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Evaluation of monoclonal antibody based immunochromatographic strip test for direct detection of *Vibrio cholerae* O1 contamination in seafood samples

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ABSTRACT

A strip test for the detection of *Vibrio cholerae* O1 was developed using two monoclonal antibodies (MAbs), *VC-223* and *VC-1226*, specific to the lipopolysaccharides of *Vibrio cholerae* O1 Inaba and Ogawa serovars. The sensitivity of the test was 5×10^5 cfu/mL which was similar to that of dot blot test. The detection limit could be improved to 1 cfu/mL of the original bacterial content after pre-incubation of the bacterium in alkaline peptone water (APW) for 12 h. Detection of *V. cholerae* O1 in various fresh seafood samples such as shrimp, blood clam, mussel and oyster could be performed directly with sensitivities ranged from 5×10^5 to 10^6 cfu/mL. After pre-enrichment of the shrimp sample in APW, the detection sensitivities increased to 10^2 to 10 CFU/mL of the original bacterial content after incubation for 12 and 24 h. However, the detection sensitivities were also depending on the content of the other bacteria that might inhibit the growth of *V. cholerae* during pre-enrichment step. The *V. cholerae* O1 strip test has advantages in speed, and simplicity in not requiring sophisticated equipment or specialized skills and the sample could be directly examined without requirement for sample processing.

1. Introduction

Vibrio cholerae, a food borne pathogen, is one of the main causes of cholera which is still a serious problem in some countries in Asia (Siddiqui et al., 2006; Lenglet et al., 2010; Ang et al., 2010), African regions (Kirigia et al., 2009) and recently in Haiti (Fraser, 2010). The epidemic and pandemic cholera in various regions was mainly caused by *V. cholerae* serogroups O1 and O139 (Chatterjee and Chaudhuri, 2003; Sack et al., 2004; Siddiqui et al., 2006).

V. cholerae serogroup O1 has two major serotypes, Inaba and Ogawa, which may alternate among cholera epidemics. The third serotype, Hikojima also exists but it is rare and unstable (Sack et al., 2004). The genes responsible for O1 antigen biosynthesis have been designated *rfb*. The mutation which defines serotypes Inaba and Ogawa is a single deletion mutation in *rfb*T gene (Stroeher et al., 1992).

However, occasional outbreaks of diarrhea in humans were caused by non-O1/non-O139 cholera Vibrios (NVCs) through the ingestion of improperly cooked seafood (Ottaviani et al., 2009) or exposure to a contaminated aquatic environment (Lukinmaa et al., 2006).

To control epidemic outbreaks, an effective method for the detection of *V. cholerae* is imperative. Traditional identification of *V. cholerae* is often achieved through the isolation of the bacteria, laborious routine microbiological and biochemical analyses. These processes require at least three working days before obtaining the results. After isolation, the serotype identification of *V. cholerae* O1 or O139 is usually performed by agglutination with antisera or monoclonal antibody specific to O1 or O139 antigens (Ito and Yokota, 1987; 1988; Qadri et al., 1994).

Various molecular-based techniques have been developed including PCR for the rapid detection of virulence and regulatory genes, which include the following: the cholera toxin enzymatic subunit (*ctx*A), zonula occludens toxin (*zot*), accessory cholera enterotoxin (*ace*), toxin-coregulated pilus (*tcp*A), outer membrane protein (*omp*U), central regulatory protein ToxR (*tox*R) and hemolysin (*hly*A). It was found that only PCR of *tox*R was suitable for the detection of all tested isolates of *V. cholerae* and *V. mimicus*. However, PCR of these genes alone could not differentiate *V. cholerae* from *V. mimicus* (Singh et al., 2002; Karunasagar et al., 2003; Blackstone et al., 2007), and using primers for the *ctx*A gene produced negative results in the analysis using qPCR for most NVCs and some *V. cholerae* O1 and *V. cholerae* O141 (Blackstone et al., 2007). Recently, a method based on PCR of the large chromosomal replication origin sequence (*ori*) of *V. cholerae* in combination with restriction fragment length polymorphism (RFLP) with respect to a *Bgl*II site (AGATCT) was used to identify and differentiate between *V. cholerae* and *V. mimicus* (Saha et al., 2006). The PCR identification targeted to *omp*W demonstrated that all of the 254 *V. cholerae* strains tested were positive (Nandi et al., 2000). Furthermore, the development of a loop-mediated isothermal amplification (LAMP) targeted to the same gene could identify all 16 isolates of *V. cholerae* O1, O139 and NVCs with a higher sensitivity than simple PCR (Srisuk et al., 2010). The primer pairs corresponding to unique stretches in the

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genes of *rfb* complex, encoding the O antigen, were designed to develop a multiplex PCR assay for specific detection of *V. cholerae* O1 and O139 (Hoshino et al., 1998).

Although the PCR methods are highly sensitive, they require expensive equipment and highly skilled personnel. Therefore, the PCR methods may not be feasible for small laboratories or for field diagnosis. For the effective control of disease outbreaks, detection methods should be both quick and easy without sacrificing specificity and sensitivity. Various immunoassays using monoclonal antibodies (MAb) (e.g., ELISA and dot-ELISA) have been developed for the detection of *V. cholerae* O1 and *V. cholerae* O139 in human stool samples (Adam et al., 1988; Qadri et al., 1994; Chaicumpa et al., 1998) and some have been further developed into simple immunochromatographic dipstick tests for *V. cholerae* O1 or/and O139 (Qadri et al., 1995; Nato et al., 2003; Mukherjee et al., 2010). However, the limit sensitivity of both test kits (10^6 and 10^7 cfu/mL respectively) was still relatively lower than that of various molecular techniques, In a previous study, we successfully produced MAbs that were specific to each serotype of *V. cholerae* including *V. cholerae* O1 (Pengsuk et al., 2011). In this study, an immunochromatographic strip test specific for *V. cholerae* O1 was developed and tested for its ability for direct detection of *V. cholerae* O1 in various fresh seafood spiked with *V. cholerae* O1. Additional step of pre-incubation of the sample in alkaline paptone water (APW) for 12 – 24 h could increase the detection sensitivity of the strip test comparable to various molecular based methods.

2. Materials and methods

2.1. Bacterial culture and antigen preparation

Sources of *V. cholerae* (70 isolates), *V. mimicus* (10 isolates), 22 isolates of other *Vibrio* species and 13 non-*Vibrio* Gram-negative bacteria used for testing the cross-reactivity of the MAbs were shown in Table 1. The bacteria were grown to the exponential phase on tryptic soy agar (TSA; Difco, Sparks, MD, USA) at 37 °C. TSA supplemented with 2% (w/v) NaCl was used to culture the *Vibrio* species. The bacteria were harvested by centrifugation at 3500 ×*g* for 10 min at 4 °C and washing with sterile phosphate buffered saline (PBS: 135 mM NaCl, 15 mM sodium phosphate, pH 7.2). The bacterial pellets were washed twice and suspended with sterile PBS, adjusted to an OD of 1 at 600 nm (approximately 10^9 cfu/mL of *V. cholerae* as determined by plate counting) and finally heat-killed at 60 °C for 60 min. The resulting bacterial suspensions were divided into aliquots and stored at −70 °C until further use.

Various biochemical tests for the identification of the bacteria were performed in a conventional format. All 70 *V. cholerae* isolates are positive for oxidase, Voges-Proskauer, sucrose fermentation, lysine decarboxylase and ornithine decarboxylase. They are negative for D-glucose, lactose and myo-inositol fermentations and also negative for arginine dihydrolase. The PCR of *omp*W for the confirmation of *V. cholerae* was conducted as described by Srisuk et al. (2010).

2.2. Monoclonal antibody preparation

MAbs specific to the *V. cholerae* O1 *VC-223* was obtained from previous study (Pengsuk et al., 2011), and a new MAb (*VC-1226*) specific to *V. cholerae* O1 was produced from a mouse immunized with *V. cholerae* O1 Inaba and Ogawa (VC1 and VC11) by the same protocol as described previously (Pengsuk et al., 2010). Both MAbs bound to *V. cholerae* O1 serovars Inaba and Ogawa similarly. 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. Preparation of the immunochromatographic test strip

The VC O1 test strip was prepared by the Pacific Biotech Co. Ltd. (Petchaboon province, Thailand). The MAbs *VC-223* was conjugated to colloidal gold particles (diameter = 10 nm) and sprayed onto glass fiber pads 3 μL/cm before being dried at 40 °C overnight. MAbs *VC-1226* (1 mg/mL) was micro-sprayed onto nitrocellulose membranes (8-μm pore size) at 1 μL/cm at the positions that would become the captured test line (T) 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 MAb *VC-223* conjugated with colloidal gold at the opposite end upstream of the test line. The sample pad was placed on top and 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. 1B). The assembly was cut into 4.5 mm wide strips that were housed individually in a plastic case (Fig. 1C and D) that was stored in a desiccated plastic bag.

2.4. Specificity testing

V. cholerae O1 Inaba, Ogawa, O139, O141, NVC, *Vibrio* spp. and various Gram-negative bacteria at 10^7 cfu/mL were diluted with application buffer (1% Tween80, 1 mg/mL gelatin in PBS pH 7.2), and 100 μL of the diluted solutions was 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 T 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 T and C positions, whereas a negative result yielded a reddish-purple band at position C only (Fig. 2).

2.5. Detection sensitivity testing in various fresh seafood samples

Heat killed *V. cholerae* O1 at 10^4 to 10^7 cfu/mL diluted in application buffer (100 μL) was applied to the strip test and the results were observed at 15 min after application. To determine possible interference of various types of food sample on detection, ice cold storage shrimp *Penaeus vannamei*, alive oyster, mussel, blood clam were obtained from local market in Bangkok. They were washed several times with distilled water, then the flesh was dissected out, minced and homogenized in application buffer at ratio of 1:10 (weight:volume). Heat killed *V. cholerae* O1 Inaba (VC1) ranged from 10^4 to 10^7 cfu/mL, was spiked into the homogenates then 100 μL of the homogenates was applied to the strip test.

2.6. Detection of V. cholerae O1 after pre-incubation in alkaline peptone water (APW)

V. cholerae O1 Inaba (VC1) was spiked into 10 mL of the 2% NaCl APW at dilutions from 0 to 10^7 cfu/mL and incubated at 37 °C. Aliquots of 1 mL from each tube at 0, 3, 6, 12, 24 h incubation periods were collected and immediately heat killed at 60 °C for 60 min before storage at −20 °C prior to testing with strip test and dot blotting. Each homogenate sample was mixed with application buffer at ratio 1:1 before 100 μL was applied to the strip test. In comparison with dot blotting, the homogenate (1 μL) was also applied to each square of the nitrocellulose membrane and processed for dot blotting using MAb specific to *V. cholerae* O1(*VC-223*), and MAb specific to *Vibrio* spp. (*VC-201*) (Pengsuk et al., 2011) as described above.