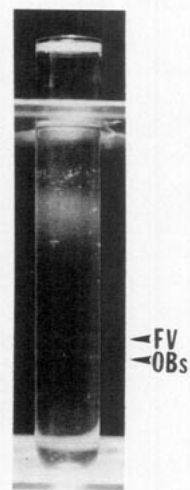


FIG. 2. Isodensity banding of *Penaeus monodon*-type baculovirus and occlusion bodies, purified from postlarvae of *P. monodon*, in caesium chloride (CsCl) gradient (30–60%). Density of virus (FV) is 1.28–1.29 g/ml and density of occlusion bodies (OBs) is 1.32–1.33 g/ml.

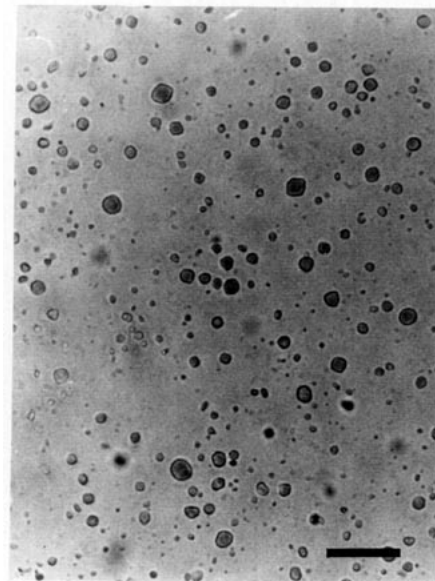


FIG. 4. Following purification from CsCl gradient, *Penaeus monodon*-type baculovirus occlusion bodies were observed under light microscope. The samples were stained with 0.1% eosin. Bar = 50 μ m.

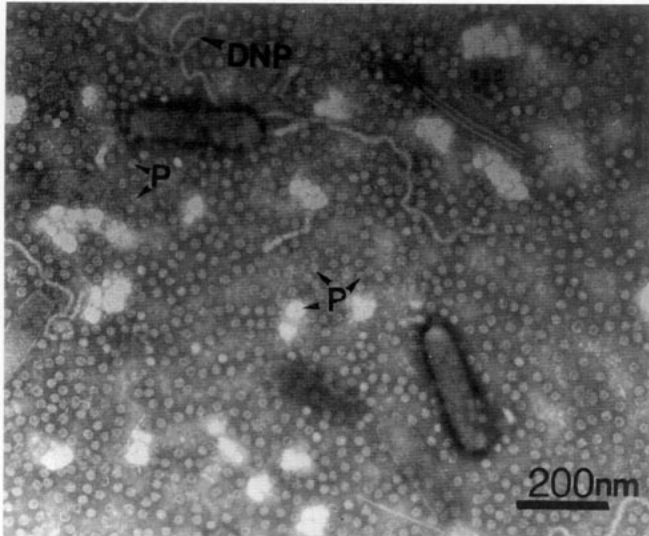


FIG. 5. Nucleocapsid of *Penaeus monodon*-type baculovirus and the fragments of deoxyribonucleoprotein (DNP) exuded from nucleocapsid. Magnification 60,000 \times . P, polyhedrin

was obtained when DIG-dUTP-labeled DNA of MBV produced from PCR reacted with purified MBV DNA fragment (Fig. 9 A1 and B1). Similar result was obtained when the probe reacted with DNA extracted from MBV-infected postlarvae (Fig. 9, A3, B3, and C2). In contrast, when DNA of MBV-uninfected larvae was detected a negative result was observed (Fig. 9, C1, C3, and D1-3). These results may demonstrate that PCR product obtained from the present study is originated from a portion of polyhedrin gene of MBV.

DISCUSSION

The present study showed that purification of MBV can be achieved by using caesium chloride gradient techniques. In the purified fractions, except virions, fragments of DNA that may be exuded from the nucleocapsid of MBV were also observed when negative stain for electron microscopy was used.

Similar exudation was also observed when *Heliothis zea* single nuclear polyhedrosis virus (SNPV) was purified (Kelly *et al.*, 1983). This SNPV-exuded material was demonstrated to be associated with basic proteins constituting a deoxyribonucleoprotein (DNP) (Burley *et al.*, 1982; Kelly *et al.*, 1983). The diameter of DNP observed from SNPV was approximately 10 nm, which is similar to the DNA fragment obtained from MBV in the present study.

Our results also demonstrated that occlusion bodies (OBs) of MBV are fragile under mechanical agitation. Therefore when following centrifugation for purification OBs, the crystal polyhedrin and polyhedral virions would be observed simultaneously.

Although *Baculovirus penaei* is also an occluded baculovirus, it possesses a different buoyant density. The present study showed that the densities of MBV virions and occlusion bodies were 1.28–1.29 and 1.32–1.33 g/ml, respectively. However, the buoyant density of *B.*

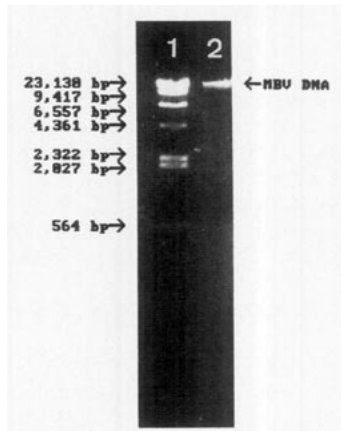


FIG. 6. Detection DNA of *Penaeus monodon*-type baculovirus in 1% agarose. Lane 1, λ DNA/*Hind*III marker; Lane 2, MBV DNA.



FIG. 7. Detection the products of polymerase chain reaction (PCR) for DNA of *Penaeus monodon*-type baculovirus. Lane 1, λ DNA/*Hind*III marker; Lane 2, PCR products of MBV DNA.

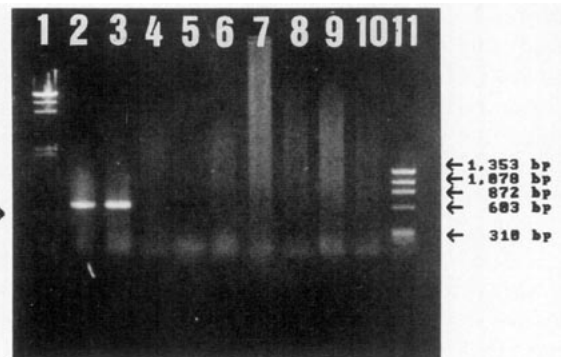


FIG. 8. Detection DNA of *Penaeus monodon*-type baculovirus in MBV-infected shrimp and DNA from various shrimp tissues by polymerase chain reaction. Lane 1, λ DNA/*Hind*III marker; Lane 2 and 3, MBV-infected postlarvae; Lane 4–7, MBV-uninfected postlarvae; Lane 8, DNA of *P. monodon*; Lane 9, DNA of *P. japonicus*; Lane 10, DNA of *P. orientalis*; Lane 11, P1X174 DNA/*Hae*III marker.

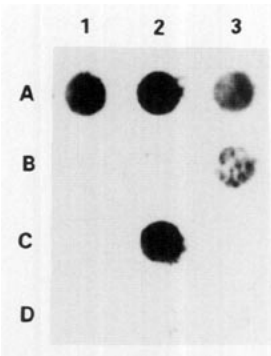


FIG. 9. Chemiluminescent detection of MBV DNA fragment on dot blot of DIG-labeled PCR-produced DNA fragment of *Penaeus monodon*-type baculovirus (MBV). Various purified DNA were blotted onto positively charged nylon membranes and then hybridized with the labeled probe. Well A1, purified MBV DNA; well A2, purified MBV DNA; well B1, negative control of unhybridized PCR product of MBV DNA; well B2, unhybridized BMV DNA. The wells A3, B3, and C2 were MBV-infected postlarvae and C1, C3, and D1-3 were MBV uninfected postlarvae.

penaei reported by Bruce *et al.* (1991) is 1.32 g/ml, which is similar to the density of MBV occlusion bodies.

Agarose gel electrophoresis of the purified DNA of MBV revealed a single band of high molecular weight. The molecular weight of MBV DNA was compared to *Eco*RI-cleaved fragments and estimated to be 100–200K bp (Chang *et al.*, 1991).

This is the first report of the detection of MBV using the polymerase chain reaction. Two primers used in this study were designed according to conserved sequences of the insect baculovirus polyhedrin gene. Purified PCR product of DNA fragments of MBV as a hybridization probe was demonstrated to be able to react with purified MBV DNA. It is therefore suggested that polyhedrin genes of insect baculoviruses and the genome of MBV may possess similar conserved sequences. The result of chemiluminescent detection may also confirm that the DNA product of PCR obtained from the present study is originated from MBV. The excellent amplification for MBV DNA fragment presented in this paper may provide sequence information for the preparation of a DNA probe which could be used to accurately diagnose MBV infection of *P. monodon*.

Based on fresh smears and histopathological observations, the detectable stage for MBV in *P. monodon* is postlarvae 2 (PL2) (Chen *et al.*, 1989). The use of PCR and DNA probes may contribute to an early diagnosis of MBV infection in larval shrimp. Understanding the properties of the nucleic acid of MBV may be beneficial to be development of preventive or curative measures for MBV infection in host shrimps.

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