Iranian Journal of Fisheries Sciences 21(6) 1367-1382 2022
DOI: 10.22092/ijfs.2022.128211

Research Article

Efficiency of monovalent *Vibrio alginolyticus* formaldehyde-killed vaccine on the immune responses and protection of Asian seabass (*Lates calcarifer*) juveniles against Vibriosis

Ahangarzadeh M.[1]; Houshmand H.[1*]; Kakoolaki S.[2]; Sepahdari A.[2]; Ghorbanpoor M.[3]; Ajdari A.[4]; Nazemroaya S.[1]; Zabayeh Najafabadi M.[1]; Torfi Mozanzadeh M.[1]; Sadat Sadr A.[1]

Received: February 2022 Accepted: May 2022

Abstract

Vibrio alginolyticus causes severe health problems in marine fish production each year. Among various therapeutic strategies, vaccination is the most economic, efficient and environmentally-friendly approach against microbial infections. Evaluation of formalin-killed *Vibrio alginolyticus* vaccine aquired from native isolates was carried out using a total of 200 Asian seabass juveniles (55±6.43 g), which were divided among three groups, including (I) control (non-vaccinated), (II) vaccinated with killed *V. alginolyticus* and (III) killed vaccine with oral booster. Fish were vaccinated intraperitoneally. Blood samples were taken from fish in each group at three, five, and eight weeks after immunization to assess the antibody levels against *V. alginolyticus* infection. The efficacy of the killed vaccine was appraised five weeks after the start of the initial vaccination by challenging with twofold LD_{50} (3.66×10^8) equivalent of the live suspension of *V. alginolyticus* through intraperitoneal injection. The results of ELISA showed that there were not any significant differences in antibody response among different groups before vaccination ($p>0.05$). Also, the mean antibody titer of the group immunized killed-*V. alginolyticus* with oral booster was significantly higher than the other groups five weeks after the vaccination. The vaccined fish demonstrated higher survival rates than the control with relative percent survival (RPS) of 84.62 and 76.92%, respectively. It is concluded that vaccination could be an effective method to protect farmed Asian sea bass against vibriosis caused by pathogenic *V. alginolyticus*.

Keywords: Vibriosis, *Vibrio alginolyticus*, Killed vaccine, Antibody titer, Survival rate

1-South of Iran Aquaculture Research Institute, Iranian Fisheries Science Research Institute, Agricultural Research Education and Extension Organization, Ahvaz, Iran.
2-Iranian Fisheries Science Research Institute, Agricultural Research Education and Extension Organization, Tehran, Iran.
3-Department of Pathobiology, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran.
4-Offshore Fisheries Research Center, Iranian Fisheries Science Research Institute, Agricultural Research, Education and Extension Organization, Chabahar, Iran.
*Correspondence author's Email: Houshmand.hossein@gmail.com

Introduction

Diseases, especially bacterial one, will be a major challenge for expanding aquaculture in the most pioneering countries. According to available records, more than 50 marine fish species are susceptible to infection caused by various species of *Vibrio* (Woo and Gregory, 2014). Vibriosis mainly causes bacterial septicemia and major economic losses, especially in various marine fish species (Chin *et al.*, 2020). There are eight important species in the *Vibrionaceae* family that cause vibriosis in cultured marine fish, such as *V. alginolyticus*, *V. anguillarum*, *V. parahaemolyticus*, and *V. harveyi* (Haenen *et al.*, 2014; Bellos *et al.*, 2015; Liu *et al.*, 2018; Nehlah *et al.*, 2017). *V. alginolyticus* which is the most common pathogenic marine *Vibrio* species was formerly regarded as biotype 2 of *V. parahaemolyticus* (Damir *et al.*, 2013; Sadok *et al.*, 2013). The first cases of *V. alginolyticus* infection in farmed marine fish were reported in Mediterranean countries (Mohamad *et al.*, 2019). Then, the outbreaks of *V. alginolyticus* infection was also reported in Asian countries (Rameshkumar *et al.*, 2017). Cultured marine fish such as Cobia (*Rachycentron canadum*), silver seabream (*Pagrus auratus*), brown spotted grouper (*Epinephelus chlorostigma*), mullet Mugilidae sp., turbot (*Scophthalmus Maximus*), large yellow croaker (*Larimichthys crocea*), and Asian seabass were infected by *V. alginolyticus* (Krupesha Sharma *et al.*, 2012; Austin and Austin, 2016). Also, Abdallah *et al.* (2009) showed that this bacterium could induce acute infection in European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*). Among different strategies for controlling various bacterial infectious diseases in aquaculture industry, vaccination is considered as an increasingly promising and cost-effective method (Toranzo *et al.*, 2009; Hamod *et al.*, 2012; Cao *et al.*, 2018). Vaccines are used in the aquaculture industry to reduce antibiotics usage, stimulate the fish's immune system to produce antibodies, and protect them against infectious diseases (Osman *et al.*, 2009). *Vibrio* vaccine is one of the most successful methods of disease prevention in the modern aquaculture (Gudding and Van Muiswinkel, 2013). The first attempts to produce effective vaccines against fish vibriosis date back to 1991 and the first vaccine prepared for *V.*(*Listonella*) *anguillarum* was reported in 1998 (Miccoli *et al.*, 2019). *In-vitro* studies reported that *V. alginolyticus* vaccines are mainly included formaldehyde-killed, subunit, live-attenuated, and naked DNA vaccines (Cao *et al.*, 2018). In Iran, Ajdari *et al.* (2019) studied the evaluation of virulence factors in *V. harveyi* isolates from *L. calcarifer* and the protective effect of these bacterial isolates.

Effective routes for vaccine administration in fish include oral, immersion, dip or bath, anal intubation, and injection (Li *et al.*, 2015). During the past decade, Asian seabass has been considered as leading marine fish species for developing marine cage culture, especially in the Southern coasts

of Iran. This species has several advantages for culture in tropical and sub-tropical regions, such as high fecundity, fast growth rate, high tolerance to environmental conditions, particularly water salinity and high temperature, and desirable feed conversion ratio (Mozanzadeh *et al.*, 2021). This species is reported to be susceptible to vibriosis (Silva *et al.*, 2014) due to skin abrasions that often exacerbate bacterial infection (Liu *et al.*, 2015). This study describes the efficacy of the *V. alginolyticus* formaldehyde-killed vaccine in controlling vibriosis caused by *V. alginolyticus* infection in Asian seabass, *L. calcarifer*.

Materials and methods

Bacterial strain and growth condition

A local pathogenic *V. alginolyticus* isolate strain IR-Vac-V. a-2020 (NCBI accession number: MW654505) was used in this study. It was isolated from some infected cultured marine fish species, including *L. calcarifer*, common grouper (*Epinephelus coioides*), yellowfin seabream (*Acanthopagrus arabicus*), Sobaity Seabream (*Sparidentex hasta*) from South of Iran, Khuzestan province and its virulent characteristics by biochemical and molecular methods was carry out (Ahangarzadeh *et al.*, 2022). *V. alginolyticus* stock culture was preserved at -80°C in 20% (v/v) glycerol-tryptic soy broth.

Preparation of killed whole-cell vaccine

For the inactivated vaccines preparation, bacteria from frozen glycerol stocks were recovered on tryptic soy agar (TSA) for 24 h at 30°C and single colony of each strain was inoculated into TSB containing sodium chloride (3% NaCl) and incubated for 15-18 h (overnight) at 30°C (overnight) with shaking. The bacterial suspension was harvested and centrifuged at 5000×*g* at 4°C for 10 min and washed three times with PBS[1]. The bacterial cell count was done using microplate reader at 600 nm, and a final concentration was adjusted on 10^{10} colony forming units (CFU) /mL in PBS. The cells were killed by 18 h incubation with 0.5% formalin in phosphate-buffered saline (PBS) at 4°C and were washed with sterile PBS three times by centrifuging at 5000× *g* at 4°C for 10 min to ensure the formalin was entirely removed (Li *et al.*, 2015). The pellets were re-suspended in sterile PBS to 10^{10} CFU/ mL and maintained at 4°C until used (Liu *et al.*, 2015; Nehlah *et al.*, 2017; Aly *et al.*, 2021). Then, the inactivated bacteria suspension was homogenized with equal volumes of Freund incomplete adjuvant (Baharafshan, Iran) (Habeeb *et al.*, 2007).

Sterility test

This test was done by cultivation of a prepared vaccine on TSA & TCBS medium, which was then incubated at 37°C for 24 h and examined for bacterial growth (Bahnasawy *et al.*, 2019).

[1] Phosphate-Buffered Saline

Safety test

This test was performed according to Bahnasawy *et al.* (2019) by intraperitoneally inoculation of fish with the prepared vaccine and their monitoring for two weeks to ensure that there was no infection occurred.

Experimental designed and vaccination strategy

Two hundred Asian sea bass (55±6.43 g), were obtained from the South of Iran, Bushehr province. The experimental fish were acclimatized for two weeks before the experiments. The fish were divided randomly into three groups with 60 fish per group with three repetitions (20 fish per repetition). The fish were kept in 300-l tanks supplied with aerated seawater and fed three times a day with a commercial pelleted diet. Throughout the experimental period, temperature was kept at 28±2°C, and salinity was maintained at 43±2 ppt. Three experimental groups including I) control (n=60) without vaccine and injected by PBS, II) fish (n=60) immunized by killed *V. alginolyticus* vaccine with adjuvant (FKC) and III) fish (n=60) immunized by killed *V. alginolyticus* vaccine with adjuvant and oral booster (10^9 CFU/g of feed) (FKC+Booster). In vaccine treated groups (group II and III), fish were injected by intraperitoneally at a dose of 0.1 ml/fish. For oral vaccination as booster in III group, fish were fed with pellets coated with formalin killed whole-cell vaccine for 7 days from the third-week of post-injection vaccination, then they were fed with the control diet (Li *et al.*, 2015; Aly *et al.*, 2021).

Fish sampling

Fish were anesthetized using 2-Phenoxyethanol (300 ppm). Before and after vaccination (three, five, and eight weeks), blood samples were obtained from the caudal vein of nine fish per group. After clotting the blood overnight at 4°C, serum samples were collected by centrifugation at 3500× g for 10 min. Sera were stored at -80°C until further analysis (Li *et al.*, 2015; Bahnasawy *et al.*, 2019).

Preparation of rabbit anti-Asian sea bass immunoglobulin

Purified Asian sea bass immunoglobulin M (IgM) was prepared with ammonium sulphate. Two adult male rabbits were immunized with 1 mL of Asian sea bass Ig in 50% Freund's complete adjuvant intramuscularly. The second injection was carried out after 14 days, in the same manner, using Freund's incomplete adjuvant. Negative control was injected using PBS. Blood was obtained from the ear marginal vein two weeks after the second immunization, and antisera were collected (Kalbasi *et al.*, 2000).

Development of ELISA for titration of antibodies

The specific immune response after vaccination with *V. alginolyticus* formaldehyde-killed vaccine was evaluated using the indirect-ELISA (enzyme-linked immunosorbent assay) to determine the IgM antibody levels

against *V. alginolyticus* according to Firdaus-Nawi *et al.* (2013).

ELISA plate (Nunc, Denmark) was covered with 100 µL sonicated *V. alginolyticus* formaldehyde-killed (100 µg/mL) antigen diluted (1:60) in coating buffer (carbonate bicarbonate with pH=9.6) for 18 h at 4°C. Then the plates were washed three times with PBS Tween-20 (washing solution) and the reaction was blocked with blocking buffer, PBST+5% Skim milk for 2 h at room temperature (25ºC). Then, the wells were washed three times with the washing solution. Serum diluted with PBST+0.1% Skim milk to 1:100 was added into the wells and incubated for 1 h at room temperature, and the wells were washed four times with the washing solution. The plates were incubated for 1 h at room temperature with Rabbit anti-sea bass IgM diluted with PBST +0.1% Skim milk to 1:300, and the wells were washed four times with the washing solution. Mouse anti-rabbit IgG conjugate diluted with PBST+0.1% Skim milk was added. The plates were incubated for 1 h at 25ºC. After being washed four times (with the washing solution substrate solution (Tetramethyl benzidine with H_2O_2) was added and left to react for 15 min at 25ºC. The enzyme reaction was stopped by adding 50 µ of 2N H_2SO_4. The absorbance at 450 nm was measured with an automated microplate reader. The OD values were transformed to S/P ratio based on the results on serum samples for both the negative and positive controls in duplicate using the following equation:

S/P (%) = (OD of sample − OD of negative control)/ (OD of positive control − OD of negative control) × 100. (El-Jakee *et al.*, 2008; Jung and Rautenschlein, 2020).

Pathogenic challenge

The efficacy of *V. alginolyticus* formalin-killed vaccine was determined by the relative percent survival (RPS) through the challenge test with the virulent strain of *V. alginolyticus* five weeks after vaccination. A fresh 24 h bacterial culture of a virulent *V. alginolyticus* was used for the experimental challenge. For evaluation of vaccine potency, fishes in each experimental group were inoculated with 0.1 ml of an overnight culture of Virulence *V. alginolyticus* suspended in PBS at a concentration of approximately twice the lethal dose via intraperitoneal injection five weeks after vaccination (Setyaningsih *et al.*, 2020). Each symptom and mortalities were monitored daily for two weeks after the challenge in all experiments. To confirm the disease, from the dead fish, was sampled for re-isolation of bacteria (Li *et al.*, 2015; Nehlah *et al.*, 2017; Bahnasawy *et al.*, 2019).

The survival rates and the protection of Asian sea bass vaccinated with vaccine preparations were evaluated

according to the following formula described by Amend (1981):

RPS = [l - (% mortality in vaccinated fish/ % mortality in control fish)] x 100

Statistics

One-way analysis of variance (ANOVA) by Duncan's post hoc test was carried out to compare numerical data among groups. All data were presented as Mean± SE in which the significant differences were determined at $p<0.05$.

Results

Sterility and safety test

The vaccine safety and sterility assessments showed that no bacteria grew on the cultured plates after 48 h incubation, and fish did not show any disease symptoms during the two-week post-vaccination indicating safety of vaccines.

Protection against pathogenic challenge

The cumulative mortality of control and vaccinated groups in two weeks after challenge with virulent *V. alginolyticus* isolate was shown in Figure 1.

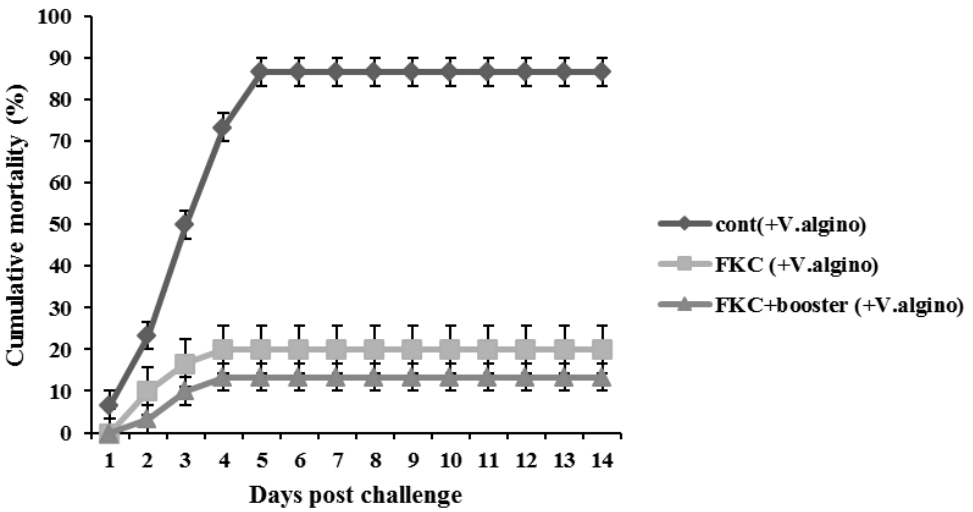

Figure 1: Cumulative mortality of different groups with the pathogenic strain of *V. alginolyticus* via ip route.

The mortality rate in control group was 86.66±3.33% (mean± SE). In contrast, the mean mortality rate in groups II and III were 13.33±3.33% and 20.0±5.77%, respectively. As a result, the highest and lowest mortality percentages were recorded in the control and the formalin-killed (FKC) vaccine with the oral booster, respectively (Fig. 2).

Fish vaccinated with *V. alginolyticus* vaccines (FKC and FKC+ booster) displayed a better survival rate than control non- vaccinated fish groups with RPS of 76.92±6.66 and 84.62±3.84%, respectively.

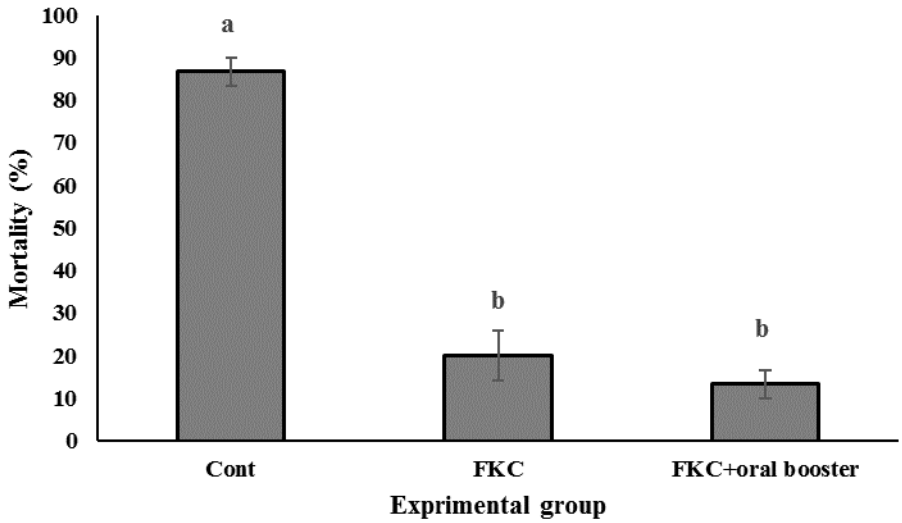

Figure 2: Mortalities percentage (Mean+SE) in control and vaccinated groups, following experimental infection with *V. alginolyticus*. Different letters indicate significant differences between treatments (p<0.05).

The survival of the treatment groups was significantly higher than the control (p<0.05). Fish vaccinated by the intraperitoneal method by FKC without booster exhibited a satisfactory survival (76.92%), while better protections were achieved in the trials of fish vaccinated under the intraperitoneal with oral booster regime (survival rate= 84.62%) (Fig. 3).

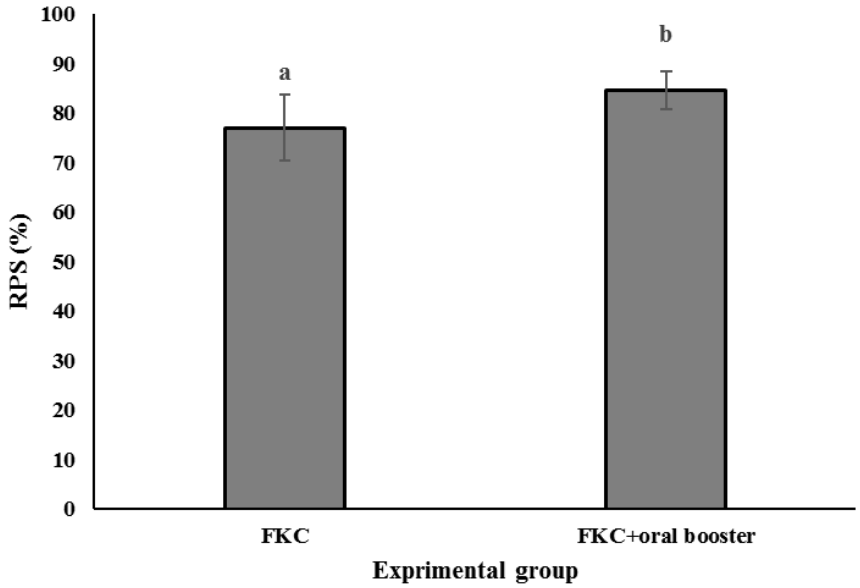

Figure 3: Survival percentage (Mean+ SE) in experimental groups challenged with virulent *V. alginolyticus* isolate. Different letters indicate significant differences between treatments (p<0.05).

The specific immune response in Asian sea bass was evaluated by measuring serum antibody titer using the ELISA three times, including before and four- and eight-weeks post-vaccination. There was no significant difference in antibody response among groups before treatment (p>0.05).

The highest antibody titers against *V. alginolyticus* were observed five weeks after vaccination in both FKC (126.43±5.53) and FKC+oral booster (96.74±2.88) groups, and the lowest antibody titer was detected three weeks after vaccination.

Statistical analysis showed that fish vaccinated with *V. alginolyticus* formalin-killed (FKC) vaccine and FKC+ oral booster had significantly higher antibody titer than control fish at all three sampling times (p<0.05). Fish vaccinated with FKC and oral booster displayed the highest antibody levels (126.43±5.53) five weeks after vaccination (p<0.05). However, there were no significant differences between the two vaccinated groups (FKC & FKC+ oral booster) five weeks after vaccination (p>0.05) (Fig. 4).

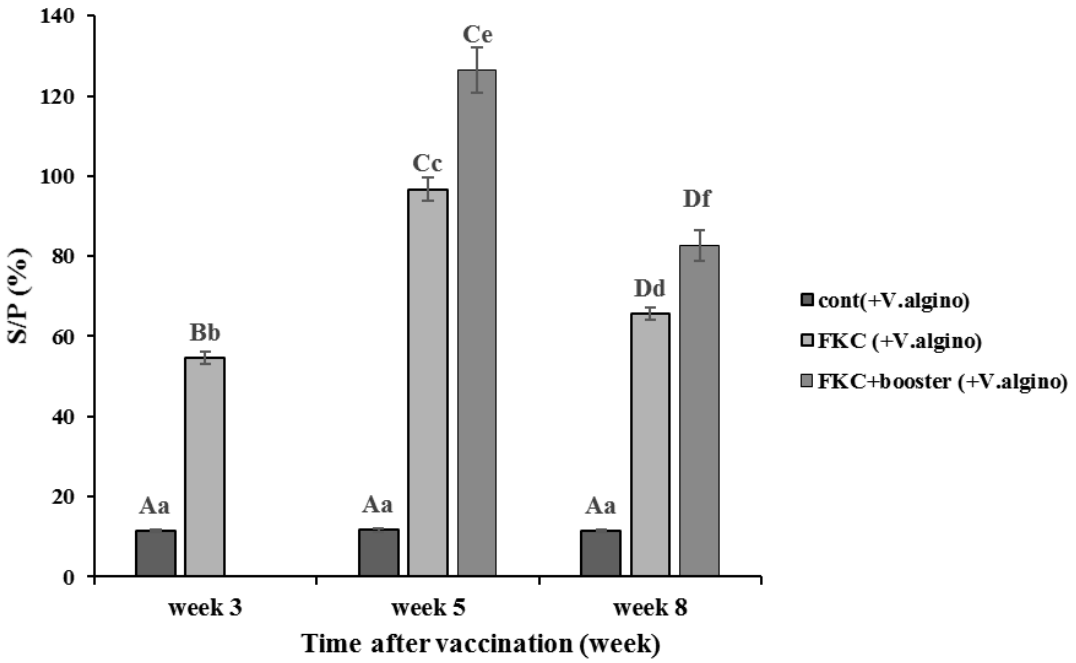

Figure 4: Serum ELAISA antibody titer (S/P %) of Asian sea bass against *V. alginolyticus* (mean±SE) in three, five and eight weeks after vaccination. The Capital letters in the same color columns show a significant difference within each week (p<0.05), and the small letters in the same color columns show a significant difference between weeks (p<0.05).

Discussion

Vaccination has become a routine method for preventing the fish diseases in intensive mariculture and has become safe and cost-effective for fish disease prevention compared to the other therapeutic strategies (Gudding, 2014; Aly *et al.*, 2021). Researchers report that formalin-killed vaccines are currently the most popular vaccines used in

cultured fish and provide a robust protection against vibriosis in many marine fish species (Colquhoun and Lillehaug, 2014; Lillehaug, 2014). To our best knowledge, there are no previous reports of vibrio vaccines in Asian sea bass in Iran.

The RPS is an important index to evaluate the immune effect of vaccines. This study showed that *L. calcarifer* vaccinated with the FKC+oral booster exhibited better protection (84.62%) than the FKC vaccine (76.92%) after challenge test. The present study results agreed with Li *et al.* (2015), who reported that formalin-killed whole-cell bacteria provided 80% protection against *V. alginolyticus* in silver sea bream three weeks after the vaccination.

Also, Nehlah *et al.* (2017) were developed vaccines based on the conserved antigens of *Vibrio* sp., namely outer membrane protein K (OmpK) and outer membrane protein W (OmpW) in hybrid grouper juvenile. They reported that RPS was 100% in rOmpK and it was 63% in rOmpW group.

Furthermore, vaccination of zebrafish with a live attenuated vaccine of *V. alginolyticus* provided a protection about 71.2% (Zhou *et al.*, 2020). Similar to the present study, Bahnasawy *et al.* (2019) showed that the level of protection after challenge with virulent *V. alginolyticus* in sea bream by formalin vaccine was 91.66%, while it was 83.33% in vaccinated fish groups produced by heat-killed vaccine. Therefore, higher values of RLP[1] were reported in formalin vaccinated fish than in fish vaccinated by heat-killed. Also, the challenge with *V. alginolyticus* isolate at four weeks after vaccination of fish with DNA vaccine containing *flaA* gene of this virulent isolate showed that the vaccine's protection rate is 88% (Liang *et al.*, 2011). Aly *et al.* (2021), in the efficiency of Monovalent and Polyvalent *V. alginolyticus* and *V. parahaemolyticus* vaccines on protection in Gilthead sea bream, against vibriosis, showed that fish vaccinated with the *V. alginolyticus* monovalent vaccine displayed RPS of 100% and the RPS for the polyvalent vaccinated fish groups were 91.7% against virulent *V. alginolyticus*, and these results were also supported by Li *et al.* (2015). Also, Pang *et al.* (2018) reported more than 70% survival rate in orange-spotted grouper vaccinated IP with *V. alginolyticus* FKC. Previous studies confirmed these results by defining a promising and excellent vaccine that provides relative percent survival of about 70% and 80%, respectively (Chettri *et al.*, 2015). Similarly, Rømer Villumsen *et al.* (2015) reported 78-80% survival, and Fredriksen *et al.* (2013) reported 77.5% survival following fish vaccination. Improving survival and increasing the survival rate after vaccination is beneficial to farmers as increases production (Mohamad *et al.*, 2021). The researchers suggested that RLP provided more than 60% of acceptable protection (Chettri *et al.*, 2015), so the FKC and

[1] Relative Level of Protection

FKC+oral booster vaccines used in this study are excellent vaccines.

The present study results showed that the fish had a specific antibody titer against *V. alginolyticus* before vaccination, possibly due to the constant presence of this bacteria in aquatic environments. These results are consistent with those of Ajdari *et al.* (2019) and Ahangarzadeh *et al.* (2015). Similarly, Crosbie and Nowak (2004) reported that the Asian sea bass fish had a specific antibody titer against *V. harveyi* before vaccination. Crosbie and Nowak (2004) explained that the natural antibody against bacterial pathogens is produced due to contact of normal fish with these bacteria present in the fish environment. High levels of natural antibodies against bacterial pathogens are usually produced due to the constant of the fish with these pathogens, which are normally present in water and reside in the intestinal tract of fish. The present study results showed that the FKC+oral booster vaccine augmented significant antibody production in Asian sea bass vaccinated against *V. alginolyticus*.

The results also showed that the antibody titer of *V. alginolyticus* had the highest value in all vaccinated groups at five weeks, then at eight weeks, and the lowest at three weeks after immunization. Likewise, Ajdari *et al.* (2019) demonstrated that vaccination of Asian sea bass with IP administration of *V. harveyi* vaccine increased the S/P (%) of antibody titer at four weeks as well as Halimi *et al.* (2019) showed similar results for antibody titer against *L. garvieae* after vaccination by chitosan-alginate coated oral vaccine enhanced on days 20 and 40 in rainbow trout.

Similar results have been reported in Gilthead Seabream vaccinated with monovalent and polyvalent *V. alginolyticus* and *V. parahaemolyticus* vaccines showing the antibody titers in two, four, and eight weeks after vaccination in the vaccinated groups were significantly different from the control (Aly *et al.,* 2021). The antibody titers against *V. alginolyticus* showed the highest rate in the monovalent vaccine in four weeks compared to two and eight weeks after vaccination (Li *et al.*, 2015; Aly *et al.,* 2021), in the protective evaluation of different vaccinating modalities against *V. alginolyticus* in Silver sea bream, showed that the vaccinated groups were significantly different from the control group in all vaccination methods. However, the highest level of specific antibody titer was observed in LPS-vaccinated fish, and then relevant higher values of agglutinating antibody titer were found in the fish vaccinated with FKC.

In agreement with the findings of the present study, in a study to evaluate the protection of the recombinant *V. alginolyticus* vaccine, it was found that the highest level of antibody titer was released four weeks after vaccination and then began to decrease in the juvenile hybrid grouper that was vaccinated with the recombinant *V. alginolyticus* vaccine (Nehlah *et al.*, 2017).

In general, the immune response to vaccination depends on the physiological condition of the fish, the bacterial species from which the vaccines are made, the methods of vaccination, and the dosage of vaccine (Lillehaug, 2014). Similar to the previous findings, injection method via IP was the most common and effective means of fish vaccination. Vaccination is an effective effort to protect farmed sea bass against vibriosis caused by pathogenic *V. alginolyticus*, which is consistent with the study by Li *et al.* (2015). These findings suggest that at least a single injection seems necessary to achieve effective protection against vibriosis (Li *et al*., 2015).

In conclusion, the results of the present study showed that *V. alginolyticus* formalin-killed vaccine prepared from pathogenic native isolates could provide acceptable protection and good antibody titer against *V. alginolyticus* in Asian sea bass fish. Furthermore, IP injections of formalin-inactivated whole-cell bacterins could offer effective protection with oral vaccination in Asian seabass.

Acknowledgements

We thank the Iranian Fisheries Science Research Institute (IFSRI) for funding (No. 24-74-12-029-981164) for this study. The authors are also grateful for the assistance made by the staff of Bandar-e- Imam Khomeini Marine Fish Research Station for their assistance.

References

Abdallah, F.B., Chaieb, K., Kallel, H. and Bakhrouf, A., 2009. RT-PCR assays for in vivo expression of *Vibrio alginolyticus* virulence genes in cultured gilthead *Dicentrarchus labrax* and *Sparus aurata*. *Annals of Microbiology,* 59(**1**)**,** 63-67. DOI: 10.1007/BF03175600

Ahangarzadeh, M., Ghorbanpour Najafabadi, M., Peyghan, R., Houshmand, H., Sharif Rohani, M. and Soltani, M., 2022. Detection and distribution of virulence genes in *Aeromonas hydrophila* isolates causing infection in cultured carps. *Veterinary Research Forum,* 13(**1**)**,** 55-60. 10.30466/vrf.2020.115998.2761

Ahangarzadeh, M., Peyghan, R., Ghorbanpour, M., Sharif Rohani, M. and Soltani, M., **2015**. *Role of Aeromonas hydrophila in bacterial septicemia of cultured carps in Khouzestan Province and investigation on protectivity of bacterin (s) prepared from acute isolate(s) of it.* PhD Thesis, Shahid Chamran University of Ahvaz. Veterinary Faculty. *Aquatic Animals Healths*. 199 P. [In Farsi]

Ajdari, A., Peyghan, R., Ghorbanpour, M., Ahangarzadeh, M. and Mirbakhsh, M., **2019**. *Evaluation of virulance factores in Vibrio harveyi isolates from Cultured Asian seabass (Lates calcarifer) and investigation on protective effect of virulant isolate bacterin.* PhD Thesis, Shahid Chamran University of

Ahvaz. Veterinary Faculty. Aquatic Animals Healths. 153 P. [In Farsi].

Aly, S.M., Eissa, A. E., ElBanna, N.I. and Albutti, A., 2021. Efficiency of monovalent and polyvalent *Vibrio alginolyticus* and *Vibrio Parahaemolyticus* vaccines on the immune response and protection in gilthead sea bream, *Sparus aurata* (L.) against vibriosis. *Fish & Shellfish Immunology,* 111**,** 145-151. DOI: 10.1016/j.fsi.2020.10.011

Amend, D.F., 1981. Potency testing of fish vaccines. *Fish biologics: serodiagnostics and vaccines,* 49**,** 447-454,

Austin, B. and Austin, D.A., 2016. *Bacterial Fish Pathogens: Disease of Farmed and Wild Fish*, Springer International Publishing. DOI: 10.1007/978-3-319-32674-0

Bahnasawy, M., El-Bakry, K., El-Safy, M. and El-Borsh, D., 2019. Use of vaccines in controlling bacteria fish diseases caused by *Vibrio anuiliticus*. *African Journal of Biological Sciences,* 15(**1**)**,** 87-100. DOI: 10.21608/AJBS.2019.64002

Bellos, G., Angelidis, P. and Miliou, H., 2015. Effect of temperature and seasonality principal epizootiological risk factor on vibriosis and photobacteriosis outbreaks for Eeuropean sea bass in Greece (1998-2013). *Journal of Aquaculture Research and Development,* 6(**338**)**,** 10.4172,

Cao, J., Zhang, J., Ma, L., Li, L., Zhang, W. and Li, J., 2018. Identification of fish source *Vibrio alginolyticus* and evaluation of its bacterial ghosts vaccine immune effects. *Microbiology Open,* 7(**3**)**,** e00576-e00576. DOI: 10.1002/mbo3.576

Chettri, J.K., Jaafar, R.M., Skov, J., Kania, P.W., Dalsgaard, I. and Buchmann, K., 2015. Booster immersion vaccination using diluted *Yersinia ruckeri* bacterin confers protection against ERM in rainbow trout. *Aquaculture,* 440**,** 1-5. DOI: 10.1016/j.aquaculture.2015.01.027

Chin, Y.K., Al-saari, N., Zulperi, Z., Mohd-Aris, A., Salleh, A., Silvaraj, S., Mohamad, A., Lee, J., Zamri-Saad, M. and Ina-Salwany, M. Y., 2020. Efficacy of bath vaccination with a live attenuated *Vibrio harveyi* against vibriosis in Asian seabass fingerling, *Lates calcarifer*. *Aquaculture Research,* 51(**1**)**,** 389-399. DOI: 10.1111/are.14386

Colquhoun, D.J. and Lillehaug, A., 2014. Vaccination against Vibriosis. *In:* Gudding, R., Lillehaug, A. & Evensen, Ø. (eds.) *Fish Vaccination.* Hoboken, New Jersey: John Wiley & Sons, Ltd, 172-184. DOI: 10.1002/9781118806913.ch15

Crosbie, P.B.B. and Nowak, B.F., 2004. Immune responses of barramundi, *Lates calcarifer* (Bloch), after administration of an experimental *Vibrio harveyi* bacterin by intraperitoneal injection, anal intubation and immersion. *Journal of Fish Diseases,* 27(**11**)**,** 623-632. DOI: 10.1111/j.1365-2761.2004.00575.x

Damir, K., Irena, V.S., Damir, V. and Emin, T., 2013. Occurrence, characterization and antimicrobial susceptibility of *Vibrio alginolyticus* in the Eastern Adriatic Sea. *Marine Pollution Bulletin,* 75(**1-2**)**,** 46-52. DOI: 10.1016/j.marpolbul.2013.08.008

El-Jakee, J., Marzouk, M.S., Mahmoud, N., Maha, A., Elghobashy, H., Fitzsimmons, K. and Diab, A.S., 2008. Trials to create Edwardsiellosis native vaccines for freshwater fish in Egypt. 8th International Symposium on tilapia in Aquaculture, October 12-14 2008 Cairo, Egypt. *The Central Laboratory for Aquaculture Research*, 1143-1155.

Firdaus-Nawi, M., Yusoff, S.M., Yusof, H., Abdullah, S.Z. and Zamri-Saad, M., 2013. Efficacy of feed-based adjuvant vaccine against *Streptococcus agalactiae* in *Oreochromis* spp. in Malaysia. *Aquaculture Research,* 45(**1**)**,** 87-96. DOI: 10.1111/j.1365-2109.2012.03207.x

Fredriksen, B.N., Olsen, R.H., Furevik, A., Souhoka, R.A., Gauthier, D. and Brudeseth, B., 2013. Efficacy of a divalent and a multivalent water-in-oil formulated vaccine against a highly virulent strain of *Flavobacterium psychrophilum* after intramuscular challenge of rainbow trout (*Oncorhynchus mykiss*). *Vaccine,* 31(**15**)**,** 1994-1998. DOI: 10.1016/j.vaccine.2013.01.016

Gudding, R. and Van Muiswinkel, W.B., 2013. A history of fish vaccination: science-based disease prevention in aquaculture. *Fish and Shellfish Immunology,* 35(**6**)**,** 1683-1688. DOI: 10.1016/j.fsi.2013.09.031

Gudding, R., 2014. Vaccination as a Preventive Measure. *In:* Gudding, R., Lillehaug, A. & Evensen, Ø. (eds.) *Fish Vaccination.* John Wiley & Sons, Ltd. DOI: 10.1002/9781118806913.ch2

Habeeb, F., Stables, G., Bradbury, F., Nong, S., Cameron, P., Plevin, R. and Ferro, V.A., 2007. The inner gel component of Aloe vera suppresses bacterial-induced pro-inflammatory cytokines from human immune cells. *Methods,* 42(**4**)**,** 388-393. DOI: 10.1016/j.ymeth.2007.03.005

Haenen, O., Fouz Rodríguez, B., Amaro González, C., Isern, M., Mikkelsen, H., Zrnčić, S., Travers, M.-A., Renault, T., Wardle, R. and Hellström, A., 2014. Vibriosis in aquaculture. 16th EAFP Conference, Tampere, Finland, 4th September 2013. *Bulletin of the European Association of Fish Pathologists,* 34(**4**)**,** 138-147, https://roderic.uv.es/bitstream/handle/10550/41263/100643.pdf?sequence=1

Halimi, M., Alishahi, M., Abbaspour, M.R., Ghorbanpoor, M. and Tabandeh, M.R., 2019. Valuable method for production of oral vaccine by using alginate and chitosan against *Lactococcus garvieae*/*Streptococcus iniae* in rainbow trout (*Oncorhynchus*

mykiss). *Fish & Shellfish Immunology*, 90**,** 431-439. DOI: 10.1016/j.fsi.2019.05.020

Hamod, M.A., Nithin, M.S., Shukur, Y.N., Karunasagar, I. and Karunasagar, I., 2012. Outer membrane protein K as a subunit vaccine against *V. anguillarum*. *Aquaculture*, 354-355**,** 107-110. DOI: 10.1016/j.aquaculture.2012.03.033

Jung, A. and Rautenschlein, S., 2020. Development of an in-house ELISA for detection of antibodies against *Enterococcus cecorum* in Pekin ducks. *Avian Pathology*, 49(**4**)**,** 355-360. DOI: 10.1080/03079457.2020.1753653

Kalbasi, M.R., Soltani, M., Pourbakhsh, S.A. and Adams, A., 2000. Humoral immune response of cultured Persian sturgeon (*Acipenser persicus*) to four different *Aeromonas hydrophila* antigens. *Archives of Razi Institute*, 51(**1**)**,** 75-83. DOI: 10.22092/ARI.2000.109198

Krupesha Sharma, S.R., Rathore, G., Verma, D.K., Sadhu, N. and Philipose, K.K., 2012. *Vibrio alginolyticus* infection in Asian seabass (*Lates calcarifer*, Bloch) reared in open sea floating cages in India. *Aquaculture Research*, 44(**1**)**,** 86-92. DOI: 10.1111/j.1365-2109.2011.03013.x

Li, J., Ma, S. and Woo, N.Y.S., 2015. Vaccination of Silver Sea Bream (*Sparus sarba*) against *Vibrio alginolyticus*: Protective Evaluation of Different Vaccinating Modalities. *International Journal of Molecular Sciences*, 17(**1**)**,** 40. DOI: 10.3390/ijms17010040

Liang, H.Y., Wu, Z.H., Jian, J.C. and Huang, Y.C., 2011. Protection of red snapper (*Lutjanus sanguineus*) against *Vibrio alginolyticus* with a DNA vaccine containing flagellin flaA gene. *Letters in Applied Microbiology*, 52(**2**)**,** 156-161. DOI: 10.1111/j.1472-765X.2010.02981.x

Lillehaug, A., 2014. Vaccination Strategies and Procedures. *In:* Gudding, R., Lillehaug, A. & Evensen, Ø. (eds.) *Fish Vaccination.* DOI: 10.1002/9781118806913.ch12

Liu, S., Li, E., Cai, Y., Wang, S., Ren, Z., Li, Q., Guo, W., Wu, Y. and Zhou, Y., 2018. Isolation, identification and pathogenicity characterization of *Vibrio ponticus* from the golden pompano *Trachinotus ovatus*. *Aquaculture*, 496**,** 285-290. DOI: 10.1016/j.aquaculture.2018.04.065

Liu, X., Wu, H., Liu, Q., Wang, Q., Xiao, J. and Zhang, Y., 2015. Skin-injured Zebrafish, *Danio rerio*, are more Susceptible to *Vibrio anguillarum* Infection. *Journal of the World Aquaculture Society*, 46(**3**)**,** 301-310. DOI: 10.1111/jwas.12188

Miccoli, A., Saraceni, P.R. and Scapigliati, G., 2019. Vaccines and immune protection of principal Mediterranean marine fish species. *Fish & Shellfish Immunology*, 94**,** 800-809. DOI: 10.1016/j.fsi.2019.09.065

Mohamad, N., Amal, M.N.A., Yasin, I.S.M., Zamri Saad, M., Nasruddin, N.S., Al-saari, N., Mino, S. and Sawabe, T., 2019. Vibriosis in cultured marine fishes: a review. *Aquaculture,* 512**,** 734289. DOI: 10.1016/j.aquaculture.2019.734289

Mohamad, A., Zamri-Saad, M., Amal, M.N., Al-saari, N., Monir, M.S., Chin, Y.K. and Md Yasin, I.S., 2021. Vaccine Efficacy of a Newly Developed Feed-Based Whole-Cell Polyvalent Vaccine against Vibriosis, Streptococcosis and Motile Aeromonad Septicemia in Asian Seabass, *Lates calcarifer*. *Vaccines,* 9(**4**). DOI: 10.3390/vaccines9040368

Mozanzadeh, M.T., Safari, O., Oosooli, R., Mehrjooyan, S., Najafabadi, M.Z., Hoseini, S.J., Saghavi, H. and Monem, J., 2021. The effect of salinity on growth performance, digestive and antioxidant enzymes, humoral immunity and stress indices in two euryhaline fish species: Yellowfin seabream (*Acanthopagrus latus*) and Asian seabass (*Lates calcarifer*). *Aquaculture,* 534**,** 736329. DOI: 10.1016/j.aquaculture.2020.736329

Nehlah, R., Firdaus-Nawi, M., Nik-Haiha, N., Karim, M., Zamri-Saad, M. and Ina-Salwany, M., 2017. Recombinant vaccine protects juvenile hybrid grouper, *Epinephelus fuscoguttatus*× *Epinephelus lanceolatus*, against infection by *Vibrio alginolyticus*. *Aquaculture International,* 25(**6**)**,** 2047-2059. DOI: 10.1007/s10499-017-0172-8

Osman, K.M., Mohamed, L.A., Rahman, E.H.A. and Soliman, W., 2009. Trials for vaccination of Tilapia fish against Aeromonas and Pseudomonas infections using monovalent, bivalent and polyvalent vaccines. *World Journal of Fish and Marine Sciences,* 1(**4**)**,** 297-304,

Pang, H., Qiu, M., Zhao, J., Hoare, R., Monaghan, S.J., Song, D., Chang, Y. and Jian, J., 2018. Construction of a *Vibrio alginolyticus* hopPmaJ (hop) mutant and evaluation of its potential as a live attenuated vaccine in orange-spotted grouper (*Epinephelus coioides*). *Fish & Shellfish Immunology,* 76**,** 93-100. DOI: 10.1016/j.fsi.2018.02.012

Rameshkumar, P., Nazar, A.K.A., Pradeep, M.A., Kalidas, C., Jayakumar, R., Tamilmani, G., Sakthivel, M., Samal, A.K., Sirajudeen, S., Venkatesan, V. and Nazeera, B.M., 2017. Isolation and characterization of pathogenic *Vibrio alginolyticus* from sea cage cultured cobia (*Rachycentron canadum* (Linnaeus 1766) in India. *Letters in Applied Microbiology,* 65(**5**)**,** 423-430. DOI: 10.1111/lam.12800

Rømer Villumsen, K., Koppang, E.O. and Raida, M.K., 2015. Adverse and long-term protective effects following oil-adjuvanted vaccination against *Aeromonas salmonicida* in rainbow trout. *Fish & Shellfish Immunology,* 42(**1**)**,** 193-203. DOI: 10.1016/j.fsi.2014.09.024

Sadok, K., Mejdi, S., Nourhen, S. and Amina, B., 2013. Phenotypic characterization and RAPD

fingerprinting of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* isolated during Tunisian fish farm outbreaks. *Folia Microbiologica,* 58(**1**)**,** 17-26. DOI: 10.1007/s12223-012-0174-x

Setyaningsih, S.U., Kusdarwati, R., Rozi and Handijatno, D., 2020. The Effectiveness of Vaccines in Gurame (*Osphronemus goramy*) and Challenged *Aeromonas hydrophila*. *IOP Conference Series: Earth and Environmental Science,* 441(**1**)**,** 012027. 10.1088/1755-1315/441/1/012027

Silva, Y.J., Costa, L., Pereira, C., Mateus, C., Cunha, A., Calado, R., Gomes, N.C., Pardo, M.A., Hernandez, I. and Almeida, A., 2014. Phage therapy as an approach to prevent *Vibrio anguillarum* infections in fish larvae production. *PLoS ONE,* 9(**12**)**,** e114197. DOI: 10.1371/journal.pone.0114197

Toranzo, A.E., Romalde, J.L.B.M. and J.L.B., 2009. Present and future of aquaculture vaccines against fish bacterial diseases. *In:* C., R. & B., B. R. (eds.) *The use of veterinary drugs and vaccines in Mediterranean aquaculture 2003/05/21-23, Izmir (Turkey).* Zaragoza CIHEAM

Woo, P.T.K. and Gregory, D.W.B., 2014. *Diseases and Disorders of Finfish in Cage Culture, 2nd Edition*, CABI. 523-546. DOI: 10.1079/9781780642079.0000

Zhou, S., Tu, X., Pang, H., Hoare, R., Monaghan, S.J., Luo, J. and Jian, J., 2020. A T3SS Regulator Mutant of *Vibrio alginolyticus* Affects Antibiotic Susceptibilities and Provides Significant Protection to *Danio rerio* as a Live Attenuated Vaccine. *Frontiers in Cellular and Infection Microbiology,* 10**,** 183-183. DOI: 10.3389/fcimb.2020.00183