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Immunoreactivity Inactive *Edwardsiella Tarda* whole Cell Vaccine on Koi fish (*Cyprinus carpio*).

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ABSTRACT

Vaccination is one of the most effective methods to cope with Edwardsiellosis disease problems caused by *Edwardsiella tarda* bacteria in koi fish cultivation (Cyprinus carpio). This conduct possesses high economic value as vaccination is capable of improving non-specific and specific fish immune systems. Therefore, it is necessary to conduct research in order to understand the immunoreactivity of inactive *E. tarda* vaccine in koi fish (*C. carpio*) infected with *E. tarda* bacteria. Vaccine production was performed using head kill method at 100 ° C for 15-20 minutes or 60 ° C for 30-60 minutes. To find immunoreactivity vaccine, researchers conducted observations on immunohistochemistry, antibody titer, and RPS. Based on obtained result, C treatment using vaccine 10^8 cell / mL dose produced the highest IC value 64.67% in kidney and 78.10% in spleen. Antibody titer value was 2.11. RPS value post-vaccination was 94.12% and post-challenge test was 83.33%. It is hoped that this research result to be applied koi fish cultivation. It can also be used as a source of information for further research.

Keywords: *Cyprinus carpio Edwardsiella tarda*, Immunohistochemistry, Antibody Titer, RPS

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INTRODUCTION

The development of disease prevention in fish aquaculture tend to study preventive approach using vaccination strategy specifically capable of protecting fish from certain pathogens. Based on various fish disease control efforts, it is proven that vaccination is the best disease control (Passarela, 2006). Purwaningsih et al. (2014) stated that vaccination is an attempt to increase the immune response to certain pathogens based on two elements of adaptive immunity. The two elements are specificity and memory. For culture production on the other hand, especially koi fish (*Cyprinus carpio*), which has high economic value (Utami, 2013, Zubaidah, 2013) is much constrained due to bacterial diseases such as Edwardsiellosis disease caused by *E. Tarda* bacterial infection (Firma et al., 2013; Li et al., 2015) and common disease prevention using antibiotics.

Edwardsiellosis disease is a disease that causes blood clots on the fins and small bleeding on body surface which led to mortality especially to koi fish (*Cyprinus carpio*). Infections often occur when the host experience immunosuppressed due to stress or degradation of water quality during fish seed stage (Dangeubun et al., 2013; Sumiati, 2015). A new immune response is perfectly formed after the fish matured (Suhermanto et al., 2011).

One alternative of disease prevention is increasing the immune system in a specific and non-specific manner. The use of vaccines is thought to be more effective compared to using immunostimulants that work only on non-specific immunities in test organisms, in contrast to vaccines that can work on both non-specific and specific immune systems in examined fish. It may provide antibody memory for specific pathogens (Hazzulli et al., 2015; Song Lin et al., 2015; Pratiwi, 2016). According to Setiawan et al., (2012). Vaccination may enhance specific and non-specific immunity of a fish.

However, no research has been conducted on the utilization of inactive *E. tarda* whole cell vaccine to prevent *Edwardsiellosis* disease plaguing koi fish infected with *E. tarda* bacteria. It is not known immunoreactivity of *E. tarda* vaccine using the heat-killed method at 100 ° C for 15-20 minutes in koi fish. Immunoreactivity is antigen ability to bind into an appropriate antibody. Good vaccine criterion to use is to have a high immunoreactivity in the host. The higher the immunoreactivity level the better the vaccine would be(Setyawan et al., 2012). Therefore it is necessary to conduct immunoreactivity research of inactive *E. tarda* whole cell vaccine on koi fish (*Cyprinus carpio*) studied by increasing number of immunohistochemistry, antibody titer, and RPS value.

MATERIALS AND METHODS

Research Material

Tools used in vaccines manufacture are ose needles, water shaker, centrifuge, water bath, petri dish, autoclave, bunsen, test tube, reaction tube rack, hot plate, refrigerator, micropipette, and centrifuge. Tools used in pure *E. tarda* culture are petri dish, Erlenmeyer 500 ml, 100 ml measuring cup, digital scale, ose, bunsen, autoclave, and incubator. In test fish examinations and blood observation basin, aquarium, scoop net, glass object, glass cover, microscope, aerator, measuring glass, beaker glass, microplate, and micropipette were used.

The ingredients used in vaccine preparation are pure bacteria *E. tarda* and *phosphate buffer saline* (PBS). Materials used in pure *E. tarda* culture were obtained from the collection of East Kalimantan Fish Quarantine Institute of East Java in the form of Merck *Tryptone Soy Agar* (TSA) media, *Tryptone Soy Broth* (TSB), aquades, alcohol and Na-fis 0.9%. Ingredients used for blood observation are koi fish blood (*Cyprinus carpio*), Turk's solution, 3.8% Na citrate, spuit, *eppendorf*, *E. tarda* vaccine, paper label, tissue, mask, and gloves.

Research Design

The research method used is an experimental method. The design used was RAL (Completely Randomized Design) consisting of 4 treatments and 3 replicates. Positive control (bacterially infected fish) and

negative control (uninfected and unvaccinated fish) were used as comparison material. The main parameters in this study were increased immunohistochemistry, antibody titer and relative percent survival (RPS) in koi fish (*C. carpio*).

Test Fist Preparation

The test fish used in this study is koi fish (*C. carpio*) sized at 10-12 cm. 120 fishes were obtained from cultivators in Blitar, East Java. Prior to conducting the study, the test fish were kept for 1 week for acclimation process.

Vaccine Manufacture

Preparation of vaccine manufacture is conducted by heating method referring to Alifuddin method(2002) by heating bacterial culture aged 24 hours at 100 ° C for 15-20 minutes or 60 ° C for 30-60 minutes. After the heating process, bacterial cultures are centrifuged at 3500-500 rpm for 15-20 minutes to separate pellets and supernatant. The separated pellet with supernatant was washed with phosphate buffer saline (PBS) for 3 times. Obtained pure pellet vaccine was whole cell vaccine.

To test the quality of the vaccine, a viability test was conducted by culturing the vaccine using *broth hert infusion agar* (BHIA). The vaccine is eligible for use should in bacterial growth does not occur in BHIA media.

Vaccination

Inactivate vaccine from intact *E. tarda* cell were produced using following concentrations: A: 10^4 cell/mL; B: 10^6 cell/mL; C: 10^8 cell/mL; D: 10^{10} cell/mL ; and K: *shoulen broth*. Vaccinations were given to test fish as much as 0.1 ml/fish (Hardi et al., 2013) using vaccine injection methods in intraperitoneal section according to koi fish treatment for of 0.1 mL/fish(Hardi et al., 2013). Before conducting vaccination process, fish was maintained for 10 days. Vaccination process was conducted on day 0, day 8 (booster), and one-week post-booster. On day 16 test challenge was conducted by injecting bacteria E. tarda 10^6 cell/mL.

Challenge Test

Bacterial infections were performed after 2 weeks after vaccination by injecting 10^6 cell/mL *E. tarda* as much as 0.1 ml/fish (Lukistyowati and Morina, 2012) in intraperitoneal section. Fish cultivation continued for 2 weeks until blood sampling and organ retrieval was conducted on the koi fishes.

Immunohistochemistry

Kidney and spleen tissue used for Immunohistochemical preparates (CPI) were taken from fish organ after 2 weeks of cultivation post-challenge test. CPI staining is used to detect the presence of *E. tarda* antigen in *head squash* preparates (smear cells). The antigen distribution will be visualized in brown when *E. tarda* emerge. Preparates processed through paraffinization process were fixed using cold methanol for 2-4 minutes, and 3% peroxidase blocking solution (H_2O_2) for 30 minutes, then washed with 3x phosphate buffer saline (PBS) for 5 minutes each. Nonspecific blocking binding was performed by spreading the tissue using 1% Fetal bovine serum (FBS) for 30 minutes then washed with PBS, then the tissue was treated with primary antibody (monoclonal antibody, anti-*E. tarda* mouse) and incubated for one night at 4 ° C and then washed again with PBS. The tissue was exposed to *Biotinylated universal secondary antibody* (secondary anti-mouse antibody) for 30 minutes, it is then washed using PBS 3x for 5 minutes each process. The tissue was dried and treated with Trekavidin-HRP for 30 minutes, then washed again with PBS 3x for 5 minutes each process and dried for 3 minutes. Chromogen 3,3'-*diaminobenzidine* (DAB) was dropped on preparates and left for 15 seconds. After administration of DAB, it is immersed in aquades and dried for 3 min followed by blue core counterstain using Mayer's *hematoxylin* for 25 s. The preparates are then washed using distilled water and followed by a dehydration process using 100% ethanol and clearing using Xylol. Preparates in glass object was mounted using permount and covered using glass cover.

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Antibody Titer

Observation of antibody titers was performed to observe antibodies produced by fish after vaccination (Purwaningsih, 2013). Measurements of antibody titers were performed before vaccination, 2nd week after vaccination (after booster), and week 4 (2 weeks after challenge test). The observation was performed on blood plasma or fish blood serum post- centrifugation test for agglutination test in a microplate. Antibody binding can be detected by conducting direct agglutination method. Blood serum is diluted according to treatment. According to Purwaningsih, (2013) should the serum contains antibodies, then when the serum is administered with antigens, antigen-antibody complex agglutination would occur. Antibody Titer measurement was conducted using agglutination method using microplate. 50 µl PBS solution is inserted into from 2nd to 12th wellbore. 50 µl of blood serum was inserted into the 1st wellbore as a positive control. The serum to be measured is 50 µl at the 2nd wellbore and homogeneous using a micropipette. Serial dilution was conducted from 2nd – to 11th wellbore. The 12th well was only filled with PBS as a negative control. 50 µl. *E. tarda* bacteria (10^6 cells / ml concentration) of 50 µl were inserted from 1st to 12th wellbore which was mixed with serum and PBS-saline. The microplate was covered using plastic wrap to avoid evaporation and was incubated at 37 ° C for 24 hours. According to Pratiwi, (2016) the presence of clumps such as fog in a microplate hole is a positive result of the formation of antibody titers.

Relative Percent Survival (RPS)

The survival rate (SR) post challenge test is calculated into Relative Percent Survival (RPS) value to obtain effectiveness of vaccination using Ellis (2001) formula:

$$RPS = 1 - \frac{\%\ \text{vaccinated fish mortality}}{\%\ \text{control fish mortality}} \times 100$$

RESULTS AND DISCUSSION

Immunohistochemistry (IHK)

Based on CPI test result, vaccinated fish had higher CPI values than control fish without vaccination (table 1). CPI is a method for detecting or identifying cells that are specifically based on antigenic structures or components and cellular products with complex reactions between antigens. In other words, immunohistochemistry can be used as a tool to detect immunoreactivity levels of specific antibodies produced by fish from the vaccination process. It aims to discover that antibodies work in fish rather than nonspecific natural antibodies from fish, but from specific antibodies from the results of vaccine induction.

Table 1. IHK Value on Koi Fish (*Cyprinus carpio*)

Treatment	Kidney Immunoratio±SD (%)	Spleen Immunoratio ± SD (%)
Control	17.57 ± 2.15	25.30 ± 1.35
A	24.73 ± 2.86	37.33 ± 2.49
B	53.00 ± 9.14	61.83 ± 3.10
C	64.67 ± 8.73	78.10 ± 6.22
D	32.83 ± 1.46	48.10 ± 1.90

Information: Dosage K: 0 cell/ml; A: 10^4 cell/ml; B: 10^6 cell/ml; C: 10^8 cell/ml; D: 10^{10} cell/ml

CPI observation in this study used two organ tissues in the form of kidney and spleen. Kidneys produce antibodies or immune cell sources including lymphoid cells that play a role in the induction and elaboration of the immune response. Based on the results of the IHK for kidney and spleen organ, spleen possesses higher average CPI value compared to a kidney. C (10^8 cell/ml) treatment had the highest CPI value of 64.67 ± 8.73 on kidney and 78.10 ± 6.22 on the spleen. This occurs because the spleen is the site where B cells activation occur to differentiate into plasma B and B memory cells (Sari et al., 2013). Plasma cells secrete antibodies specific to antigens which are captured by APC (Uribe et al. , 2011). Memory cells serve as memory

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reminders and constructors according to antigen structure (Setyawan et al., 2012). The lowest CPI value occurred in treatment A (10^4 cell/ml) in each organ. The small CPI value in treatment A indicates that vaccine dosage in treatment A is not optimal as to stimulate a specific immune response.

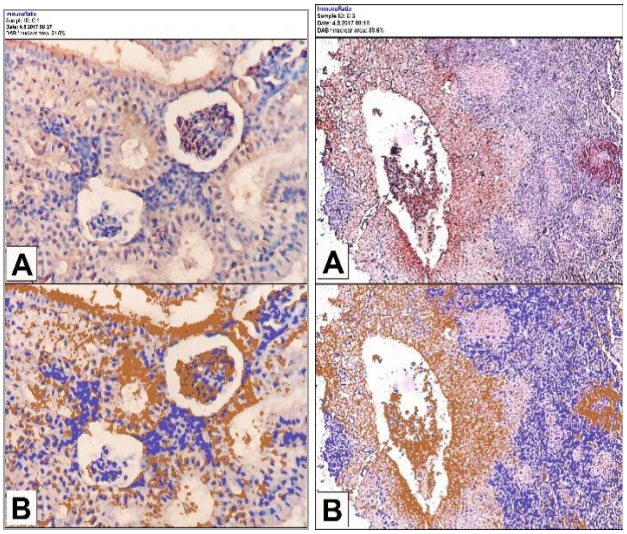

Figure 1. A). Kidney histology (left: 100x magnification) and spleen (right: 40x magnification) CPI staining results; B). Histology of the kidney (left) and spleen (right) results of the Immunoratio analysis.

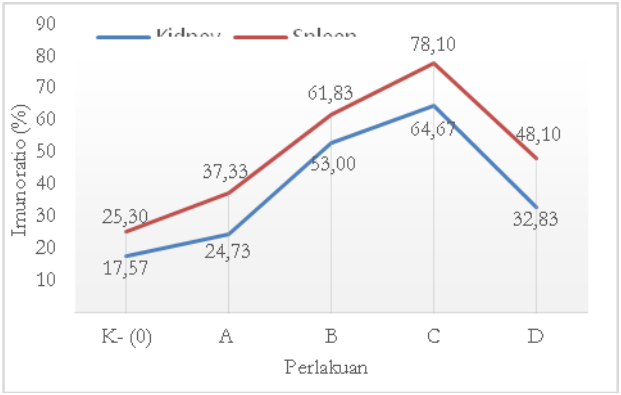

Figure 2. Histogram of CPI values on kidneys and spleen.

The change in golden brown color on the CPI test results indicate cross reaction between immunogenic proteins with a specific immune system (Yanuhar, 2011). Immunogenic proteins are proteins that can trigger active immune system complex should protein is inserted or exposed to an organism. Usually these immunogenic proteins enter the body of the organism in the form of antigen. Should the vaccine dose is too small during vaccination process it would result in small reactions that occur between vaccines acting as immunogenic proteins with a specific immune system of koi fish (C. carpio). Therefore, an immune response to treatment A (10^4 cell/ml) is ineffective in order to produce a specific immune response at the time of the vaccination process.

Treatment C (10^8 cell/ml) has the biggest CPI value compared to treatment D (10^{10} cell/ml) which has a higher dose. It means that in this study, vaccine overdose might cause lacking in formation evolution of a specific immune response produced by fish. The immune response process of fish can recognize antigens by introducing *antigen presenting cells* (APC) consisting of macrophages, B lymphocytes, and dendritic cells. Macrophages break down the antigen into smaller fragments and are presented to T lymphocyte cells via the second class Major histocompatibility complex (MHC), and when T cells capture the antigen via the T-cell receptor (TCR) and activated T cells, it would secrete cytokines such as IL-2, IL-4, IL-6, and IL-10 to differentiate into plasma B cells and memory cells. Should large amounts of cytokine compounds were produced in a short time, it would result in the occurrence of cytokine strom, where fish experience a decrease in health or stress.

This is why dosage in treatment D is not very effective reacting with fish-specific immunity. Thus treatment of C (10^8 cell/ml) in this study was the best vaccine dosage or optimum limit to produce vaccine immunoreactivity with a high specific immune response on koi fish (Cyprinus carpio) when infected with *E. Tarda* with immunohistochemical detection.

Antibody Titer

The results of antibody titer test in this study exhibits that koi fish (*C. carpio*) vaccinated with O-antigen from inactive *E. tarda* bacteria had higher adaptive immune response compared to non-vaccinated control fish, indicating that the vaccine was able to improve bactericidal ability in serum towards *E. tarda* disease, especially fish specific immunity (Sumiati, 2015) which is identified by clumping of serum mixture, phosphate buffer saline (PBS), and bacteria (Purwaningsih et al., 2014).

Table 2. Antibody Titer value on koi fish (-log2)

Treatment	Time (Day)				Mean
	7	14	28	42	
K (0)	1.81	1.81	0.90	1.20	1.43
A (10^4)	1.81	2.11	2.11	1.51	1.88
B (10^6)	1.81	2.41	2.11	1.81	2.03
C (10^8)	1.81	2.71	2.11	1.81	2.11
D (10^{10})	2.11	2.11	1.81	1.51	1.88

Blood sampling for antibody titer testing was performed 4 times, on days 7; Day-14; Day-28 (1 week post infection); and day-42 (2 weeks post infection). Treatment C (10^8 cell/ml) had the highest average antibody titer value during the study (2.11). The average value of antibody titer value in treatment C indicates that the dose of vaccine in treatment C is the most effective dose to trigger vaccine immunoreactivity with increased specific immune response against *E. tarda* bacteria. The value of antibody titer for all treatments increased on the 14th day or the first 2 weeks of vaccination (Sugiani et al., 2013; Trilia et al., 2014). Due to the introduction of the fish immune system that responds to the production of specific antibodies to recognize vaccine structure given in the form of antigens entering fish flesh. It will be then phagocytosed by macrophages and in turn macrophages will send signals to T lymphocytes through MHC II. Activated T cells will produce cytokines to trigger B cells activation that occurs in the spleen. Those B cells will differentiate into B plasma cells and B memory cells. B Plasma cells secrete antibodies specific to antigens captured by APC (Uribe et al., 2011), and B memory cells serves to remember the structure of antigens entering fish system should infection occurs again (Yanuhar, 2011). The production and introduction of antibodies specific to the antigen last several weeks (Sumiati, 2015), where T *heper* cells and B cells still perform the introduction of foreign molecules or antigens. The introduction of antigens by these antibodies is influenced by factors such as fish species, water temperature, vaccine doses, and inactivation methods (Romstad et al., 2012).

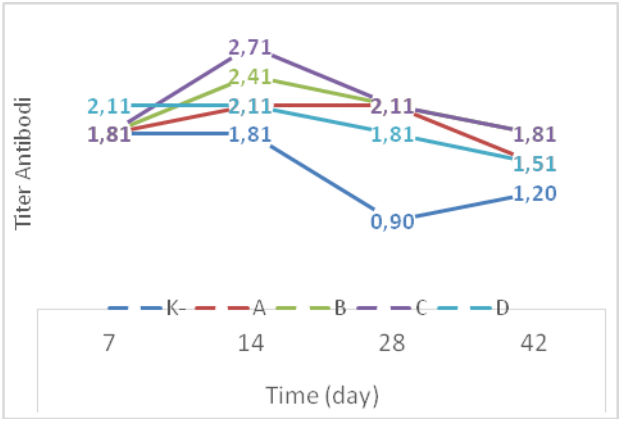

Figure 3. Histogram of antibody titers value on koi fish (C. carpio) vaccinated during the study

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After 1 week of *E. tarda* bacteria infection, antibody titer value decreases in each treatment. It occurs because the specific antibodies produced during vaccination are used to fight incoming *E. tarda* bacteria antigen. Compared to non-vaccinated control fish, the mean value of antibody titer on vaccinated fish was above control fish. It indicated that the dose of vaccine administered during vaccination process affected the fish's immune system during the challenge test. In the last week of treatment (day 42), on average all the antibody titer values in test fish decreased. Antibody titer decrease rate in each treatment returned to the normal level before the challenge test, especially on treatment C with antibody titer value of 1.81. This proves that the administration of vaccines can boost fish immune system, especially when specific bacterial infection occurs. Overall, the results obtained on the average value of antibody titer were directly proportional to the CPI value C as best treatment (10^8 cell/ml).

Relative Percent Survival (RPS)

Relative Percent Survival (RPS) value of koi fish (*Cyprinus carpio*) post vaccination and infection showed mixed results in each treatment. One of the factors that influence RPS value in this study is the dose of vaccine given. As explained earlier, overly low or high doses are not very effective in increasing the immune response to *E. tarda* bacterial infection.

Treatment	Post Vaccination	Post Infection
	RPS (%)	RPS (%)
A	88.23	50.00
B	94.12	66.67
C	94.12	83.33
D	82.35	41.67

The relative percent survival (RPS) is the relative percentage of the ratio between mortality rate of vaccinated fish and control fish. Based on RPS value after vaccination, all treatments had values above 80%. The highest RPS value was in treatment B (10^6 cell/ml) and C (10^8 cell / Ml) with RPS of 94.12%. It indicates that vaccine administration in test fish did not cause significant mortality and proved that the vaccine resulted from inactivation of *E. tarda* bacteria had no pathogenic effect. In addition, a few percent of fish death after vaccination is due to inadequate doses of vaccine given, resulting in fish stress and decreased health.

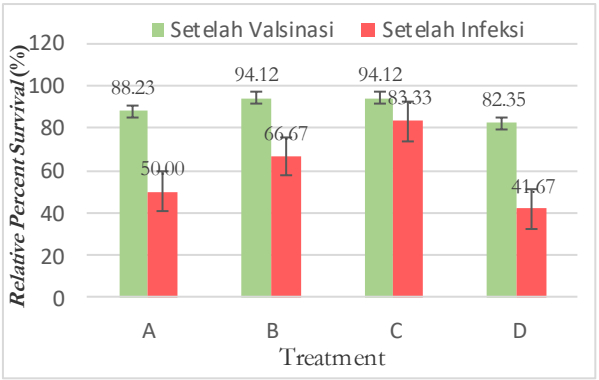

Figure 4. RPS histogram after vaccination and after E. *tarda* bacteria infection

Figure 5. Phase of wound healing (red circle) in fish body during treatment

After challenge test, RPS value decreased for each treatment. The highest RPS value remained at treatment C (83.33%). Based on overall results of SR and RPS, treatment C (10^8 cell/ml) had the best results compared to other treatments. According to several studies on *E. tarda* vaccine, 10^7 CFU/ml doses yields 100% RPS in carp (Li et al., 2015); 10^8 CFU/ml doses yields the highest RPS of 78% in *Paralichthys olivaceous* fish. A vaccine is effective when it has an RPS value of> 50% (Sukenda et al., 2014), control mortality rate for at least 60%, while vaccinated fish mortality rate less than 24% (Purwaningsih et al., 2014). It means that the vaccinated fish stability is higher than that of unvaccinated fish.

CONCLUSION

Inactive *E. tarda whole cell* vaccine with 10^8 doses is capable of increasing koi's immunoreactivity (*Cyprinus carpio*) infected by *E. tarda bacteria*. Improvement in fish-specific immunity parameters and relative percent survival rate of 83.33% after *E .tarda* infection for 2 weeks.

Letter of Gratitude

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