

# Cloning shrimp *Baculovirus penaei* DNA and hybridization comparison with *Autographa californica* nuclear polyhedrosis virus

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## ABSTRACT

A significant degree of specific homology between *Baculovirus penaei* (BP) - from infected shrimp feces and hepatopancreas - and *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) of insects was observed by dot blot hybridization. A genomic library of BP was constructed and some clones showed partial homology with AcMNPV DNA. Two of these clones were further characterized and one of them hybridized specifically with baculovirus DNA. This baculovirus DNA may be employed as a probe for detecting BP infected shrimp.

## INTRODUCTION

The Baculoviridae include a variety of rod-shaped viruses with covalently closed DNA (size: 88 to 153 kbp) that typically infect arthropods (Blissard and Rohrmann, 1990). Most published information on baculoviruses is related to those that infect insects. Insect baculoviruses, such as *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV), have been well studied in the past decade because of their importance in pest control programs and, more recently, as vectors for gene expression systems (Luckow and Summers, 1988).

*Baculovirus* infections in penaeid shrimp have caused much concern in commercial shrimp farms. *Baculovirus penaei* (BP), the most important cause of baculovirus disease in penaeid shrimp in the Americas, was first reported from *Penaeus duorarum* in the Gulf of Mexico (Couch, 1974a). Since then, the number of

reports of host species and geographic range of BP has increased considerably (Lightner and Redman, 1991; Lightner, 1992).

Both BP and AcMNPV are type A baculoviruses, or nuclear polyhedrosis viruses (NPV), which contain several virus particles (virions) embedded within a proteinaceous (polyhedrin) occlusion body. Type A baculoviruses are grouped according to the number of nucleocapsids per envelope. BP has a single nucleocapsid per envelope and is, therefore, referred to as SNPV, while *A. californica* nuclear polyhedrosis viruses have more than one (multiple) and are referred to as MNPV.

BP causes serious epizootics during the larval and post-larval life stages, which often lead to high cumulative mortality rates (Lightner *et al.*, 1985; Lightner, 1988). Diagnosis is made by demonstration of one or more tetrahedral occlusion bodies within the hypertrophied nuclei of the epithelial cells of the hepatopancreatic tubules in slide or squash preparations of shrimp larvae (Couch, 1974b; Lightner *et al.*, 1985). Occlusion bodies are eventually eliminated along with feces after lysis of infected hepatopancreatic cells. Therefore,

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checking fecal pellets for the presence of occluded bodies is an important diagnostic procedure to detect and eliminate adults (broodstock) carrying virus in some commercial farms.

Our knowledge regarding DNA from BP is limited. Summers (1977) concluded from electron microscope studies that the BP genome is a circular molecule with approximately 100 kbp. He also suggested that some cross immunoreactivity might exist between the polyhedrin protein of BP and AcMNPV. More recently, Bruce *et al.* (1991) published a detailed methodology for BP purification and DNA extraction.

## MATERIAL AND METHODS

### Shrimp specimens and feces

Fresh frozen adults of *Penaeus vannamei* were obtained from a commercial shrimp farm located in the state of Rio Grande do Norte, Northeastern Brazil and stored at  $-20^{\circ}\text{C}$  upon arrival at the laboratory in São Paulo. The hepatopancreas was removed and kept separately.

Shrimp feces were collected *in loco* by siphoning fecal pellets from maturation tanks with suspected BP infected *P. vannamei* broodstock. Infection was confirmed after light microscopy examination of typical BP occlusion bodies in fecal samples. Feces were immediately frozen, shipped to São Paulo and kept at  $-20^{\circ}\text{C}$  until needed.

### BP purification and DNA extraction

The virus was purified from whole hepatopancreas and subsequently DNA was extracted according to Bruce *et al.* (1991). Minor modifications were introduced for the purification of BP from feces. Feces were resuspended in TN buffer (0.01 M Tris-HCl, 0.10 M NaCl, pH 8.0) and filtered through a series of 200  $\mu\text{m}$  and 80  $\mu\text{m}$  mesh nylon screens. After centrifugation (4,000 rpm, 10 minutes), the virus was further purified by CsCl gradient as described by Bruce *et al.* (1991). DNA was extracted from purified nucleocapsid with phenol-chloroform and ethanol precipitated.

### DNA cloning

BP genomic DNA was restriction digested with *EcoRI*, *BamHI* and *PstI*, used singly or in combination of pairs and cloned in pBluescript SK+ plasmid (pBISK+, from Stratagene), using a standard procedure (Sambrook *et al.*, 1989).

## Dot blot hybridization

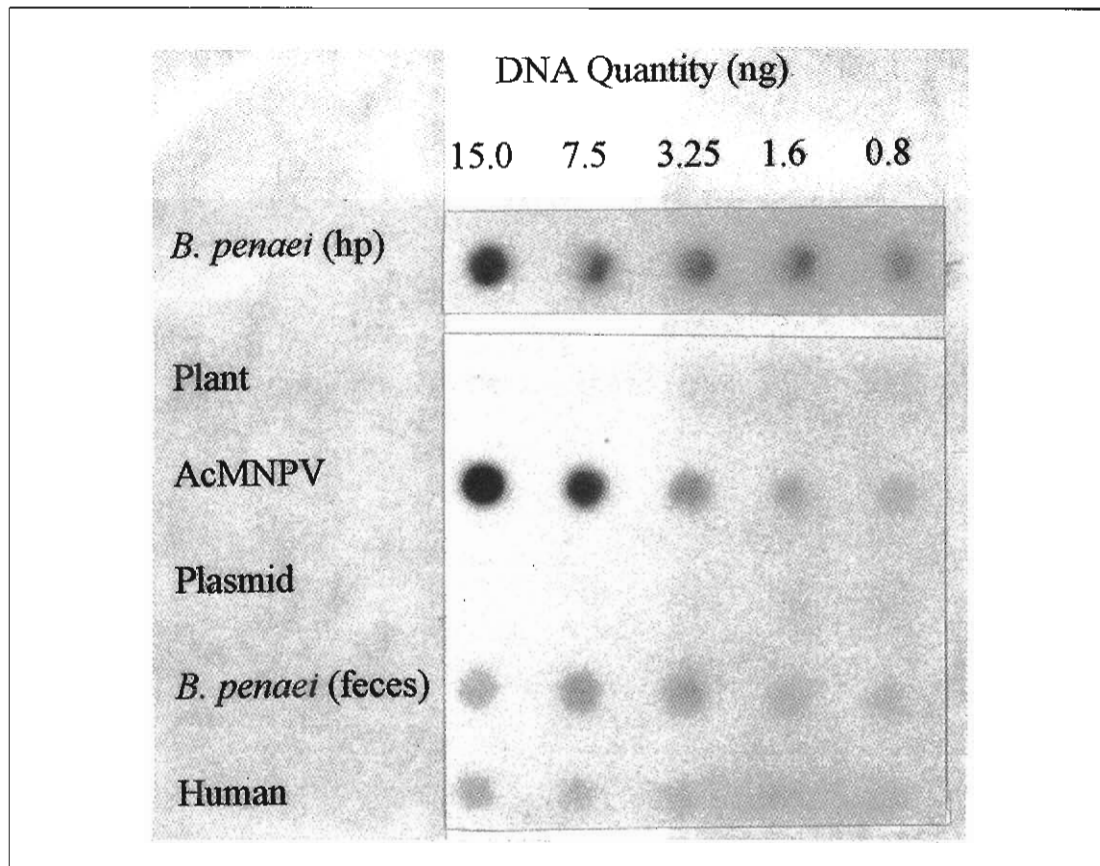
The DNA was immobilized on membranes according to a procedure described by Kafatos *et al.* (1979), with minor modifications. The DNA samples (400  $\mu\text{l}$ ) were denatured by adding 3 M NaOH (40  $\mu\text{l}$ ) at  $65^{\circ}\text{C}$  for 30 minutes and then neutralized with 2 M  $\text{NH}_4\text{OAc}$  (400  $\mu\text{l}$ ).

A nylon filter (Hybond, Amersham), presoaked in 1 M  $\text{NH}_4\text{OAc}$ , was assembled in a Hybri-dot (BRL) manifold and rinsed thoroughly under vacuum with 1 M  $\text{NH}_4\text{OAc}$ . A 250  $\mu\text{l}$  aliquot of each of the denatured DNAs was immobilized onto the filter. The filters were then air dried and baked at  $80^{\circ}\text{C}$  for two h. The filters were prehybridized for 4 h at  $65^{\circ}\text{C}$  using a hybridization mixture (5x SSC: 0.15 M NaCl, 0.015 M sodium citrate; 1% defatted milk; 1% SDS). A probe of whole AcMNPV DNA was radiolabeled to a high specific activity ( $10^8$  cpm/ $\mu\text{g}$  DNA) by the multiprime DNA labeling method as described by Feinberg and Vogelstein (1983). The filters were hybridized for 24 hours and washed in 2x SSC at room temperature (2x 5 minutes) followed by two 30 minutes washes at  $65^{\circ}\text{C}$  in 2x SSC, 1% SDS. They were then exposed overnight to X-ray film at  $-70^{\circ}\text{C}$ . The autoradiograms was scanned with an Ultra XL-Pharmacia LKB densitometer. The relative homology between the different DNAs and AcMNPV was estimated using self-hybridized AcMNPV DNA as a standard.

## RESULTS

Inspection of all distinct bands obtained after purification through a CsCl gradient supports previous observations by Bruce *et al.* (1991). Small amounts of BP occlusion bodies could be recovered from the CsCl gradient at a density of 1.33 g/ml, either from feces or whole hepatopancreas. The BP genomic DNA was extracted and analyzed by agarose gel electrophoresis. This DNA was consistently degraded (not shown).

In order to evaluate the homology between BP and AcMNPV genomic DNA, a dot blot analysis was carried out using AcMNPV DNA as a probe (Figure 1). Densitometry of the autoradiograms allowed the quantification of the hybridization signals of the DNA samples in relation to AcMNPV DNA. Four replicates were made and 15.0 and 7.5 ng of DNA were used, so that bias and deviation due to background could be avoided. AcMNPV DNA hybridizes strongly with BP DNA, either extracted from feces or hepatopancreas, with a similar intensity (Figure 1 and Table I). There was also a weak hybridization with human DNA, but no significant hybridization with plant (*Arabidopsis thaliana*) DNA or



**Figure 1** - Hybridization of AcMNPV genome with DNA from different origins. Samples of genomic DNAs were denatured and immobilized on nylon filters. Filters were hybridized against  $^{32}\text{P}$ - labeled AcMNPV DNA. (hp) BP extracted from shrimp hepatopancreas. Plant DNA is from *Arabidopsis thaliana*. Plasmid is pBISK+ from Stratagene.

with bacterial plasmid. The results indicate some specific homology between BP and AcMNPV DNA.

The BP DNA purified from shrimp hepatopancreas was digested with restriction enzymes and ligated with pBluescript SK+ vector. These samples were used to transform *E. coli* (strain XL1-Blue) and approximately 220 clones containing inserts were screened. The DNA from most of these clones were extracted and analyzed in agarose gels. As a result, we obtained a partial genomic library from BP DNA with 85 independent and distinct clones, with an average of approximately 2 kbp per inset.

Several clones of this BP genomic library hybridize with AcMNPV DNA (Figure 2). These clones were further analyzed, in order to check for homology specificity. Both clones pBac9 and pBac19 hybridized with AcMNPV (Figure 3) and BP DNAs, but while pBac19 seems to be specific for the baculovirus genome, pBac9 hybridized strongly with human and shrimp DNA. There was no hybridization of any of the clones tested with plant DNA. Considering the insert from pBac9 (~ 400 bp) as a single DNA sequence, the

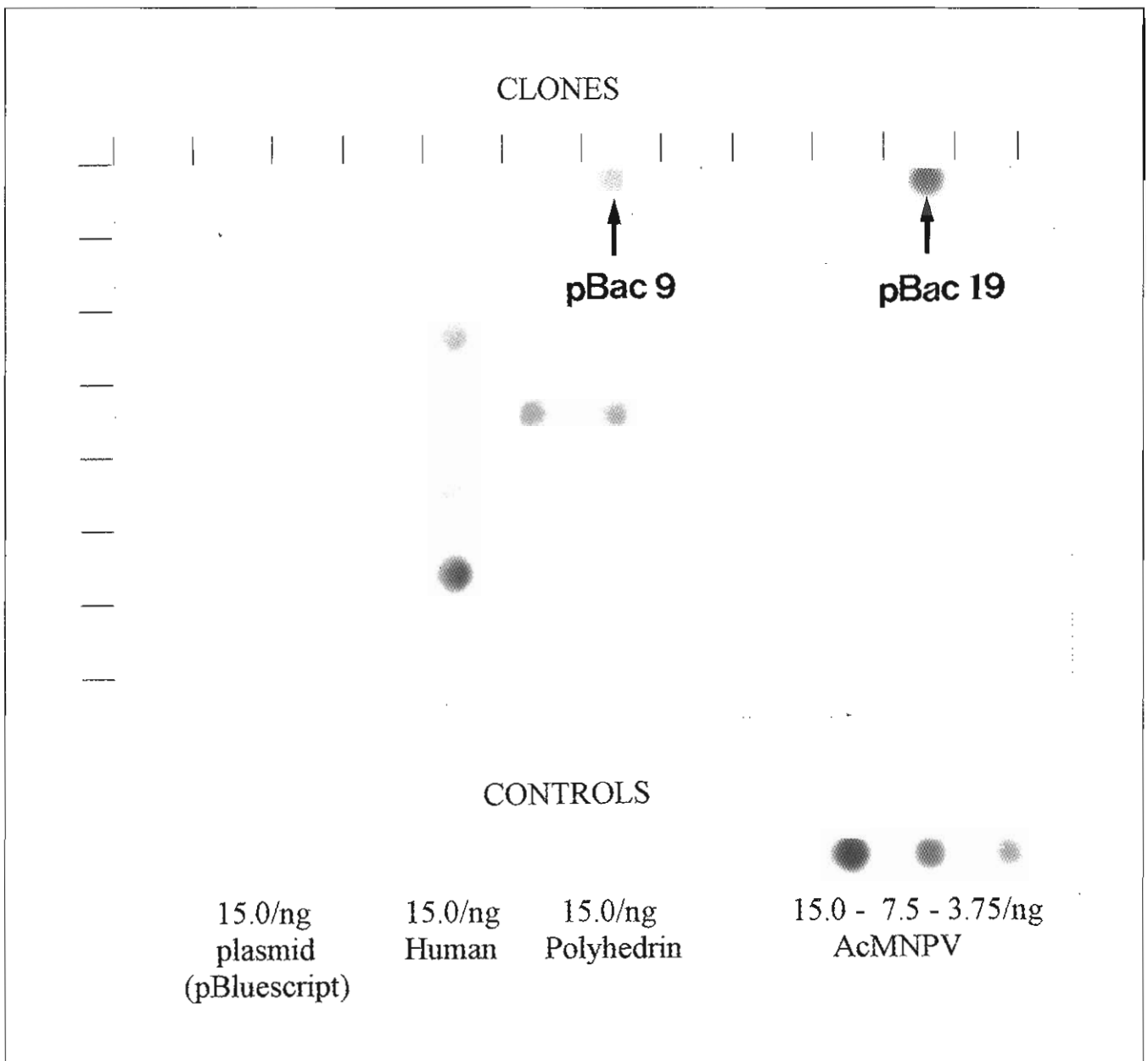
**Table I** - Hybridization of AcMNPV DNA with BP DNA.

DNA source	Hybridization (%)*		Average (%)
	DNA quantity		
	15.0 ng	7.5 ng	
BP Hp**	17.0 ± 3.3	28.0 ± 5.2	23.2 ± 6.0 <sup>a</sup>
Feces	-	24.0 ± 5.4	
Human	10.0 ± 4.0	8.0 ± 5.0	9.0 ± 4.8 <sup>b</sup>
Plant ( <i>A. thaliana</i> )	0.7 ± 0.3	0.8 ± 0.5	0.8 ± 0.3 <sup>c</sup>
plasmid (pBISK+)	1.0 ± 0.4	1.0 ± 0.3	0.9 ± 0.3 <sup>c</sup>

\*Results of four replicates, obtained by densitometry of experiments like the one shown in Figure 1.

\*\*BP extracted from hepatopancreas (HP) or feces.

a,b,c are statistically different ( $p < 0.01$ ), according to the Student-Newman-Keuls test.



**Figure 2** - Analysis of hybridization of AcMNPV genome with different clones from the partial BP library. DNA samples of BP library clones were denatured and immobilized on nylon filter. The filter was hybridized against <sup>32</sup>P- labeled AcMNPV DNA. The bottom lane shows control DNAs from different origins, as indicated. Plasmid is pBISK+. The seven other lanes have 84 independent clones for the BP library (15.0 ng/dot). Arrows indicate the clones which were further analyzed (pBac 9 and pBac 19).

quantification of the data presented in Figure 3b indicates that there are approximately 1,000 to 10,000 copies of this sequence in shrimp or human DNA, while 1 to 10 copies are present in the BP genome.

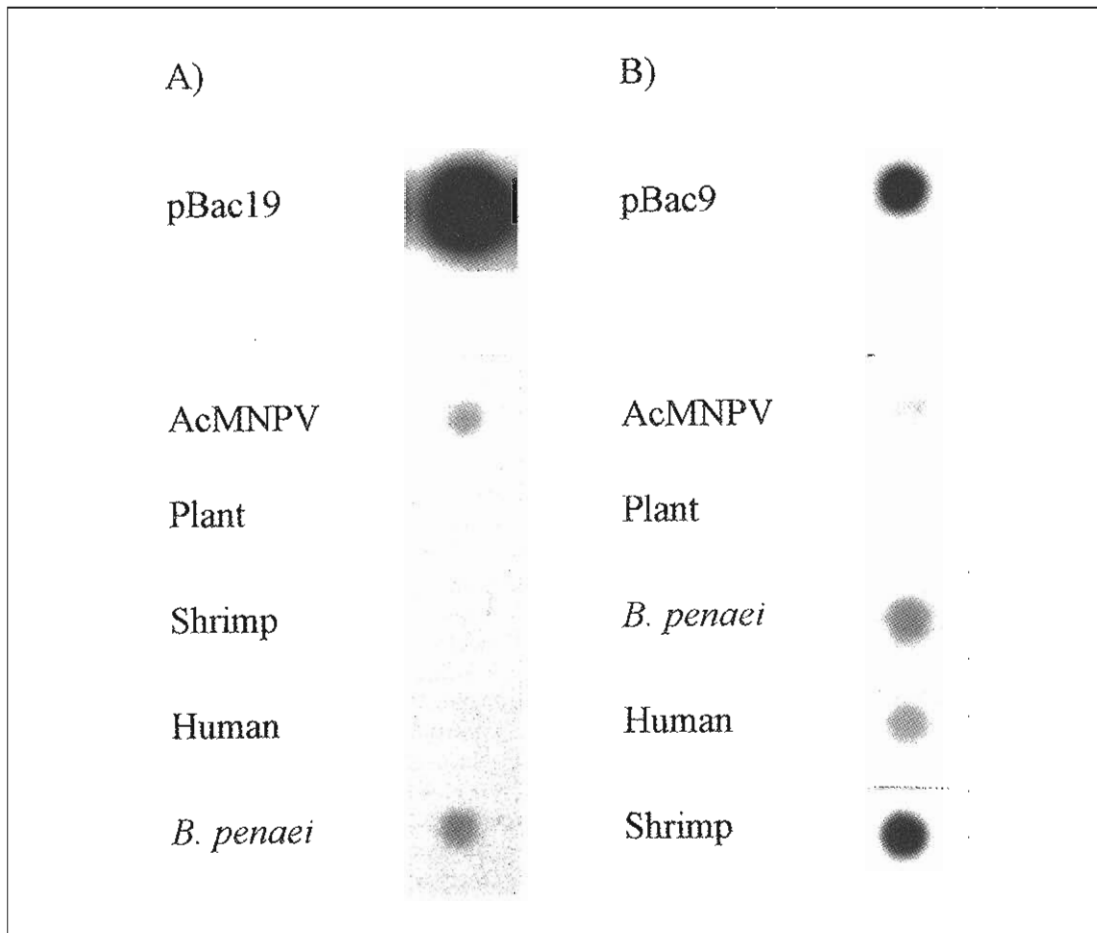
## DISCUSSION

The absence of an *in vitro* system for the propagation of BP requires that this virus be collected from infected shrimp specimens. Although BP infection in cultured marine shrimp has been reported from some

Brazilian shrimp farms (Bueno *et al.*, 1990; Lightner and Redman, 1991), large numbers of BP infected specimens are not readily available.

DNA degradation prevented the construction of the viral restriction map and determination of the actual size of BP genome. Such degradation was also observed by Bruce *et al.* (1991) and it has been suggested to be due to the action of nucleases present in the samples.

Homology among different insect baculoviruses has been reported. Jewell and Miller (1980) reported a non significant homology (less than 0.2%)



**Figure 3** - Analysis of the specificity of DNA hybridization of two different clones from the BP DNA library. Samples of DNA (15.0 ng) were denatured and immobilized on nylon filters. Filters were hybridized against  $^{32}\text{P}$ -labeled pBac19 DNA (A) and pBac9 (B) DNA. Plant DNA is from *Arabidopsis thaliana*.

between SNPVs (two viruses) and MNPVs (four viruses), but when only MNPVs were considered the homology among them increased to approximately 1%. Possee and Kelly (1988) reported a 70% homology between *Panolis flammea* NPV (PfnNPV) and *Mamestra brassicae* NPV (MbNPV) but only 2% when each of these were compared to AcMNPV.

An extensive homology study on insect baculovirus (10 MNPVs, 3 SNPVs, 4 Granulosis viruses and 1 non occluded virus) using the dot blot technique was reported by Smith and Summers (1982). According to these authors, the homology among MNPVs varied from less than 1% to 12%, except between *Heliothis armigera* NPV (HaNPV) and MbNPV for which it was 90%.

The significant hybridization (23%) between BP and AcMNPV genomes (Figure 1, Table I) suggests that a homology between both baculoviruses can be found, regardless of the taxonomic distance between their respective hosts. The differences between our results and those reported by others may be due to different hybridization protocols. Our experiments were made in the absence of carrier DNA. Maeda *et al.* (1990) also

reported a high homology (above 50%) for two insect baculoviruses that were different according to phenotype (multiple and single NPV), host specificity and restriction map analysis. Maeda *et al.* (1990) did not make use of carrier DNA and the stringency of their hybridization protocols was similar to that used here. It is possible that carrier DNA (normally vertebrate DNA) has some homology with AcMNPV DNA and it traps the probe during hybridization. This is in agreement with the significant hybridization of AcMNPV and human DNA shown in Figure 1 and Table I.

Several clones from the BP genomic library hybridized with AcMNPV DNA. These clones may represent genomic segments of BP homologous to some DNA segments of AcMNPV. It is evident that detailed molecular characterization is required in order to establish the relatedness between the DNA of these two viruses. Among them, at least one of these clones (pBac9) may contain a DNA sequence which is also present, in a repetitive manner, in human and shrimp DNA. Another clone, however, showed a very strong specificity hybridization with baculovirus DNA. This

feature makes this clone, pBac19, a potential DNA probe for diagnosis of BP infection in fecal pellets from broodstock shrimp.

Although the levels of homology between BP and AcMNPV, as presented in hybridization methods, are not definitive, the data indicate that both baculoviruses share homology between gene segments, despite the taxonomic divergence between their hosts.

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## RESUMO

*Baculovirus penaei* (BP) infecta camarão marinho causando importantes perdas em fazendas comerciais de camarão nas Américas. Estamos estudando este vírus ao nível molecular de modo a obter dados comparativos com outros baculovírus. Um nível significativo de homologia entre genomas de BP - proveniente de hepatopâncreas e fezes de camarão - e de baculovírus de inseto (AcMNPV) foi observado por hibridação. Uma biblioteca genômica de BP foi construída e alguns clones mostraram homologia parcial com DNA de AcMNPV. Dois desses clones foram melhor caracterizados e um deles hibrida especificamente com DNA de baculovírus. Este clone pode ser empregado como sonda de DNA na detecção de camarão infectado com BP.

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