

Development of Co-agglutination Method for Viral Nervous Necrosis from Grouper (*Epinephelus* sp.) in Batam

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Abstract: Viral nervous necrosis (VNN) has been reported to infect larvae and juvenile of humpback grouper, and until now, this virus is one of main problem for humpback grouper. Co-agglutination test proved to be a simple, rapid and reliable diagnostic test suitable for use in the field or laboratory without any special apparatus. The study aimed to study application of a co-agglutination test using *Staphylococci* sensitized with specific antibody for the diagnostic of VNN in grouper. First, brain and eye organ samples from diseased fish are homogenized with phosphate buffer saline (PBS) of pH 7.2. Then, the supernatant is collected after centrifugation at 8,000 rpm for 20 min, and finally one drop of the supernatant and one drop of anti VNN antibody sensitized *Staphylococci* suspension are mixed on a glass slid and observation of the agglutination is performed after 5-10 min. The result shows that co-agglutination technique detects positive VNN in the brain and eye samples. The co-agglutination technique may provide valid result in a very short time as compared with complex method, such as enzyme-linked immunosorbant essay (ELISA) and fluorescent antibody technique (FAT) that require a high cost. Thus, this test can detect VNN faster, simple and more economic.

Key words: Co-agglutination, VNN, grouper.

1. Introduction

Immunodiagnostic test using *Staphylococci* co-agglutination has been recognized for its ability to detect bacterial and viral pathogen in fish [1-3]. This method is proven to have many advantages for its character being simple, fast, accurate and inexpensive especially for field application. The co-agglutination test for diagnosing of fish disease could be used to detect the specific viral antigen in extracts of whole organ or affected tissue [3].

Viral nervous necrosis (VNN) has been reported to infect larva and juvenile of humpback grouper [4], and until now, this virus is one of main problem for humpback grouper. VNN is one of infection disease that was causing serious damage to the marine culture

industries, especially for the hatchery and the culture of humpback grouper [5-7]. The indication of fish infected by virus is differed by the age; on 45 days to four months age, the fish will stay at the base, swim up site down, slowly move and sometime suddenly startle, and if usually do not want to eat in 3-5 days, the fish will be dead.

The diagnose for VNN disease usually use the polymerase chain reaction (PCR) conventional method which generally needs much time and real-time PCR which needs more expensive cost [8]. The co-agglutination test method that used in the research is the serology method test which needs less time and less cost. This method proved to have an advantage with simple characteristic, fast, accurate and cheap, especially for fields application [9]. But, in Indonesia, this technique has not gotten attention and sufficient development. Until now, there has been none study of

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co-agglutination implementation to detect the virus fish pathogen in Indonesia. Nevertheless, this method is really suitable to apply in Indonesia, because of limited tools and chemicals of laboratories in Indonesia.

The co-agglutination method, principally, is the technique that the antibody immunoglobulin is tied with the protein A from *Staphylococcus aureus* which has big size. When the couple of *S. aureus* immunoglobulin G tied with the antigen target, the huge complex is made although the antigen is small, and so this can be used to detect the organ antigen without planting it in the artificial media so that it can be seen with eye or light microscope (the enlargement of 10 times objective lens). This method is known as the antibody sensitized *Staphylococci* method or super-agglutination. Thus, the paper aimed to detect the VNN from grouper (*Epinephelus* sp.) by co-agglutination method.

2. Materials and Methods

2.1 Immunity Serum Preparation

Polyvalent immune serum was obtained from rabbits which have been immunized with a VNN vaccine. The vaccine used in this study is the vaccine killed VNN (KoVax Ltd. product) [10]. The antiserum harvest was done after five weeks.

2.2 Immunoglobulin Preparation

Separation of immunoglobulin is done with ammonium sulfate precipitation method [2]. Equal amount of immune serum was added to 50% ammonium sulfate (pH 8.0) slowly while stirring by stirrer for 30 min. The suspension was centrifuged at 6,000 rpm for 30 min, the supernatant was discarded and the pellet resuspended again in physiological saline to initial volume and re-precipitated using ammonium sulfate. This process was repeated up to three times. Precipitate/final pellet was re-dissolved in phosphate buffered saline (PBS) to a number of initial volume and placed in a dialysis membrane. Aliquot is

washed by immersing in dialysis membrane PBS of pH 7.2 solution for 48 h, with replacement of PBS solution every 12 h.

2.3 Preparation of Protein A

S. aureus isolates used were from the collection of the Faculty of Veterinary Medicine, University of Gadjah Mada. Isolates were propagated in tryptone soya agar (TSA) plate, harvested and centrifuged at 3,000 rpm for 15 min according to Saharia and Prasad [2] in three times. The final pellet was added 0.5% formalin, PBS of pH 7.2 and left overnight at room temperature. Aliquot then was heated at a temperature of 80 °C water bath for 15 min and immediately cooled in the refrigerator. Final suspension is centrifuged at 3,000 rpm for 15 min and the pellet was added PBS of 1:10 (w/v).

2.4 Coupling of Immunoglobulin G and Protein A

Suspension of *S. aureus* and immunoglobulin G with volume of 1:1 is homogenized. After incubation for overnight at room temperature, the suspension was centrifuged at 1,500 rpm for 20 min. Supernatant was discarded, PBS was added to return to the initial suspension volume and was used as a reagent for the co-agglutination test.

2.5 Positive Control

Used as positive control was vaccine killed of VNN (KoVax Ltd. product). For the negative control, used was physiological saline (0.85%).

2.6 Co-agglutination Test Procedure

Co-agglutination test was carried out using the method by Yoshimizu and Kimura [3]. Samples from organ were homogenized with a ratio of 1:1, 1:2 and 1:4 with PBS of pH 7.2. The homogenate was centrifuged at 8,000 rpm for 20 min. The filtrate/supernatant from samples was dropped on the slide and added with co-agglutination test kit with an equal volume (1:1). Observation of the agglutination was performed after 5-10 min.

3. Results and Discussion

The method by using co-agglutination kit to detect viral disease in fish is done for the first time in Indonesia. The application of co-agglutination shows that the test kit produced is able to detect VNN from organ fish infected. The detection of VNN in affected brain and eye showed positive co-agglutination reaction (Figs. 1 and 2), while detection of VNN in control showed negative (Fig. 3). The agglutination reaction occurred within 5-10 min and the optimal reaction was observed within 30 min. The test result shows there is a sand on glass slide and the suspension shows the agglutination. Brain and eye specimen



Fig. 1 Positive result in brain VNN test.

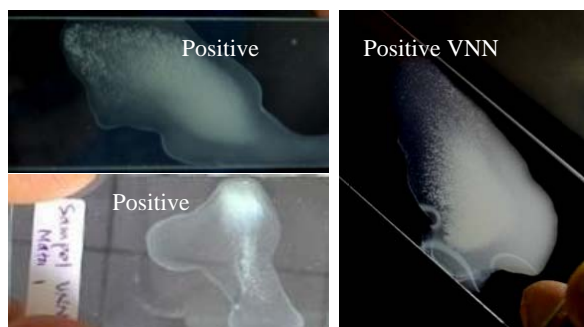


Fig. 2 Positive result in brain and eye VNN test.



Fig. 3 Negative result in VNN test, if there is no VNN virus.

prepared from diseased fish showed positive agglutination.

The co-agglutination test developed by Kronvall [10] was further standardized, and the agglutination reaction was visible within 2 min, whereas he observed the agglutination reaction within 10 min. Co-agglutination has also been used for detection of infectious pancreatic necrosis virus in the tissue sample of Atlantic salmon [9]. The most important use of the co-agglutination test in the diagnosis of fish disease would be to detect the specific viral antigen in the tissue of infected fish and it was found that viral antigen could be detected in extract of whole organ or affected tissue [3].

4. Conclusions

The development of co-agglutination test kit produced is able to detect VNN in brain and eye organ sample of affected fish. This test proved to be a simple, rapid and reliable diagnostic procedure in the field without any special apparatus.

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