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Immunogenicity of the 89 kDa Toxin Protein from Extracellular Products of Streptococcus in *Oreochromis niloticus*

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ABSTRACT

The development of vaccines is crucial in order to reduce economic loss due to infectious disease outbreaks in the aquaculture industry. The aim of this study was to evaluate *Oreochromis niloticus* immune responses after the administration of 89 kDa toxin protein, extracellular products (ECP89) of the *Streptococcus agalactiae* bacteria. Nile tilapias weighing approximately 25 g received 0.1 mL fish^{-1} intraperitonial injection doses with 2 μg mL^{-1} ECP89 (ECP89-2), 4 μg mL^{-1} (ECP89-4), 6 μg mL^{-1} (ECP89-6) and 8 μg mL^{-1} (ECP89-8). The positive controls were fish injected with the whole-cell *S. agalactiae* vaccine (WCV) and the crude ECP *S. agalactiae* bacteria vaccine (ECPV). The negative controls were those injected with PBS solution. The fish were kept for 20 days with a population density of 30 fish 195 L^{-1} water. The observation of immune responses were done every 5 days. The results of the study showed that the administration of ECP89-4, ECP89-6 and ECP89-8 could increase specific and non-specific immune response significantly higher ($p<0.05$) compared to ECP89-2, ECPV and PBS, but lower than the ones treated with WCV. It could be concluded that the ECP89 is able to increase specific and non-specific immune response in the Nile tilapia. Therefore, it has a great potential to be developed as a vaccine against *S. agalactiae* infection.

Key words: Extracellular products, immunogenicity, Nile tilapia, *Streptococcus agalactiae,* toxin protein

INTRODUCTION

Infectious diseases caused by pathogenic organisms are an important issue that has caused a lot of financial loss in the aquaculture industry. One of the infectious diseases that often attacks Nile tilapia is streptococcosis, caused by *Streptococcus agalactiae* which is highly pathogenic. This disease could cause 90% mortality 6 days post-injection in toxicity tests (Evans *et al.*, 2006) and 60% mortality in Nile tilapia cultivation in South Sumatera, Indonesia (Yuasa *et al.*, 2008). Streptococcus cause sporadic disease in Nile tilapia breeding industry in China from 2001-2006 and led to a cumulative mortality by 5-15% at the farm (Li *et al.*, 2009). In the large-scale, cultivation of the streptococcus outbreaks continue to occur with a high mortality (30-80%) in 2009-2011 (Chen *et al.*, 2012). While, toxicity test with crude ECP *S. agalactiae* cause pathological abnormalities and eventually death occurs up to 14 after infectian (Hardi *et al.*, 2013).

The development of vaccines is one of the solutions against the pathogen as a sustainable prevention method to control this emerging disease. Various fish vaccines have been developed for streptococcal diseases. Killed and modified-killed vaccines developed for streptococcal diseases have been well documented in previous studies such as *S. difficile* (Eldar *et al*., 1995), *Streptococcus* sp. (Akhlaghi *et al*., 1996) and *S. iniae* (Klesius *et al*., 1999). Killed vaccine composed of concentrated extracellular products and formalin-killed *S. agalactiae* whole cells (Evans *et al*., 2004).

Besides outer membrane proteins as a source of immune protective immunogens, recent increasing interests have been paid on extracellular secretory proteins (Song *et al*., 2013). The extracellular secretory proteins easily activate host's immune response since they are secreted out of cells and are easily contacted with the host (Zhang *et al*., 2012). Thus, investigation on protective immunogens from extracellular proteins may provide efficient candidates for development of vaccines (Song *et al*., 2013). It has been employed in various studies to effectively investigate not only immune response between host and pathogen, but also antigenic proteins of importance for vaccine developments (Shin *et al*., 2007; Ni *et al*., 2010). The utilization of crude, the extracellular products (ECP) of *S. agalactiae*, which contain toxins as a vaccine has been investigated by Pasnik *et al*. (2005) and Klesius *et al*. (2000, 2007). Although, the vaccine is effective, the specific components important for efficacy are not known.

Identification of toxin proteins ECP *S. agalactiae* isolates $N_{14}G$ was performed by Amrullah *et al*. (unpublish), the result showed that the ECP bacterium *S. agalactiae* consists of three proteins with molecular weight of 76.52, 89 and 132.92 kDa. Of the three proteins, the 89 kDa protein is a toxin in tilapia. Therefore, in this study, the immune response of tilapia on protein 89 kDa toxin was evaluated.

MATERIALS AND METHODS

ECP isolation: The ECP was isolated from non-haemolytic type of $N_{14}G$ *S. agalactiae* from the collection at Research Institute for Freshwater Aquaculture, Bogor, Indonesian Ministry of Marine Affairs and Fishery, Indonesia. The test-isolate was innoculated in a Brain Hearth Infussion Agar (BHIA) medium. The ECP isolation was based on the maximum population growth pattern, 72 h post innoculation in a BHI medium (Klesius *et al*., 1999; Evans *et al*., 2004). Neutral Buffer Formalin (NBF) was then added to the ECP, resulting in a 3% concentration which was stored at 27°C for 24 h. After inactivation using the formalin, it was centrifuged at a speed of 10,000 rpm for 30 min at a temperature of 4°C. The supernatant which contained the ECP was stirred using a shaker and filtered through a sterile 0.22 µm milipore filter (membran solution MS® CA syringe filter) and was then stored at -20°C.

Fractionation of ECP protein: The fractionation of the *S. agalactiae* bacterial toxoid protein was conducted to separate the proteins found in the bacterial toxin according to their molecular weight. The protein fractionation was done using the SDS-PAGE method (Laemmli, 1970). Before the fractionation, the ECP was concentrated using a 6 mL vivaspin concentrator centrifuge (vivaspin centrifugal concentrator, GE Healthcare Bio-Sciences AB Björkgatan 30 751 84 Uppsala Sweden) at a speed of 12,500 rpm at 4°C. About 100 µL of the concentrated ECP were diluted in 50 µL buffer then separated using SDS-PAGE with a gel concentration of 12%. After the electrophoresis, the gel was taken and sliced at a width of 7-10 mm from the left and right sides. The gel slices were prepared for silver staining. The result of the staining was used as a pattern for the cutting of the acrylamide gel.

Protein isolation from the acrylamide gel: Isolation of the toxin protein from the result of the SDS-PAGE acrylamide gel fractionation was conducted using an electro-eluter (Bio-rad Model 422). An SDS-PAGE result sample weighing 89 kDa was sliced and put into a glass tube which was attached to the module in the form of small pieces. Elution was done on the 8-10 mA glass^{-1} tube for 3-5 h.

Immunogenicity testing of toxin protein: The study was conducted by keeping the Nile tilapia±25 g fish^{-1} in 100×65×30 cm fiberglass tanks with a density of 30 fish tank^{-1}. The toxin protein tested was the *S. agalactiae* ECP protein toxin weighing 89 kDa (ECP89) which was obtained through the fractionation. There were four treatment doses namely: 2 μg mL^{-1} (ECP89-2), 4 μg mL^{-1} (ECP89-4), 6 μg mL^{-1} (ECP89-6) and 8 μg mL^{-1} (ECP89-8). The ECP89 vaccine was diluted with PBS according to the treatment doses and the concentrations were confirmed by measuring the absorbance at 280 nm using a thermo scientific Nanodrop 2000 Spectrophotometer. Two positive controls used; formalin inactivated whole-cell *S. agalactiae* vaccines (WCV) and *S. agalactiae* crude ECP vaccine (ECPV). While, PBS solution used as negative control. Each fish was injected 0.1 mL fish^{-1} intraperitoneally (i.p.) according to its treatment dose. Seven days after the initial injection, boosters were given using the same method and doses as the initial injections. The fish were then tended for 20 days and immunological observations were done every 5 days.

The fish immunological signs observed were the differential leucocyte counts (Blaxhall and Daisley, 1973), the phagocytic activity (Anderson and Siwicki, 1995), the serum lysozyme activity (Ellis, 1990) and the indirect ELISA (Shelby *et al*., 2002) with some modifications.

Statistical analysis: One way ANOVA and Duncan's *post hoc* test were run for each experiment using the statistical program SPSS 16.0. Significant differences between groups were accepted at $p<0.05$.

RESULTS

Fractionation and protein isolation: The SDS-PAGE results showed 3 protein bands with molecular weight of 76.52, 89 and 132.92 kDa. The protein band with the molecular weight of 89 kDa (ECP89) was sliced and isolated using electro-elution, yielding pure protein which was then used as the toxin protein (Fig. 1).

Spesific antibody titer by indirect ELISA: The ELISA results (optical density; OD at 450 nm) antibody levels Nile tilapia increased on day 5 after the injection of the ECP89 and remained elevated on day 10 after the booster until day 20 post-injection (Fig. 2). Increased fish antibodies OD levels at the same ECP89 treatment with WCV and ECPV treatment and higher than that of PBS. On day 5-20, treatment ECP89-6 and ECP89-8 showed higher antibody OD levels ($p<0.05$) compared to treatment ECP89-4, ECPV and PBS. Fish antibodies OD levels of ECP89-6 and ECP89-8 was not significantly ($p>0.05$) up to day 20, as well as between treatment ECP89-8 with WCV on day 5 and 15 was not significantly too ($p>0.05$).

Serum lysozyme activity: Five days post injection, lysozyme activity serum of Nile tilapia increased in all treatment groups except in ECP89-2 and PBS (Fig. 3). On day 5 and 15, lysozyme increase significantly in ECP89-4, ECP89-6 and ECP89-8 but no significantly found in lysozyme

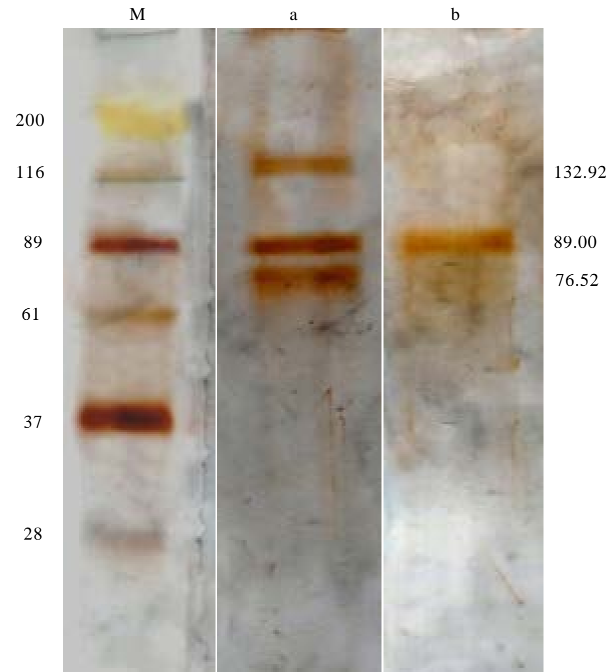

Fig. 1: Fractionation of the toxin protein from the bacteria *S. agalactiae* using the SDS-PAGE method. a: Protein was concentrated using a vivaspin to produce a protein band with a molecular weight of 76.52, 89 and 132.92 kDa, b: Results of the isolation and purification of the 89 kDa protein toxin using the electro eluter is shown in and M: Marker is marked

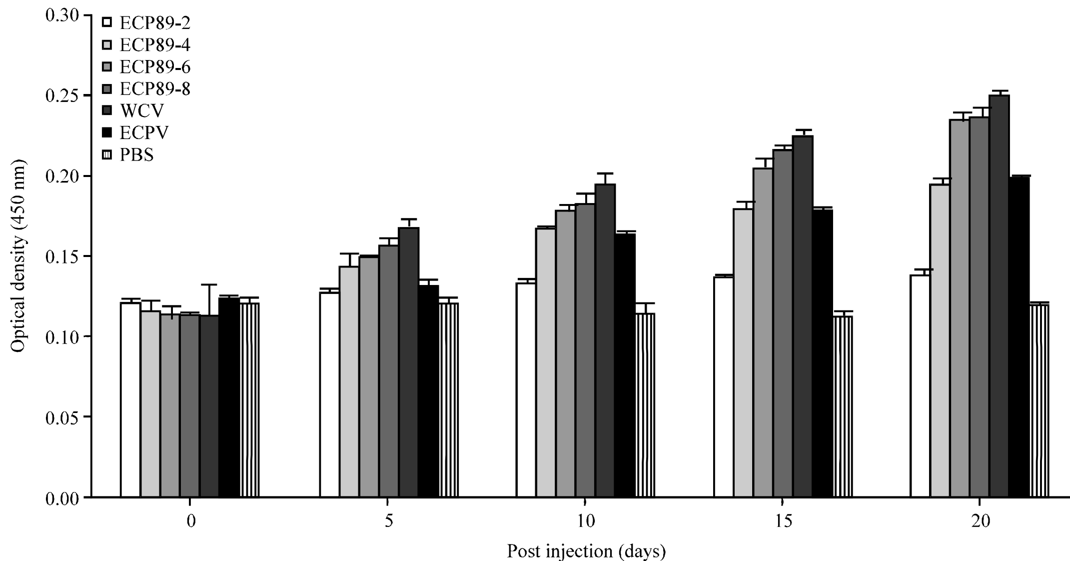

Fig. 2: Spesific antibody titer by indirect ELISA at 450 nm of the Nile tilapia after injected 0.1 mL fish^{-1} i.p. doses using 2 μg mL^{-1} ECP89 (ECP89-2), 4 μg mL^{-1} (ECP89-4), 6 μg mL^{-1} (ECP89-6) and 8 μg mL^{-1} (ECP89-8). The positive controls were Nile tilapias injected with the whole-cell *S. agalactiae* vaccine (WCV) and the ECP *S. agalactiae* bacteria vaccine (ECPV). The negative controls were those injected with PBS solution

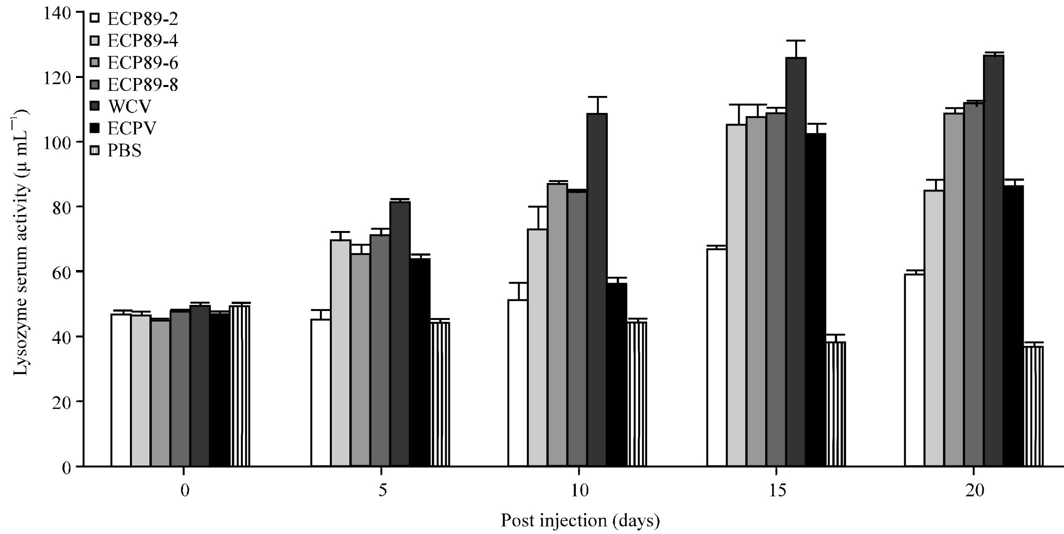

Fig. 3: Serum lysozyme activity level of the Nile tilapia after injected 0.1 mL $fish^{-1}$ i.p. doses using 2 μg mL^{-1} ECP89 (ECP89-2), 4 μg mL^{-1} (ECP89-4), 6 μg mL^{-1} (ECP89-6) and 8 μg mL^{-1} (ECP89-8). The positive controls were Nile tilapias injected with the whole-cell *S. agalactiae* vaccine (WCV) and the ECP *S. agalactiae* bacteria vaccine (ECPV). The negative controls were those injected with PBS solution

among ECP89-4, ECP89-6 and ECP89-8. On 10 and 20 days post injection, lysozyme of ECP89-6 and ECP89-8 were higher ($p<0.05$) than ECP89-2, ECP89-4, ECPV and PBS but lower than WCV ($p<0.05$).

Differential leucocyte counts: The distribution of monocytes in each treatment showed a trend of increased monocyte percentage up to day 10, stabilization and then decrease on day 20 (Fig. 4). The monocytes increase for the ECP89-4, ECP89-6, ECP89-8 and WCV treatments were higher than the other treatments on day 5 and day 15 ($p<0.05$). On day 20, the increase in all ECP89 treatments, ECPV and WCV treatments were higher than in the PBS treatment ($p<0.05$). The distribution of neutrophils in each of the treatments showed an increase since the vaccination until day 20. The increase in neutrophil numbers in the ECP89-4, ECP89-6, ECP89-8 and WCV treatments after initial and booster injections were significantly higher ($p<0.05$) than in ECP89-2, ECPV and PBS treatments (Fig. 5).

The increase in the percentages of neutrophils and monocytes cause a decrease in the percentage of lymphocytes. The distribution of the lymphocyte percentage in the treatments (Fig. 6) showed decrease in all treatments including the PBS. Observations from day 5-20 showed that the lymphocyte percentages in the ECP89-2, ECPV and PBS treatments were statistically higher ($p<0.05$) than in the other treatments.

Phagocytic activity: The phagocytic activity on day 5 rose and reached its peak on day 15 and then fell on day 20 (Fig. 7). In general, the phagocytic activity increases shown by ECP89-4, ECP89-6, ECP89-8 treatments were higher than those shown by ECPV and PBS treatments ($p<0.05$) but lower than that shown by the WCV treatment ($p<0.05$).

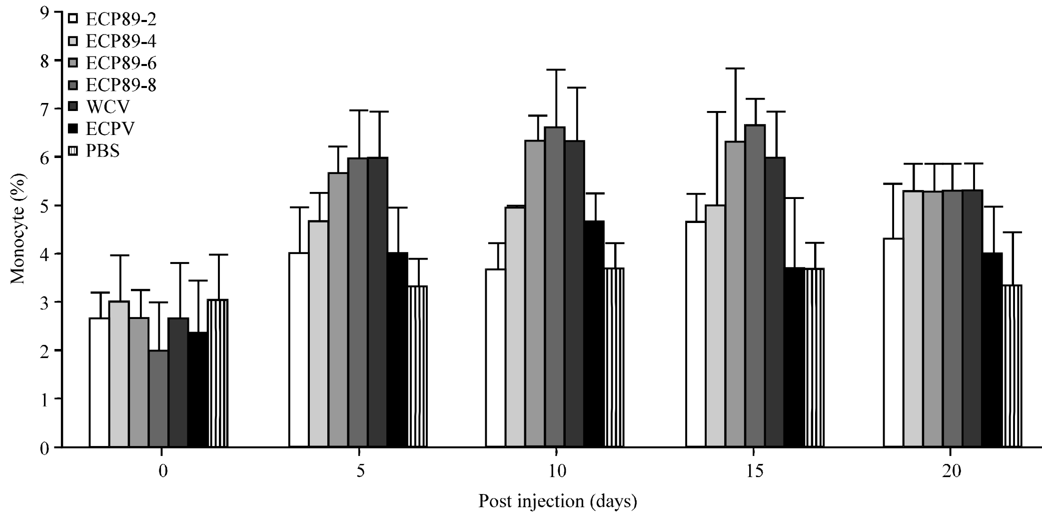

Fig. 4: Monocytes level of the Nile tilapia after injected 0.1 mL fish^{-1} i.p. doses using 2 μg mL^{-1} ECP89 (ECP89-2), 4 μg mL^{-1} (ECP89-4), 6 μg mL^{-1} (ECP89-6) and 8 μg mL^{-1} (ECP89-8). The positive controls were Nile tilapias injected with the whole-cell *S. agalactiae* vaccine (WCV) and the ECP *S. agalactiae* bacteria vaccine (ECPV). The negative controls were those injected with PBS solution

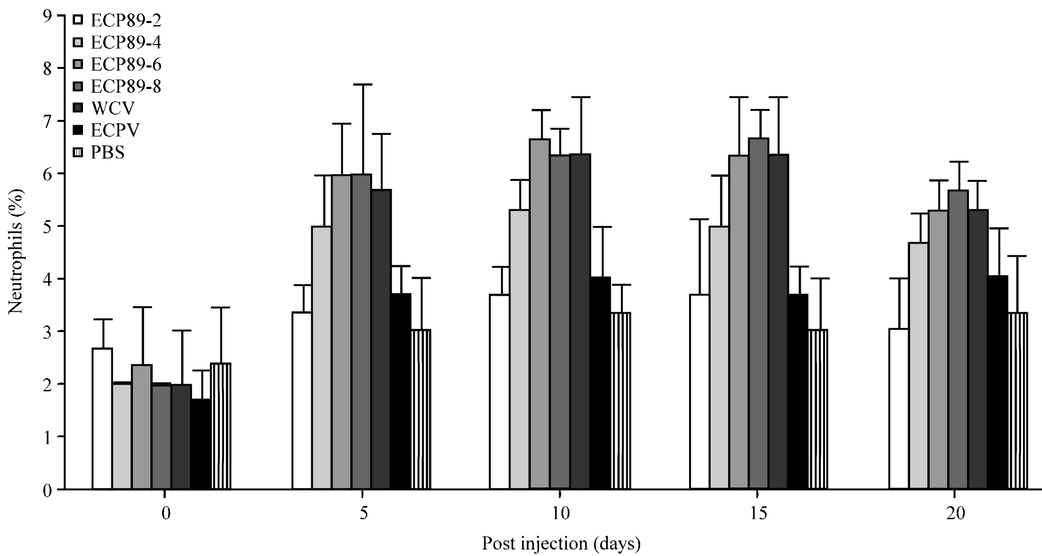

Fig. 5: Neutrophils level of the Nile tilapia after injected 0.1 mL i.p. doses using 2 μg mL^{-1} ECP89 (ECP89-2), 4 μg mL^{-1} (ECP89-4), 6 μg mL^{-1} (ECP89-6) and 8 μg mL^{-1} (ECP89-8). The positive controls were Nile tilapias injected with the whole-cell *S. agalactiae* vaccine (WCV) and the ECP *S. agalactiae* bacteria vaccine (ECPV). The negative controls were those injected with PBS solution

DISCUSSION

The findings of the present study suggest that ECP 89 has been proven to have an effect on fish's immune responses. The fish antibodies OD levels showed higher results in ECP89-4, ECP89-6

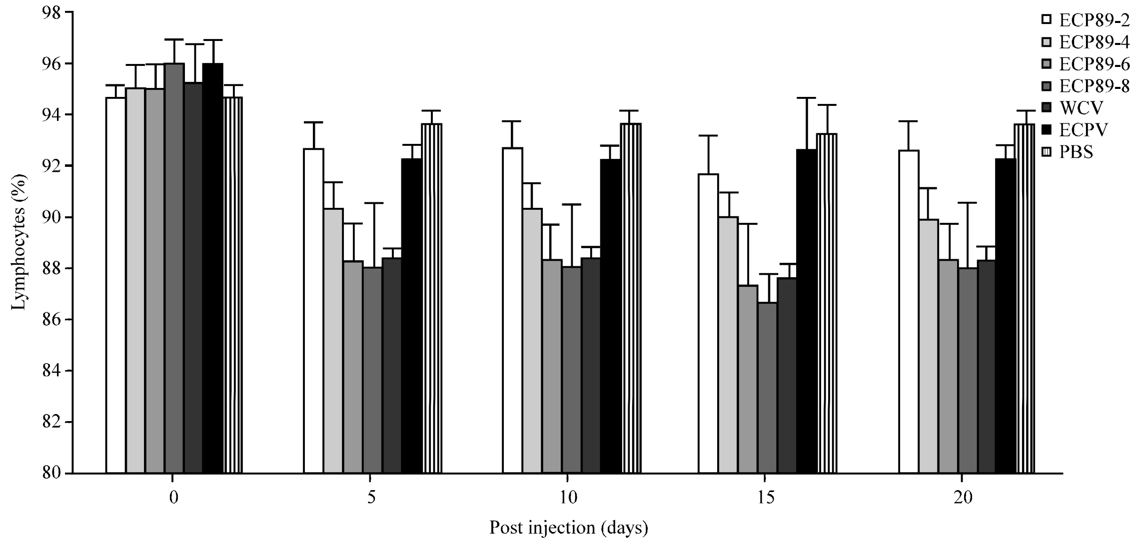

Fig. 6: Lymphocytes level of the Nile tilapia after injected 0.1 mL fish^{-1} i.p. doses using 2 μg mL^{-1} ECP89 (ECP89-2), 4 μg mL^{-1} (ECP89-4), 6 μg mL^{-1} (ECP89-6) and 8 μg mL^{-1} (ECP89-8). The positive controls were Nile tilapias injected with the whole-cell *S. agalactiae* vaccine (WCV) and the ECP *S. agalactiae* bacteria vaccine (ECPV). The negative controls were those injected with PBS solution

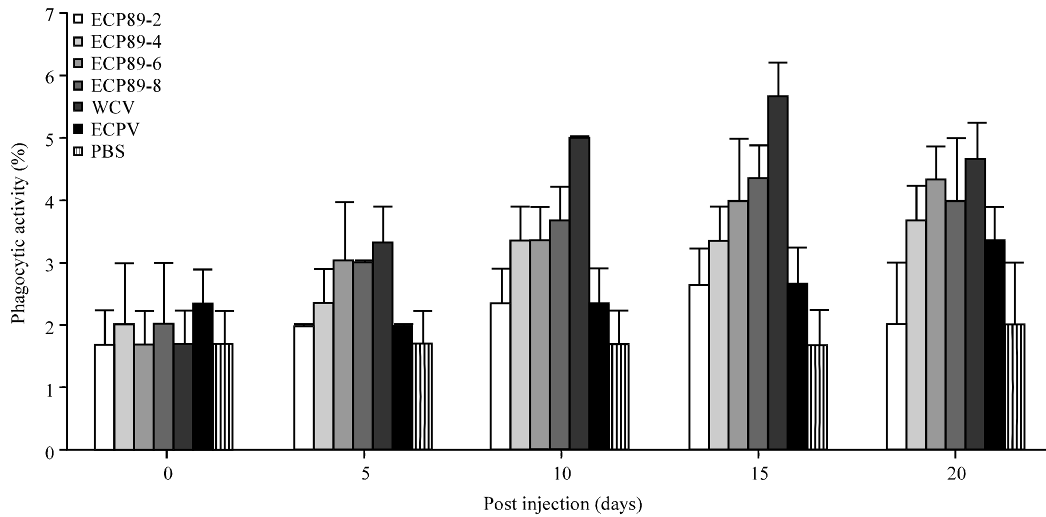

Fig. 7: Phagocytic activity level of the Nile tilapia after injected 0.1 mL fish^{-1} i.p. doses using 2 μg mL^{-1} ECP89 (ECP89-2), 4 μg mL^{-1} (ECP89-4), 6 μg mL^{-1} (ECP89-6) and 8 μg mL^{-1} (ECP89-8). The positive controls were Nile tilapias injected with the whole-cell *S. agalactiae* vaccine (WCV) and the ECP *S. agalactiae* bacteria vaccine (ECPV). The negative controls were those injected with PBS solution

and ECP89-8 treatments compared to that of the PBS treatment (Fig. 2). Differential leucocyte counts, phagocytic activity and serum lysozyme activity also showed increases and were found higher in ECP89-4, ECP89-6 and ECP89-8 treatments compared to those of the PBS treatment

(Fig. 3-7). In general, all the test parameters underwent significant increases after the administration of the 89 kDa and they were sustained until day 20 post injection. The contrast was seen in the negative control which was injected with PBS. The study results showed that ECP89 is a strong immunogenic. The Nile tilapia was able to identify the antigen and produce responses in the form of increased non-specific and specific response, a rise in the fish's antibody. Different results were shown by the PBS treatment which was not immunogenic, it did not stimulate any hematologic or immunologic increases.

The use of ECP89 could increase specific immune response which is demonstrated by the specific antibody titer by indirect ELISA. The treatment using ECP89 and using the whole-cell vaccine could both increase the fish's antibody titer. The increase could already be observed 5 days post vaccination. Artificial immune responses in the teleost group can be detected in a few days or even 4-6 weeks after the initial infection or inflammation (Press and Evensen, 1999) depending on the environmental temperature.

As with specific immune responses, ECP-6 and ECP-8 demonstrated the highest increase in neutrophil and monocyte percentages and phagocytic activity and lysozyme and have proven to play the same role as the whole cell vaccine, whereas ECP89-2 is possibly unable to stimulate an adequate immune response. The increase of neutrophil percentage and sustained until day 20 indicated that ECP89 is an antigenic and it can induce neutrophil consistent with previous studies (Mutoloki *et al*., 2006) and suggest that the importance of the ECP89 when an antigen enter the nila tilapia body. This shows that the administration of ECP89 in correct doses is able to increase non-specific cellular immune responses in fish optimally which play an important role in the initial response, being involved in the process of elimination and destruction of pathogens. This is similar to the role played by the *S. agalactiae* whole-cell vaccine and the findings were similar in non-specific immune response parameters. Innate immune host defense mechanisms can be activated during pathogenic bacterial infections (Wang *et al*., 2010). These responses are ediated by macrophages through the production of various mediators such as nitric oxide, reactive oxygen species and roinflammatory cytokines.

The study above supports previous studies which showed that the administration of vaccines not increase specific immune responses only but also non-specific immune too (Chung and Secombes, 1987; John *et al*., 2002; Harikrishnan *et al*., 2012; Wang *et al*., 2013). While, the non-specific immune response is a crucial response in the fish's immune system (Ellis, 1989).

There have been numerous studies about vaccines that employed whole cells and they have given satisfactory results (Eldar *et al*., 1995; Pasnik *et al*., 2005). Likewise, the use of proteins as vaccines have shown rapid development (Hamod *et al*., 2012; Yu *et al*., 2013; Marancik *et al*., 2013; Aviles *et al*., 2013; Wang *et al*., 2013). However, very limited studies have attempted to use ECP vaccines such as the one conducted by Pasnik *et al*. (2005), especially using the purified protein from ECP fractionation as evaluated in this study. Whereas, ECP are important virulence factors of fish pathogens and are often sufficiently immunogenic to provide protection against challenge after inoculation (Pasnik *et al*., 2005). Further ECP can be the key to improve cross-protection with modified bacteria *S. agalactiae* (containing ECP) provides protection to test the homologous and heterologous challenge in tilapia (Evans *et al*., 2004; Nho *et al*., 2011).

CONCLUSION

Based on the study that has been conducted, it is concluded that ECP89 is able to increase the Nile tilapia's specific immune response through an increase in its antibody titer and also its

non-specific immune response which was shown by the following parameters; differential leucocyte counts, serum lysozyme activity and phagocytic activity. The results of this study showed that ECP89 is a vaccine candidate which is promising to be developed. Further study is needed to assess the ECP89's efficacy in controlling infection of *S. agalactiae* in Nile tilapia.

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