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Simultaneous and rapid detection of white spot syndrome virus and yellow head virus infection in shrimp with a dual immunochromatographic strip test

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A B S T R A C T

A strip test for the dual detection of white spot syndrome virus (WSSV) and yellow head virus (YHV) was developed using monoclonal antibodies (MAbs) specific to the WSSV major envelope protein VP28 (W1 and W30) and the YHV nucleocapsid protein p20 (Y19 and Y21). The MAbs W30 and Y19 were conjugated with colloidal gold and sprayed onto a glass fiber pad that was placed adjacent to a sample chamber. The MAbs W1 and Y21 and the goat anti-mouse immunoglobulin G (GAM) antibody were sprayed onto a nitrocellulose membrane in strips at positions designated W, Y and C, respectively. These test strips were placed in plastic cases and stored desiccated in a plastic bag. The test strips were assessed for their ability to detect WSSV and YHV simultaneously using pleopods sampled from shrimp. A pleopod homogenate in application buffer 100 μl was applied to the sample chamber to flow through the nitrocellulose membrane strip, and antibody–protein complexes could be observed within 15 min. In sample from shrimp infected with WSSV and/or YHV, viral protein bound to the colloidal gold-conjugated MAbs. These complexes were captured by the MAbs at the W and/or Y test lines, resulting in the appearance of reddish-purple coloured bands. Any unbound colloidal gold-conjugated MAbs migrated pass the W and Y lines would be captured by the GAM antibody, forming a band at position C. When samples not containing WSSV and YHV proteins or containing viral proteins at below the detection limit of the test, only the band at position C was observed. The sensitivity of the test was comparable to dot blot tests using single MAbs, and ~500-fold less sensitive than a 1-step PCR test for WSSV and 1000-fold less sensitive than an RT-PCR test for YHV. Despite this lower sensitivity, the dual strip test has advantages in speed and simplicity in not requiring sophisticated equipment or specialized skills. The ability to co-detect WSSV and YHV provides simultaneously cost savings.

1. Introduction

White spot syndrome virus (WSSV) and yellow head virus (YHV) are highly infectious viruses of the most common penaeid species cultured commercially. These viruses have caused severe economic losses, particularly in Thailand where they have resulted in 1 billion dollars of losses over the past decade (Flegel, 2006). Various molecular methods have been developed for the diagnosis of these viruses, including PCR for WSSV (Takahashi et al., 1996; Lo et al., 1996; Srisala et al., 2008); RT-PCR for YHV (Wongteerasupaya et al., 1997; Cowley et al., 2004); qRT-PCR for YHV (Dhar et al., 2001; Ma et al., 2008); and loop-mediated isothermal amplification (LAMP) for WSSV (Jaroenram et al., 2009) and RT-LAMP (Mekata et al., 2009) for YHV. Of these methods, both PCR and qPCR have been used widely for both detection and research due to their high levels of sensitivity and specificity.

Immunologically-based diagnosis methods using both polyclonal antibodies (PAbs) and monoclonal antibodies (MAbs) have been developed for the detection of WSSV (Nadala et al., 1997; Nadala and Loh, 2002; Poulos et al., 2001; Anil et al., 2002; Liu et al., 2002; Chaivisuthangkura et al., 2004, 2010) and YHV (Nadala et al., 1997; Sithigorngul et al., 2000, 2002). However, these immunologically-based techniques can only be performed in a laboratory by well-trained personnel. Further improvements of such tests lead to the development of immunochromatographic strip tests that are available for the detection of WSSV (Powell et al., 2006; Sithigorngul et al., 2006) and YHV (Sithigorngul et al., 2007). The beneficial features of these kits are their simplicity and convenience; the results can be obtained quickly without sophisticated tools or specialized skills. Furthermore, this method can be used to screen individual shrimp or pooled shrimp samples to

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confirm relatively high levels of viral infection (in comparison to PCR-based methods) and can be used easily for monitoring the infection of both viruses by the farmers themselves. In the case of single strip tests which were developed for detection of WSSV and YHV (Sithigorngul et al., 2006, 2007), PAbs were used as the main components of the captured antibodies in the test lines of the test strips. The PAbs were typically less reliable than MAbs in terms of quality control in addition to being limited in quantity. In this report, new MAbs that are specific to WSSV (W1 and W30) and YHV (Y21) were produced and used to replace the PAbs in the test lines of the strip tests developed previously (Sithigorngul et al., 2006, 2007); and both strips were combined into a dual strip test for convenient detection of both viruses using a single preparation.

2. Materials and methods

2.1. Viral preparation

Penaeus (*Litopenaeus*) *vannamei* infected with YHV naturally, weighing between 10 and 15 g were obtained from farms in the Banglen district of Nakhonpathom province in the central part of Thailand. *P. vannamei* infected with WSSV naturally weighing between 10 and 15 g were obtained from farms in the Songkhla and Prajuabkirikhan provinces in the southern part of Thailand. Distal segments of two pleopods taken from infected shrimp were homogenized in 100 μl 0.3 M phosphate buffered saline (2× PBS, pH 7.2) and 0.5 ml aliquots of supernatant from these homogenates were stored at −70 °C until tested.

2.2. Monoclonal antibody preparation

MAbs specific to the WSSV major envelope protein VP28 (W1, W30) obtained from a mouse immunized with recombinant VP28 as described previously (Chaivisuthangkura et al., 2004) and a MAb specific to the YHV nucleocapsid protein p20 (Y21) obtained from a mouse immunized with partially purified YHV as described previously (Sithigorngul et al., 2002). MAb (Y19) specific to YHV p20 has been described previously (Sithigorngul et al., 2002). Hybridomas producing the MAbs were grown in Hybridoma-SFM serum-free media (Gibco, Carlsbad, CA, USA) and the MAbs were purified using a Protein G-agarose column (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's instructions. The eluted antibodies were dialyzed in phosphate buffer (PB: 10 mM phosphate buffer, pH 7.3) and the antibody concentration was adjusted to 1 mg/ml.

2.3. Dot blot

Pleopod homogenates from either WSSV- or YHV-infected shrimp were diluted serially using pleopod homogenates from uninfected shrimp and the dilutions were spotted onto nitrocellulose membranes. The membranes were placed in solution containing either a single or pair of MAbs specific to WSSV or YHV, and MAbs bound to viral protein were detected as described previously (Sithigorngul et al., 2002; Chaivisuthangkura et al., 2004). Any observed increase in detection sensitivity when using pairs of antibodies indicated that the MAbs bound to non-overlapping epitopes.

2.4. Preparation of the dual immunochromatographic test strip

The dual test strip was prepared by the Pacific Biotech Co. Ltd. (Petchaboon province, Thailand). The MAbs W30 and Y19 were each conjugated to colloidal gold particles (diameter = 10 nm) and sprayed onto glass fiber pads at 5 and 3 μl/cm before being dried consecutively at 40 °C overnight. MAbs W1 (0.4 mg/ml) and Y21 (1 mg/ml) were each micro-sprayed consecutively onto nitrocellulose membranes (8-μm pore size) at 1 and 1.5 μl/cm at the positions that would become the captured test lines for WSSV (W) and for YHV (Y) on the completed strips (Fig. 1A). Goat anti-mouse IgG antibody (0.8 mg/ml) was micro-sprayed onto the same nitrocellulose membranes at 1 μl/cm at position that would become the captured control lines (C). The membranes were then dried at 40 °C overnight. For kit assembly, the nitrocellulose membranes were combined with the glass fiber pad containing W29 and Y19 conjugated with colloidal gold at the opposite end downstream of the control line. The sample pad was placed anterior to the glass fiber pad at the site of sample application wells, and the absorption pad was placed at the posterior end next to the control line for collecting the excess liquid (Fig. 1A). The assembly was cut into 4.5 mm wide strips that were housed individually in a plastic case (Fig. 1C) that was stored in a desiccated plastic bag.

2.5. Specificity testing

One pleopod from *P. vannamei* either uninfected or WSSV- or YHV-infected naturally (weighing between 10 and 15 g) was homogenized in PBS (50 μl/pleopod). This homogenate was then diluted at 1:5 with application buffer (30 mM Tris, 336 mM NaCl, 9 nM EDTA, 1% Triton X-100, pH 9.3), and 100 μl of the diluted supernatant solutions were applied to the sample well of individual test strips so that they would flow chromatographically along the nitrocellulose strip test and pass lines at positions W, Y and C before entering the absorption pad (Fig. 1B). The test results could be observed within 15 min of applying the sample. A positive result yielded reddish-purple bands at the W and/or Y and C positions, whereas a negative result yielded a reddish-purple band at position C only (Fig. 2). Similar tests were performed using pleopod homogenate mixtures of WSSV- and YHV-infected shrimp at a 1:1 ratio. To determine the test specificity, pleopod samples from Taura syndrome virus- (TSV) or infectious hypodermal and hematopoietic necrosis virus- (IHHNV) infected shrimp were also tested in the same manner.

To determine possible interference of between the two viruses in their binding capacity to the corresponded MAbs, the homogenate from shrimp infected with WSSV was diluted serially with the homogenates from shrimp infected with YHV or from uninfected shrimp before application to the strips to compare the detection sensitivity. The opposite experiments using homogenate from shrimp infected with YHV and diluted serially with homogenates from shrimp infected with WSSV or from uninfected shrimp were performed in the same fashion.

2.6. Comparison of the sensitivity of the strip test with dot blotting and PCR

Supernatant solutions from pleopod homogenates of WSSV- or YHV-infected *P. vannamei* were diluted serially with uninfected *P. vannamei* pleopod homogenates (in PBS for dot blotting or in the application buffer for strip tests and PCR). Samples (1 μl) taken from each dilution were tested by dot blotting as described previously using MAbs specific to either WSSV or YHV. Nucleic acid was extracted from the same shrimp homogenate (100 μl) using a High Pure viral nucleic acid kit (Roche Molecular Biochemicals) and diluted serially with nucleic acid extracted from uninfected shrimp. These samples were tested for the presence of WSSV DNA by PCR using primers VP28F and VP28R yielding an amplicon 633 bp in length (Chaivisuthangkura et al., 2004) or the presence of YHV RNA by RT-PCR using primers YHV10F and YHV114R designed