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Inactivation of *Streptococcus iniae* in substitution of chemicals and drugs to develop vaccine for mono-sex Nile tilapia

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Abstract
Streptococcus iniae is an important pathogen that can cause a broad range of disease in aquatic animals. To avoid the use of antibiotics and drugs, it is critical to identify protective antigens for developing highly effective vaccines against this pathogen. Vaccination is the most effective means of preventing infectious diseases; however, few vaccines are effective against *Streptococcus iniae* (*S. iniae*) in mono-sex Nile Tilapia. This work presents an efficacious and safe vaccine against *S. iniae* infections in monosex Nile tilapia (*Oreochromis niloticus*). The vaccine candidate *S. iniae* F-1 strain administered by intraperitoneal (i.p.) injection, and consisted of inactivated antigens; both the vaccinated and non-vaccinated fishes were challenged intraperitoneally with *S. iniae* (1×10^7 CFU ml^{-1}) isolates and PBS (negative control). Peripheral blood samples were collected for SDS-PAGE, phagocytosis and agglutination assays. Present results indicated that immunoglobulin M (*IgM*) was maximally expressed in the low-amperage electric current inactivated (ECKC) vaccinated group at 3 months post-secondary vaccination (PSV). Phagocytic activity and index increased significantly in (ECKC) vaccinated group. Furthermore, fish in (ECKC) vaccinated group exhibited significantly elevated agglutination titers compared to fish in the control group, in which almost no agglutination reaction was detected. In the efficacy test, the vaccinated fishes had a significant increase in RPS (69 and 89, respectively); the percentage mortality declined from 83 ± 0.6 and 74 ± 0.7 in challenged and control fishes to 25% ± 0.8% and 8% ± 0.8% in vaccinated and challenged fish groups, respectively. Furthermore, the level of protection observed in the field trial closely resembled that achieved on a laboratory scale. Therefore, EC-killed showed the highest molecular weight 31 kDa in SDS-PAGE and Western blot and increased RPS (91%), suggested that the EC-killed cells of *S. iniae* could play an important role in immunizing mono-sex Nile tilapia. The EC-killed cells of *S. iniae* will may safe and long-lasting protection against streptococcosis.

Keywords: *S.iniae*; Mono-sex Nile tilapia; inactivated vaccine; agglutination; SDS-PAGE; Western blot; Phagocytosis.

1. Introduction
Streptococcus iniae (*S. iniae*) [1], a Gram-positive bacterium, induces streptococcosis, a disease characterized by meningoencephalitis, systemic septicemia, and skin lesions [2], this disease can lead to heavy mortality [3]. *S. iniae* affects many cultured fish species such as rainbow trout [4-7], and grouper [8] of high commercial value, tilapia are widely cultured in Southeast Asia. Many diseases threaten the tilapia industry, and vaccination remains the most effective means of reducing mortality. Unfortunately, vaccines especially those based on recombinant antigens or inactivated pathogens are not usually able to confer protection on their own [9].
Streptococcus iniae is highly pathogenic in freshwater, marine, and euryhaline fish, and is highly lethal: outbreaks may be associated with 30–50% mortality [3]. In tilapia, *S. iniae* causes meningoencephalitis, with symptoms including lethargy, dorsal rigidity, and erratic swimming behavior; death follows in a matter of days [2,3].
Several antibiotics; Amoxicillin, erythromycin, furazolidone, and oxytetracycline, have been used successfully to treat *S. iniae* infection in fish [10]. Vaccination against *S. iniae* has been attempted with limited success as it only provides up to 6 months' immunity [10].
Much work has been done to examine the heat resistance of pathogenic bacteria such as *Escherichia coli* O157:H7 [11, 12]; *Salmonella sp.*[13] and *Listeria monocytogenes* [13,14]. Low

Amperage electric current using pulsed electric fields, which are non-thermal and non-chemical has been tested for inactivation of bacteria [15-18]. Ozone and UV light are efficient and produce harmless derivatives but were more costly [19-21].
To the best of our knowledge, till date there is no report on the immune response of mono sex Nile tilapia to LAEC inactivated *S. iniae* vaccines. Hence, this study aimed to develop an inactivated vaccine in substitution of chemicals and drugs against *S. iniae* infection and to evaluate its protective response by assessing immunity in vaccinated mono-sex Nile tilapia.

Materials and methods

Fish husbandry

Juvenile mono-sex Nile tilapia (15 ± 3 g) was obtained from a commercial fish farm in Jessore. The fish were habituated for two weeks in aerated sea water at 20°C to 25 °C in 500 l tanks prior to experiments. During the experiment, fishes were maintained with standard culture conditions and fed twice a day with commercial pelletized diet. Also, fishes were confirmed negative for *S. iniae* infection by culturing fish tissue in the brain heart infusion (BHI) agar (BD, USA).

Bacterial culture and inactivation of bacteria

The *S. iniae* F-1 strain was cultured on tryptic soy agar (TS) with 1.5% NaCl (TSN) at 25 °C for 48 h. Colonies were subcultured onto brain heart infusion agar with 1.5% NaCl (BHIN) at 25 °C for 24 h. The cell concentration was then adjusted to an optical density of 3 (OD_{610} = 3; 10^{10} CFU/mL). The inactivated whole-cell vaccine was prepared as in (Tsai *et al., 2013*) [22] with slight modifications. The bacterial pellet was collected by centrifugation (5000 × *g* for 10 min at 4 °C), washed twice in sterile PBS (pH 7.4) and resuspended in 30 ml of PBS. Following inactivation, the bacterial pellet was re-suspended at a final concentration of 10^{10} CFU/mL in PBS containing 0.1 to 1.0 % of all chemicals. Formalin (0.2, 0.4 and 0.6%), ethanol (0.2, and 0.6%), chloroform (0.2, 0.4 and 0.6%), Na_2SO_4 (0.2 and 0.6%), $CuSO_4$ (0.2, and 0.6%), KCl (0.2, 0.4 and 0.6%), phenol (0.2, and 0.6%) and EDTA (0.2, and 0.6%) were used as inactivating chemicals. To investigate the promoting effect of low amperage electric current (ECKC), inactivation was performed with 1, 3, 5, 7 and 9 mA for 5 min; heat 70ºC for 10 min and 100ºC for 30 min; Chemical with heat (0.4% phenol + 100 ºC for 30 min, 0.3% formalin + 100ºC for 10 min,0.6% formalin 100ºC for 50 min) after those treatment, the culture was incubated at 37ºC for 2 days to examine inactivation.

Virulence test (LD_{50})

S. iniae was used in the experimental infection of tilapia to determine the LD_{50} dosage. Eight groups of 20 fish were tested. Through the serial dilution method, different concentrations of cells (10^{-1} to 10^{-7}) or sterile PBS were intra-peritoneally injected into tilapia. Ten (10) µl bacterial solutions from different concentrations were cultured onto TSN agar at 25 °C for 24–48 hours, notably, each cell concentration was run in duplicate. Mortalities were recorded daily for 14 days after challenge. *S. iniae* was re-isolated from the liver, spleen, kidney, and brain of moribund and dead tilapia after challenge.

Vaccination-Preparation of fish anti- *S. iniae* sera

Fishes, maintained at 20°C to 25 °C were divided into two groups (n = 100) each in duplicate. One group was vaccinated intra-peritoneally (IP) with 100µl of 1×10^7 CFU ml^{-1} of *S. iniae*, while the other group (control) received the same quantity of PBS alone. The booster dose was injected after two week's interval. The mortality were recorded for a period of 6 weeks; the dead fish were examined to confirm the infection by the re-isolation of the pathogen from kidney, spleen, liver, brain, gill, skin on BHI agar. The blood sample was collected from live fishes once in two weeks at regular intervals, from both the vaccinated and control fishes. The blood was allowed to clot for 1 h at 25 °C and then centrifuged at 1000 × *g* for 10 min. The collected serum was stored at -70 °C until assayed for antibody titer and western blotting.

Preparation of rabbit anti- *S. iniae* sera and administration

A rabbit was immunized with an intravenously administered ECKC-killed PBS-diluted suspension with *S. iniae* cells (1×10^7 CFU ml^{-1}) twice a week with consecutive doses of 0.2, 0.4, 0.8, and 1.0 ml)[10]. One week after the last injection, the rabbit was bled from the ear vein. Two weeks later, the immunization procedure was repeated, now with 1.0 ml doses throughout. The antisera was collected and stored at -20 °C.

Agglutination assay

Sixteen (16) days after challenge, surviving fish were randomly removed from each of the replicate tanks and blood samples collected following anesthetizing with MS-222. Approximately 100 ml serum/sample was collected following centrifugation at 1000g for 5 min then stored frozen at -80°C for subsequent determination of agglutinating antibody titers to *S. iniae* by modifying the method of Chen and Light (1994)[23]. *S. iniae* inactivated with formalin 0.4%, ethanol 0.6%, Na_2SO_4 0.6%, KCl 0.6%, ECKC 7mA for 5 min and heat 70°C for 10 min were grown in brain–heart infusion broth (BHI) for 24 h. The cells were centrifuged at 3000g. The resulting cell pellet was washed twice in 0.85% saline solution and suspended in saline solution to an optical density of 0.8 at 540 nm. Starting with a dilution of 1:10 (10-µl serum and 90 µl PBS), two-fold serial serum dilutions were made in 96-well round bottom microtiter plates by adding 50 Al of diluted serum into the remaining wells plated with 50 µl of PBS. Thereafter, 50 µl of bacterial cell suspension was added to each well; thus, the initial serum dilution was 1:20. The plates were covered with plastic film and incubated at room temperature for 16–18 h. The agglutination end point was established as the last serum dilution where cell agglutination was visible after incubation. Agglutination titers were reported as log10 of the reciprocal of the highest serum dilution showing visible agglutination as compared to the positive control. Prior to the bacterial challenge, four fish from each of the replicate tanks were bled to determine if they were negative for antibody to to *S. iniae*.

Denaturing poly-acrylamide gel electrophoresis (SDS–PAGE) and immunoblotting analysis

For the protein extraction, 25 ml of *S. iniae* BHI broth culture was centrifuged for 15 min for 5000 × *g*. Thereafter the pellet was washed twice with distilled water and sonicated for three times at 30 W for 30 s with frequent incubation on ice for 20Sce. *Inactivated S. iniae* cells were washed by PBS to remove released free antigen molecules, such free antigen inhibit cell agglutination reaction. Diluted antigen at MCFALAND No. 2, with PBS to achieved reactive antigen suspension. To prepare poly-acrylamide gel electrophoresis (SDS–PAGE) gel 14% separating gel and 4% stacking gel were used (Tris-HCl CPH, 10% SDS, 30% Acrylamide mix,

TEMED and 25% APS, Ammonium perisulfate). SDS-PAGE machine filled with loading buffer (50 ml of 10% SDS add with 450 ml Distilled water). 20 µl of each inactivated and control samples were pulled on the gel and 5 µl of known markers were used. Then electricity 10 to 50 mA were used for 30 min. Electricity line was stopped when the blue marker was gone at bottom of the glass on green part.

Gel separated smoothly from SDS-PAGE machine by using hand and washed using transparent buffer (Glycin, Tris, Methanol and DW) with stirrer. The proteins were electrophoretically transferred to nitrocellulose paper (0.45 µm pore size, BioRad) by using a semi-dry apparatus (Bio- Rad) as described by Towbin *et al.*, 1979 [24] after blocking with 1% skim milk at 4^0C overnight, the membrane was reacted with rabbit anti *S. iniae* F-1 serum. The blotted membranes were blocked with blocking buffer and then incubated separately with primary antibody (diluted 1:1000 in iBlot antibody diluting mixture) and then with goat anti-rabbit IgG (diluted 1:2000 in iBlot antibody diluting mixture) secondary antibody. Then the blots were immune-stained to produce a color reaction by using iBlot Western Detection Chromogenic kit (Invitrogen, USA).

Phagocytosis analysis

PBLs at a density of 10^7 cells/mL were cultured on coverslips in L15 medium containing 20% FBS at 27 °C for 5 h; 10^7 CFU/mL bacteria were then seeded on coverslips and incubated at 27 °C for 1 h. Coverslips were stained in a dilution 1:10 (v/v) solution of Giemsa (Merck) for 45 min at 25 °C, and cells were observed with an Olympus CX21 microscope (magnification 1000×). By using the each coverslip there 200 cells were examined. The phagocytic activity (PA) and phagocytic index (PI) were calculated as previously described [25,23].

$$\text{Phagocytic ratio} = \frac{\text{No. of phagocytic cells with engulfed bacteria}}{\text{No. of phagocytes}} \times 100$$

$$\text{Phagocytic index (PI) \%} = \frac{\text{No. of phagocytic cells}}{\text{Total no. cells}} \times 100$$

Data were analyzed using one-way ANOVA and differences between treatments were examined using Tukey's multiple comparisons test (SAS Institute, Cary, NC, USA). Statistical significance was considered at p values of 0.05, 0.01, and 0.001.

Leukocyte isolation

Peripheral blood from 10 groupers was collected in EDTA-containing tubes, diluted 1:3 (v/v) in PBS (145 mM NaCl, 1.4 mM NaH_2PO_4, 8 mM Na_2HPO_4, pH 7.5), and overlaid on a layer formed by 3 mL of Histopaque-1077 (Sigma). Gradients were centrifuged at 400 × g at 25 °C for 30 min. Cells at the interface were harvested, washed in PBS, and then re-suspended in 1 mL of L15 medium.

Vaccinated fish challenge assay

Injection (i.p.) challenge tests were done 6 weeks post immunization and the control fish were challenged accompanied with vaccine groups at the respective time points. The *S. iniae* dose for i.p. challenge test was 1×10^7 CFU ml^{-1} and 1×10^5 CFU ml^{-1}. Control fish has been injected and challenged with PBS. The cumulated mortality was recorded 12 days post challenge (Table 2). *E. tarda* was confirmed by analysis of kidney isolates cultured on Salmonella Shigella agar (SS agar, Nissui) and the infection of *S. iniae* was confirmed by observation of black pigments. Slide agglutination titer was also performed to confirm *S. iniae* using fish antiserum mixed with bacterin on PBS; visible granular clumps (agglutination) were observed under the microscope. The cause of death and pathological signs were verified by re-isolation of bacteria from kidney sample of freshly dead fish or survivors. Relative percentage survival, RPS [26] was calculated from the end cumulative mortalities as:

$$\textbf{RPS (\%)} = \left(1 - \frac{\textbf{mortality of vaccinated group}}{\textbf{mortality of unvaccinated control group}}\right) \times 100$$

Statistical Analysis

The mean (±SD) of assayed parameters were calculated for each group. Two-sample Student's *t*-tests were used to compare values between individual experimental and control groups. Measurements were considered significant if $P < 0.05$.

3. Results

Inactivation of *S. iniae*

The antigenicity of inactivated bacterium *Streptococcus iniae* was examined after treated with different chemicals and heat (Fig. 1). Formalin 0.2%, ethanol 0.2%, 0.6%, chloroform 0.2%, 0.4%, Na_2SO_4 0.2%, $CuSO_4$, 0.2%, and KCl 0.2, 0.4%, phenol 02%, 0.4%, EDTA 0.2% and ECKC 1, 3, 5mA for 5 min showed low killing activity where the bacteria treated with the different concentration of these chemicals and treatments still remained alive after 24h (Fig. 1).

Formalin 0.4%, ethanol 0.6%, chloroform 0.6%, Na_2SO_4 0.6%, $CuSO_4$, 0.6%, phenol 0.6%, EDTA 0.4%, heat 70°C for 10 min and ECKC 7mA for 5 min showed moderate killing activity in 24h (Fig. 1).

Complete killing with Formalin 0.6%, KCl 0.6%, EDTA 0.6%, and ECKC 9mA for 5 min at 12h after treatment was observed (Fig. 1).

Rapid inactivation just after the addition of chemicals or treatment was resulted by heat 100°C for 30 min and chemicals with heat (0.4% phenol + 100 ºC for 30 min, 0.3% formalin + 100ºC for 10 min,0.6% formalin 100ºC for 50 min) (Fig. 1).

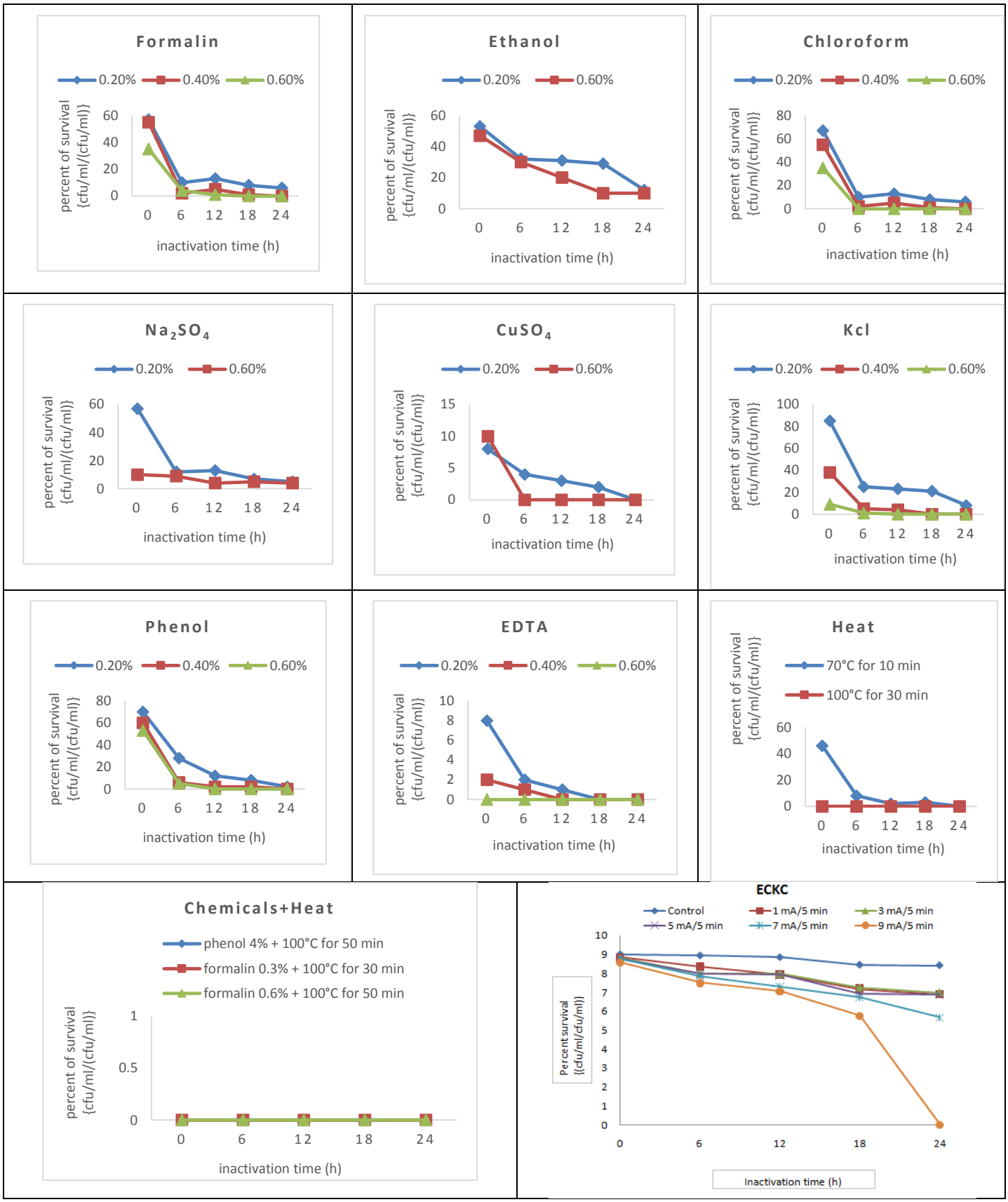

Fig 1: Inactivation of *Streptococcus iniae* with different chemicals, heat and low amperage electric current.

Agglutinating antibody titer

Serum agglutination titers of selected six vaccinated groups (formalin 0.4%, ethanol 0.6%, Na_2SO_4 0.6%, KCl 0.6%, ECKC 7mA for 5 min and heat 70°C for 10 min) showed a significantly higher agglutination titer than that of the control group. Antibody titers for a group of antigens with (ECKC 7mA for 5 min) was significantly higher than those of *S. iniae* inactivated with formalin 0.4%, ethanol 0.6%, Na_2SO_4 0.6%, KCl 0.6%, ECKC 7mA for 5 min and heat 70°C for 10 min at 16 days post challenge. No significant differences were observed among the values for ethanol 0.6%, Na_2SO_4 0.6%, KCl 0.6%, ECKC 7mA for 5 min and heat 70°C for 10 min. The serum agglutination titers were significantly increased for a group of antigens with formalin 0.4% (Table.1).

Table 1: Mean antibody titer of mono-sex Nile tilapia with inactivated *S. iniae* at 16 days post challenge.

Inactivated Condition of *S. iniae*	Log 10 of antibody titer**
Control	-
Formalin 0.4%	4.10721
Ethanol 0.6%	2.90309
Na_2SO_4 0.6%	2.90309
KCl 0.6%	3.20412
ECKC 7mA for 5 min	4.40824
Heat 70°C for 10 min	3.80618

*Means in the same column with different superscripts are significantly different at $P < 0.05$.
**Values are means of 10 determinations per treatment.

3.2. Phagocytosis assay

In vitro phagocytosis was performed in vaccinated and control groupers to evaluate cellular immunity. Leukocytic phagocytosis was analyzed by Giemsa staining, which can be used to observe phagocytosed *S. iniae* (Table 3). PA values peaked at 1 month PSV for antigens with *S. iniae* and antigens with formalin 0.4%, ECKC 7mA for 5 min (87.5% and 91.0%, respectively); PI values were 185.05 ± 4.45 and 223.04 ± 6.23, respectively (Fig. 2A and B).

Table 3: Phagocytic ratio analyzed by Giemsa staining, phagocytosed *S. iniae*

	CON	FKC	EKC	NSKC	KKC	ECKC	HKC
15 D	6	77.5	43.5	32.5	27.5	92.5	67.5
1 M	7.5	80	47.5	37	33.5	94.5	69
2 M	7	82.5	55	37.5	36	95.5	73.5
3 M	7.5	85.5	61	42.5	38.5	97.5	76

Protection of fish after vaccination

To compare the efficacy of differently-inactivated vaccines, the final concentrations of the vaccines during injection (i.p.) immunization were adjusted to 1×10^7 CFU ml^{-1} and 1×10^5 CFU ml^{-1}. There were no abnormalities in any of the vaccinated or control fish before challenge. The fish in each group were injected intraperitoneally (i.p.) and challenged after 6 weeks post immunization. The cumulative mortality of the challenged fish was 88-52% for the control group. The RPS value of the fish immunized by ECKC 7mA for 5 min was 91-77, i.e. much higher than that of the fish immunized by formalin 0.4% (RPS = 73-62) or other vaccinated group (Table 2). In addition, all efficacy trials the dead fish showed clinical signs typical of Edwardsiellosis disease. No pathogen other than *S. iniae* was isolated from dead fish.

Table 2: Challenge with inactivated *S iniae* mono-sex Nile tilapia (*Oreochromis niloticus*).

Condition	Challenge dosecells/fish	Total fish	Dead fish	Number of abnormal fish	Mortality (%)	Abnormality (%)	RPS (%)
Control	PBS	25	22	15	88	60	-
Formalin 0.4%	1×10^7 CFU ml^{-1}	25	6	2	24*	8	73
Ethanol 0.6%	,,	25	14	5	56	20	36
Na_2SO_4 0.6%	,,	25	18	4	72	16	18
KCl 0.6%	,,	25	19	3	76	12	14
ECKC 7mA for 5 min	,,	25	2	0	8*	0	91
Heat 70°C for 10 min	,,	25	14	3	56	12	36
Control	PBS	25	13	11	52	44	-
Formalin 0.4%	1×10^5 CFU ml^{-1}	25	5	1	20*	4	62
Ethanol 0.6%	,,	25	12	3	48	12	7.7
Na_2SO_4 0.6%	,,	25	12	3	48	12	7.7
KCl 0.6%	,,	25	10	7	40	28	23
ECKC 7mA for 5 min	,,	25	3	0	12*	0	77
Heat 70°C for 10 min	,,	25	9	4	36	16	31

Asterisk: significantly *(P<0·05)* lower than control.

Antigenic protein recognition of vaccinated fishes

The SDS-PAGE and Western blot profile of *S. iniae* protein (Fig. 2) reveals that the rabbit anti-Ps serum is more effective in capturing antigenic protein when compared to the fish anti-Ps. About 4 fragments (molecular weight of approximately 100 kDa, 68 kDa, and 40 kDa) are clearly detected in which 60 kDa fragments is more prominent. However, the fish anti-Ps sera captured the 68 kDa protein fragment.

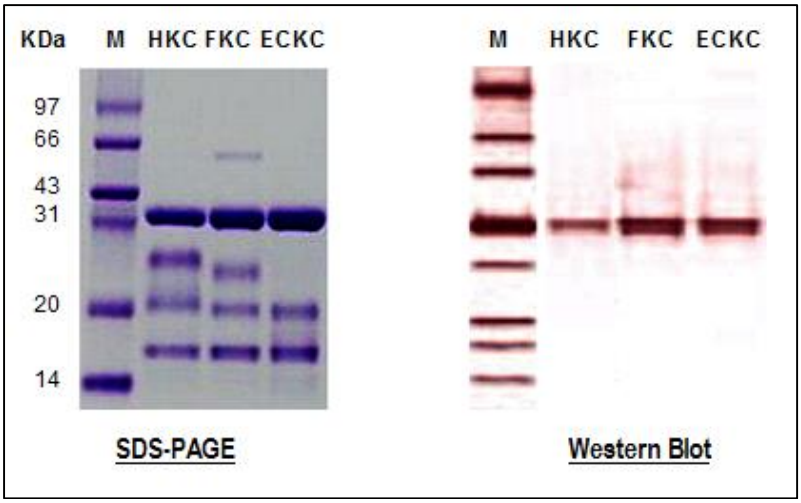

Fig 2: The SDS-PAGE and Western blot profile of *S. iniae*.

4. Discussion

The first killed vaccine was developed to prevent trout losses caused by *S. iniae* infection [27]. Killed vaccines are generally administered through injection to achieve high efficacy [27]. Previous research demonstrated that bath immunization of tilapia resulted in RPS values that were 2-fold lower than those achieved using i.p. vaccination [28]. In a field trial [27] have reported that the mortality of rainbow trout intraperitoneally immunized with a formalin-killed *S. iniae* vaccine was 5%, but exceeded 50% in non-immunized rainbow trout. Similarly, the results of this study demonstrated that i.p. injection of a formalin-killed *S. iniae* vaccine provides a higher level of protection in groupers (RPS, 97.5%-100%) after *S. iniae* challenge; such a vaccine can provide excellent efficacy against *S. iniae* infection in fish. Oil-based vaccines administered to fish provide long-term protection [29]. Protection conferred by the tested vaccine lasted a minimum of 6 months, as determined in the laboratory challenge. As streptococcosis poses a threat to groupers during the summer (due to high water temperature), 6-month duration of immunity should protect groupers against *S. iniae* infection during this critical period [29].

It is well known that vaccination with an oil-adjuvant antigen stimulates the expression of certain immune-related genes [30]. This work assessed gene expression in PBLs by RT-qPCR analysis, and examined portions of the adaptive and innate immune responses. In the peripheral blood of groupers, immunoglobulin expression increased 14 days PPV and peaked at 3 months PSV. This suggests that activation of an immune response is complex and time consuming.

Il-1α is a pleiotropic molecule produced by activated macrophages and blood monocytes [31]. TNF-α has been shown to eliminate pathogens by enhancing various cellular responses including phagocytosis and chemotaxis; it also increases the production of effector molecules, reactive oxygen species, and nitrogen intermediates [32-34]. Phagocytosis aids in the destruction of extracellular pathogens. This phenomenon was evident in groupers in which *il-12* and *tnf-α* expression were up-regulated after vaccination, and phagocytic activity was elevated in the two vaccinated groups compared to the control group. These results suggested that the inactivated *S. iniae* vaccine could enhance the innate immune response in groupers, and regulate the expression of immune-related genes. Furthermore, analytical results indicated that PA and PI values were markedly higher in vaccinated groups than in the control group. This finding suggested that the inactivated *S. iniae* vaccine may have increased the number of phagocytic cells in the peripheral blood, and enhanced non-specific defense mechanisms.

Vaccine efficacy is time consuming and requires many controls which were evaluated through the challenge experiments [35]. TNF-α has been proposed as a biomarker for monitoring fish health and vaccine efficacy [36,37]. Therefore, the present study demonstrated the feasibility of using transcriptional biosignatures (*il-12* and TNF-*α* mRNA expression) as indicators of vaccine efficacy, which could improve conventional immunological evaluation methods and allow for more efficient testing of vaccines. The expression of immune-related genes after vaccination provides valuable insight into immune mechanisms against *S. iniae* in groupers. Further work is necessary to determine the functions of these immune response genes during *S. iniae* infection. As humoral immunity plays an important role in fish [38], this study also analyzed the fish-specific antibody titer against *S. iniae* whole cells by agglutination tests. We found that the agglutination titer under laboratory conditions is higher than that observed in the field trial after vaccination, which may have resulted from fish of different genetic backgrounds responding differently to vaccination.

5. Conclusions

This work may develop an efficacious and safe vaccine against *S. iniae* infection in farmed mono-sex Nile tilapia. The vaccine produced a detectable specific antibody that offered protection against *S. iniae* infection for at least 6 months. Mortality of vaccinated mono-sex Nile tilapia remained below 8%, while RPS exceeded 91%, implying that mono-sex Nile tilapia treated with the tested vaccine mounted a protective immune response against *S. iniae*. Moreover, the tested vaccine significantly elevated innate and adaptive immune responses, and regulated the expression of immune-related genes.

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7. References

1. Pier GB, Madin SH. *Streptococcus iniae* sp. nov., a beta hemolytic streptococcus isolated from an Amazon freshwater dolphin, *Inia geoffrensis*, Internation Journal of Systemic Bacteriology. 1976; 26:545-553.
2. Austin D, Austin B. Characteristics of the diseases. Bacterial fish pathogens: disease of farmed and wild fish. Springer Press, Berlin, 2008,18.
3. Scheld WM, Craig WA, Armstrong D, Hughes JM. (Eds.). Emerging infections ASM Press, Washington 1999; 3:53-65.
4. Eldar A, Bejerano Y, Bercovier H. *Streptococcus shiloi* and *Streptococcus difficile*: two new streptococcal species causing a meningoencephalitis in fish Current Microbiology 1994; 28:139-143.
5. Lahav D, Eyngor M, Hurvitz A, Ghittino C, Lublin A, Eldar A. *Streptococcus iniae* type II infections in rainbow trout *Oncorhynchus mykiss* Disease of Aquatic Organism 2004; 62:177-180.
6. Kvitt H, Colorni A. Strain variation and geographic endemism in *Streptococcus iniae* Disease of Aquatic Organism 2004; 61:67-73.
7. Shoemaker CA, Evans JJ, Klesius PH. Density and dose: factors affecting mortality of *Streptococcus iniae* infected tilapia (*Oreochromis niloticus*) Aquaculture2000; 188:229-235.
8. Arthur JR. Ogawa A brief overview of disease problems in the culture of marine finfishes in east and southeast Asia K.L. Main, C. Rosenfeld (Eds.), Aquaculture health management strategies for marine fishes, The oceanic institute, Makapu'u point, HI, USA, 9-31.
9. Tafalla C, Bogwald J, Dalmo RA. Adjuvants and immunostimulants in fish vaccines: current knowledge and future perspectives Fish Shellfish Immunology 2013; 35:1740-1750.
10. Agnew W, Barnes AC. *Streptococcus iniae*: an aquatic pathogen of global veterinary significance and a challenging candidate for reliable vaccination Veterinary Microbiology 2007; 16:122(1-2):1-15.
11. Ahmed NH, Conner DE, Huffman DL. Heat-resistance of

Escherichia coli O157:H7 in meat and poultry as affected by product composition. Journal of Food Science. 1995; 60:606-610.
12. Kotrola JS, Conner DE, Mikel WB. Thermal inactivation of *Escherichia coli* O157:H7 in cooked turkey products, Journal of Food Science. 1997; 62:875-905.
13. Roberts TA, Baird-Parker AC, Tompkin RB. Micro-organisms in foods. 5. Microbiological specification of food pathogensChapman and Hall, London, United Kingdom, 1996, 105-114.
14. Farber JM, Brown BE. Effect of prior heat shock on heat resistance of *Listeria monocytogenes* in meat, Applied Environmental Microbiology 1999; 56:1584-1587.
15. Manolache S, Somers EB, Wong AC, Shamamian V, Denes F. Dense medium plasma environments: a new approach for the disinfection of water, Environmental Science and Technology 2001; 35:3780-3785.
16. Wouters PC, Bos AP, Ueckert J. Membrane permeabilization in relation to inactivation kinetics of *Lactobacillus* species due to pulsed electric fields Applied Environmental Microbiology 2001; 65:5364-5371.
17. Rowan NJ, Macgregor SJ, Anderson JG, Cameron D, Farish O. Inactivation of *Mycobacterium paratuberculosis* by pulsed electric field Applied Environmental Microbiology 2001; 67:2833-2836.
18. Wouters PC, Dutreux N, Smelt JPP, Lelieveld HLM. Effecets of pulsed electric fields on inactivation kinetics of *Listeria innocua* Applied Environmental Microbiology 1999; 67:3092-3101.
19. Blatchley E, Dumoutier N, Halaby TN, Levi Y, Laine JM. Bacterial responses to ultraviolet irradiation Water Science and Technology 2001; 43:179-186.
20. Christopher M, Richard L. Oxidation behavior of aqueous contaminants in the presence of hydrogen peroxide and filter media, Journal of Hazardous Materials. 1995; 41:105-116.
21. Smith RJ, Kehoe SC, Mcguigan KG, Barer MR. Effect of simulated solar disinfection of water on infectivity of *Salmonella typhimurium* Letter of Applied Microbiology 2000; 31:284-288.
22. Tsai MA, Wang PC, Cao TT, Liao PC, Liaw LL, Chen SC. Immunoprotection of glyceraldehydes-3-phosphate dehydrogenase(GAPDH) from*Lactococcus garviease* against Lactococcosis in tilapia, Journal of General Applied Microbiology. 2013; 59:437-449.
23. Chang CF, Chen HY, Su MS, Liao IC. Immuno-modulation by dietary β-1, 3-glucan in the brooders of the black tiger shrimp *Penaeus monodon* Fish Shellfish Immunology 2010; 10:505-514.
24. Towbin H, Staechelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, Proceeding of Natural Academic Science USA 1979; 76:4350-4.
25. Sano H, Hsu DK, Apgar JR, Yu L, Sharma BB, Kuwabara L. Critical role of galectin-3 in phagocytosis by macrophages, Journal of Clinical Investigation. 2003;112:389-397.
26. Croy TR. Amend DF. Immunization of sockeye salmon (*Oncorhynchus nerka*) against vibriosis using the hyperosmotic infiltration technique Aquaculture 1977; 12:317-325.
27. Eldar A, Horovitz A, Bercovier H. Development of a vaccine against *S. iniae* infection in farmed rainbow trout. Veterinary Immunology and Immunopathology 1997; 56:175-183.
28. Evans JJ, Klesius PH, Shoemaker CA. Efficacy of Streptococcus agalactiae (group B) vaccine in tilapia (*Oreochromis niloticus*) by intraperitoneal and bath immersion administration Vaccine 2004; 22:3769-3773.
29. Midtlyng PJ, Reitan LJ, Speilberg L. Experimental studies on the efficacy and side-effects of intraperitoneal vaccination of Atlantic salmon (Salmo salar L.) against furunculosis Fish Shellfish Immunology 1996; 6:335-350.
30. Jiao XD, Cheng S, Hu YH, Sun L. Comparative study of the effects of aluminum adjuvants and Freund's incomplete adjuvant on the immune response to an *Edwardsiella tarda* major antigen Vaccine 2010; 28:1832-1837.
31. Bird S, Zou J, Wang T, Munday B, Cunningham C, Secombes CJ.Evolution of interleukin-1α, Cytokine Growth Factor Review 2002; 13:483-502.
32. Cunha FQ, Assreuy J, Moss DW, Rees D, Leal LM, Moncad S. Differential induction of nitric oxide synthase in various organs of the mouse during endotoxaemia: role of TNF-alpha and IL-1-beta Immunology 1994; 81:211-215.
33. Kisich KO, Higgins M, Diamond G, Heifets L. Tumor necrosis factor alpha stimulates killing of *Mycobacterium tuberculosis* by human neutrophils Infection and Immunology 2002; 70:4591-4599.
34. Ma X. TNF-alpha and IL-12: a balancing act in macrophage functioning Microbes Infection 2001; 3:121-129.
35. Bridle AR, Koop BF, Nowak BF. Identification of surrogates of protection against yersiniosis in immersion vaccinated Atlantic salmon, PLOS ONE 2012; 7(7):e40841. doi:10.1371/journal.pone.0040841.
36. Lam FWS, Wu SY, Lin SJ, Lin CC, Chen YM, Wang HC. The expression of two novel orange-spotted grouper (*Epinephelus coioides*) TNF genes in peripheral blood leukocytes, various organs, and fish larvae Fish Shellfish Immunology 2011; 30:618-629.
37. Kadowaki T, Harada H, Sawad Y, Kohchi C, Soma GI, Takahashi Y. Two types of tumor necrosis factor-a in bluefin tuna (*Thunnus orientalis*) genes: Molecular cloning and expression profile in response to several immunological stimulants Fish Shellfish Immunology 2009; 27:585-594.
38. Ooyama T, Kera A, Okada T, Inglis V, Yoshida T. The protective immune response of yellow tail *Seriola quinqueradiata* to the bacterial fish pathogen *Lactococcus garvieae* Disease of Aquatic Organism 1999; 37:121-126.