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Research Note

Application of Monoclonal Antibody Based ELISA for Determining Circulating Specific Antibody in Rohu (*Labeo rohita*)

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ELISA is a powerful diagnostic technique, widely used in health management. Usually it is applied as a direct tool to detect specific pathogens, employing antigen specific monoclonal antibodies (MAbs) or specific antiserum as detecting antibody. In fish health management, it is extensively used for the detection of specific pathogens especially bacteria and virus.

Recently due to the advent of MAbs specific to different fish immunoglobulins, ELISA has been employed for detecting and quantifying circulating pathogen specific antibodies in fishes. ELISA has been developed to detect and quantify specific antibodies against different pathogens in many fishes employing anti fish Ig MAbs (Zilberg & Klesius, 1997; Bobadilla et al., 2004; Rathore et al., 2008). Anti Ig MAb based ELISA has been employed to study the efficacy of vaccination in fishes also (Stromsheim et al., 1994; Coeurdacier et al., 1997; Wagner et al., 2001). In the present study an indirect ELISA was standardized for the specific detection of anti-*Aeromonas hydrophila* Ig titre in rohu, post infection and post vaccination.

Monoclonal antibody to rohu immunoglobulin (F2D9) employed for the development of immunodot described in our previous report (Suresh Babu et al., 2014) was used in the present study. *Labeo rohita* (rohu) maintained in 500 l FRP tanks at the freshwater fish farm of College of Fisheries, Mangalore was used for the study. Artificial pelleted feed (30% protein) was given to the fish throughout the experimental period.

A. hydrophila isolate obtained from Aquaculture Department, College of Fisheries, Mangalore was grown on tryptic soya broth (TSB) overnight at room temperature and the culture was washed with PBS three times by centrifuging at 3500 rpm. The pellet was resuspended in 15 ml PBS and stored as aliquots at -20ºC. Rohu (n=3) weighing around 200 g were injected (intramuscularly) with 0.1 ml of the antigen (*A. hydrophila* at ~10^8 cells ml^{-1}). Serum was obtained from moribund fishes by clotting the blood collected from caudal vein and centrifuging the blood at 6000 rpm for 10 min and the serum stored at -20°C. The fish serum was streaked on Aeromonas isolation media and incubated overnight at 37ºC for 48 h in order to confirm the infection by reisolating the bacteria. Serum from all the infected fishes were pooled and stored at -20ºC. Rohu (n=2) were immunized with PBS alone and the serum collected on 15th day was used as control. The serum of control fishes was also subjected to Aeromonas isolation procedure.

For immunization 2 mg of antigen (*A. hydrophila*) was subjected to denaturation by 0.1% SDS treatment followed by boiling at 100ºC. The

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denatured protein was dialyzed at 4°C against phosphate buffered saline (pH 7.2) using a low molecular weight dialysis membrane (12 KD cut off, Sigma, USA) and was concentrated to 2 ml by shifting the membrane pouch to sucrose. The concentrated protein was stored at -20°C for further use and the protein content was measured using spectrophotometry employing a kit (Genei Bangalore, India) following Lowry's method. Rohu (n=3) weighing around 200 g were immunized (intramuscularly) with denatured *A. hydrophila* bactrin with Freund's Complete Adjuvant (1:1 ratio) at 100 µg kg^{-1} body weight. Serum was collected from the fish on the 5th and 15th day post immunization (dpi).

ELISA for detection of *A. hydrophila* specific rohu Ig was carried out as per Furuta et al. (1995). The bacterin used for immunization was used as the antigen and was coated on the ELISA plate at ~1 µg $well^{-1}$ using carbonate-bicarbonate buffer (pH 9.5) and incubated overnight at 4°C. After washing off the unbound antigen with wash buffer (0.05% Tween 20 in PBS), free sites on the wells were blocked by 200 µl blocking solution (5% skimmed milk powder in PBS) for 1 h at room temperature. After washing, the fish sera (Control, Infected and Immunised) were diluted with wash buffer as doubling dilutions from 1: 100 to 1: 12, 800 and 100 µl of each dilution was added to the plate in duplicates. The fish sera were incubated for 3 h and plate was washed three times with wash buffer. MAb (F2D9) was added and incubated for 1 h at room temperature. The plate was washed three times with wash buffer and rabbit anti mouse IgG peroxidase (Bangalore Genie) at 1: 2000 dilutions in washing buffer was added and incubated for 1 h at room temperature. The plate was washed 3 times with wash buffer and 100 µl of substrate; tetramethylbenzidine (TMB) /H_2O_2 (Bangalore Genei, Bangalore, India) diluted in distilled water (1:20), was added and incubated for 10 min. Reaction was stopped by adding 100 µl of 1 M H_2SO_4 and absorbance was measured at 450 nm using an ELISA reader (Bio-Tek instruments, USA).

During the infection studies, clinical signs such as distended abdomen, loose scales, lesions on the body especially at the tail region were observed from 3rd day post injection. Similar clinical signs were reported in *A. hydrophila* infected rohu by Nayak et al. (1999). Mortality of all fish occurred between 5 to 7 days of post infection. Shome et al. (2005) reported the clinical manifestation of the disease within 72 h in Indian Major Carps followed by death after 4-6 days of infection. In the present study, *A. hydrophila* colonies could be reisolated from the moribund fish using Aeromonas selective agar (Hi Media, Mumbai). The control fishes and immunized fishes did not show any clinical signs and mortality pattern, indicating fish death due to *A. hydrophila* infection.

Both vaccinated fish sera and infected fish sera were subjected to an antibody capture ELISA to demonstrate the ability of the MAb to determine antigen (*A. hydrophila*) specific antibodies. MAb F2D9 was employed for ELISA because of its higher sensitivity in ELISA (data not shown). From the ELISA titration values (Fig. 1 and 2) it is evident that there is a clear difference in the antibody titre values between control, immunized and infected sera. There is a decline in antibody titre from the 5th dpi sera to the 15th dpi sera in the case of immunized fishes and the infected sera showed the titre values in between. As the dilution of the test sera increased, absorbance decreased linearly ranging from 1:100 to 1600. Since the absorbance values did not vary considerably after 1:1600 dilutions the optimum dilution of rohu sera for detecting the specific Ig can be limited to this dilution. From the figure it is clear that the absorbance of 0.1 units and above only show marked difference in titration and hence 0.1 absorbance can be considered as the lower limit for the *A. hydrophila* positive sera. Moreover control fish serum shows absorbance values well below this value. Similar results were reported by many workers for different anti fish Ig MAbs employing different antigens. Rathore et al. (2008) also demonstrated similar ELISA curves in sera collected from *Edwardsiella tarda* infected fishes using anti-rohu Ig MAbs. Van der Hejden et al. (1995) demonstrated the suitability of the anti-European eel Ig MAbs to detect antigen specific antibodies using similar ELISA titration curves. Zilberg & Klesius (1997) demonstrated the antigen specificity of catfish immunoglobulin employing an anti-cat fish IgM

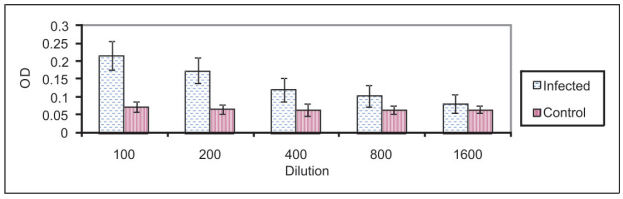

Fig. 1. Absorbance (OD) measured employing ELISA for two fold serially diluted sera obtained from infected and control fishes.

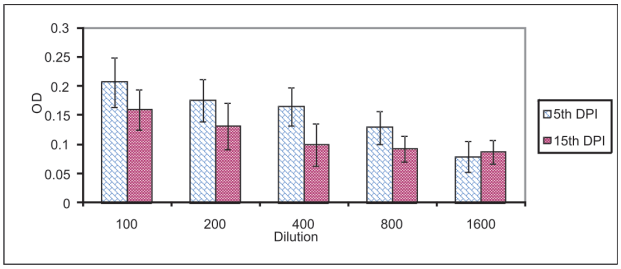

Fig. 2. Absorbance (OD) measured employing ELISA for two fold serially diluted sera obtained from immunised fishes.

MAb based ELISA.

Since the MAb used in this study is specific for rohu immunoglobulin, similar ELISA can be developed for other pathogens to determine the pathogen specific antibody titre. More over the ELISA procedure is also compatible to determine the efficacy of vaccines in rohu for various pathogens. Further the sensitivity of the MAb can be enhanced by using the purified anti-rohu Ig MAb conjugated with the substrate as the primary antibody

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