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Infectivity and Pathogenicity of *Baculovirus penaei* (BP) in Cultured Larval and Postlarval Pacific White Shrimp, *Penaeus vannamei*, Related to the Stage of Viral Development

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Abstract

The infectivity and pathogenicity of the penaeid shrimp virus, *Baculovirus penaei* (BP), is influenced by the stage of viral development. This study consisted of a preliminary experiment which showed that nonoccluded virus administered *per os* is infective, followed by two infectivity experiments. In the first phase of each infectivity experiment, mysis stage larvae of *Penaeus vannamei* were inoculated with BP and samples of infected tissue were collected at various times postinoculation (p.i.). These samples were then used to inoculate either mysis or postlarval stage *P. vannamei* in the second phase of each experiment. Viral inocula prepared from patently infected tissues consistently produced infections in both mysis and postlarval stage shrimp. Inocula prepared from prepatently infected tissue collected 12–18 h p.i. produced infections in some but not all replicate cultures, while inocula prepared from tissues collected earlier than 12 h p.i. were not infective. Viral development in mysis stage larvae was substantially delayed and the mortality was significantly lower when the viral inocula were prepared from prepatently compared to patently infected tissues. BP appears to be most pathogenic to larvae that have been exposed to inocula prepared from tissues that had recently developed a high prevalence of patent infections or about 72 h p.i.

Keywords: baculovirus, BP infections, *Penaeus vannamei*, infectivity and pathogenicity, viral development

Introduction

Baculovirus penaei, commonly known as BP and designated as PvSNPV (Francki et al., 1991), was first described in pink shrimp, *Penaeus duorarum*, from the northern Gulf of Mexico (Couch, 1974). BP is known to infect at least 14 species of penaeid shrimp and can cause serious epizootics with high mortality of larval and postlarval stages in some of those species (Lightner and Redman, 1991). BP is responsible for annual economic losses in cultured penaeid shrimp in the Americas and Hawaii due to mass mortalities that occur in the hatchery phase of production (Stuck and Overstreet, 1994). BP also commonly infects a substantial number of wild brown shrimp, *P. aztecus*, from the northern Gulf of Mexico (Overstreet, 1994); however, the mechanism of transmission and effects of the virus on wild shrimp populations are not known.

Several studies have investigated the replication cycle of BP in penaeid shrimps. Couch (1989) speculated that virus particle assembly and occlusion into viral polyhedra, characteristic of a patent infection, occurred within 24 h (h) postinoculation (p.i.) in pink shrimp. The timing of these events roughly coincided with similar events described in insect baculoviruses. Bruce et al. (1994) and Stuck and Wang (1996) reported that patent infections, characterized by the presence of viral polyhedra, in fresh squash preparations of hepatopancreas, first developed 18–24 h p.i. and increased to 100% prevalence 48–72 h p.i. among experimentally infected *P. vannamei*. In those studies, molecular and histological diagnostic methods were also used to detect prepatent infections in some shrimp as early as 12 h p.i.

While information is now available on the time course for BP replication in shrimp, the time p.i. at which newly replicated virus becomes infective is not known. The objectives of this study were to determine the stage of viral replication at which the virus becomes infective, assess pathogenicity of inocula prepared from prepatently and patently infected tissues, and when applicable, compare those results with similar information on insect baculoviruses. Information obtained from this study should be useful in prevention and control of BP epizootics in commercial culture operations and provide insights to understanding possible mechanisms of transmission and pathogenicity of the virus in both cultured and wild populations of penaeid shrimps.

Materials and Methods

Culture Methods for Shrimp

High health nauplii of *P. vannamei* from the U.S. Marine Shrimp Farming Program (USMSFP) Population 1 (Wyban 1992) were obtained from one of three sources: The Oceanic Institute (OI), Makapuu Point, Honolulu, Hawaii; Waddell Mariculture Center (WMC), Bluffton, South Carolina; or Gulf Coast Research Laboratory (GCRL), Ocean Springs, Mississippi. Nauplii were reared to the desired test age generally following the procedures described by Stuck and Overstreet (1994). Culture water was prepared from well water that was passed through a reverse osmosis unit (Aquaculture Supply Company, Dade City, Florida) to which hw-Marinemix (Hawaiian Marine Imports, Houston, Texas) and EDTA-Na_2 (10 ppm) was added. The diatom *Chaetoceros gracilis* was provided *ad libitium*

to nauplii, protozoeae, and early mysis stage shrimp while late mysis and protozoeae were fed brine shrimp nauplii. The salinity of all cultures was maintained at 30 ± 2 ppt and the temperature at 27 ± 2°C.

Infectivity of Nonoccluded Viral Particles (NOVPs)

A preliminary experiment was conducted to determine if nonoccluded viral particles (NOVPs) of BP were infective. Viral stock used in this, and the subsequent infectivity experiments, was originally collected from broodstock and juveniles of *P. vannamei* in Ecuador (Overstreet et al., 1988) and then passed through several lots of shrimp larvae from USMSFP Population 1. Approximately 45 mg of patently infected tissue was homogenized in 12 ml of culture water using a hand held tissue homogenizer. A similar concentration of BP-free *P. vannamei* tissue was also homogenized for use as a negative control. The homogenate prepared from the infected tissue was divided into four aliquots of equal volume. One aliquot, which served as the positive control, was not centrifuged. The other three aliquots were centrifuged at either 1000*g* for 5 min, 10,000*g* for 10 min, or 40,000*g* for 30 min. A portion of the supernatant from each aliquot was then carefully recovered so as not to disturb the pellet. Two milliliters of supernatant from each of those aliquots and 2 ml of the infected and noninfected whole homogenates were then used to inoculate five separate groups of 9-day-old (PL9) postlarvae that were reared from nauplii obtained from OI. Each group consisted of 100 ± 20 postlarvae stocked into a 7.6-liter glass tank that was maintained according to the procedures outlined by Overstreet et al. (1988) for bioassay of BP. After 7 days, fresh squashes prepared from a sample of shrimp (n = 17–23) from each tank were examined for patent infections following the procedures described by Overstreet et al. (1988). The same diagnostic procedure for detection of patent infections was used in the subsequent infectivity experiments. Differences in the infectivity of whole homogenates and supernatants were tested for significance using Pearson's chi-square statistical test.

Infectivity Experiment One

Two infectivity experiments, each consisting of two phases, were conducted. In both experiments, samples collected at various times p.i. from a mass experimental infection (phase 1) were subsequently used to inoculate a second group of larvae or postlarvae (phase 2) in a bioassay similar to that described by Overstreet et al. (1988). In the first phase of experiment 1, nauplii obtained from WMC were stocked (100–125 nauplii per liter) into two 200-liter glass tanks that were maintained in a common circulating water bath and reared to a mysis II stage. One culture was inoculated with a homogenized highly infective viral stock at a concentration of approximately 10 mg per liter and the second culture, which served as the control, was inoculated with a similar concentration of BP-free shrimp homogenate. Replicate samples, each consisting of 50–100 mg of shrimp, were taken from both cultures at 0, 8, 12, 15, 18, 24, 36, 48, 72, 144, 240, and 408 h p.i. The sampled shrimp were purged in clean culture water for 1 h, sieved, and then briefly blotted (3–5 s) with absorbent paper to remove excess external moisture. The samples were then weighed, placed in polypropylene vials, frozen in liquid nitrogen, and stored at 270°C for later use in phase 2 of the experiment. Samples collected at 24 h p.i. were inadvertently lost during

processing. Fresh squashes of 15–20 larvae from each phase 1 sampling period were examined for patent BP infections.

In phase 2, nauplii obtained from OI were reared to a mysis I stage and then stocked into 42 separate 1-liter Imhoff cones. Seven treatments, each consisting of six cones containing 100 larvae, were inoculated with approximately 10 mg per liter of infective material prepared by homogenizing the samples collected at 0, 8, 12, 15, 18, 72, and 408 h p.i. from BP-inoculated shrimp during phase 1 of the experiment. A subsample of larvae (n = 10) from three cones in each treatment were used to monitor the prevalence of patent infections at 18, 36, 68, and 144 h p.i. The other three cones in each treatment were used to determine mortality at the termination (144 h p.i.) of the bioassay.

Infectivity Experiment Two

The procedures used during phase 1 of the second infectivity experiment were the same as those described for the first experiment, except nauplii were obtained from OI, and samples were collected at 0, 4, 8, 12, 15, 18, 24, 40, 53, 72, and 168 h p.i. A subsample (n = 10–15) of shrimp from each sampling period were diagnosed for patent BP-infections by examination of fresh squashes and 8–17 shrimp from each sampling period between 0 and 24 h p.i. were diagnosed for BP using a PCR-based procedure (Wang et al., 1996). Results from the PCR-based diagnostics of this group of shrimp have been reported elsewhere (see Stuck and Wang, 1996; Table 1). In phase 2 of the second experiment, nauplii obtained from GCRL were reared to PL1 and then stocked into 24 1-liter Imhoff cones. Eight treatments, each consisting of three replicate cones containing 75 postlarvae, were inoculated with approximately 10 mg per liter of infective material prepared from homogenates of samples collected at 0, 4, 8, 12, 15, 18, 24, and 72 h p.i. during phase 1 of the second experiment. The bioassay was terminated at 144 h p.i., at which time mortality and the prevalence of patent infections in a subsample (n = 20) of postlarvae from each cone was determined. ANOVA was used to test for significant differences in the mortality data obtained at the termination of each trial.

Results

Infectivity of NOVPs

In the preliminary study, cultures inoculated with either supernatant or whole homogenates prepared from patently infected shrimp developed an infection during the 7-day bioassay (Fig. 1). The infectivity of inocula centrifuged at 40,000g decreased significantly as compared to the other infective treatments (chi-square statistical test $P \leq 0.01$). Patent BP infections were not detected in the negative control.

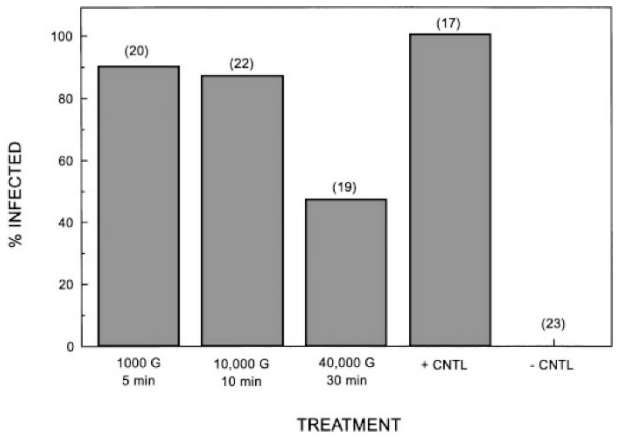

Figure 1. Infectivity of inocula prepared from whole homogenates of BP-infected tissues (+ cntl), uninfected tissues (– cntl), and supernatants of BP-infected tissues obtained at different centrifugal forces. Numbers in parentheses represent number of shrimp examined for patent BP infections.

Infectivity Experiments

In phase 1 of the first infectivity experiment, patent infections were first detected in some shrimp at 24 h p.i., and developed in all shrimp examined by 240 h p.i. (Fig. 2). Inocula prepared with shrimp tissues collected 12 h p.i. or later from phase 1 caused patent infections in the second phase of the experiment (Table 1). The initial appearance and prevalence of patent infections in phase 2 was prolonged when the inoculum was prepared from prepatently infected shrimp tissue. Inocula prepared from the 18-h p.i. shrimp tissue caused patent infections in only one of the three replicate cones in that treatment. There was a significant difference (ANOVA, $P \leq .01$) in the mortality caused by the different inocula. Mortality was highest in the treatment exposed to inoculum prepared from 72-h p.i. shrimp tissue.

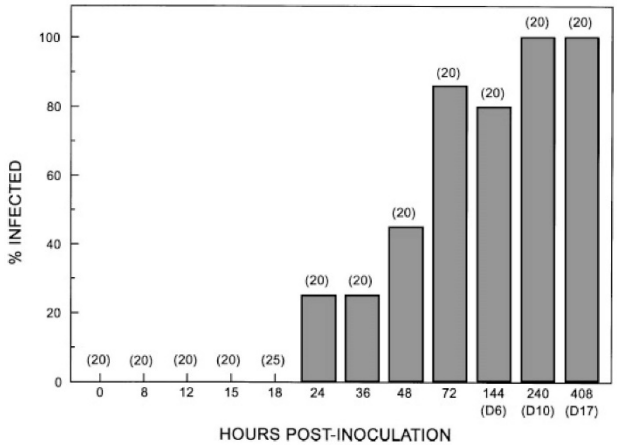

Figure 2. Infectivity experiment 1, phase 1. Prevalence of infection among mysis stage larvae during the course of an experimental BP-infection. Numbers in parentheses represent number of shrimp examined for patent BP infections.

Table 1. Infectivity Experiment 1, Phase 2

Source of inoculum (time p.i.)	Prevalence of infection in % at				Percentage of mortality
	18 h	36 h	68 h	144 h	144 h
	0	0	0	0	27
0 h	0	0	0	0	12
	0	0	0	0	9
	0	0	0	0	20
8 h	0	0	0	0	46
	0	0	0	0	19
	0	0	0	88	23
12 h	0	0	10	13	22
	0	0	0	81	36
	0	0	10	100	41
15 h	0	0	50	100	42
	0	0	30	100	41
	0	0	0	0	18
18 h	0	0	0	0	21
	0	10	30	100	66
	10	100	100	100	91
72 h[a]	10	100	100	100	82
	20	100	100	100	80
	0	100	88	100	52
408 h[a]	10	90	100	100	72
	20	100	100	100	51

Note: Prevalence of infection (n = 10 shrimp per replicate) and 144 h p.i. mortality among replicate cultures of mysis stage larvae. Cultures were inoculated with BP-infected tissues that were collected from different times postinoculation (p.i.) during the first phase of the experiment.

a. Inoculum prepared from patently infected shrimp.

In phase 1 of the second experiment, prepatent infections were detected by PCR-based diagnostics 12–15 h p.i. Patent BP infections were first observed at 24 h p.i. and the prevalence of infection reached 100% by 72 h p.i. (Fig. 3). Inocula prepared from shrimp collected at 15 h p.i. or later caused patent infections in some of the replicate cultures in phase 2 of the second experiment (Table 2). The observed 144-h p.i. mortality was highest in the group inoculated with material taken at 12 h p.i.; however, differences among groups were not significant (ANOVA, $P \leq 0.01$).

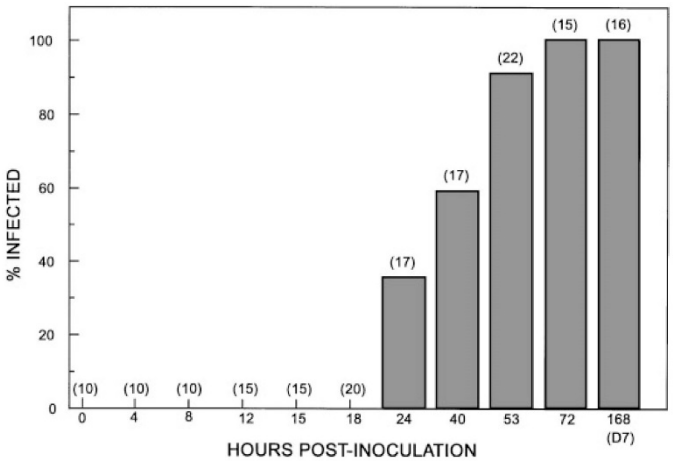

Figure 3. Infectivity experiment 2, phase 1. Prevalence of infection among mysis stage larvae during the course of an experimental BP-infection. Numbers in parentheses represent number of shrimp examined for patent BP infections.

Discussion

In the later stages of replication in BP, mature newly assembled virions become occluded in a proteinaceous matrix which forms the viral polyhedra characteristic of a patent infection (Couch, 1989, 1991). Viral inocula prepared from patently infected tissues containing occluded viral particles (OVPs), as well as OVPs purified on a sucrose gradient, produce infections when administered *per os* (Overstreet et al., 1988; Stuck, unpublished data). The infectivity of inocula prepared from prepatently infected tissues depends on the ability of free, preoccluded, or nonoccluded virus particles (NOVPs), to cause infections when administered *per os*. Summers (1977) reported that supernatants recovered from low speed centrifugation of BP-infected tissue homogenates contained only NOVPs. Our ability to produce experimental infections using the supernatants obtained from both low and high speed centrifugation of BP-infected tissue homogenates supports previous observations on the infectivity of NOVPs in *P. vannamei* (see Overstreet, 1994). Wood et al. (1993) reported that NOVPs of the insect baculovirus Ac-MNPV are highly infectious when administered *per os* to neonate larvae of *Trichoplusia ni*.

The time course for development of BP infections in *P. vannamei* observed in the first phase of both infectivity experiments conducted during this study is similar to previous observations for shrimp and insects. Bruce et al. (1994) and Stuck and Wang (1996) reported that patent BP infections were first detectable 18–24 h p.i., and prepatent infections, diagnosed by molecular methods, were detectable as early as 12 h p.i. Granados and Williams (1986) reported that virogenic stroma and progeny virus in many species of insects first appear 8 to 12 h p.i. and viral occlusion bodies develop 16 to 24 h p.i.

Table 2. Infectivity Experiment 2, Phase 2

Source of inoculum (time p.i.)	Prevalence of infection in % at 144 h	Percentage of mortality at 144 h
	0	8
0 h	0	0
	0	7
	0	20
4 h	0	15
	0	16
	0	17
9 h	0	1
	0	5
	NA	NA
12 h	0	23
	0	89
	45	12
15 h	0	15
	45	0
	0	5
18 h	5	17
	0	17
	100	15
24 h[a]	100	16
	90	9
	100	21
72 h[a]	95	16
	90	19

Note: Prevalence of infection (n 5 20 shrimp per replicate) and 144 h p.i. mortality among replicate cultures of postlarval shrimp. Cultures were inoculated with BP-infected tissues that were collected from different times postinoculation (p.i.) during the first phase of the experiment. NA, data not available due to loss of replicate culture.

a. Inoculum prepared from patently infected shrimp.

Results obtained from the second phase of both infectivity experiments conducted during this study showed that the time required for establishment of a patent BP infection is influenced by the stage of development of the virus in the inoculum. Differences in infectivity of inocula prepared from prepatently and patently infected tissues observed during this study are probably related to the replication cycle of the virus. Couch (1989, 1991) proposed a time sequence of replication events for BP that paralleled those of insect baculoviruses. Recent investigations by Bruce et al. (1994) and Stuck and Wang (1996) on replication of BP in shrimp support Couch's assumptions. In the present study, the time p.i. that BP-infected tissues first became infective corresponded with the appearance and envelopment (12–16 h p.i.) of recently replicated nucleocapsids in insects as reported by Granados and Williams (1986).

In our study, inocula prepared from patently infected tissues consistently produced infections, whereas inocula prepared from prepatently infected tissues caused infections in some but not all of the replicate cultures within the individual time periods assayed. Tissues known to contain nonoccluded virus (12–18 h p.i.), as determined by PCR-based diagnostics, failed to produce patent infections in several groups of mysis stage and postlarval shrimp. A possible cause for the apparent inconsistent infectivity of prepatently infected tissues may be related to a dose response. Overstreet (1994) reported that the dose of viral material used in an experimental BP infection can significantly influence the infectivity of an inoculum. He described a threshold effect in which a small dose of a viral inoculum was not infective, whereas a 103 dose of the same inoculum was highly infective. Inocula prepared from prepatently infected tissues may contain a low number of NOVPs that is near the threshold value that can cause an infection. In insect baculoviruses, the production of patent viral infections is also known to be affected by the initial dose or multiplicity of infection (m.o.i.) of a viral inoculum (Brown and Faulkner, 1975). In our study, the m.o.i. of inocula prepared from prepatently infected tissues (,12 h p.i.) may be insufficient to produce infections.

The low and inconsistent infectivity of inocula prepared from prepatently infected tissues might also be attributed to stability of NOVPs during prolonged storage in the freezer. The viral material collected during the first phases of both infectivity experiments was stored at 270°C for 4 to 10 months before it was used during the second phase bioassays. Although Overstreet (1994) found no significant relationship between virulence of patently infected tissues and the length of time that the tissue was frozen, the stability and infectivity of recently replicated NOVPs of BP is unknown. Jarvis and Garcia (1994) found that frozen insect baculoviruses OPVs retain their infectivity during long-term storage as long as they are not exposed to light. However, Shapiro and Ignoffo (1969) observed a significant loss of infectivity of enveloped NOVPs of the insect baculovirus HzSNPV after 15 days at 5°C. NOVPs of BP when used as a *per os* inoculum may also be less stable in an aqueous environment than OVPs.

The apparent prolonged developmental period for the virus we observed when using prepatently infected tissues as an inoculum may be due to a dose response and not to low infectivity of NOVPs. An inoculum prepared from prepatently infected tissues may contain sufficient NOVPs to initially cause infections in only a few individuals in a culture. These few infected individuals, which may not be represented in the small subsamples used for viral diagnosis, can then serve as a source of infection for other shrimp in the culture. After several rounds of replication and transmission, each typically requiring 24–72 h, the entire culture may become infected. Overstreet (1994) has reported that a single infected shrimp can, over a period of a few days, infect many or all of the other shrimp in a container. He also speculated that the lengthy prepatent periods observed in shrimp that were inoculated with tissues with long standing BP infections was due to the small numbers of NOPVs present in these tissues.

Mortality resulting from BP infection appears to be influenced by both host age when initially exposed to BP and the stage of viral development in the tissues used to prepare an inoculum. Although inocula prepared from prepatently (≥15 h p.i.) and patently infected tissues both produced infections in postlarval shrimp, there was no consistent difference

in the mortality caused by these different inocula. The high 144-h mortality observed in the group of postlarvae inoculated with 12 h p.i. tissue was likely due to a temporary loss of aeration to replicate cones in that group. Although the prevalence of infection among some groups of mysis stage larvae inoculated with prepatently infected tissues was 100%, 144-h mortality in these groups was significantly lower than in larvae that were inoculated with patently infected tissues. These differences may simply reflect the extended period required for establishment of patent infections when using prepatently infected tissues as an inoculum and do not necessarily indicate reduced pathogenicity of NOVPs compared to OVPs.

The pathogenic effects of BP have been shown to be age-dependent (Overstreet et al., 1988; LeBlanc and Overstreet, 1990); larvae and early postlarvae are more susceptible to viral induced mortality than older shrimp. Previous studies (Bruce et al., 1994; Stuck and Overstreet, 1994; Stuck and Wang, 1996) have reported that larvae and young postlarvae typically develop a high prevalence of infection by 72 h p.i. Kelly et al. (1978) reported that maximum titers of an insect baculovirus from *Heliothis armigera* developed 72 h p.i. and then gradually decreased until 144 h p.i. BP appears to be most pathogenic to larvae that have been exposed to inocula prepared from tissues that had recently developed a high prevalence of patent infections, or about 72 h p.i.

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