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In situ detection of Australian gill-associated virus with a yellow head virus gene probe

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Abstract

A digoxigenin-labeled gene probe for yellow head virus (YHV) was used to detect gill-associated virus (GAV) in Penaeus monodon from Australia via in situ hybridization. In GAV-infected shrimp, positively reacting tissues included: lymphoid organ, gills, antennal gland, and cuticular epithelium of the stomach. This demonstrates that a YHV probe can be used as a diagnostic tool for GAV and supports previous suggestions that these two viruses are closely related. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Yellow head virus (YHV) is a rod-shaped, enveloped RNA virus, first observed in Thailand in the early 1990s. It has caused significant mortality in populations of Penaeus monodon (Chantanachookin et al., 1993; Wongteerasupaya et al., 1995; Flegel et al., 1995). Clinical signs of affected shrimp characteristically include a discolored, yellow cephalothorax, and the virus infects primarily hemocytes, connective tissue, and tissues of the lymphoid organ and gills (Lightner, 1996). A YHV-like virus, named gill-associated virus (GAV), has been reported in Australia (Spann et al., 1997). GAV is
very similar to YHV; it causes nearly 100% mortalities and has the same primary target tissues. However, GAV-infected *P. monodon* do not have the yellow cephalothorax commonly found in association with YHV-infection. Comparison of three regions of ORF1b, total 1.7 kb, of YHV and GAV showed 80–85% homology in nucleotide sequence (Cowley et al., 1999). Recently, the sequence analysis of GAV genome revealed that GAV is related to arteri- and coronaviruses (Cowley et al., 2000).

Traditional methods for the diagnosis of YHV and GAV are based on clinical signs and histopathology. These methods are not highly specific, since a number of viruses can cause similar effects. Recently, in situ hybridization has been developed for both YHV and GAV (Tang and Lightner, 1999; Spann et al., unpublished data). Here we report that, based on the high degree of homology in their nucleotide sequences, a YHV gene probe can also be used to detect GAV.

### 2. Materials and methods

#### 2.1. GAV-infected *P. monodon*

Healthy sub-adult *P. monodon* (12–20 g) were injected with GAV tissue homogenate prepared as previously described from moribund *P. monodon* collected from a farm in northern Queensland (Spann et al., 1997). Juvenile specific-pathogen-free *P. vannamei* (1–2 g) were injected with a tissue homogenate prepared from *P. monodon*, which were collected in Queensland, Australia during 1995. The shrimp were kept at \(-70^\circ\text{C}\). The homogenate was prepared by homogenizing shrimp tissues in buffer (0.02 M Tris–HCl, pH 7.4, 0.4 M NaCl; 1 g/10 ml), and it was then centrifuged at 3000 \(\times\) g for 30 min. The supernatant was diluted with sterile 2% saline to generate an inoculum. A volume of 50 \(\mu\)l of inoculum was injected into each shrimp. The infected shrimp were fixed with RF-fixative (Hasson et al., 1997) when gross signs of disease, such as lethargy and red body coloration, were observed. The fixed shrimp were then processed, embedded in paraffin, and sectioned according to standard methods.

#### 2.2. YHV gene probe labeling and in situ hybridization

The nucleotide sequence of the YHV probe has been deposited in GenBank (accession no. AF148846). The probe labeling with digoxigenin-11-dUTP and the subsequent in situ hybridization procedures used were in accordance with those described by Tang and Lightner (1999). Tissue sections (4 \(\mu\)m thick) from YHV-infected and specific-pathogen-free *P. vannamei* were used as positive and negative controls for in situ hybridization, respectively.

### 3. Results and discussion

A digoxigenin-labeled YHV gene probe was used for in situ hybridization with the GAV-infected *P. monodon*. The probe reacted to lymphoid organ that was necrotic and disorganized in tubule structure from the severe infection (Fig. 1A). The gills also reacted...
positively, with the reaction localized in the cytoplasm (Fig. 1B). In the stomach, a strong reaction was found in the cuticular epithelium, and a less intense response was seen in the underlying spongy connective tissue (Fig. 1C). For the antennal gland, the fibrous connective tissues reacted positively to the probe while the epithelial cells in the tubules did not (Fig. 1D).

Juvenile *P. vannamei*, injected with a tissue homogenate generated from moribund *P. monodon* collected in Australia in 1995, became moribund 6 days after injection, and only 33% of shrimp survived at day 14 after injection. The histological analysis showed a viral infection characteristic of YHV. Further in situ hybridization studies with a YHV gene probe showed that the positively reacting lymphoid organs had normal tubular structure; neither spheroids nor tubule disorganization were found (Fig. 2A). Positive reactions with this virus were also found in the gills (Fig. 2B), in the hemolymph within the hepatic artery (Fig. 2C) and in connective tissues. The hepatopancreas tubules did not react with the probe, but the surrounding connective tissues were positive (Fig. 2C). The reactions of muscle cells were negative (data not shown). Based on the virus origin, histopathology, in situ hybridization, and the fact that this virus is highly pathogenic, this YHV-like virus is likely to be closely related to GAV. These data showed that the presumed GAV can infect *P. vannamei* and cause mortality.

Fig. 1. In situ hybridization of tissues from GAV-infected *P. monodon* with a digoxigenin-labeled YHV gene probe. The dark blue precipitate indicates a positive reaction. (A) Lymphoid organ, (B) gills, (C) cuticular epithelium of stomach, (D) antennal gland. Scale bar = 10 μm.
Fig. 2. In situ hybridization of tissues from presumably GAV-infected P. vannamei with a digoxigenin-labeled YHV gene probe. The dark blue precipitate indicates a positive reaction. (A) Lymphoid organ, (B) gills, (C) hepatic artery. Scale bar = 20 μm.
In the past, the tissue distribution of shrimp viruses has relied on electron microscope (EM). However, EM analysis is highly technical and time-consuming and, thus, cannot be used for routine diagnosis. In contrast, in situ hybridization provides more specificity in that the positive reaction occurs only with target virus or closely related viruses. Also, the method is relatively easy to perform and can be used routinely by diagnostic laboratories. In this report, we demonstrated that a YHV probe, with 81% homology to the corresponding region of GAV (GenBank AF227196), can be used to detect GAV through in situ hybridization.

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