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Development and application of antibody microarray for lymphocystis disease virus detection in fish

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ABSTRACT

Lymphocystis disease virus (LCDV) is the causative agent of lymphocystis disease affecting marine and freshwater fish worldwide. Here an antibody microarray was developed and employed to detect LCDV in fish. Rabbit anti-LCDV serum was arrayed on agarose gel-modified slides as capture antibody, and Cy3-conjugated anti-LCDV monoclonal antibody (MAbs) was added as detection antibody. The signals were imaged with a laser chip scanner and analyzed by corresponding software. To improve the sensitivity, different substrate binders (poly-L-lysine, MPTS, aldehyde, APES and agarose gel modified slides, and commercially available amino-modified slides), markers (fluorescein isothiocyanate, Cy3, horseradish peroxidase, biotin or colloidal gold) conjugated to anti-LCDV Mabs, and storage time of the antibody were assessed. The results showed that the antibody microarrays based on agarose gel-modified slides gave a lower detection limit of 0.55 μg/ml of LCDV when Cy3 and HRP conjugated anti-LCDV MAbs were used as detection antibody; and the lowest detectable LCDV protein concentration was 0.0686 μg/ml when streptavidin–biotin conjugated to anti-LCDV MAbs served as detection antibody. The developed antibody microarray proved to have a high specificity for LCDV detection and a shelf-life of more than 8 months at −20 °C. Furthermore, the LCDV detection results of the microarray in fish gills or fins ($n = 50$) presented a concordance rate of 100% with enzyme-linked immunosorbent assay (ELISA) and 98% with immunofluorescence assay technique (IFAT). These results revealed that the developed antibody microarray could serve as an effective tool for diagnostic and epidemiological studies of LCDV in fish.

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1. Introduction

Lymphocystis disease virus (LCDV) is the causative agent of lymphocystis disease (LCD) affecting approximately 140 species of marine and freshwater fish worldwide (Lang, 1994; Plumb, 1993). Fish infected with LCDV exhibit characteristic external symptoms of wart-like nodules on the body skin, fins, mouth and gills, causing a decrease in commercial value (Tidona and Darai, 1997). In addition, diseased fish are more susceptible to secondary infection by other microorganisms, resulting in high mortalities and great economic losses for aquaculture worldwide (Chinchar, 2002; Iwamoto et al., 2002). Since there are currently no available treatments for LCD, rapid and accurate detection of LCDV is crucial in prevention and control of lymphocystis disease.

Diagnosis of LCD is generally based on typical skin lesion observation. At present, the techniques used frequently for LCDV detection include polymerase chain reaction (PCR), real-time PCR, virus isolation and neutralization using cell lines, and several immunological techniques based on anti-LCDV antibody (Garcíarosado et al., 2002; Kitamura et al., 2006; Cano et al., 2006, 2007). PCR and real-time PCR are of specific, sensitive, rapid and cost-effective; however, their performances require highly trained personnel and specialized equipments. The isolation of viral pathogens in cell cultures has been regarded as the "gold standard" for their convenience and accuracy, but this approach is often slow and requires considerable technical expertise. Immunological techniques (ELISA, immunofluorescence assay technique, Western-blot) based on anti-LCDV antibody are sensitive and specific, but time-consuming with complicated operation. Antibody microarray-based analysis is a cost-effective approach that yields reproducible results and can allow replicate analyses in a single assay run. As a next-generation tool, antibody microarrays are increasing in popularity, for they offer unparalleled throughput, minimal reagent consumption and sensitive detection of multiple targets simultaneously (Angenendt, 2005); and test results could be read by naked eyes when combined with immunoenzyme techniques or immunogold-sliver staining. However the accuracy conferred by this method is linked with the quality of antibodies employed closely, and there are issues relating to cross-reactivity and antibody availability, both of which are crucial for sensitive and specific strain typing particularly (Mahesh et al., 2008). Given the listed benefits, antibody microarrays have been and are being

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developed that have growing potential for clinical, biothreat, and point-of-care applications (Huelseweh et al., 2006; Lian et al., 2010). For disease diagnosis in aquaculture, the antibody microarray for white spot syndrome virus (WSSV) detection in shrimp have been developed (Xu et al., 2011), however for fish viral detection there are, to our knowledge, no reports of antibody microarray diagnostics so far.

This paper described the development and application of a low-density antibody microarray for LCDV detection in fish tissue extracts, as well as improvement of antibody microarray sensitivity. With the prepared antibody-microarray-integrated system, the entire testing procedures could be completed in less than 2 h, and the multiple samples from diverse sources could be tested in a single assay without the need for labeling. As a supplementary technique for detection of LCDV in fish, the developed antibody microarray is convenient, accurate, sensitive and time-saving, and suitable for diagnostic and epidemiological detection to LCDV.

2. Materials and methods

2.1. Preparation of samples

The LCDV-free flounders (*Paralichthys olivaceus*, 15 ± 1 cm in length) tested by PCR (Zhan et al., 2010) were obtained from a fish farm located in Qingdao of Shandong province, China; LCDV-infected flounders (20 ± 2 cm in length) with various-sized nodules on body surface were from a fish farm in Hebei province. Common sea bass (*Lateolabrax japonicus*, 23 ± 2 cm) and sting fish (*Sebastes schlegeli*, 20 ± 2 cm) were from a fish farm in Shandong province. All these fish were shipped in chill containers, and then anaesthetized with tricaine (0.065 mg/ml) (MS-222, Sigma–Aldrich, St. Louis, US). The tissues (gill, fins, skin, lymphocystis nodules and internal organs) were taken and frozen at −80 °C. Sampled fish fin or gill (target organs of LCDV) homogenates were used for LCDV detection. The fish gills or fins (∼1 g) were washed with TNE buffer (50 mmol/l Tris, 100 mmol/l NaCl, 1 mmol/l EDTA, pH 7.4) for 5 min at a ratio of 10% (w/v), homogenized and suspended in TNE buffer at 4 °C. The suspension was subjected to rapid freeze–thaw (3×), ultrasonication, and centrifuged at 500 × *g* and then 1800 × *g* for 20 min at 4 °C, respectively. The homogenates of gills and fins from the same fish were pooled and stored at −80 °C until use.

Purified LCDV solution was prepared from lymphocystis nodule homogenate using the method developed previously (Cheng et al., 2006) and resuspended with 0.01 mol/l PBS (pH 7.4), and the viral protein concentration was measured 0.878 mg/ml. Graded reagents and MilliQ grade water were used throughout the experiment.

All studies were conducted in accordance with institutional, national and international standards on animal welfare.

2.2. Antibody production and purification

Rabbit anti-LCDV antibody (as capture antibody of the microarray) and rabbit anti-mouse Ig antibody (as positive control) were prepared by immunizing adult New Zealand rabbits with purified LCDV and mouse Ig in conventional methods respectively (Huang et al., 2005). One week after the last injection, blood was taken from the rabbit and the rabbit antiserum was purified by the Ampure PA kit (GE Healthcare, Fairfield, US) following the manufacturer's protocol. The reactivity of LCDV with rabbit antiserum was determined by immunofluorescence assay technique (IFAT) using pre-immune serum as control (Cheng et al., 2006).

Four anti-LCDV MAbs, 3G3, 2B6, 1D7 and 2D11 developed previously (Cheng et al., 2006), were produced in ascites by injecting the hybridoma clone into the peritoneal cavity of Balb/c mice, and purified in the same way above-mentioned. These MAbs were then labeled with Cy3 (GE Healthcare, Fairfield, US) according to the manufacturers' instructions and used as detection antibody after mixed in equal proportion.

2.3. Antibody microarray fabrication for LCDV detection

2.3.1. Optimal concentration of capture antibody

Agarose gel modified slides were prepared following the methods of Xu et al. (2011). Rabbit anti-LCDV antibody was diluted at the concentration range of 0.5 μg/ml to 1.5 mg/ml, and spotted on agarose gel-modified slides as capture antibody in 3 × 3 matrix respectively. The slides were put in humid chambers at 37 °C for 2 h to complete the immobilization of antibodies and then washed 3 times with PBST (PBS containing 0.5% Tween-20) for 5 min each. Slide surface was blocked with 3% bovine serum albumin (BSA) in PBS for 1 h at 37 °C. After 3 washes, LCDV diluent at 0.1 mg/ml was incubated with the capture antibody, and the antigen–antibody complex formed was identified by Cy3-conjugated anti-LCDV antibody. Detection results were measured by laser chipscanner at 532 nm and quantified with software EcoscanCHS. The optimal concentration of capture antibody was confirmed when the relative signal value of the microarray tended to be stable.

2.3.2. Integration of antibody microarray

The capture antibody, 0.5 mg/ml rabbit anti-LCDV antibody in printing buffer (PBS containing 50% glycerol) was spotted onto the agarose gel-modified slide with a spot volume of 20–50 nl. The microarray contained eight groups deposited in 4 × 4 matrixes and 2 × 4 arrangement on one slide. In each matrix, the printing buffer as negative control and the anti-mouse Ig antibody as positive control were spotted to form four replicates, respectively, and the capture antibody was spotted in eight replicates. To perform multiplexed assays on the same slide, the microarrays were compartmentalized into eight subarrays by Super PAP Pen or silicone gasket. After arraying, the slides were incubated in humid chambers at 37 °C for immobilization of antibodies and then blocked by 3% BSA. The antibody microarrays were washed and allowed to dry, and stored at 4 °C or −20 °C until use.

2.4. LCDV detection

Detection samples, 40 μl per matrix, were added on the antibody microarray. The slides were put in a humid chamber at 37 °C for 15–30 min and then washed 3 times, followed by incubation with Cy3-conjugated anti-LCDV antibody at 37 °C for 30 min. The microarrays were washed again and imaged by laser chip scanner, and fluorescence signals were quantified with software EcoscanCHS. The fluorescence intensity of negative control was used as the background value, and the mean fluorescence intensity value of the eight replicates of capture antibody in one matrix was determined as the relative signal value after subtracting the corresponding background value.

2.5. Detection limit of antibody microarray

Purified LCDV (0.878 mg/ml) was diluted 1:50–6400 with PBS, and added to the integrated antibody microarrays in 40 μl per matrix. The detection limit of the antibody microarray was defined as the lowest LCDV concentration that could be detected reliably and positively and also a measure of sensitivity. For improvement of detection limit, the supports of microarray, markers labeled to anti-LCDV MAbs, and shelf life of antibody microarray, were assessed. In addition, the detection limit of the antibody microarray was compared with that of indirect sandwich enzyme-linked immunosorbent assay (ELISA) (Luo et al., 2009). The 96-well EIA plates (Corning, New York, USA) were coated with 50 μl rabbit

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