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Development of a highly sensitive lateral immunochromatographic assay for rapid detection of *Vibrio parahaemolyticus*

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ABSTRACT

Vibrio parahaemolyticus is widely present in brackish water all over the world, causing infections in certain aquatic animals. It is also a foodborne pathogen that causes diarrhea in humans. The aim of this study is to develop an immunochromatographic lateral flow assay (LFA) for rapid detection of *V. parahaemolyticus* in both aquatic products and human feces of diarrheal patients. Two monoclonal antibody (MAb) pairs, GA1a-IC9 and IC9-KB4c, were developed and proven to be highly specific and sensitive to *V. parahaemolyticus*. Based on the two MAb pairs, two types of LFA strips were prepared. Their testing limits for *V. parahaemolyticus* culture were both 1.2×10^3 CFU/ml. The diagnostic sensitivities and specificities were both 100% for the 32 tested microbial species, including 6 *Vibrio* species. Subsequently, the LFA strips were used to test Whiteleg shrimps and human feces. The type II strip showed a higher diagnostic sensitivity. Its sensitivity and specificity for hepatopancreas and fecal samples from 13 Whiteleg shrimps and fecal samples from 146 human diarrheal patients were all 100%. In conclusion, our homemade type II LFA is a very promising testing device for rapid and convenient detection of *V. parahaemolyticus* infection not only in aquatic animals, but also in human diarrheal patients. This sensitive immunochromtographic LFA allows rapid detection of *V. parahaemolyticus* without requirement of culture enrichment.

1. Introduction

Vibrio parahaemolyticus is a Gram-negative, curved, rod-shaped bacterium widely present in brackish water. Many aquatic animals, including *Penaeus orientalis*, tiger prawns, abalones and Iberian toothcarp, can be infected by *V. parahaemolyticus* (Alcaide et al., 1999; Liu et al., 2000). *V. parahaemolyticus* has been reported as a common cause of foodborne diseases worldwide (McLaughlin et al., 2005; Wang et al., 2007). In China, *V. parahaemolyticus* has been the leading cause of foodborne outbreaks and bacterial infectious diarrhea in coastal regions (Lin et al., 2011). In Japan, it has been found to account for 20–30% of all food poisoning cases (Alam et al., 2002). The typical symptom of *V. parahaemolyticus* infection is watery diarrhea, which usually occurs within 24 h (Newton et al., 2014). However, the etiology of diarrhea can be difficult to determine based on observing clinical symptoms alone. Besides *V. parahaemolyticus*, diarrhea can be caused by a variety of bacteria, viruses, and parasitic organisms. Hence, laboratory testing is a necessity for confirmation purposes.

The most common method for diagnosis of *V. parahaemolyticus* infection is conventional bacteria isolation and identification, which is currently still considered as the "gold standard". Another microbiological method widely used for detecting *V. parahaemolyticus* is the International Organization for Standardization (ISO) cultural method, where two selective media [thiosulfate–citrate–bile salts–sucrose agar (TCBS) and triphenyltetrazolium chloride soya tryptone agar (TSAT)] are employed (International Organization for Standardization, ISO, 1990; Hara-Kudo et al., 2001). However, the conventional microbiological methods are very time-consuming and usually take longer than one week. In addition, the test results can be affected by many factors, such as bacterial load of specimens and the experience of laboratory technicians. Also, when the culture of the target bacteria is contaminated, the results may be confusing and misleading.

Recently, molecular approaches for detection of *V. parahaemolyticus* have been developed significantly. Many PCR-based methods have been introduced by targeting specific genes of *V. parahaemolyticus* (Kim et al.,

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1999; Di Pinto et al., 2005). They are generally less time-consuming and more sensitive than conventional microbiological methods. But these molecular methods require professional personnel and equipment, and still need several hours to give a test result. Lateral flow assay (LFA), on the other hand, is a newly developed technique that can obtain the results within only a few minutes. Moreover, it needs neither trained technicians nor expensive equipment. LFA, an immunoreaction-based assay, is developed by targeting specific epitopes of protein antigens. So far, several rapid tests have been developed for the detection of pathogens in aquatic products (Sithigorngula et al., 2007; Sheng et al., 2012; Liu et al., 2015). However, due to their low testing sensitivities, the process of bacterial culturing is usually required before those immunoassays. The report on LFA for direct detection of *V. parahaemolyticus* without requirement of culture enrichment has not been seen yet.

In this study, two monoclonal antibody (MAb) pairs targeting *V. parahaemolyticus* were successfully prepared. The colloidal gold test strips were developed to establish a rapid and accurate diagnostic method for *V. parahaemolyticus* infection both in aquatic products and in human feces of diarrheal patients.

2. Materials and methods

2.1. Microbes

V. parahaemolyticus, V. alginolyticus, *V. cholerae*, *V. vulnificus*, *V. harveyi*, *V. fortis*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Edwardsiella ictaluri*, *Bacillus subtilis*, *Shewanella putrefaciens*, *Enterobacter cloacae*, *Streptococcus iniae* were provided by the Pathogen Center of National and Local Joint Engineering Laboratory for Disease Control and Prevention Technology in Aquaculture Animals (Sichuan, China). Other microbes, including *Staphylococcus aureus*, *Helicobacter pylori*, *Aeromonas caviae*, *Stenotrophomonas maltophilia*, *Candida tropicalis*, *Aeromonas hydrophila*, *Citrobacter freundii*, *Candida albicans*, *Staphylococcus epidermidis, Salmonella paratyphi B*, *Salmonella typhi*, *Serratia marcescens*, *Proteus mirabilis*, *Escherichia coli, Staphylococcus haemolyticus*, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* were preserved in the laboratory of Artron BioResearch Inc. (Burnaby, Canada). All the microbes were cultured according to the standard methods.

2.2. Animal immunization

The *V. parahaemolyticus* cells were inactivated at 56 °C for 30 min, followed by determination of protein concentration using bicinchoninic acid kit (Sigma-Aldrich, USA). Female BALB/c mice (8 weeks old), provided by Laboratory Animal Center of Shandong University, were immunized intraperitoneally with 50 μg (approximately 10^6 CFU), 100 μg, 150 μg and 200 μg, respectively, of whole *V. parahaemolyticus* cells emulsified in Freund's complete adjuvant (Sigma-Aldrich, USA). After four and eight weeks, the mice were injected intraperitoneally with the same amount of antigen emulsified in Freund's incomplete adjuvant (Sigma-Aldrich, USA). At ten weeks, the bloods of immunized mice were collected individually by tail vein puncture method, followed by centrifuging at 1500*g* to prepare sera samples. The antibody titers of sera were determined by indirect enzyme-linked immunosorbent assay (ELISA). The mouse with highest titer was selected as the spleen cell donor and received a final intraperitoneal immunization with *V. parahaemolyticus* cells alone (without any adjuvant).

2.3. Hybridoma production

Mouse myeloma Sp2/0 cells, used as fusion partners, were cultured and propagated in RPMI-1640 culture medium (Gibco, ThermoFisher, USA) containing 10% fetal bovine serum (FBS) (Hyclone, GE Healthcare, USA). Three days after the final immunization, the mice were euthanized by cervical dislocation and the splenocytes were isolated. The freshly harvested splenocytes were then fused with the myeloma Sp2/0 cells at a ratio of 5:1 by 50% (*w*/*v*) polyethyleneglycol 4000 (Sigma-Aldrich, USA). Subsequently, the cells were re-suspended in Hypoxanthine-Aminopterin-Thymidine (HAT) medium (Sigma-Aldrich, USA), seeded in 96-well tissue culture plates and incubated in a humidified incubator with 5% CO_2 at 37 °C for 2 weeks. Clones were kept in Hypoxanthine Thymidine (HT) medium (ThermoFisher, USA) for 2 more weeks. After selection by indirect ELISA, the desired cell lines were cloned three times by limiting dilution to stable monoclones.

2.4. Indirect ELISA

The 96-well microplates were coated with 100 μl 5 μg/ml *V. parahaemolyticus* cells and incubated at 4 °C overnight. Plates were blocked for 2 h with blocking buffer, phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA), at 37 °C and washed three times in PBST (PBS/0.05% Tween-20). 100 μl of hybridoma culture supernatants, the positive control (serum of immunized mice), and the negative control (Sp2/0 culture), were accordingly added to the plates and incubated at 37 °C for 1 h. Plates were washed four times with PBST and incubated with 100 μl horseradish peroxidase conjugated goat anti-mouse immunoglobulin (IgG-HRP) (Santa Cruz Biotechnology, USA) for 30 min at 37 °C. Finally, plates were washed five times with PBST and incubated with 100 μl 3,3′,5,5′-Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA (Sigma-Aldrich, USA) in dark for 15 min at room temperature. The reaction was terminated by supplementing 50 μl H_2SO_4 solution (1 M) and the absorbance values were determined at 450 nm by BioTek™ ELx808™ Absorbance Microplate Readers (ThermoFisher, USA).

2.5. MAb production

MAbs against *V. parahaemolyticus* were produced by traditional ascitic fluid method. Cells of desired monoclones in density of 1×10^6 cells/0.5 ml PBS were injected intraperitoneally into each mouse, which had been previously injected with 0.5 ml Pristane (Sigma-Aldrich, USA) two weeks earlier. Their ascites were harvested about ten days later when their abdomens were completely enlarged and their skins were extended. The supernatants were collected after centrifugation of the ascites at 2900*g* for 30 min. The antibodies were precipitated by 50% saturation of ammonium sulfate solution, followed by purification by protein G column (GE Healthcare, USA) affinity column chromatography according to the manufacturer's protocol.

2.6. MAb characterization

The purity of prepared MAbs was analyzed by SDS-PAGE. The titers of MAbs were determined by indirect ELISA. The titer of an antibody solution was defined as the highest dilution that could give a positive reaction against the antigen. The immunoglobulin subclass was determined by a commercial Mouse Typer Sub-isotyping Kit (Bio-Rad, USA) according to the manufacturer's protocol. The affinity constant (K_{aff}) of MAbs were determined by indirect ELISA according to the method described previously (Beatty et al., 1987).

2.7. Preparation of colloidal gold and colloidal gold-MAb conjugate

Colloidal gold was prepared according to a previous report (Grabar et al., 1995). Briefly, 100 ml of 0.01% (*w*/*v*) $HAuCl_4$ (Sigma-Aldrich, USA) in a 250 ml siliconized flask was heated to boiling in a microwave oven, and 1.4 ml 1% trisodium citrate (Sigma-Aldrich, USA) was added. The solution was allowed to gradually cool down and then stored at 4 °C in a dark-colored glass bottle. The pH of the colloidal gold was adjusted to 8.4 with 1% (*w*/*v*) potassium carbonate. The capture MAb (30 μg) was added dropwise into 10 ml colloidal gold solution on a magnetic stirrer