Fish and Shellfish Immunology Reports 4 (2023) 100078

Contents lists available at ScienceDirect

Fish and Shellfish Immunology Reports

journal homepage: www.sciencedirect.com/journal/fish-and-shellfish-immunology-reports

Protective effects of immersion immunization of koi with *Escherichia coli* DH5α carrying DNA vaccine against koi herpesvirus

Jauharotul Afifah, Sukenda Sukenda, Alimuddin Alimuddin, Hasan Nasrullah, Sri Nuryati *

Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University (Bogor Agricultural University), Dramaga, Bogor 16680, Indonesia

ARTICLE INFO

Keywords:
DNA Vaccine
Immersion
Koi
Koi herpesvirus

ABSTRACT

This study aims to immunize the koi fingerlings immersion using the formalin-killed and freeze-dried E. coli DH5α carrying plasmid for the KHV DNA vaccine. 200 fish on each tank in a total water volume of 20 L. Each tanks consists of different vaccination group: PBS as control (10 mL; C), empty E. coli DHα (10 mL at 108 CFU mL-1; E), formalinkilled E. coli DHα:ORF81 (10 mL at 108 CFU mL-1; KE), freezedried E.coli DHα:ORF81 without formalin inactivation (10 mL at 108 CFU mL-1; FE), and formalin-killed and then freeze-dried E. coli DHα:ORF81 (10 mL at 108 CFU mL-1; KFE). The bath vaccination was conducted for 1 × 30 min. For the challenged test, fish were immersed with the 100 mL of LD50 dose of KHV (10–2 dilution from the KHV stock) for 30 min. The vaccination using E. coli DH5α:ORF81 could significantly modulate the innate and adaptive immunity of the fish and result in higher fish survival after KHV infection. The vaccination using formalin-killed or formalin-killed and freezedried E. coli DH5α:ORF81 will be further developed as an alternative to the costefficient koi or carp fingerlings vaccination method.

Introduction

Indonesia's ornamental live fish trade was valued at more than 33 US $ billion in 2019 [1]. Koi fish (*Cyprinus rubrofuscus*), is one of the most popular ornamental fish commodities due to their variety of colors and patterns. However, this lucrative industry is continuously afflicted by disease outbreaks. Cyprinus herpesvirus-3 (CyHV-3) or koi herpesvirus (KHV) are the major pathogenic viruses in carp and koi cultures [2]. The term koi herpesvirus disease (KHVD) refers to KHV infection in carp and koi. Large mortality from this viral infection can range from 80% to 100%, with clinical signs including reddish patches and sores and tissue damage in the gills [3,4]. Furthermore, KHV causes greater mortality in fingerlings than in adult fish. Preventing a potential disease outbreak before it occurs is the best option for KHVD management since the virus could be latent in fish [5].

DNA vaccination is among the most promising strategies to protect fish from less either or KHV infection. Our previous studies showed that most vaccination by DNA vaccine encoding KHV's glycoprotein gene provided high levels of protection in common carp and koi. Fish injected with the purified DNA vaccine resulted in relative percent survival (RPS) of 90.48% after challenge in koi [6] and up to 78.9% in common carp after vaccination by oral feeding [7]. However, numerous physico-chemical and biological barriers in the gastrointestinal tract must be surmounted for oral vaccination. [8]. Vaccination through injection, on the other hand, is unpleasant for fish and is difficult to apply to the fingerlings. The immersion technique by bath vaccination is especially well-suited for immunizing small fish because it is less invasive and reduces handling [9,10]. It is also provided with the fish with the vaccine taken up by the gills, skin, and gut and processed by the immune system, where the resulting response may direct to protection [10].

For immersion delivery, immersed DNA vaccines were typically in the form of pure plasmids or plasmids encapsulated with nano-carriers [11,12]. However, these techniques will be costly for future mass applications in commercial koi and common carp hatcheries and farms. In this study, we propose to immunize the koi fingerlings immersion using the formalin-killed and freeze-dried *E. coli* DH5α carrying plasmid for the KHV DNA vaccine. This vaccine preparation will be easier and less costly compared to the purified or encapsulated DNA vaccine. The protective effects of the vaccinations were evaluated in this study by assessing the immunological parameters after vaccination and after the virus challenge. This preliminary study provided valuable information for the development of a more effective and efficient vaccination in koi

* Corresponding author.
E-mail addresses: jauhaafifa@gmail.com (J. Afifah), sukenda@apps.ipb.ac.id (S. Sukenda), alimuddin@apps.ipb.ac.id (A. Alimuddin), nasrullah.ipb@gmail.com (H. Nasrullah), sri_nuryati@ipb.ac.id (S. Nuryati).

https://doi.org/10.1016/j.fsirep.2022.100078
Received 12 August 2022; Received in revised form 28 November 2022; Accepted 13 December 2022
Available online 28 December 2022

Table 1
Primers used in the study.

Gene	Sequence (5′−3′)	Properties	Reference
orf81	F: TTACCGGTATGGCCTCCACTTCAA CCGCT R: TAGAGATCGTCCCAGTCCTTGTT TAG	Plasmid-specific primer	[6]
igm	F: CACAAGGCGGGAAATGAAGA R: GGAGGCACTATATCAACAGCA	qPCR	[2]
ifnγ	F: CGATCAAGGAAGATGACCCAGTC R: GTTGCTTCTCTGTAGACACGCTTC	qPCR	[2]
tnfα	F: GCTGTCTGCTTCACGCTCAA R: CCTTGGAAGTGACATTTGCTTTT	qPCR	[24]
β-actin	F: AGAGTATTTACGCTCAGGTGGG R: CCTTCTTGGGTATGGAGTCTTG	qPCR and internal control	[24]
KHV *sph*	F: GACACCACATCTGCAAGGAG R: GACACATGTTACAATGGTCGC	KHV detection	[4]

and carp against KHV.

Materials and methods

KHV isolation and filtrate preparation

Pathogenic KHV isolates were obtained from naturally-infected common carp at farmers' net cage culture in Jatiluhur, West Java. The KHV DNA was extracted from the gills of infected fish using the Puregene® Core kit A (Qiagen, USA) according to the kit's manual. The extracted DNA was amplified using the PCR method using specific primers for KHV detection [4]. The primer used in the study was presented in Table 1. The virus filtrate was prepared from the positive samples. Briefly, one gram of gill was minced using a sterile mortar and then dissolved with 9 mL of sterile phosphate-buffered saline (PBS, pH 7.4). The suspension was centrifuged at 1500 × g at 4 °C for 15 min and then filtered through a 0.45 μm sterile filter (Merck, USA). The filtrate was stored at −80 °C before use. Virus filtrate was then serially diluted from the stock using PBS for pathogenicity and lethal dose 50 (LD_{50}) test.

E. coli DH5α and DNA vaccine

The DNA vaccine used in this study was constructed from the *orf-81* of the KHV glycoprotein gene [13]. The plasmids were transformed into *E. coli* DH5α vector (*E.coli* DHα:ORF81) and stored at −80 °C after PCR confirmation with plasmid-specific primers [6]. The verified stock was cultured on 200 mL 2 × YT broth media with kanamycin (50 μg mL^{-1}) for 18 h at 37 °C and 15 × g. Empty *E. coli* DH5α (without the *orf-81* plasmid) was cultured in 2 × YT broth + kanamycin for control. The bacteria were harvested by centrifugation at 2300 × g for 5 min at 4 °C and then verified by PCR amplification.

Formalin inactivation and freeze-drying

The *E. coli* DHα:ORF81 (10^8 CFU mL^{-1}) was inactivated with 0.3% formalin (v/v) and incubated for 24 h at 37 °C. After incubation, the bacteria were harvested by centrifugation at 5300 × g for 5 min. The pellets were washed with PBS (pH 7.4) three times. The complete inactivation was verified by a viability test on agar plates at 37 °C for 24 h. The preparation of freeze-dried *E. coli* was from the previous report from the 10^8 CFU mL^{-1} of *E. coli* DHα:ORF81, and empty *E.coli* DHα [14]. The 15% skim milk was used as the protectant. The freezing temperature was at −80 °C for 36 h, then transferred to −20 °C for storage.

Fish immunization and KHV infection

The experimental fish were four-week-old koi fingerlings (average bodyweight= 0.5 ± 0.01 g). Fish were KHV-free as verified by the PCR method [4]. Fish were distributed into six 60 × 30 × 30 cm^3 tanks, with a density of 200 fish/tank in a total water volume of 20 L. Each tank consisted of different vaccination groups: PBS as control (10 mL; C), empty *E. coli* DHα (10 mL at 10^8 CFU mL^{-1}; E), formalin-killed *E. coli* DHα:ORF81 (10 mL at 10^8 CFU mL^{-1}; KE), freeze-dried *E.coli* DHα: ORF81 without formalin inactivation (10 mL at 10^8 CFU mL^{-1}; FE), and formalin-killed and then freeze-dried *E. coli* DHα:ORF81 (10 mL at 10^8 CFU mL^{-1}; KFE). The bath vaccination was conducted for 1 × 30 min. After vaccination, fish were placed into six 40 L aquariums for each corresponding vaccination group with a density of 30 fish per aquarium (n = 6 tanks per group). Fish were then reared for 28 days in the controlled indoor facility to ensure that no KHV infection occurred during the experiment. Fish were fed with commercial feed (40% protein) twice a day *at satiation*. Water and waste were cleaned daily. After 28 days postvaccination, 30 fish were immersed with the 100 mL of LD_{50} dose of KHV (10^{-2} dilution from the KHV stock) for 30 min inside the new tank and then put back into the rearing tank.

A sampling of immunological parameters

Body serum was randomly collected from fish from each group (n = 3) on 0, 7, 14, 21, and 28 days post-vaccination (dpv) and 7, 14, and 21 days after the virus challenge (dpc). The preparation of serum are referring to our previous study [15]. Briefly, body serum was collected aseptically from the anesthetized fish and diluted at 1:4 using PBS-T (PBS pH 7.4 + 0.05% Tween-20). Serum was obtained after centrifugation at 5000 × *g* for 10 min, then stored at −20 °C for lysozyme activity and antibody titers analysis. The lysozyme activity was conducted by incubating 100 μL of blood serum with 100 μL *Micrococcus lysodeikticus* suspension (0.4 mg/mL in 0.1 M PBS at 25 °C). The lysozyme was determined at 0 min and 30 min after incubation. The antibody titers were measured using indirect ELISA refers to [16]. Both parameters were analyzed by spectrophotometry method at an optical density of 450 nm.

Immune-related genes expression analysis

The gene expression was analyzed by real-time PCR (qPCR). The level of *igm* mRNA was measured after vaccination to evaluate early antibody expression, and the levels of the *tnfα* and *IFNγ* mRNA were analyzed after the challenge test to evaluate the immune responses related to the virus infection [2]. The kidney was pooled from fish (n = 3) in each corresponding group into a single microtube with a total of 20–25 mg. Total RNA was extracted from samples using GENEzol™ (Geneaid, Taiwan) according to the product instructions. Synthesis of cDNA was conducted from 100 ng μL^{-1} total RNA using the Revertra® Ace qPCR RT Mastermix kit with gDNA removal (Toyobo, Japan). The qPCR reaction was conducted in a total reaction volume of 20 μL using the SensiFAST SYBR® High-ROX (Bioline, UK) enzyme mix and run by the StepOne® Real-Time PCR machine (Thermo Scientific, USA). The amplifying program was set as follows: 95 °C for two min, 40 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 10 s. The *β-actin* gene was chosen as an optimal reference gene for relative quantification and data normalization based on its stable expression in previous KHV-related studies [17–19].

Statistical analysis

The data were presented as means ± SD and statistical analysis using one-way analysis of variance (ANOVA) with Tukey's post hoc test (significance level $p < 0.05$). Genes expression analysis was conducted according to the $2^{-\Delta\Delta CT}$ method [20] after normalized with *β-actin* gen and measured as the fold-change expression to the expression at PBS treatment.

A

Group	SR after vaccination (%)	SR after KHV infection (%)	RPS (%)
C	100	16.67±5.77[a]	-
E	100	41.11±3.85[b]	-
KE	100	87.78±1.92[c]	85.33±2.31[a]
FE	100	80.00±3.33[c]	76.00±4.00[a]
KFE	100	83.33±3.33[c]	80.00±4.00[a]

B

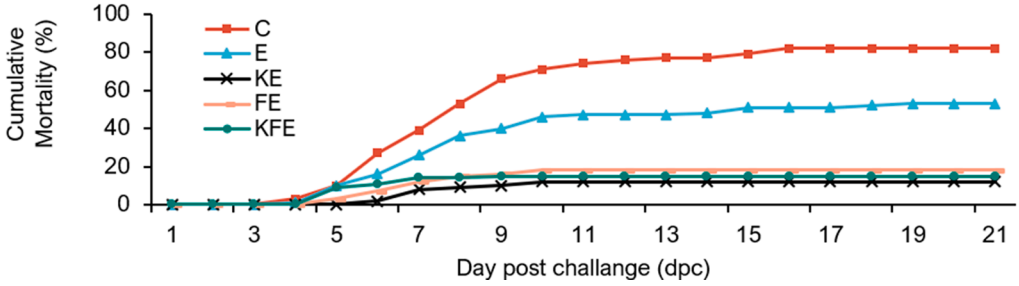

Fig. 1. **(A)** Survival rate (SR) and relative percent survival (RPS), and **(B)** Cumulative mortality, of koi fingerlings after immersion with *E. coli* DH5α carrying KHV DNA vaccine, and after being challenged with koi herpesvirus. **Vaccination group: C** (PBS control), **E** (Empty *E. coli* DHα), **KE** (Formalin-killed *E. coli* DHα:ORF81), **FE** (Freeze-dried *E. coli* DHα:ORF81 without formalin inactivation), and **KFE** (Formalin-killed and freeze-dried *E. coli* DHα:ORF81). Data are presented as mean±*S*.D. Cumulative mortality was analyzed from fish%total mortality at the sampling points. Different letters showed a significant difference between treatments with α=0.05.

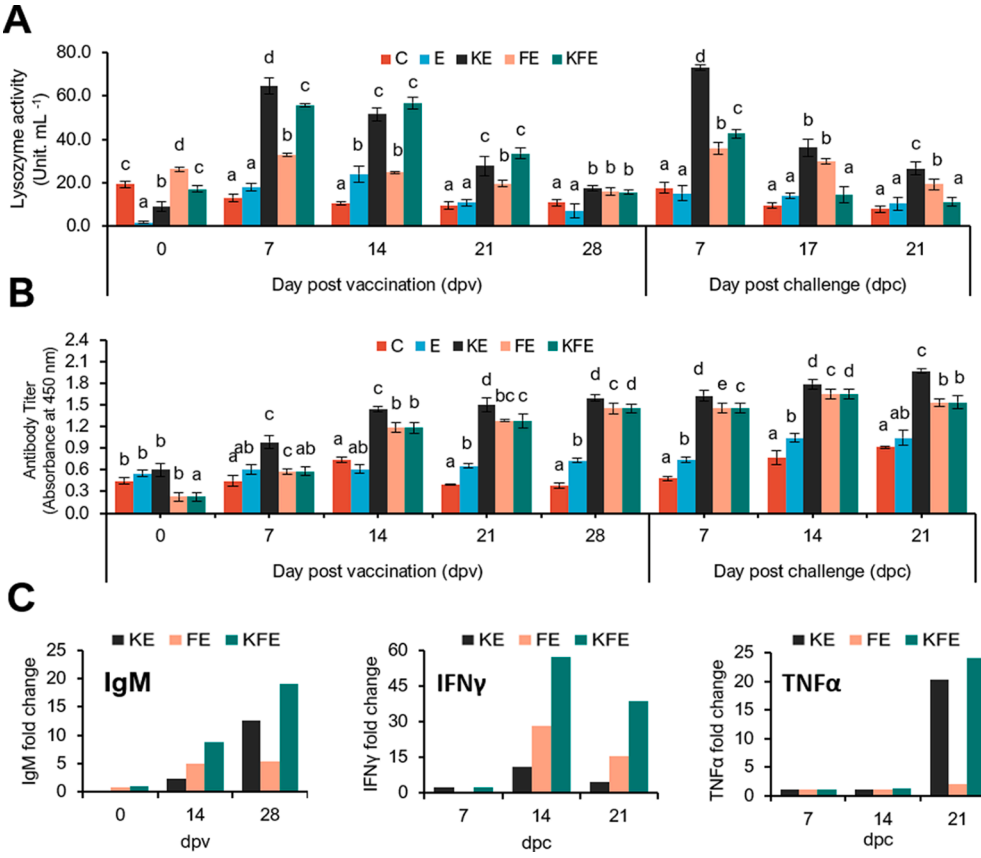

Fig. 2. **(A)** Lysozyme activity, **(B)** Antibody titer, and **(C)** Immune gene expression of koi fingerlings after immersion with *E. coli* DH5α carrying KHV DNA vaccine, and after being challenged with koi herpesvirus. **Vaccination group: C** (PBS control), **E** (Empty *E. coli* DHα), **KE** (Formalin-killed *E. coli* DHα:ORF81), **FE** (Freeze-dried *E. coli* DHα:ORF81 without formalin inactivation), and **KFE** (Formalin-killed and freeze-dried *E. coli* DHα:ORF81). Data are presented as mean±SD. Gene expression was analyzed from the pooled sample (n = 3) and presented as the fold-change to the expression at PBS treatment. Different letters showed a significant difference between treatments with α=0.05.

Results

The results of this study demonstrated that immersion immunization of koi fingerling with *E. coli* DHα:ORF81, whether it was formalin killed and/or freeze-dried, resulted in higher survival cumulative mortality, lysozyme activity, antibody titer, and immune genes expression compared to the non-vaccinated fish. The fish mortality was presented at Fig. 1. There was no mortality observed after vaccination in all treatments. Significant fish mortality was observed after 5–15 days of KHV infection in control and empty *E. coli* DH5α. Non-vaccinated control had the highest mortality ($p<0.05$), followed by empty *E.coli* DH5α ($p<0.05$), and then other *E. coli* DH5α:ORF81 groups. All treatments of *E. coli* DH5α:ORF81 had higher survival compared to control with more than 75% of RPS value. However, the RPS value was not different among

E. coli DH5α:ORF81 treatments ($p>0.05$).

For the immune parameters (Fig. 2), the lysozyme activity and the antibody titer were significantly modulated in *E. coli* DH5α:ORF81 groups after vaccination and after the KHV challenge. The lysozyme activity of empty *E. coli* DH5α was relatively similar to the control postvaccination and after the KHV challenge ($p>0.05$). The highest level of lysozyme activity was observed at 7 days postvaccination and 7 days after the KHV challenge in formalin-killed *E. coli* DH5α:ORF81 treatment ($p<0.05$). Formalin-killed *E. coli* DH5α:ORF81 also have higher antibody titer after 21 days of vaccination compared to other treatments. Its levels remained high after 21 days after the KHV challenge($p<0.05$).

Although higher than PBS, the *igm* mRNA of formalin-killed *E. coli* DHα:ORF81 was relatively lower than KFE treatment (formalin-killed and freeze-dried *E. coli* DHα:ORF81) at 14 and 28 days postvaccination. In KFE treatment, the expression of *tnfα α* and *ifnγ* mRNA was highly observed after the challenge test.

Discussion

For small fish, the immersion method through bath administration is a convenient vaccine delivery for large-scale immunization [21]. In immersion delivery, DNA vaccines were typically submerged in the form of pure plasmids or plasmids capsulated with nano-carriers [11,12]. However, these manufacturing techniques will relatively not be cost-efficient for application in koi or carp fingerlings. Therefore, in this study, we used the bath administration of koi fingerlings using *E. coli* DH5α carrying *orf-81* of the KHV glycoprotein gene as a DNA vaccine as an alternative method. Another idea is using *E. coli* DH5α as a vaccine carrier to induce a better protective immune response since rapid tissue clearance and on-site DNA degradation are barriers to DNA vaccination [21].

In oral vaccination, the first absorption and processing of antigens occur exclusively in the gut [9,22]. In immersion immunization, several organs and cells (including the gut) may take in and process the antigens [10]. As a result, immersion vaccination was proposed as the optimal delivery strategy to elicit mucosal responses since it would likely produce a broader mucosal response [23]. This process may induce localized innate immune responses and further induces the systemic response and production of specific antibodies [23]. In the current study, fish immersion with *E. coli* DH5α:ORF81 showed better immune responses compared to non-vaccinated fish and empty *E. coli* DH5α. It is reflected by the higher levels of lysozyme activity, antibody titer, and immune gene expression after vaccination and KHV infection. The high presence of *igm* mRNA and specific antibody titers in body serum was also observed after 14 days of vaccination of *E. coli* DH5α:ORF81 groups.

Formalin-killed *E. coli* DHα:ORF81 generally had higher lysozyme activity and antibody titer after vaccination and KHV infection among *E. coli* DHα:ORF81 groups, while killed and freeze-dried *E. coli* DHα: ORF81 (KFE) showed higher *igm* and innate immune gene expression. Although there were variations in antibody expression and immune levels among the *E. coli* DHα:ORF81 groups, the fish survival and RPS are not significantly different ($p>0.05$). In addition, empty DHα showed improved survival compared to the control fish after the KHV challenge. This might happen due to the improvement of immunity by the antigenic *E. coli* DHα cells/proteins. These results highlight the critical relevance of various innate defensive mechanisms in fish protection that are modulated after immersion vaccination [9,10]. This study also showed the advantages of freeze-drying vaccine antigen preservation on *E. coli* DHα:ORF81 was not showed a significant improvement in fish protection compared to non-freeze-drying treatment.

Taking together, the vaccination using *E. coli* DH5α:ORF81 could significantly modulate the innate and adaptive immunity of the fish and resulted in higher fish survival after KHV infection. The vaccination using formalin-killed or formalin-killed and freeze-dried *E. coli* DH5α: ORF81 will be potentially further developed as an alternative to the cost-efficient vaccination method for koi or carp fingerlings. However, the mechanisms underlying the improved protection, the fate of antigenic plasmid, and plasmid expression inside the fish after vaccination still needed to be further studied.

Ethical approval

This study was approved by the Animal Ethics Committee Faculty of Veterinary Medicine, IPB university (ethical number: 052/KEH/SKE/XII/2021)

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

I have shared my data at the attach file

Acknowledgments

This research was supported by the Ministry of Finance of The Republic of Indonesia through LPDP Grant Batch 152, in 2020, with ID number 202001113015844. The authors thank Dr. Enny Kusrini from the Research Center for Ornamental Fish Aquaculture, Depok, Indonesia, for providing the experimental fish. Thank you to Yodi Husein, Muhammad Firdaus, and Dendi Hidayatullah for their great technical help in collecting and preparing the KHV isolates. Thank you to Siska Aliyas Sandra for your kind support in the qPCR analysis.

References

[1] MENKOMARVES, Performance Report of Year 2020. Deputy for Maritime Resources Coordination, Coordinating Ministry for Maritime Affairs and Investment, The Republic of Indonesia, 2020.
[2] C.W.E. Embregts, R. Tadmor-Levi, T. Veselý, D. Pokorová, L. David, G. F. Wiegertjes, M. Forlenza, Intra-muscular and oral vaccination using a Koi Herpesvirus ORF25 DNA vaccine does not confer protection in common carp (Cyprinus carpio L.), Fish Shellfish Immunol. 85 (2019) 90–98, https://doi.org/10.1016/j.fsi.2018.03.037.
[3] D.K. Gomez, S. Joon, H. Jang, S. Phil, C.H. Choresca, J. Eun, J. Hyung, J. Woo, S. Chang, Detection of koi herpesvirus (KHV) from koi (Cyprinus carpio koi) broodstock in South Korea, 311 (2011) 42–47. https://doi.org/10.1016/j.aquaculture.2010.11.021.
[4] OIE, Infection with koi herpesvirus. Man. Diagnostic Tests Aquat, Anim, 2019, pp. 1–18.
[5] K.E. Eide, T. Miller-morgan, J.R. Heidel, M.L. Kent, R.J. Bildfell, S. Lapatra, G. Watson, L. Jin, Investigation of Koi Herpesvirus Latency in Koi, J. Virol. 85 (2011) 4954–4962, https://doi.org/10.1128/JVI.01384-10.
[6] S.A. Chairunnisa, S. Nuryati, A. Alimuddin, S. Murtini, A. Santika, D.H. Yanti, EFFICACY OF GP-11 KHV DNA VACCINE IN Cyprinus carpio haematopterus, Indones. Aquac. J. 11 (2016) 31–39.
[7] A.B. Rouf, S. Nuryati, A. Alimuddin, S. Sukenda, Efficacy of GP-11 KHV DNA Vaccine in Common Carp (Cyprinus carpio) through Feed by Different Frequency of Administration, Omni-Akuatika 16 (2020) 1–10, https://doi.org/10.20884/1.oa.2020.16.1.768.
[8] J.E.V. Ramirez, L.A. Sharpe, N.A. Peppas, Current state and challenges in developing oral vaccines, Adv. Drug Deliv. Rev. 15 (2018) 116–131, https://doi.org/10.1016/j.addr.2017.04.008.Current.
[9] J. Ma, T.J. Bruce, E.M. Jones, K.D. Cain, A review of fish vaccine development strategies: conventional methods and modern biotechnological approaches, Microorganisms 7 (2019), https://doi.org/10.3390/microorganisms7110569.
[10] J. Bøgwald, R.A. Dalmo, Review on Immersion Vaccines for Fish : an Update 2019, Microorganisms 7 (2019) 1–28, https://doi.org/10.3390/microorganisms7120627.
[11] C. Zhang, Y. Zheng, Y. Gong, Z. Zhao, Z. Guo, Y. Jia, G. Wang, B. Zhu, Evaluation of immune response and protection against spring viremia of carp virus induced by a single-walled carbon nanotubes-based immersion DNA vaccine, Virology 537 (2019) 216–225, https://doi.org/10.1016/j.virol.2019.09.002.
[12] F. Hu, Y. Li, Q. Wang, B. Zhu, S. Wu, Y. Wang, W. Zeng, J. Yin, C. Liu, S. M. Bergmann, C. Shi, Immersion immunization of koi (Cyprinus carpio) against cyprinid herpesvirus 3 (CyHV-3) with carbon nanotube-loaded DNA vaccine, Aquaculture 539 (2021), 736644, https://doi.org/10.1016/j.aquaculture.2021.736644.

[13] S. Nuryati, A. Alimuddin, S. Sukenda, R.D. Soejoedono, A. Santika, F.H. Pasaribu, K. Sumantadinata, Construction of a DNA vaccine using glycoprotein gene and its expression towards increasing survival rate of KHV-infected common carp (Cyprinus carpi), J. Natur Indones. 13 (2010) 47–52.
[14] L. Yang, Y. Ma, Y. Zhang, Freeze-drying of live attenuated Vibrio anguillarum mutant for vaccine preparation, Biologicals 35 (2007) 265–269, https://doi.org/10.1016/j.biologicals.2007.03.001.
[15] S. Sukenda, R. Rahman, K. Nisaa, D. Hidayatullah, A. Vinasyiam, The efficacy of Streptococcus agalactiae vaccine preparations, administered to tilapia broodstock, in preventing streptococcosis in their offspring, via transfer of maternal immunity, Aquac. Int. 26 (2018) 785–798, https://doi.org/10.1007/s10499-018-0252-4.
[16] A.A. Aonullah, S. Nuryati, S. Murtini, Efficacy of koi herpesvirus DNA vaccine administration by immersion method on Cyprinus carpio field scale culture, Aquac. Res. 48 (2016) 2655–2662, https://doi.org/10.1111/are.13097.
[17] S. Klafack, L. Schröder, Y. Jin, M. Lenk, P.Y. Lee, W. Fuchs, J.C. Avarre, S. M. Bergmann, Development of an attenuated vaccine against Koi Herpesvirus Disease (KHVD) suitable for oral administration and immersion, Npj Vaccines 7 (2022) 1–10, https://doi.org/10.1038/s41541-022-00525-6.
[18] R. Machat, L. Pojezdal, J. Gebauer, J. Matiasovic, R. Tesarik, H. Minarova, N. Hodkovicova, M. Faldyna, Early immune response of two common carp breeds to koi herpesvirus infection, Fish Shellfish Immunol. 128 (2022) 206–215, https://doi.org/10.1016/j.fsi.2022.07.064.
[19] M. Reichert, E. Borzym, M. Matras, J. Maj-Paluch, M. Stachnik, M. Palusinska, Down-regulation of MHC class I mRNA expression in the course of KHV infection, J. Fish Dis. 39 (2016) 1253–1256, https://doi.org/10.1111/jfd.12451.
[20] K. Livak, T. Schmittgen, Analysis of relative gene expression data using real- time quantitative PCR and the 2-ΔΔCT Method, Methods 24 (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.
[21] B. Zhu, G.L. Liu, Y.X. Gong, F. Ling, G.X. Wang, Protective immunity of grass carp immunized with DNA vaccine encoding the vp7 gene of grass carp reovirus using carbon nanotubes as a carrier molecule, Fish Shellfish Immunol. 42 (2015) 325–334, https://doi.org/10.1016/j.fsi.2014.11.026.
[22] L.C. Cui, X.T. Guan, Z.M. Liu, C.Y. Tian, Y.G. Xu, Recombinant lactobacillus expressing G protein of spring viremia of carp virus (SVCV) combined with ORF81 protein of koi herpesvirus (KHV): a promising way to induce protective immunity against SVCV and KHV infection in cyprinid fish via oral vaccination, Vaccine 33 (2015) 3092–3099, https://doi.org/10.1016/j.vaccine.2015.05.002.
[23] D. Parra, F.E. Reyes-Lopez, L. Tort, Mucosal immunity and B cells in teleosts: effect of vaccination and stress, Front. Immunol. 6 (2015) 1–12, https://doi.org/10.3389/fimmu.2015.00354.
[24] S. Nuryati, F.W. Alsani, H. Nasrullah, O. Carman, Y.P. Hastuti, E. Kusrini, Alimuddin, Immune related genes expression analysis in Koi fish after vaccinated with koi herpes virus DNA vaccines, Indones. Aquac. J. 15 (2020) 1–7, https://doi.org/10.15578/iaj.15.1.2020.25-32.