Effect of Injection Vaccination against *Pseudomonas fluorescens* on Specific and Non-Specific Immune Response of Nile Tilapia (*Oreochromis niloticus*) Using Different Prepared Antigens

Adel Attia1, Salah Mesalhy2, Youssif Abdel Galil1 and Mohamed Fathi3*

1Bacteriology, Immunology and Mycology Department, Faculty of Veterinary Medicine, Zagazig University, Egypt
2Pathology Department, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt
3Aquaculture and Genetic improvement, World Fish Center, Abbassa, Sharkia, Egypt

Abstract

The current work aimed to study four different prepared *Ps. fluorescens* antigens to develop the best adequate strategy to control such infection in cultured Nile tilapia. One thousand and fifty Nile tilapia (*Oreochromis niloticus*) were divided to 5 equal groups and used for vaccination trial. Fish in groups 1-5 were injected intraperitoneal with 0.2 ml from each of sterilized saline, Formalin killed bacterin, Extracellular product (ECP) suspension, Sonicated cells (SC) suspension and mixture of ECP & SC suspension; respectively. At 1, 2, 4, 6, and 8 weeks post vaccination, ten fish from each group were randomly used for the collection of whole blood and tested for nitro blue tetrazolium (NBT), neutrophil adherence tests, lysozyme activity and the serum bactericidal test.

The NBT, Neutrophil adherence and lysozyme activity of vaccinated fish showed significant increases in all immunized groups in comparison with control at 1, 2 and 4 weeks post vaccination. Serum bactericidal activity and Antibody titer were significantly increased in all immunized groups at all periods of experiment. Mixture of Sonicated and extracellular product vaccine showed the best serum bactericidal activity and antibody titer against *Ps. fluorescens*.

The relative percent of survival (RPS) after challenge with *Ps. fluorescens* at 4, 6 and 8 weeks post vaccination was significantly increased in all immunized groups in comparison with control. There are significant increases in RPS among group immunized with a mixture of sonicated and extracellular product antigen than other three immunized groups at 4 weeks only. The higher values of the relative percent of survival was seen in the mixture of sonicated and extracellular product antigen followed by formalin killed antigen, sonicated cell antigen and then extracellular product antigen.

It could be concluded that all prepared vaccines are efficient against *Ps. fluorescens* infection, however a mixture of sonicated and extracellular product antigen seemed superior to other vaccines especially in bactericidal activity, antibody titer and RPS against *Ps. fluorescens*.

Keywords: *Pseudomonas fluorescens*; Vaccine; *Oreochromis niloticus*; Immune response; Protection

Introduction

Bacterial diseases among fish caused by a variety of pathogens and represent a significant economic problem in the commercial aquaculture [1]. *Pseudomonas fluorescens*, are Gram-negative bacteria of the families Pseudomonadaceae, among the recognized bacterial pathogens that commonly associated with reared aquaculture species [2]. *Ps. fluorescens* is a pathogen for a wide range of fish species including Indian major carps (Labeo rohita, Catla catla, and Cirrhisus mrigala), grass carp Ctenopharyngodon idellus), black carp (Mylopharyngodon piceus), and tilapia (Oreochromis) [3-8]. Infection of fish by *Ps. fluorescens* leads to the development of the so called Red Skin Disease, which is characterized by hemorrhage, scale falling off, and fin ulceration. Disease outbreaks often occur under stress conditions. The use of vaccines in the aquaculture industry was important in reducing economic losses which occur as a result of disease [9,10] and in the reduction in use of antibiotics [11]. A number of different types of vaccines have been developed in fish against Gram negative, such as whole cell (WC) [12,13] outer membrane protein (OMP) [14], extracellular products (ECPs), lipopolysaccharide (LPS) preparations [15] and also biofilms [16]. Although the vaccinations are efficient, their mode of action remains unclear to determine its efficiency. Vaccination can be done by several ways where the injection route reliably delivers a small, known amount of antigen directly to the fish that most likely to be effective and provide protection of long duration [17]. Although vaccines have provided varying degrees of protection in fish, still until now no commercial vaccine available for *Ps. fluorescens* [2].

In this work, four different *Ps. fluorescens* antigens were prepared and assayed to prevent *Ps. fluorescens* infections in cultured Nile tilapia.

Materials and methods

Fish

One thousand and fifty apparently healthy, Nile tilapia (*O. niloticus*) of both sexes were collected from the World Fish Center, Abbassa, Egypt and checked to be free from *Ps. fluorescens*. Fish were used for vaccination trial and treated of the ethic committee. Fish were divided

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*Corresponding author: Mohamed Fathi Mohamed, Aquaculture and Genetic improvement, World Fish Center, Abbassa, Sharkia, Egypt, Tel: +2 01020 43 125; Fax: +2 055 3405578; E- mail: m.fathi@worldfishcenter.org*

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to 5 equal groups (each 210 fish) and each group subdivided into three equal replicates that kept in 15 fiber glasses (3 × 0.6 × 0.5 m). Fish, throughout the experiment, fed on a balanced diet and provided with fresh water that partially replaced and air using electrical air pumping compressors with temperature adjusted at 26 ± 1°C.

**Bacterial strains**

The pathogenic strain, *Ps. fluorescens* was obtained, as reference strains, from the Fish Health Laboratory at The World Fish Center, Abussa, Egypt.

**Preparation of *Ps. fluorescens* antigens**

Preparation of formalin killed bacterin: A formalin-killed vaccine was prepared as previously described [18]. *Ps. fluorescens* was grown in Trypticase Soy Broth (TSB; Difco) at 28°C for 24 h. Bacterial cells were killed by addition of formalin to achieve a final concentration of 0.7% and incubated for 3h at 25°C and then at 4°C overnight. Cells were collected by centrifugation at 6500g for 30 min at 4°C and washed three times with phosphate buffered saline (PBS; pH 7.4), and then they were re-suspended in PBS at a final concentration of 1 × 109 cells/ml.

Preparation of extracellular product (ECP): The supernatants that obtained after centrifuging 24h old cultures of *Ps. fluorescens* in brain heart infusion broth were filtered (0.22 µm). The toxic activities of ECP were neutralized with 0.5% formalin overnight at 4°C. Formalin was neutralized by the addition of a 15% solution of sodium metabisulphite (10 mL/L-1 of inactivated culture supernatants) with overnight incubation at room temperature [19].

Preparation of sonicated cell (SC): *Ps. fluorescens* was grown on brain heart infusion agar (BHI; Difco Laboratories, Detroit, MI, USA) at 28°C for 24h. Bacterial cells were collected by centrifugation at 6500g for 30 min at 4°C and washed three times with Sodium phosphate buffered saline (PBS; 10 mM Sodium phosphate buffer, 150 mM NaCl, pH 7.0) and re-suspended in PBS at 106 cells ml-1. The suspension were kept on ice and sonically lysed with two 10 sec bursts using a probe sonicator with power level at 60 W. The sonicated cells were stored at 20°C [20].

**Immunization trial:** The fish of groups 1, 2, 3, 4 and 5 were injected intraperitoneally with 0.2 ml from each of sterilized saline (0.85 %), Formalin killed bacterin (corresponding to 4 × 109 cfu/ml), ECP suspension (corresponding to 4 × 1010 cfu/ml), SC suspension (corresponding to 4 × 1011 cfu/ml) and 0.2 ml mixture of ECP and SC suspension (corresponding to 4 × 1012 cfu/ml), respectively.

**Blood sampling and analysis:** At 1, 2, 4, 6, and 8 weeks post vaccination (pv), ten fish were randomly collected from the control (1) and treatment groups (2-5). The fish were anesthetized by immersion in water containing 0.1 ppm tricaine methane sulfonate (MS-222). Whole blood (0.5 ml) was collected from the caudal vein of each fish using syringes (1 ml) and 27-gauge needles that were rinsed in heparin (15 unit ml-1), to determine the NBT, and neutrophil adherence tests. A further 0.5 ml blood-sample was centrifuged at 1000 x g for 5 min in order to separate the plasma that stored at -20°C to be used for lysozyme activity test. For separation of serum, blood samples (0.5 ml) were withdrawn from the fish caudal vein and transferred to Eppendorf tubes without anticoagulant. The blood samples were centrifuged at 3000 g for 15 min and the supernatant serum was collected and stored at -20°C until used for the serum bactericidal test.

Nitroblue tetrazolium activity (NBT): Blood (0.1 ml) was placed in microtiter plate wells, to which an equal amount of 0.2% NBT solution was added and incubated for 30 min at room temperature. A sample of NBT blood cell suspension (0.05 ml) was added to a glass tube containing 1 ml N,N-dimethyl formamide and centrifuged for 5 min at 3000 rpm. The supernatant fluid was measured in a spectrophotometer at 620 nm in 1 ml cuvettes [21].

**Adherence/NBT assays:** NBT-glager adherant assays were performed by placing single drops of blood (0.1 ml) on 2 glass cover slips and incubating them for 30 min at room temperature. The cover slips were then gently washed with phosphate buffered saline (PBS). Drops (0.1 ml) of 0.2% NBT were placed on microscope slides and covered by a cover slip, then incubated at room temperature for 30 min with the NBT solution. The activated neutrophils were then counted under a microscope (400 x) [22].

**Lysozyme activity:** The lysozyme activity was measured using the turbidity assay. Chicken egg lysozyme (Sigma) was used as a standard and 0.2 mg ml-1 lyophilised Micrococcus lysiodekticus in 0.04 M sodium phosphate buffer (pH 5.75) was used as substrate. Fifty ml of serum was added to 2 ml of the bacterial suspension and the reduction in the absorbance at 540 nm was determined after 0.5 and 4.5 min incubation at 22°C. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 min-1 [23].

**Serum bactericidal activity (SBA):** Bacterial cultures of *P. fluorescens* were centrifuged, and the pellet was washed and suspended in phosphate buffered saline (PBS). The optical density of the suspension was adjusted to 0.5 at 546 nm. This bacterial suspension was serially diluted (1:10) with PBS five times. The serum bactericidal activity was determined by incubating 2 ml of the diluted bacterial suspension with 20 ml of the serum in a micro-vial for 1 h at 37°C. PBS replaced the serum in the bacterial control group. The number of viable bacteria was determined by counting the colonies after culturing on tryptic soy agar plates for 24 h at 37°C [24].

**Serum antibody titer (Agglutination test):** Serum antibody titer was measured using the agglutination protocol described utilized by Klesius et al. [25]. *Ps. fluorescens* were grown in TSB for 24 h at 28°C, harvested by centrifugation at 2000 g for 15 min, and washed with PBS. Bacteria were washed in phosphate buffered saline (PBS) twice more before adjusting the bacterial suspension concentration to each separated bacteria to 1.0 × 105 CFU of bacterial cell/ml. The agglutination test was assayed in 96-well U-bottom microtiter plates. Tilapia sera (15 µl) were serially diluted two-fold in PBS. Each Pseudomonas sp. suspension (15 µl) was applied to each well prior to incubating plates overnight at room temperature (28°C). Agglutination titers were reported as log10 of the reciprocal of the highest dilution of the sera showing agglutination of bacteria. Positive (Pseudomonas infected serum) and negative (normal serum) were included in each plate as controls.

**Challenge test:** Thirty fish from each treatment (10 from each replicate) were collected at 4, 6 and 8 weeks and subjected to experimental infection with *Ps. fluorescens*.

*Ps. fluorescens* were grown in TSB for 24 h at 28°C with shaking on an orbital shaker (100 revolutions per minute) [25]. Fish were e.p. injected with 4×105 CFU/fish. Following challenge, mortality was monitored and recorded daily for 15 days. Cumulative percent mortality and the relative percent of survival were calculated [26]. Freshly dead fish were cultured for the presence of *Ps. fluorescens* to confirm the cause of mortality using standard bacteriology procedures.

\[ RPS = \frac{1-(\text{percent of mortality in immunized group})}{\text{percent of mortality in control group}} \times 100. \]
Figure 1: Non-specific immunological parameters of Nile tilapia immunized by different Ps. fluorescens antigens by injection.

<table>
<thead>
<tr>
<th>Period (week)</th>
<th>Test</th>
<th>Control</th>
<th>Formalin killed vaccine</th>
<th>Sonicated cell vaccine</th>
<th>Extracellular product vaccine</th>
<th>Sonicated &amp; extracellular product</th>
</tr>
</thead>
<tbody>
<tr>
<td>One</td>
<td>Colony count</td>
<td>82.60±4.95</td>
<td>77.40±4.78</td>
<td>78.20±2.67</td>
<td>76.40±2.92</td>
<td>72.00±3.44</td>
</tr>
<tr>
<td></td>
<td>Antibody titer</td>
<td>2.80±0.37</td>
<td>4.00±0.34</td>
<td>4.20±0.37</td>
<td>4.00±0.32</td>
<td>4.80±0.20</td>
</tr>
<tr>
<td>Two</td>
<td>Colony count</td>
<td>81.60±4.85</td>
<td>63.00±2.98</td>
<td>58.80±3.54</td>
<td>56.80±2.92</td>
<td>57.40±3.45</td>
</tr>
<tr>
<td></td>
<td>Antibody titer</td>
<td>2.20±0.20</td>
<td>5.20±0.37</td>
<td>5.20±0.38</td>
<td>5.40±0.24</td>
<td>6.80±0.58</td>
</tr>
<tr>
<td>Four</td>
<td>Colony count</td>
<td>80.80±4.59</td>
<td>56.40±2.32</td>
<td>48.20±2.78</td>
<td>45.80±2.20</td>
<td>46.20±1.45</td>
</tr>
<tr>
<td></td>
<td>Antibody titer</td>
<td>2.40±0.24</td>
<td>6.20±0.37</td>
<td>6.40±0.24</td>
<td>6.00±0.45</td>
<td>7.80±0.37</td>
</tr>
<tr>
<td>Six</td>
<td>Colony count</td>
<td>81.20±5.76</td>
<td>54.00±1.87</td>
<td>47.80±1.39</td>
<td>48.40±0.40</td>
<td>58.40±1.61</td>
</tr>
<tr>
<td></td>
<td>Antibody titer</td>
<td>2.40±0.24</td>
<td>7.40±0.24</td>
<td>7.20±0.37</td>
<td>6.80±0.20</td>
<td>8.00±0.32</td>
</tr>
<tr>
<td>Eight</td>
<td>Colony count</td>
<td>84.00±3.78</td>
<td>64.80±3.83</td>
<td>54.20±3.15</td>
<td>59.20±3.72</td>
<td>66.00±2.91</td>
</tr>
<tr>
<td></td>
<td>Antibody titer</td>
<td>2.20±0.20</td>
<td>7.00±0.32</td>
<td>6.60±0.24</td>
<td>6.20±0.37</td>
<td>7.60±0.51</td>
</tr>
</tbody>
</table>

*The column or row with the same letters has no significant difference.*
*Capitals letter compare between the different periods within the same treatment.*
*Small letters compare between the different treatments within the same period.*

Table 1: Serum bactericidal activity and antibody titer against Ps. fluorescens of Nile tilapia immunized by using several Ps. fluorescens antigens.

**Statistical analysis:** Analysis of Variance (ANOVA) and Duncan's multiple Range Test [27] was used to determine the differences between treatments. The mean values were significant at the level of (P < 0.05). Standard errors, of treatment-means, were estimated. All the statistics were carried out using Statistical Analysis Systems (SAS) program [28].

**Results**

The NBT, Neutrophil adherence and lysozyme activity of vaccinated fish through the injection route against *Ps. fluorescens* showed significant increases in all immunized groups in comparison with control group at 1, 2 and 4 weeks post vaccination, while at 6 and 8 weekspv a non-significant increase were seen in same groups (Figure 1).

Serum bactericidal activity of immunized Nile tilapia against *Ps. fluorescens* by different antigens showed significant increases in all immunized groups in comparison with control group at all periods of experiment except at 1st week pv. Antibody titer of experimented Nile tilapia was significantly increased in all immunized groups in comparison with control at all periods. Mixture of Sonicated and extracellular product antigen showed the best values of serum bactericidal activity and antibody titer (Table 1).

The percentage of mortalities after challenge with *Ps. fluorescens* at 4, 6 and 8 weeks post vaccination were significantly decreased in all immunized groups in comparison with control. There is no significant difference between the immunized groups at all periods. Also, no significant differences in-between the different periods within the same treatment except in formalin killed antigen which showed a significant decrease values at 6 and 8 weeks than at 4 week post vaccination. The most decreased values showed in mixture of sonicated and extracellular product antigen followed by formalin killed antigen and sonicated cell antigen then extracellular product antigen (Figure 2 and Table 2). The relative percent of survival after challenge with *Ps. fluorescens* at 4, 6 and 8 weeks post vaccination showed significant increases in all immunized groups in comparison with control. There are significant increases in
group immunized with a mixture of sonicated and extracellular product antigen than other three immunized groups at 4 weeks only. The higher values of the relative percent of survival was seen in the mixture of sonicated and extracellular product antigen followed by formalin killed antigen, sonicated cell antigen then extracellular product antigen (Table 2).

**Discussion**

The way of the antigen preparation and the route of administration to fish are principal factors in obtaining effective vaccination [29]. During past 1–2 decades, much attention has been given to develop suitable vaccine based on antigenic nature of used strains and route of administration for controlling diseases in aquaculture [30–33].

The route of delivering the vaccine and the ambient water temperature are essential factors for successful vaccination of fish [34].

Although several vaccine delivery methods are available, injection is the most effective and the most reliable [35]. Non-specific immune parameters viz. lysozyme activities, NBT activity and neutrophils adherence in fish are important defense mechanism to protect it from bacterial infections but there is a paucity of information on the influence of vaccine on the activity of non-specific immune mechanisms [36]. Lysozyme, as an important innate immune response in fish [37] and secreted by monocytes, neutrophils and macrophages [38], is a cationic enzyme defined as muramidase which catalyses the hydrolysis of β-1, 4-glycosidic bond, and might cause the lysis of bacteria [39], also Lysozyme activity induces phagocytosis by activating polymorph nuclear leucocytes and macrophages and by opsonisation [40].

In the present study, there was a significant difference in the lysozyme activity, NBT activity and neutrophils adherence between the control and immunized groups in the 1st, 2nd and 4th weeks of immunization. The same results were reported by Dash et al [36].

In fish, bactericidal activity is considered one of the major defense mechanisms in the early stages of microbial infections [41]. In the present study, serum bactericidal activity of immunized Nile tilapia against *Ps. fluorescens* was significantly increased in all immunized groups in comparison with control group at all periods of experiment except at 1th week post vaccination. On the other hand, in a parallel study, serum bactericidal activity analyses of vaccinated fish (32%) against *Edwardsiella tarda* was 2.3-fold lower than unvaccinated fish (72%) [42]. Sun et al. [43] showed that, the survival rates of *E. tarda*, and *V. anguillarum* sera from fish vaccinated with *E. tarda*, and *V. anguillarum*, were significantly (P<0.05) lower, suggesting that each of the vaccinations had significantly enhanced serum bactericidal activity. Also similar vaccine-induced serum killing effect against *V. anguillarum* has been observed previously in Atlantic cod [44].

Some fish species will produce protective antibody response to

![Figure 2: Accumulative Mortalities of Nile tilapia immunized by injection with different *Ps. fluorescens* antigens A. 4 weeks, B. 6 week and C. 8 weeks post vaccination.](image-url)

<table>
<thead>
<tr>
<th>Period (week)</th>
<th>Mortality %</th>
<th>RPS %</th>
<th>Mortality %</th>
<th>RPS %</th>
<th>Mortality %</th>
<th>RPS %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Four</td>
<td>73.33±4</td>
<td>45.00±2.89</td>
<td>38.33±3.33</td>
<td>40.00±2.89</td>
<td>30.00±0.00</td>
<td></td>
</tr>
<tr>
<td>Six</td>
<td>68.33±1.67</td>
<td>31.67±0.45</td>
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### Table 2: Mortalities percentage and relative percent of survival among Nile tilapia immunized by injection with different *Ps. fluorescens* antigens.

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The mixture of Sonicated and extracellular product antigens showed the best values of bactericidal activity and antibody titer against Ps. fluorescens followed by formalin killed antigen and sonicated cell antigen then extracellular product antigen. Bjornsdottir et al. [58] showed that, a significant antibody response against sonicated bacterial cells was detected after vaccination but a significant response was not observed against the ECP antigens before or after challenge with Aeromonas salmonicida. ECP antigens were reported to has a stronger immune response to Photobacterium damselae spp. piscicida [59]; Vibrio harveyi [60] and Flavobacterium psychrophilum [61].The extracellular products (ECP) of bacteria is a factor responsible for a number of biological effects including immunostimulatory activities in different animals including fish [62].

The percentage the relative percent level of survival after challenge with Ps. fluorescens at 4, 6 and 8 weeks post vaccination were significantly increased in RPS in all immunized groups in comparison with control. Some results indicated that immunity is produced by systemic injection (IP or IM) of the ECP and FKCI1ECP vaccines [63]. The RPS values obtained for Y. ruckeri using the ECP-vaccine ranged between 74.0 and 81.4%. These values are similar to or lower than RPS values reported previously for the vaccine against yersiniosis on the rainbow trout [64,65].

The percentage the relative level of survival after challenge with Ps. fluorescens at 4, 6 and 8 weeks post vaccination were significantly increased in RLS in all immunized groups in comparison with control. Some results indicated that immunity is produced by systemic injection (IP or IM) of the ECP and FKCI1ECP vaccines [63]. The RPS values obtained for Y. ruckeri using the ECP-vaccine ranged between 74.0 and 81.4%. These values are similar to or lower than RPS values reported previously for the vaccine against yersiniosis on the rainbow trout [61,65]. The higher values of the relative percent of survival was seen in the mixture of sonicated and ECP antigen followed by formalin killed antigen, sonicated cell antigen then extracellular product antigen. Unpurified ECP has been shown to be protective when ‘toxoided’ by formalin and chloroform and then established with lysine [66]. S. salar immunized with Aeromonas salmonicida sp. achromogenes ECP were found to elicit better protection than formalin killed whole-cells [67]. The mixture of killed-cells and concentrated ECPs from Jeju-45 led to significant protection against the homologous isolate of S. iniae in olive flounder, followed by killed cells then the ECPs.

It could be concluded that, although all prepared vaccines are efficient against Ps. fluorescens infection through injection route, a mixture of sonicated and extracellular product antigen seemed superior to other vaccines especially in bactericidal activity, antibody titer and RPS against Ps. fluorescens. However, as injection vaccination is difficult to apply in a large scale, further trials on Nile tilapia using same vaccines through immersion is advised to tested applicability and the cost-effectiveness for the commercial use.

References


