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NOTE

Detection of White Spot Disease Virus (WSDV) Infection in Shrimp Using *in Situ* Hybridization

Viruses are among of the most important pathogens in the crustacea, especially shrimp. Among various viruses of shrimp, white spot disease virus (WSDV) is demonstrated to be associated with high mortalities in the cultured shrimp, particularly in Asia (Woongteerasupaya *et al.*, 1996). The virus possesses a wide range of host specificity; it is pathogenic to several species of shrimp, such as *Penaeus monodon* (Fabricius), *Penaeus japonicus* (Bate), *Penaeus penicillatus* (Alcock), *Penaeus orientalis* (Kishinouye), *Penaeus merguiensis* (De Man), *Penaeus vannamei* (Boone), *Penaeus indicus* (Milne-Edwards), *Penaeus stylirostris* (Stimpson), and *Penaeus setiferus* (Linnaeus) (Lightner, 1996). The diseased shrimp infected with WSDV were characterized by hypertrophied nuclei in various tissues originated from meso- and ectoderm (Wang *et al.*, 1997). Currently, many diagnostic tools have been developed to detect the WSDV infection in the shrimp (Lo *et al.*, 1996; Durand *et al.*, 1997; Nunan *et al.*, 1997). In the present study, a novel approach for development of a diagnostic gene probe for WSDV of shrimp was derived, using the random amplified polymorphic DNA (RAPD) technique.

The WSD virus used in the present study was derived from a batch of juvenile *P. monodon* suffering from white spot disease, obtained from Pingtung in southern Taiwan. The stomach, gills, and lymphoid organ were removed from diseased shrimp, and homogenized at 4°C in TN buffer (0.2 M Tris-HCl, 0.4 M NaCl, pH 7.5). After being clarified 10 min at 2000*g*, the homogenized solution was laid onto 20–50% sucrose gradient and centrifuged for 1.5 h at 100,000*g*. The band from the sucrose gradient was collected and pelleted at 100,000*g* for 1 h, and resuspended in 0.5 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

The concentrated WSDV particles were diluted in solubilization buffer (50 mM Tris-HCl, 10 mM NaCl, 20 mM EDTA, 0.5% SDS, pH 8.0); proteinase K was added to a final concentration of 100 μg/ml, and then incubated at 56°C for 1 h. The DNA was then extracted by using the phenol-chloroform method and concentrated by using alcohol precipitation (Sambrook *et al.*, 1989).

Partial genomic fragments of WSDV were enriched by using the RAPD technique. For this reaction, approximately 0.5 ng virus DNA was used as template. The amplification procedures were carried out in a 100-μl reaction mixture containing 1× *Taq* polymerase buffer, 0.2 mM each dNTP, 2.5 units *Taq* polymerase, and 0.3 μg primer OPF-02 (5′-GAGGATCCCT-3′, Operon Technologies, Alameda, CA). A drop of mineral oil was added to cover the mixture. The amplification was performed in a PTC-100 thermal cycler (MJ Research Inc.) at 94°C for 5 min, and then 50 cycles (94°C 1 min, 30°C 1 min, 72°C 2 min), plus a final 5-min extension at 72°C after 50 cycles. The PCR products were analyzed in the 1.5% agarose gels containing ethidium bromide at a final concentration of 0.5 μg/ml and visualized under UV transilluminator. Many fragments were produced after the PCR reaction, and ranged from 200 to 1500 bp in size. A fragment of approximately 700 bp was subcloned into pGEM T plasmid. The clone designated as TS6 was chosen as a probe for the detection of the WSDV genome. Two primers, 6-2F, 5′-AGCAGAGGATGATATCGTAC-3′, and 6-1R, 5′-CAACACTATCATCACAGTCG-3′, were designed based on the nucleotide sequence of TS6. A nonradioactively labeled probe was prepared by PCR with WSDV DNA as template, with 6-2F and 6-1R as primers, and with substrate containing DIG-11-dUTP, by utilizing the procedures suggested by the manufacturer (Boehringer Mannheim).

DNA purified from WSDV, healthy shrimp, *P. monodon*-type baculovirus (MBV), pUC18 plasmid, a genomic clone of WSDV, and morphologically healthy, early stage WSDV infected shrimp were individually diluted, denatured, and dot blotted onto a nylon membrane followed by UV cross-linking. The membrane was then prehybridized with prehybridization solution at 42°C for 1 h, followed by hybridization with probe-hybridization solution for an additional 12 h. The detection was carried out using an alkaline phosphatase-conjugated anti-digoxigenin antibody and reactants, 5-bromo-4-chloro-3-indoyl phosphate (X-phosphate) and nitroblue tetrazolium (NBT). A positive reaction was recognized by the presence of a purple to dark purple precipitate in the nylon membrane. The results of dot blot hybridization shown in Fig. 1 revealed that 1 pg of WSDV DNA can be readily detected. No cross-reactivity was observed with uninfected shrimp DNA or *monodon*-type baculovirus (MBV). It was also found that no precipitate was observed when pUC18

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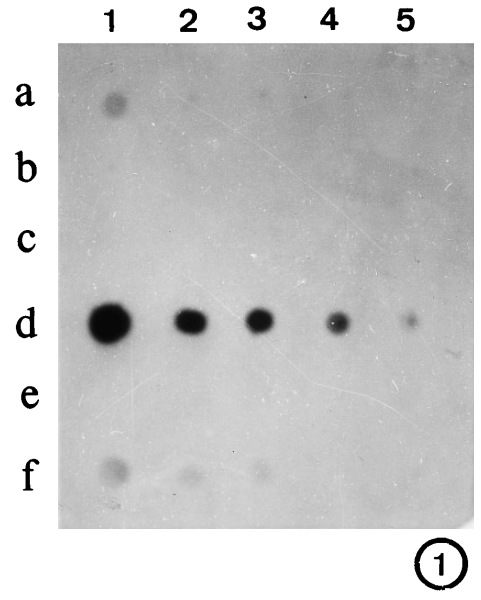

FIG. 1. Detection of different DNA dilution with WSDV DNA probe by dot blot hybridization. (a) DNA of early stage WSDV-infected giant tiger shrimp, 1 ng. (b) pUC18 plasmid, 1 ng. (c) DNA of healthy giant tiger shrimp, 100 ng. (d) Recombinant plasmid, TS6, 10 ng. (e) DNA of MBV, 100 pg. (f) DNA of WSDV, 100 pg. Numbers on the top indicate 10-fold serial dilution of DNA.

plasmid was utilized. The results shown in Fig. 1 also indicated that the presence of WSDV DNA at low levels could be detected in the diseased shrimp that was confirmed as a WSDV positive reaction by histopathological observation. In addition, the results of dot blot hybridization may suggest that the DIG-labeled WSDV genomic fragment can be used as a specific probe by using *in situ* hybridization.

The WSDV infected and uninfected shrimp were collected from the Tainan, Ilan, Pingtung, and Chiayi prefectures of Taiwan. The shrimp included *P. monodon*, *P. japonicus*, and *Metapenaeus ensis* (de Haan), and were originally determined to be infected with WSD virus by the presence of white spots on the cuticle. They were then fixed in Davidson's fixative or 10% formalin, and all histological processing followed the standard procedures as described in Bell and Lightner (1988).

Sections, 4–5 μm thick, were used for *in situ* hybridization using the DIG-11-dUTP-labeled probe according to the manual from the manufacturer (Boehringer Mannheim). The sections were deparaffined and rehydrated in a series of graded ethanol, followed by digestion with proteinase K (100 μg/ml) and then fixed in cold 4% formaldehyde. The sections were heated to 95°C for 10 min and allowed to hybridize for 12 h at 42°C in the probe hybridization solution containing 50% formamide, 1 μg/ml DIG-labeled probe, 0.2 mg/ml sonicated salmon sperm DNA, and 5% dextran sulfate, 4× SSC (0.6 M sodium chloride, 60 mM sodium citrate, pH 7.0). The detection was performed using an alkaline phosphatase-conjugated anti-DIG antibody and substrate-solution (NBT and X-phosphate). After counterstaining with 0.1% eosin Y, the sections were then dehydrated and mounted with Entellan (Merck), and then examined under bright field microscopy (Olympus MI-1).

The probe may be utilized for *in situ* hybridization to detect WSDV infection in various shrimp including *P. monodon*, *P. japonicus*, and *M. ensis* (data not shown). The results showed that the WSDV genome could be successfully detected in various tissues that originate from meso- and ectoderm of diseased shrimp, such as stomach, lymphoid organ, antennal gland, gills, nerve cord, and hematopoietic tissue (Fig. 2). Positive reactions may be recognized by a blue purple precipitate in the nuclei of the cell. The stronger precipitate could be observed in the cytoplasm of heavily infected shrimp. Our findings showed that the results of *in situ* hybridization were parallel to those obtained from histopathological observations. In contrast, no cross-reaction was found in the tissue of healthy shrimp. A negative reaction was observed in the epithelial cells of hepatopancreas tissue. The present results also showed that low levels of virus in shrimp at an early stage of infection were easily detected by using an *in situ* hybridization technique, even when the histopathological signs (H&E stain) of infection were not present. Additionally, the positive reactions of *in situ* hybridization were obtained in the hemocytes of heart from WSDV infected shrimps (Fig. 3) that were difficult to detect by classical methods.

The present study demonstrated that a novel method for development of the gene probe of WSDV was derived using the RAPD technique. The diagnostic probe could be used to detect the WSD virus in the infected shrimp using dot blot or *in situ* hybridization. No cross-reactivity was showed with uninfected shrimp DNA or *monodon*-type baculovirus (MBV). In addition, no significant difference was found for detection of different WSDV-infected shrimp such as the giant tiger shrimp (*P. monodon*), the kuruma shrimp (*P. japonicus*), and the greasy-back shrimp (*M. ensis*) using *in situ* hybridization. Furthermore, these results of diagnosis for WSDV infection in the shrimp were consistent with the previous studies that developed the gene probe using a traditional method (Durand *et al.*, 1997; Nunan *et al.*, 1997), which may suggest that the gene probe for detecting the WSDV of shrimp can be successfully developed by the RAPD method.

Many molecular probes for shrimp viruses, such as IHHNV (infectious hypodermal and hematopoietic necrosis virus) (Mari *et al.*, 1993), MBV, (Poulos *et al.*, 1994), BP-type (Baculovirus penaei) (Bruce *et al.*, 1993), and HPV (Hepatopancreatic parvo-like virus) (Mari *et al.*, 1995) for diagnosis of viral infection in shrimp have

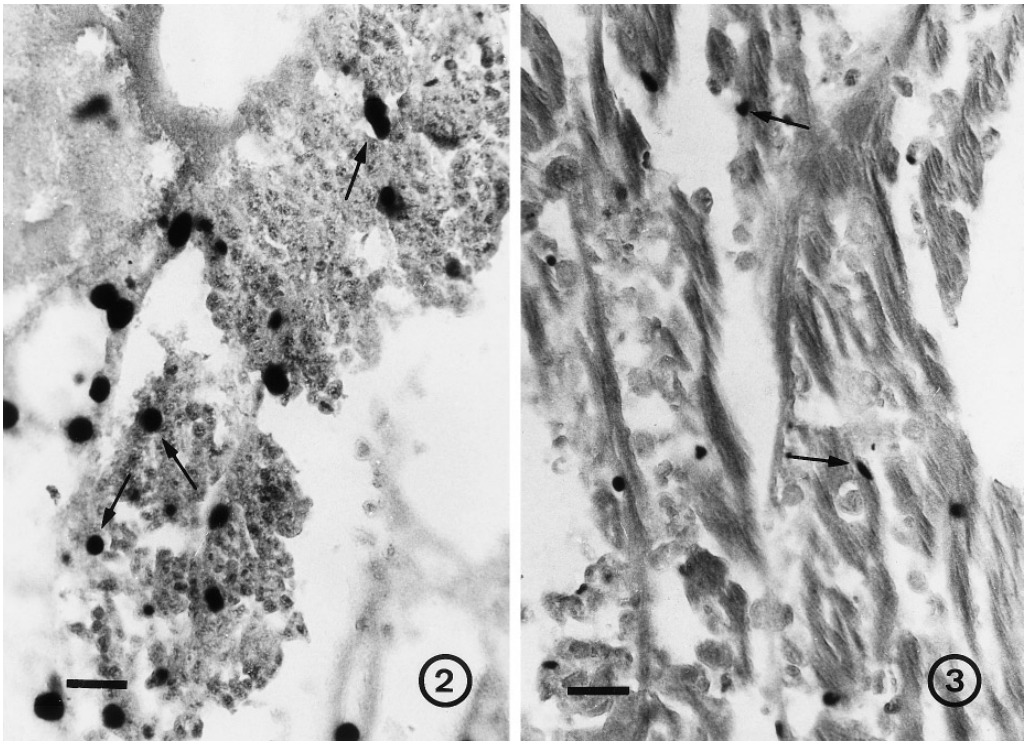

FIG. 2. Hematopoietic tissue of WSDV-infected giant tiger shrimp shows several WSDV-positive cells (arrows) scattered in the tissue using probe hybridization plus eosin Y counterstain; bar = 25 μm. **FIG. 3.** The heart of WSDV-infected giant tiger shrimp shows the hemocytes infected by the WSD virus (arrows). Probe hybridization plus eosin Y counterstain; bar = 25 μm.

been successfully used. These probes were developed by purifying the viruses, extracting the nucleic acid, digesting with restriction enzymes, and then cloning and screening the electrophoresed fragment of interest. The standard procedure demands a greater quantity of nucleic acid in order to perform a series of experiments. However, the present report demonstrated that only 0.5 ng virus DNA was used as template based on the RAPD technique, and then the diagnostic probe was constructed. The major advantage of this novel approach for gene probe development is that no mass DNA of target infectious agent needed to be available.

The RAPD technique has been used previously for identification and diagnosis of several microorganisms such as the bacterium *Mycobacterium bovis* (Rodriguez *et al.*, 1995) and the parasite *Eimeria media* (Cere *et al.*, 1996). This is the first report of a new and potentially useful diagnostic tool for detecting pathogens of shrimp, using the RAPD method to develop the gene probe. The intensive nature of aquaculture has enhanced the transmission and development of disease in cultured penaeid shrimp. Therefore, as the culture of penaeid shrimp increases, the discovery of other new infectious diseases seems a virtual certainty. In the future, it may become more easy to develop a sensitive and accurate gene probe for diagnosis of a new pathogen using the RAPD technique.

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Key Words: shrimp; white spot disease virus; RAPD; probe; *in situ* hybridization.

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