
Identification of *Koi Herpesvirus* on Koi Fish (*Cyprinus carpio*) with Immunocytochemistry Test *Streptavidin Biotin*

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KEYWORDS

KHV

Koi

Immunocytochemistry
Streptavidin Biotin

Abstract Koi herpesvirus (KHV) has been identified in common carp (*Cyprinus carpio*) and varieties of *C. carpio* such as koi carp. Handling KHV outbreak on koi fish are always late and on case KHV infection, hence the purpose of this study is to find an alternative method in identifying a particular fish diseases KHV which more simple, practical and accurate. 20 samples are positively infected by KHV were derived from Blitar, with the characteristic of them are gill slime, erosion or ulcers on the skin, movement were not controlled, and skin blister. The result of our study showed that 100% positive reaction to the ICC in smear blood, which is marked golden chocolate color. The result of immunostaining streptavidin biotin obtained that koi fish have been infected by severe KHV showed golden chocolate color on the blood smear compared to positive control. While fish have been infected by undemanding KHV infection or still in the beginning stage, the blood smear showed chocolate bluish. It can be concluded that Immunocytochemistry Test Streptavidin Biotin can be used for early identifying of Koi Herpes Virus (KHV) due to simpler producer, more practical, and accurate result.

Introduction

Koi herpesvirus (also known as Cyprinid herpesvirus 3; CyHV3) is classified as a double-stranded DNA virus belonging to the family Alloherpesviridae (which includes fish herpesviruses)(Rathore *et al.*, 2012; Zhou *et al.*, 2014), that induces a lethal acute viraemia that is highly contagious in common carp (*Cyprinus carpio*) and varieties of *C. carpio* such as koi carp and ghost carp (koi x common carp) (Way *et al.*, 2017). The experiment of Waltzek and colleagues (Cui *et al.*, 2015) revealed that KHV is indeed a herpesvirus, based on viral morphology and genetics, and is closely related to carp pox virus (Cyprinid herpesvirus 1; CyHV1) and goldfish hematopoietic necrosis virus (Cyprinid herpesvirus 2; CyHV2). Koi herpesvirus disease has been diagnosed in koi

and common carp (Hedrick *et al.*, 2000; Ogata, 2009).

In Asia, KHV has been reported in Indonesia (Sunarto *et al.*, 2002), Japan (Sano *et al.*, 2004) and Taiwan (Chen *et al.*, 2015). The first episode of mass mortalities of cultured koi (*Cyprinus carpio*) was recorded in March 2002 in Blitar, East Java. It was introduced from Surabaya, the capital city of East Java. The fish were imported from China through Hong Kong in December 2001 and January 2002. Blitar is well known as the center for koi production in the country. The koi including the infected one were distributed over the country, with Central Java, West Java and Jakarta as the main market.

The first isolation of KHV from koi and common carp in Indonesia and initial

characterization of the isolates. Clinical signs, histopathology and virion morphology are similar to those of isolates from other countries. Phylogenetic analyses using the thymidine kinase gene amplified from each isolate and from carp tissue samples collected from KHVD outbreaks throughout Indonesia indicated that the Indonesian isolates are more closely related to the Asian than the European KHV lineage (Sunarto *et al.*, 2011). Diagnose of KHV based on virus isolation and PCR has weak sensitivity (Bercovieret *al.*, 2005), and a study in farmed fish of England show almost 85%–93% of fish are seropositive by ELISA, even 1 year after disease outbreak (Taylor *et al.*, 2010). This indicates that ELISA is a valuable method of establishing previous exposure of KHV in apparently healthy fish. However, this method is not recommended as a primary diagnostic tool because it cannot determine whether fish is still infected with the virus. Cross reacting antibodies in fish serum can also lead to false positives. Based on some reasons above, we need a new diagnosis method approach for confirmation of the diagnosis of KHV, that is an immunopathology test immunohistochemistry, streptavidin biotin. Application and development biotechnology test based on antibody, also known as immunopathology immunohistochemistry streptavidin biotin for detection of DNA from KHV virus on koi fish is very important. Remember, that handling KHV outbreak on mas and koi fish are always late and on case KHV infection, fish that infected KHV looks normal (healthy) or not show clinical symptoms, but exactly they are main transmission of KHV infection because they distribute KHV continuously at waters environment around and able to infect other sensitive fish. Based on that, the purpose of this research is to apply biotechnology test based on immunopathology for early detection of KHV on fish.

Material and methods

Fish Sample Collection

In this research, we used 20 koi fishes (*C. carpio*) with the size are about 10-20 cm and showed some symptoms clinical infected with KHV, they are gill slime, erosion or ulcers on the skin, movement were not controlled, skin blister, or sometimes accompanied bleeding on the fin obtained of 2 different pools in the pool hatchery koi fish in Blitar, East Java. Bodyweight of the fish are varying; the range is between 200-300 g and 600-700 g. Immunopathology test on koi fish called immunocytochemistry *biotin streptavidin* for detecting KHV is applied on smear blood of the koi fish.

Streptavidin Biotin Koi Herpesvirus

Smear blood of koi fish (*C. carpio*) were incubated to in solution acetone, dripped with normal goat serum, and incubated in room temperature. Next, fish smear blood dripped with *anti-KHV monoclonal antibody* as primary antibodies then incubated on temperature of 37°C for 20 minutes (*Santa Cruz Biotechnology*), washed out with PBS solution for 10 minutes and directly incubated with secondary antibody and added with fluid *biotinylated secondary antibody* for 10 minutes. Samples were incubated with streptavidin-horseradish peroxidase conjugate for 5 minutes and incubated with substrate-chromogen at temperature room for 15 minutes. Then washed with distilled water for 10 minutes (*Zymed Corp.*, San Francisco, CA). Samples then dripped by counterstain hematoxylin, dehydrated, washed out with distilled water, and closed with glass cover for under observation. Smear blood that has been tested by immunopathology immunocytochemistry *streptavidin biotins* examined under microscope and analyzed on a descriptive explanation.

Results and Discussion

Visual Measurement

After 3 days adaptation, from 20 samples were taken from Blitar, just 13 samples were live and 7 samples died. The most visible anatomic pathological from the seventh dead fish is hemorrhagic and congestion in the operculum, tail fin, back fin, operculum and white nodule on the gills. This is similar with a study which demonstrated the affected fish have pale patches or blisters on the skin along with sunken eyes and increased respiratory frequency. Later the fish becomes disoriented and swim erratically prior to death. Another characteristic sign seen in diseased fish is white patches on the gills or gill necrosis (Hedrick *et al.*, 2000). The common wounds that occur is only superficial color (white milk or white grayish which is found at 1-2 mm above skin). The wound will increase in corresponding with the duration of infection. This disease is rarely occurring in young fish; thus, the search of samples was prioritized on adult fish.

Wang *et al.* (2015) revealed that the virus is located in water will permanent effective could contagious for 4 hours. Accordingly, water is one of the good mediums for spread of the virus in fish, mainly through the gills. This is similar with viruses that attack breathing on

mammals that infect network epithelium breathing and developing multiply inside. Hartman *et al.* (2004) explained that transmission of KHV is horizontal, mainly via water, but possibly via animal vectors and vomits.

Herpesvirus on fish on a general identified as cause disease start from the scale infection to infection of fatal systemic (Gilad *et al.*, 2003). This phenomenal was also seen on fish koi who will do examination/identification too experience similar symptoms with disease herpesvirus that attacks the cyprinid family. Herpesvirus could attack various size fish start from larvae up to parent, usually happen on range temperature 18°-28°C, and can cause mortality up to 80-100%. Nevertheless, herpesvirus disease was rarely found on the young fishes, it is caused the fish young still have a good defensive immunity so that able to maintain condition his body from attack of the herpes virus.

Typically, this disease is very contagious, however its attacks only limited on goldfish and koi fish. It has been proven after fish koi obtained from Blitar, after 3 days maintenance, there are 7 koi fish herpes viruses were identified, characterized with characteristic features physical on fish, similar with the experiment before (Figure 1).

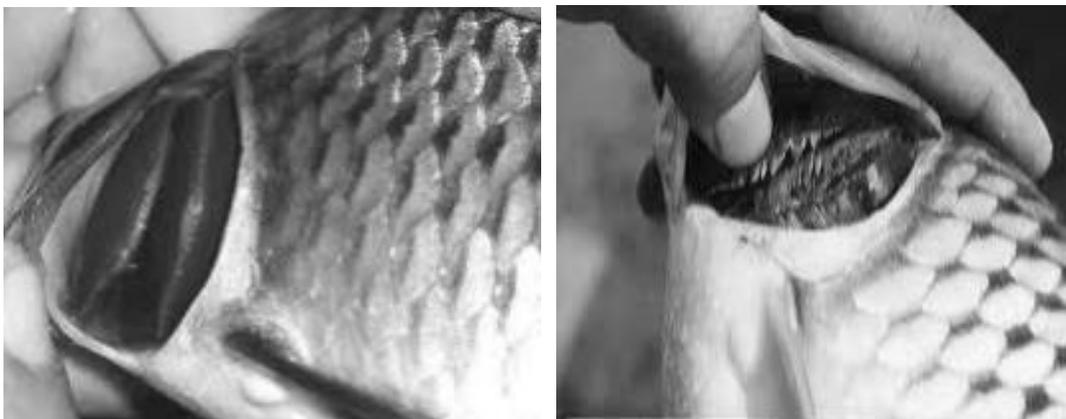


Figure 1. Infected KHV Koi Fish. a) Ulcers on fish gills. b) Haemorargic on fish gills. (Suniatmadja, 2017).

Nomenclature of this virus was not determined yet by International Committee on Taxonomy Virus (Al Rwahnih *et al.*, 2018). Virus nomenclature can base on manifestation morphology, pathogenesis or the effect off manifestation clinical, type animals used as host, antigenic properties, growth characteristics, type of cytopathic influence on cell culture or based on homology with other viruses that have already known (Waltzek *et al.*, 2005).

KHV Examination Using Immunocytochemistry Method (Streptavidin Biotin) on Blood Cell

Warford (2003) demonstrated that immunocytochemistry is a combination of immunological and cytological techniques. While the examination Immunocytochemistry itself is one kind of examination of immunological methods immunochemistry of enzymes that are specific and aims to help diagnose or detect the presence of antigens or location antigens, in tissue biopsies, preparation cytology or can be used to detect the antigen in a liquid specimen who are infected by pathogenic germs. Application of immuno (sito) chemistry in the field of animal health, among others, to establish a diagnosis of infectious diseases that affect the respiratory tract (Saxena *et al.*, 2012).

Positive samples were used in immunocytochemical examination SB technique is the blood of koi fish that is thinly reviewed with a *pull film* technique. With the results of thin reviews obtained blood smear results that can be read after the final process of *staining*. In complete results examination by Immunocytochemistry Technique can be seen on Table 1, which show 100% positive reaction to the ICC in smear blood, which is marked golden chocolate color.

Negative control of KHV test with Immunocytochemistry method showed the purple blue color, could be seen on Figure 2, and for positive control KHV test with

immunocytochemistry method with the results is golden chocolate color, could be seen on Figure 3.

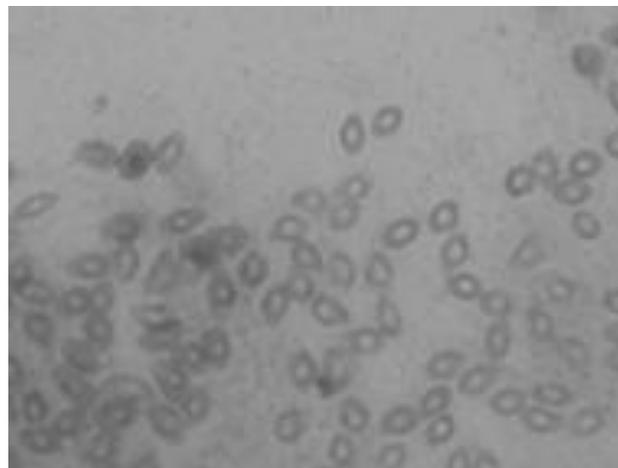


Figure 2. Negative Control of KHV Test (SB, 100x)

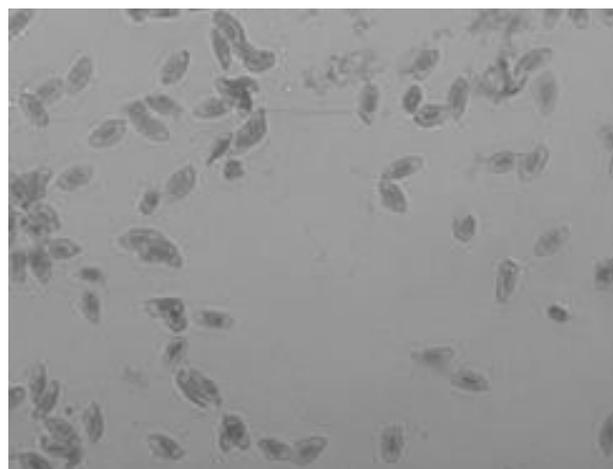
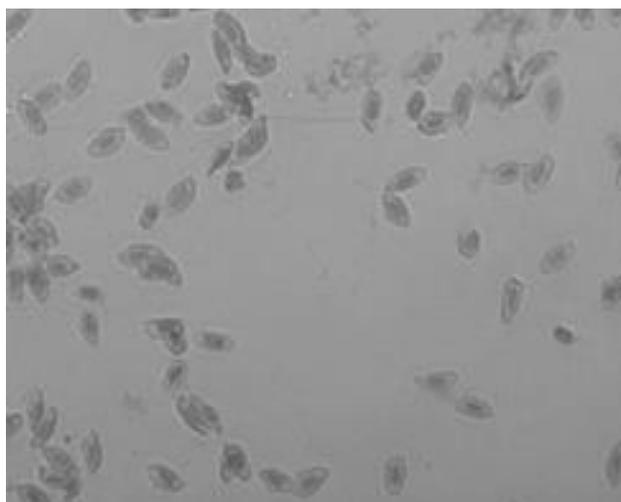


Figure 3. Positive Control of KHV Test (SB, 100x)

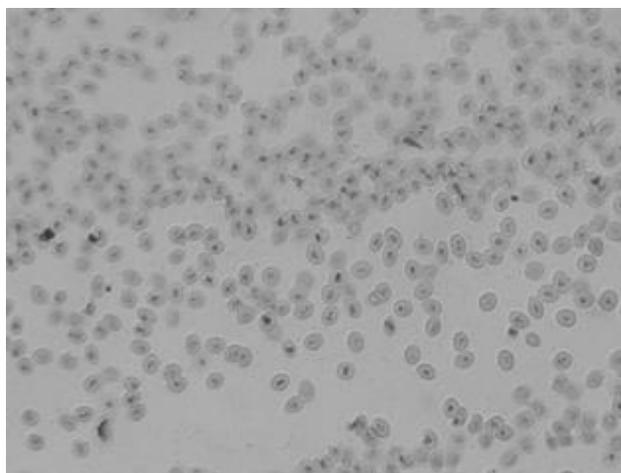
Table 1. Results of KHV test on Fish Koi with Immunocytochemistry Method

Type Sample	Target Organ	Deuteronomy		
		1	2	3
Sample 1	Blood	+	+	+
Sample 2	Blood	+	+	+
Sample 3	Blood	+	+	+
Sample 4	Blood	+	+	+
Sample 5	Blood	+	+	+
Sample 6	Blood	+	+	+
Sample 7	Blood	+	+	+

On Figure 4 (a), the blood smear showed golden chocolate color. It is mean that koi fish have been infected by KHV, while on Figure 4 (b), blood smear showed chocolate bluish, which means that fish have been infected by undemanding KHV infection or still in the beginning stage.



(a)



(b)

Figure 4. Profile of smear blood koi fish infected KHV by Staining with ICC technique Streptavidin Biotin. a) positive weight sample, the color is golden chocolate; b) positive undemanding sample, the color is chocolate (SB, 100x).

In our experiment we used the immunocytochemistry method with the Lab Vision kit. The early step namely blood

specimens mixed with EDTA for examination Anti-KHV IgG. Then we made thin smear preparations on a fixed object glass with acetone dried open in air. After dried, then painted by immunocytochemistry with streptavidin-biotin and after added Meyer's hematoxylin, we observed under microscope light. The result obtained the golden chocolate staining after dripped with substrate chromogen 3.3' diaminobenzidine (DAB) as results of positive KHV expression.

Generally, staining of immunocytochemistry is bond between antigen-antibody was attached by directly or indirectly with substance marker, and the positive reaction will be visualized caused by chromogen that binds the marker on cellular level. Chromogen is a substance who can visualize substance marker on immunocomplex bond on immunocytochemistry staining. DAB produces very insoluble product in alcohol and others solvent. DAB oxidation also cause polymerization. DAB has ability for react with osmium tetroxide, and thus it is very useful in electromicroscopy and a part of immunocytochemistry.

Conclusion

Based on our whole results, we conclude that Koi Herpes Virus (KHV) can be detected with use the immunopathology test based on biotechnology, which is immunocytochemistry with biotin streptavidin staining technique. These methods will useful for early detection of Koi Herpes Virus (KHV) on koi fish.

Acknowledgments

This research was supported by a grant from Laboratory of Water Sciences and Biotechnology, Department of Fisheries and Marine Science, University of Brawijaya Malang, and Sumber Harapan Farmers Group, Blitar, East Java.

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