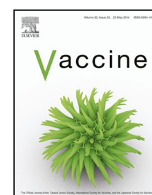
Vaccine xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine



Immune effects of the vaccine of live attenuated *Aeromonas hydrophila* screened by rifampicin on common carp (*Cyprinus carpio* L)

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ARTICLE INFO

Article history:
Received 27 November 2015
Received in revised form 19 April 2016
Accepted 25 April 2016
Available online xxx

Keywords:
Aeromonas hydrophila
Live attenuated vaccine
Immune parameter
Common carp *Cyprinus carpio*

ABSTRACT

Aeromonas hydrophila, as a strong Gram-negative bacterium, can infect a wide range of freshwater fish, including common carp *Cyprinus carpio*, and cause the huge economic loss. To create the effective vaccine is the best way to control the outbreak of the disease caused by *A. hydrophila*. In this study, a live attenuated *A. hydrophila* strain, XX1LA, was screened from the pathogenic *A. hydrophila* strain XX1 cultured on medium containing the antibiotic rifampicin, which was used as a live attenuated vaccine candidate. The immune protection of XX1LA against the pathogen *A. hydrophila* in common carp was evaluated by the relative percent survival (RPS), the specific IgM antibody titers, serum lysozyme activity and the expression profiles of multiple immune-related genes at the different time points following immunization. The results showed that the variable up-regulations of the immune-related genes, such as the pro-inflammatory cytokine *IL-1β*, the chemokine *IL-10* and *IgM*, were observed in spleen and liver of common carp injected in the vaccines with the formalin-killed *A. hydrophila* (FKA) and the live attenuated XX1LA. Specific antibody to *A. hydrophila* was found to gradually increase during 28 days post-vaccination (dpv), and the RPS (83.7%) in fish vaccinated with XX1LA, was significant higher than that (37.2%) in fish vaccinated with FKA ($P < 0.05$) on Day 28 after challenged by pathogen. It was demonstrated that the remarkable immune protection presented in the group vaccinated with XX1LA. During the late stage of 4-week immunization phase, compared with FKA and the control, specific IgM antibody titers significantly increased ($P < 0.05$) in the XX1LA group. The activity of the lysozyme in serum indicated no significant change among three groups. In summary, the live attenuated bacterial vaccine XX1LA, screened in this study, indicates the better protect effect on common carp against *A. hydrophila*, which can be applied in aquaculture of common carp to prevent from the disease outbreak in the future.

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1. Introduction

The common carp *Cyprinus carpio*, as the third most species cultured in worldwide, accounts for over 3 million metric tons a year, about 10% of annual global freshwater production in aquaculture [1,2]. The common carp was cultured for human consumption in China 3000 years ago, and now is widely cultured over 100 countries [3,4]. In addition, common carp is also an important ornamental fish species. Nevertheless, in recent years, the common carp suffered a potential environmental stress to its health in the intensive rearing pattern, and has led to a high susceptibility to various disease agents in fish.

Aeromonas hydrophila, as an important Gram-negative, motile, rod-shaped bacterial pathogen, were commonly found in aquatic environment throughout the world, and is the causative agent of epizootic ulcerative syndrome [5], which is also known as motile *Aeromonas* septicaemia (MAS) [6]. A number of fish species are found to be susceptible to *A. hydrophila*, such as common carp, goldfish (*Carassius auratus auratus*), striped catfish (*Plotosus lineatus*) and rohu carp (*Labeo rohita*) [7–10]. It has led to huge economic losses in aquaculture. However, the use of antibiotics as a preventive measure has been questioned because they can induce resistant bacteria populations, alter the gut microbiota, and bring the potential risk on human health [11,12]. In the previous studies, the various *A. hydrophila* vaccines, such as the whole bacteria killed by formalin or heat [13,14], isolated non-replicating pathogen proteins (*e.g.* the extra-cellular proteins [15], outer membrane proteins [16,17]), recombinant S-layer protein [18], lipopolysaccharide (LPS) [19], biofilms [20] and live attenuated bacteria [21] have been

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http://dx.doi.org/10.1016/j.vaccine.2016.04.075
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developed to control the spread of disease incurred by *A. hydrophila*, with varying degrees of protection against pathogen challenge. However, most vaccines indicated some defects in producing, vaccine applying or immune effect. The live attenuated vaccine is an effective agent but incurred in virulence recovery. Therefore, to screen an effective and stable live attenuated vaccine is very important for applying in aquaculture. In this study, a live attenuated *A. hydrophila* potential vaccine, XX1LA, was screened by rifampicin treatment *in vitro* condition.

2. Materials and methods

2.1. Fish

Healthy common carp (*C. carpio*) with the weight of 100 ± 17 g were obtained from the breeding farm of *C. carpio* in Zhengzhou, Henan. The fish were acclimatized at 22 ± 2 °C for 14 d before experimental manipulation in 1200-L tanks, with a photo-period of 12 h:12 h (light:dark), in which the water was filtered by an individual biofilter and aerated by oxygen filling stone. Prior to vaccine injection, the fish were anaesthetized with tricaine methane sulfonate (MS-222, Sigma) to collect the blood and then sacrificed to take out spleen and liver. Before each experiment, 5% fish were randomly selected for the examination of bacterial stability in spleen, liver and blood, and no bacteria could be detected in fish tissues examined.

2.2. Bacterial strains and growth conditions

A. hydrophila XX1, as a pathogenic strain, is isolated from the diseased common carp collected in local farm in Xinxiang city. The live-attenuated bacterium XX1LA was obtained according to the method described by Sun et al. [22]. Briefly, XX1 was cultured in Luria-Bertani broth (LB) medium to an OD_{600} of 0.8, the 50 μl of which was plated on a LB agar plate containing 1 μg/ml rifampicin (Sangon, Shanghai, China). The plate was incubated at 28 °C for 3 days. One of the colonies randomly selected from the plate, then cultured in LB medium containing 5 μg/ml rifampicin until an OD_{600} of 0.8. After 20 passages of *A. hydrophila* XX1 in LB medium containing gradually increased concentrations of rifampicin, the antibiotic-resistant strain XX1LA was able to grow in LB medium containing 250 μg/ml of rifampicin. The identity of XX1LA was verified by polymerase chain reaction (PCR) analysis using specific primers to *A. hydrophila* 16S rRNA reported previously [23].

2.3. Virulence and stability analysis of XX1LA

To examine the lethal dose 50% (LD_{50}) of XX1LA, carp were divided randomly into 6 groups (20 fish/group), and each group was injected i.p. with 200 μl of XX1LA suspension containing10^5–10^9 CFU/ml at 10-fold difference. The mortality of fish was monitored for two weeks, and LD_{50} was determined by using the Probit analysis implemented in the software SPSS 20.0 (SPSS Inc., USA).

The stability of XX1LA was determined as described previously [24]. Briefly, the examination was administered under *in vitro* and *in vivo* conditions. The mutant bacterium was cultured in LB medium until an optical density (OD) of 0.8 at 600 nm and diluted with fresh LB to 10^6 CFU/ml, and then incubated at 28 °C to OD_{600} value of 0.8. The partial cells cultured were used for LD_{50} examination, and the other cells cultured was diluted to 10^5 CFU/ml in fresh LB medium and incubated at 28 °C to 0.8 of OD_{600} as described above. The procedure was repeated ten times. The examination of XX1LA stability *in vivo* was administered by injection i.p into each carp with 100 μl 10^6 CFU/ml bacteria. The fish were monitored for the appearance of disease symptom. XX1LA was recovered from the liver, kidney, and spleen of diseased fish as described above. The recovered bacteria from the different organs were used for LD_{50} determination and for next round infection. This process was repeated ten times.

2.4. Preparation of formalin-killed A. hydrophila(FKA)

A. hydrophila was cultured in LB medium at 28 °C for 24 h, and the bacteria were harvested by centrifuging at $8000 \times g$ for 10 min, and the cells were washed and resuspended with PBS. Then formalin was added into the suspension to a final concentration of 0.6% (v/v). The suspension was incubated at 28 °C for 48 h and was tested for complete inactivation with no bacterium observed growing on LB agar plate after diluting bacterial suspension was plated. The bacteria were adjusted to a concentration of 1.0×10^7 CFU/ml for later use.

2.5. Vaccining, sampling and challenging

XX1LA was cultured in LB medium to 0.8 of OD_{600}, washed with PBS, and then resuspended in PBS to 10^7 CFU/ml. The common carp were divided randomly into three groups (180 fish/group), injected i.p. PBS, FKA and XX1LA (200 μl/fish) respectively with 1 ml disposable syringe. At 0, 7, 14, 21, and 28 dpv, the 5 fish in each group were bled aseptically after anesthetized by MS-222. The blood was clotted at 4 °C for 4 h. The serum was collected after centrifugation at $3000 \times g$ for 8 min, and stored at −20 °C until analysis. The spleen and liver in each group were taken out at 0, 1, 3, 5, 7, 14, 21 and 28 dpv, respectively, and extracted the total RNA immediately. At 14 d post-vaccination, 20 fish were taken out from the three groups respectively, and challenged with 200 μl 1×10^7 CFU/ml (ten times of LD_{50}) XX1 *via* i.p. Mortality was recorded daily up to 2 weeks and relative percentage survival (RPS) was calculated following the method of Amend [25]: RPS = (1 − the ratio of mortality percent in the vaccine group to in the control) × 100%. The experiments were performed in triplicate.

2.6. Serum lysozyme activity

Serum lysozyme activity was measured using a turbidometric method as described by Sankaran and Gurnani [26]. The 200 μl suspension of *Micrococcus lysodeikticus* (0.2 mg/ml) in 0.02 M PBS (pH 5.5) was added to 50 μl of serum previously taken in 96-well flat-bottom microtiter plate (Jetbiofil, Guangzhou, China). The absorbance at 570 nm was measured immediately after addition of bacteria and final optical density was measured after incubating for 30 min at 28 °C. Serum lysozyme activity was expressed as Unit/ml and calculated as Ey = (the first optical density − the final optical density) × volume of reaction solution/(30 min × 0.001).

2.7. Specific antibody analysis

Before the fish were immunized, five fish of each group were bled and the sera were collected as negative control. Antibody levels in fish serum against *A. hydrophila* were determined from 0 to 28 dpv using a modified ELISA method, which was described as the following: the microplate was coated for 4 h at 37 °C with 1×10^7 CFU/ml XX1 in 100 μl of coating buffer (50 mM carbonate buffer, pH 9.6). Unbound antigen was removed by washing the plate three times with 300 μl of washing buffer (0.05% Tween-20 in PBS (pH 7.4)). Then the microplate was blocked for 2 h at room temperature with 100 μl of blocking buffer (2% bovine serum albumin (BSA) in washing buffer). 2-fold serial dilutions of the sera with the first dilution 1:32 in PBS with 0.3% BSA and added to the wells in duplicate. Antibodies binding to the antigen was detected using mouse-anti-fish (common carp) IgM (developed in our lab) (1:800

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Table 1
Primers used in this study.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
β-actin	GACTTCGACAGGAG	CAAGAAGGATGGAACA
IL-1β	CGGAAGCATAAAGTGTAAAGCC	GTGAGCGAGGAAGCGGAAGA
IL-10	TGGAACCATTACTGGACGAA	TCTTTATGCTGGCGAACTCA
IgM	CCGTAACATCAGCTCAACCA	TTCTTAGCATAATCCGTCCA

dilution in washing buffer), followed by goat-anti-mouse IgG conjugated with horse-radish peroxidase (Vazyme, 1:8000 dilution in PBS with 1% BSA). Finally, 100 μl/well of TMB was added to the microplate. After incubation for 10 min at 26 °C, 50 μl stop solution (2 M H_2SO_4) was added into each well and the intensity of the ELISA reading was determined using the optical density at 450 nm with a microplate reader. Results were considered as the positive if the absorbance was at least double that in the control sera, and antibody titers were scored as the highest positive dilution. The value of serum titer was converted by Log2 [27].

2.8. Total RNA isolation, cDNA synthesis and real-time quantitative PCR

Total RNA in liver and spleen were extracted by Trizol (TaKaRa, Japan), following the instructions of manufacturer. The RNA was digested by RNase-free DNase I (Sangon, China) to eliminate genomic DNA contaminant. The quality of the total RNA was checked based on its optical density at 260 and 280 nm, measured by spectrophotometry. First strand complementary DNA (cDNA) was synthesized in 1 μg total RNA using PrimeScript RT reagent Kit (TaKaRa, Japan).

Real-time quantitative PCR (RT-qPCR) was performed by SYBR green real-time PCR mix (TaKaRa, Japan) using ABI 7500 Real-time Detection system (Applied Biosystems, USA) following the manufacturer's instructions. Primers used in PCR for each gene were listed in Table 1. The primers were designed with Primer express software (Applied Biosystems) and tested with a set of DNA and cDNA samples to make sure that the products could not be amplified from genomic DNA. The primer pairs were efficiently determined using the performing serial dilutions of reference cDNA and used to quantify the cDNA concentration. The relative expression of each immune-relative gene was determined by comparing to the expression level of β-actin gene using the $2^{-\Delta\Delta CT}$ method [28].

2.9. Statistical analysis

The value of each immune parameter was presented as mean ± standard deviation (mean ± SD). One way analysis of variance (ANOVA) was performed implemented in the software SPSS 20.0. Differences in mortality were determined in χ^2 test. The significant difference was set at $P = 0.05$.

3. Results

3.1. Virulence and stability of XX1LA

Virulence results showed that the LD_{50} of XX1LA was 4.5×10^9 CFU/ml, which was higher than that of parent strain XX1 in 200-fold, therefore, the virulence of XX1LA was reduced significantly and, generally, could not result in fish disease. To examine whether XX1LA could revert to the wild type *in vitro* and *in vivo* without selective pressure, the strain was sub-cultured ten times in LB medium and passed ten rounds in common carp respectively. The results showed that the sub-cultures and XX1LA isolated from infected fish were indistinguishable from XX1 in LD_{50}.

3.2. Protection of immunized common carp against *A. hydrophila*

Common carp were challenged with *A. hydrophila* XX1 by i.p. injection. The typical symptoms of septicemia in liver and ulcer were observed in sick fish, and the bacteria, *A. hydrophila*, were isolated from the liver of the dying fish. As shown in Fig. 1, the lethal appeared in the control and FKA groups on Day 1, when more than 10% fish died, and ceased on Day 4, when the cumulative mortalities in the control and FKA groups were reached at 71.7% and 45.0% respectively. The fish in the XX1LA group started to die on Day 2 and ceased on Day 4, when a cumulative mortality reached to 11.7%. Compared with the control, the cumulative mortality in two immunized groups reduced significant ($P < 0.05$) from 2 to 14 d post challenging in the Chi–Square test. The RPS in FKA and XX1LA group was 37.2% and 83.7% relative to the control, respectively.

3.3. Lysozyme activity in serum

The values of lysozyme activity in serum of *C. carpio* vaccinated and unvaccinated are shown in Fig. 2. The lysozyme activity decreased in serum after 7 d, both in control and vaccinated groups, and the values reached the lowest levels at 28 dpv. But no significant difference ($P > 0.05$) between vaccinated and unvaccinated group appeared at each time point.

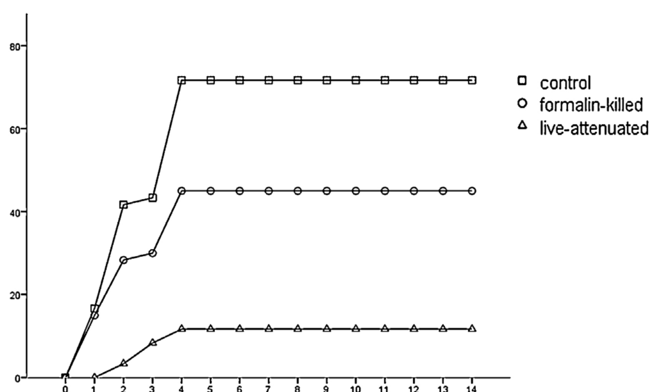

Fig. 1. Cumulative mortality of *C. carpio* in two immunized groups and the control after challenged with XX1 (i.p) at 14 dpv. *C. carpio* immunized with the vaccine of FKA or XX1LA were monitored for 2 weeks. The values were presented with mean ± SD ($n = 3$). The symbol * represents the significant difference ($P < 0.05$) between the immunized group and the control at the same time point.

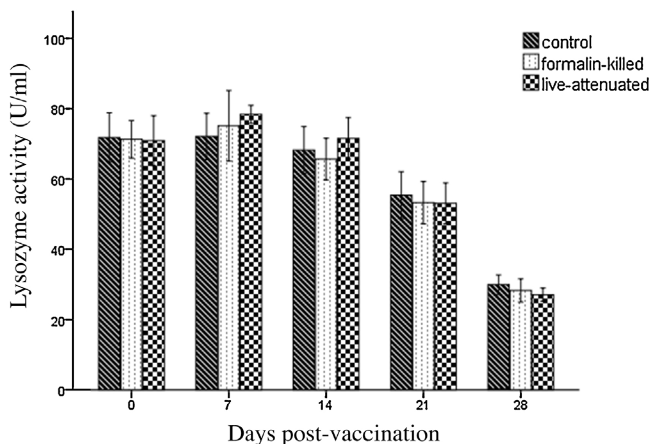

Fig. 2. The changes of lysozyme activities in serum of fish vaccinated with vaccine FKA and XX1LA, respectively. The values were presented with mean ± SD ($n = 5$). The symbol * represents the significant difference ($P < 0.05$), compared with the corresponding lysozyme activity at 0 dpv; the symbol + represents the significant difference ($P < 0.05$) in lysozyme activities between at 21 dpv and at 28 dpv.

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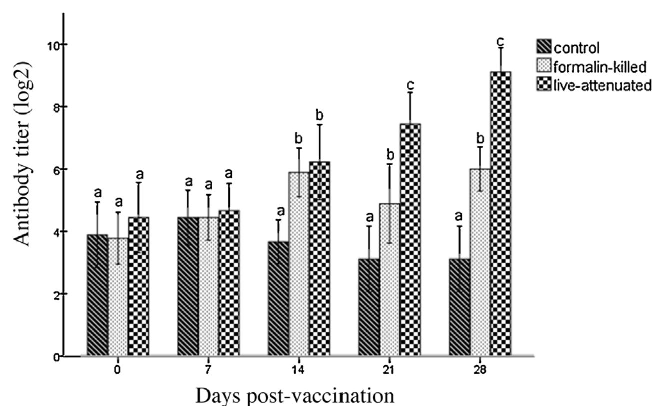


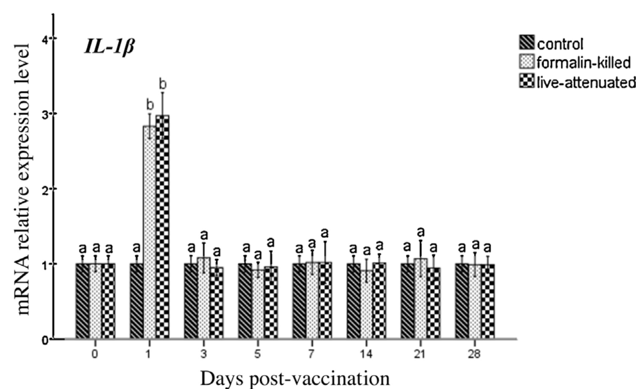
Fig. 3. Antibody titers in the serum of the vaccinated fish at 0, 7, 14, 21, and 28 dpv. Anti-XX1 antibody was determined by ELISA. Results are shown as log 2 antibody titer. Data are presented as mean ± SD ($n = 5$). The different letters (a, b and c) above the bars represent the significant difference between them ($P < 0.05$), and the same letter represents no significant difference ($P > 0.05$).

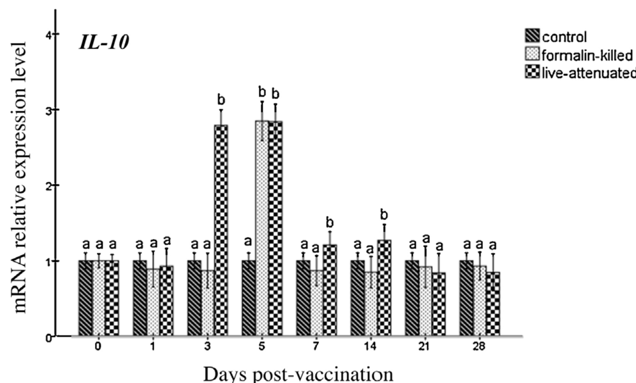
3.4. Antibody production in the vaccinated fish

ELISA analysis showed that serum antibodies against XX1 were detected to be significantly higher ($P < 0.05$) in two vaccinated groups than in the control from 14 to 28 dpv (Fig. 3), but the antibody titers between the FKA and XX1LA group indicated no significance at 14 dpv. Antibody titers in fish vaccinated XX1LA increased from 14 to 28 dpv, and the log 2 titer reached above 8.0 at 28 dpv, and was significantly ($P < 0.05$) higher than that in the FKA group from 21 to 28 dpv.

3.5. Expressions of immune-related genes in the vaccinated C. carpio

The expression changes of three immune-related genes in mRNA levels were examined, as shown in Fig. 4 and Fig. 5. Higher expression of pro-inflammatory cytokine *IL-1β* was observed in liver and spleen at 1 dpv in two vaccinated groups. The expression level of *IL-1β* in the XX1LA group increased significantly at 3 dpv in spleen, compared with that in the FKA group. The expression levels of *IL-10* were stimulated in spleen and liver after vaccination, and the up-regulations in the FKA and XX1LA groups mostly were at early periods, for example, in spleen, at 5 and 7 dpv, while in liver, at 3 and 5 dpv in the XX1LA group, only at 5 dpv in the FKA group. The expression levels of *IgM* also increased after vaccination, with the 3-fold up-regulation in spleen and liver in the XXIRM group, while in FKA group, the expression of *IgM* was up-regulated to the peak with about 1.5-fold in liver at 21 dpv, and with 2.5-fold in spleen at 14 dpv.





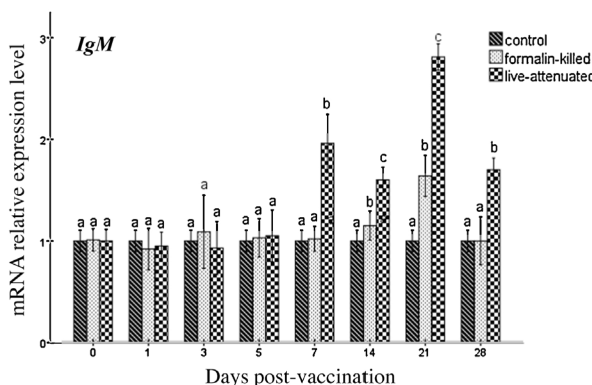


Fig. 4. The mRNA expressing patterns of immune-related gene *IL-1β*, *IL-10*, and *IgM* in liver of the vaccinated fish with the time extension. The mRNA level of each immune-related gene was normalized to that of *β-actin*, and the relative expressing levels were calculated compared to the control. The values of data are presented as mean ± SD ($n = 3$). The different letters (a, b and c) above the bars represent the significant difference between them ($P < 0.05$), and the same letter represents no significant difference ($P > 0.05$).

4. Discussion

In this study, a rifampicin-resistant *A. hydrophila* mutant, XX1LA, as a live attenuated vaccine, was screened and described. Rifampicin, a antibiotic, can kill bacteria by inhibiting RNA polymerase and blocking mRNA transcription [29]. It is known that bacterial virulence could be attenuated by the mutation to resistance against rifampicin, which was resulted in the absence of the O-chain in lipopolysaccharide in rifampicin-resistant strain [30]. However, the precise mechanism of virulence attenuation is still unclear. It has been observed that bacteria acquired resistance to rifampicin exhibit the reduced virulence [22]. At the present, several attenuated pathogens, such as mutant *Flavobacterium columnare* and *Edwardsiella ictaluri* from fish, have been screened with rifampicin. It is noted that the virulences of various bacteria are different from their respective parental strains [31,32]. In some researches, the live attenuated vaccine showed the high protection and stability obtained by rifampicin [24,33]. In this study, the virulence of XX1LA, compared to the wild type XX1, was highly attenuated due to the drastical increase of LD_{50} value. What is more, the attenuated virulence could be genetically stable through examining both *in vivo* and *in vitro*.

In this study, The RPS in FKA group (37.2%) was much lower than that in XX1LA group (83.7%) after *C. carpio* challenged by XX1. With regard to the control, the fish almost died at 1–4 dpv. A lot of proteins of *A. Hydrophila* were inactivated by formalin in FKA group and in consequence the ability of FKA that could induce immune response of fish is lower than live attenuated group. Therefore, it was demonstrated that mutant strain XX1LA confers better protection against *A. Hydrophila* than FKA in *C. carpio*.

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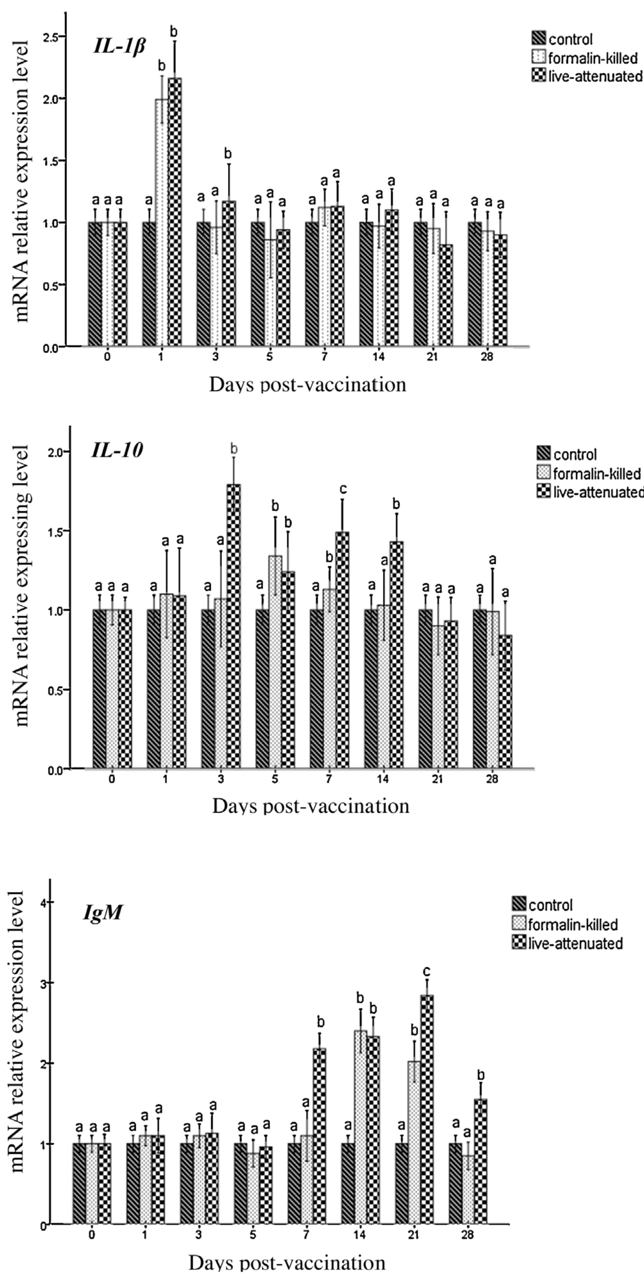

Fig. 5. mRNA expression levels of the immune-related gene *IL-1β*, *IL-10*, and *IgM* in spleen of the vaccinated fish at different time points. The mRNA level of each gene was normalized to that of *β-actin*, and the relative expression levels were calculated compared to the control. The values of data are presented as mean ± SD ($n = 3$). The different letters (a, b and c) above the bars represent the significant difference between them ($P < 0.05$), and the same letter represents no significant difference ($P > 0.05$).

Lysozyme widely exists in various fish, which could kill various microbial pathogens in fish [34–36]. In this study, the changes of lysozyme activities showed no significant difference between the vaccinated group and the control, but lysozyme activity in each group was gradually decreased with the extension of time. It was suggested that vaccination might possibly inhibit lysozyme activity in serum to some extent [37,38]. What is more, starvation might also be a critical factor to impact lysozyme activity [39]. Of course, this suggestion needs to be confirmed in the further studies.

Immunoglobulin is a critical factor in humoral immunity, which is mainly the IgM in fish. The specific antibody tilter, as an important immune parameter in serum of immunized fish, has been determined in the previous studies [40]. In our present study, significant antibody levels were detected in fish vaccinated by XX1LA with a maximum at 28 dpv. It was suggested that the XX1LA could keep the longest in immunity [41]. Antibody levels (log 2 antibody titers) in the vaccinated group increased slightly from 14 to 28 dpv during the experiment period, but the tendency was not always constant [38]. Compared to the control, antibody titers in two vaccinated groups increased significantly after 14 dpv ($P < 0.05$), and indicated the significant difference between the FKA and XX1LA group. This result was kept in agreement with the previous report [42]. It was suggested that the immunogenicity of vaccine XX1LA was higher than that of the FKA antigen.

The immune-related genes play the important role in immune protect, which are essential to investigate the responses of these genes to vaccine. It has been demonstrated that the spleen is an essential organ to generate antibody in fish [43] and the liver is also thought to function in resisting pathogen invasion [44]. In this study, the gene *IL-1β, IL-10* and *IgM* were up-regulated post-vaccination in different periods, which were postulated to play the important role in immune response. IL-1β, as a typical pro-inflammatory cytokine, is produced in several cells such as macrophages [45], which can propel the growth and proliferation of immune cells [46] and is believed to act as an important signal in the early immune response. In our study, the expression of *IL-1β* was up-regulated in liver and spleen at early period post-immunization. The similar change was observed in zebrafish *Danio rerio* vaccinated with live attenuated *V. anguillarum* [47]. It is plausible that both the FKA and live attenuated vaccine can activate the non-specific immune responses of present antigens and macrophages in spleen and liver.

The role of IL-10 is considered as an anti-inflammatory cytokine to reduce inflammation [48]. IL-10 can minimize the damage to the host by blocking chemokine receptors and inhibiting the effect of pro-inflammatory cytokines. Furthermore, IL-10 is considered to be associated with the immunoglobulin provoked by B cells [49]. As presented in our study, the expression level of *IL-10* gene increased at 3 dpv in spleen and liver in two vaccinated groups, later than *IL-1β*. The similar result was observed in previous studies [50]. Thus, the up-regulation of *IL-10* can maintain the balance between pro- and anti-inflammatory.

In accordance with specific antibody titers in serum, an up-regulation of *IgM* appeared in two vaccinated groups. It was found that the expression of *IgM* increased gently and reached the highest after 3 or 4 weeks post-vaccinated in liver or spleen in FKA and XX1LA group. It was indicated that IgM plays a key role in vaccine-induced protection. On the contrary, some researchers argued that IgM, as the major class of immunoglobulin, was not protective in preventing pathogen infection, in fish [51], which was diverse to the findings in rohu and zebrafish [17,52]. Therefore, the further study should be carried out to elucidate whether IgM could show a strong response in vaccine-induced protection.

In conclusion, in this study, the live attenuated vaccine XX1LA, derived from the pathogenic *A. hydrophila* strain XX1, achieved through the screening by the treatment of rifampicin, has been proved to be effective, as a candidate vaccine against *A. hydrophila*, by the assessment on the multiple immune parameters in *C. carpio*. It was suggested that the live attenuated vaccine XX1LA could provoke the innate and specific immune responses in fish. With respect to the application of the live attenuated vaccine XX1LA in aquaculture, it is still necessary to further study to ascertain the mutation in genomic information of the live attenuated vaccine.

Acknowledgments

This work was sponsored by Program for Innovative Research Team in Science and Technology in the University of Henan Province (Project No. 15IRTSTHN018 and 14IRTSTHN013) and the

Project for International Cooperation and Talent Plan (Project No. 144300510017). We would like to thank our colleagues for the valuable suggestions on the overall manuscript preparation.

References

[1] Saikia SK, Das DN. Feeding ecology of common carp (*Cyprinus carpio* L.) in a rice–fish culture system of the Apatani plateau (Arunachal Pradesh, India). Aquat Ecol 2009;43(2):559–68.
[2] Bostock J, Corner R. Aquaculture: global status and trends. Philos Trans R SocLond B Biol Sci 2010;(1554):2897–912.
[3] FAO. The state of world fisheries and aquaculture 2006. Rome: FAO Fisheries and Aquaculture Department; 2007.
[4] Bostock J, McAndrew B, Richards R, Jauncey K, Telfer T, Lorenzen K, et al. Aquaculture: global status and trends. Philos Trans R Soc B: Biol Sci 2010;365(1554):2897–912.
[5] Huang L, Qin Y, Yan Q, Lin G, Huang L, Huang B, et al. MinD plays an important role in *Aeromonas hydrophila* adherence to *Anguilla japonica* mucus. Gene 2015;565(2):275–81.
[6] Harikrishnan R, Rani MN, Balasundaram C. Hematological and biochemical parameters in common carp, *Cyprinus carpio*, following herbal treatment for *Aeromonas hydrophila* infection. Aquaculture 2003;221(1):41–50.
[7] Wang JL, Meng XL, Lu RH, Wu C, Luo YT, Yan X, et al. Effects of *Rehmannia glutinosa* on growth performance, immunological parameters and disease resistance to *Aeromonas hydrophila* in common carp (*Cyprinus carpio* L.). Aquaculture 2015:293–300.
[8] Sirimanapong W, Thompson KD, Kledmanee K, Thaijongrak P, Collet B, Ooi EL, et al. Optimisation and standardisation of functional immune assays for striped catfish (*Pangasianodon hypophthalmus*) to compare their immune response to live and heat killed *Aeromonas hydrophila* as models of infection and vaccination. Fish Shellfish Immunol 2014;40:374–83.
[9] Maji S, Mali P, Sn J. Immunoreactive antigens of the outer membrane protein of *Aeromonas hydrophila*, isolated from goldfish, *Carassius auratus* (Linn.). Fish Shellfish Immunol 2006;20(4):462–73.
[10] Mohanty BR, Mishra J, Das S, Jena JK, Sahoo PK. An outbreak of aeromoniasis in an organized composite carp culture farm in India: experimental pathogenicity and antibiogramstudy. J Aqua 2008;16:27–37.
[11] Verschuere L, Rombaut G, Sorgeloos P, Verstraete W. Probiotic bacteria as biological control agents in aquaculture. Microbiol Mol Biol Rev 2000;64(4):655–71.
[12] Kaskhedikar M, Chhabra D. Multiple drug resistance in *Aeromonas hydrophila* isolates of fish. Food Microbiol 2010;28:157–68.
[13] VasudevaRao Y, Das BK, Jyotyrmayee P, Chakrabarti R. Effect of *Achyranthes aspera* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. Fish Shellfish Immunol 2006;20(3):263–73.
[14] Magnadottir B. Immunological control of fish diseases. Marine Biotechnol 2010;12(4):361–79.
[15] DunhuaZhang, Pridgeon Julia W, Klesius Phillip H. Vaccination of channel catfish with extracellular products of *Aeromonas hydrophila* provides protection against infection by the pathogen. Fish Shellfish Immunol 2014;36(3):270–5.
[16] Yadav SK, Sahoo PK, Dixit A. Characterization of immune response elicited by the recombinant outer membrane protein OmpF of *Aeromonas hydrophila*: a potential vaccine candidate in murine model. Mol Biol Rep 2014;41(3):1837–48.
[17] Dash P, Sahoo PK, Gupta PK, Garg LC, Dixit A. Immune responses and protective efficacy of recombinant outer membrane protein R (rOmpR)-based vaccine of *Aeromonas hydrophila* with a modified adjuvant formulation in rohu (*Labeo rohita*). Fish Shellfish Immunol 2014;39(2):512–23.
[18] Poobalane S, Thompson KD, Ardó L, Verjan N, J Han H, Jeney G, et al. Production and efficacy of an *Aeromonas hydrophila* recombinant S-layer protein vaccine for fish. Vaccine 2010;28(20):3540–7.
[19] Dehghani S, Akhlaghi M, Dehghani M. Efficacy of formalin-killed, heat-killed and lipopolysaccharide vaccines against motile aeromonads infection in rainbow trout (*Oncorhynchus mykiss*). Glob Vet 2012;4:409–15.
[20] Siriyappagouder P, Shankar KM, Kumar BTN, Patil R, Byadgi OV. Evaluation of biofilm of *Aeromonas hydrophila* for oral vaccination of *Channa striatus*. Fish Shellfish Immunol 2014;41(2):581–5.
[21] Pridgeon JW, Yildirim-Aksoy M, Klesius PH, Srivastava KK, Reddy PG. Attenuation of a virulent *Aeromonas hydrophila* with novobiocin and pathogenic characterization of the novobiocin-resistant strain. J Appl Microbiol 2012;113(6):1319–28.
[22] Sun Y, Liu C, Sun L. Isolation and analysis of the vaccine potential of an attenuated *Edwardsiella tarda* strain. Vaccine 2010;28(38):6344–50.
[23] Altinok I, Capkin E, Kayis S. Development of multiplex PCR assay for simultaneous detection of five bacterial fish pathogens. Vet Microbiol 2008;131(3):332–8.
[24] Hu Y, Deng T, Sun B, Sun L. Development and efficacy of an attenuated *Vibrio harveyi* vaccine candidate with cross protectivity against *Vibrio alginolyticus*. Fish Shellfish Immunol 2012;32(6):1155–61.
[25] Amend DF. Potency testing of fish vaccines. Fish Biol: Serodiagnostics Vaccines 1981;49(1):447–54.
[26] Sankaran K, Gurnani S. On the variation in the catalytic activity of lysozyme in fishes. Indian J Biochem Biophys 1972;9(2):162–5.
[27] Mao Z, Yu L, You Z, Wei YW, Liu Y. Cloning: expression and immunogenicty analysis of five outer membrane proteins of *Vibrio parahaemolyticus* zj2003. Fish Shellfish Immunol 2007;23(3):567–75.
[28] Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nat Protoc 2008;3(6):1101–8.
[29] Campbell EA, Korzheva N, Mustaev A, Murakami K, Nair S, Goldfarb A, et al. Structural mechanism for rifampicin inhibition of bacterial rna polymerase. Cell 2001;104(6):901–12.
[30] Schurig GG, Roop II RM, Bagchi T, Boyle S, Buhrman D, Sriranganathan N. Biological properties of RB51; a stable rough strain of *Brucella abortus*. Vet Microbiol 1991;28(2):171–88.
[31] Arias CR, Shoemaker CA, Evans JJ, Klesius PH. A comparative study of *Edwardsiella ictaluri* parent (EILO) and *E. ictaluri* rifampicin-mutant (RE33) isolates using lipopolysaccharides, outer membrane proteins, fatty acids, Biolog, API 20E and genomic analyses. J Fish Dis 2003;26(7):415–21.
[32] Shoemaker CA, Klesius PH, Jj E. Immunization of eyed channel catfish, *Ictalurus punctatus*, eggs with monovalent *Flavobacterium columnare* vaccine and bivalent *F. columnare* and *Edwardsiella ictaluri* vaccine. Vaccine 2007;25(6):1126–31.
[33] Pridgeon JW, Mu X, Klesius PH. Biochemical and molecular characterization of the novobiocin and rifampicin resistant *Aeromonas hydrophila* vaccine strain AL09-71N+R compared to its virulent parent strain AL09-71. Vet Microbiol 2013;165(3):349–57.
[34] Wang G, Zhang S, Zhuang Z, Wang ZP. Synergistic actions of complement and lysozyme in clearance of *Escherichia coli* from amphioxus Branchiostomabelcheri. Prog Nat Sci: Mater Int 2009;19(2):179–85.
[35] Caipang CMA, Brinchmann MF, Kiron V. Short-term overcrowding of Atlantic cod, Gadus morhua: Effects on serum-mediated antibacterial activity and transcription of glucose transport and antioxidant defense related genes. Comp Biochem Physiol Part A: Mol Integr Physiol 2008;151(4): 560–5.
[36] Reyes-Becerril M, Salinas I, Cuesta A, Meseguer J, Tovar-Ramirez D, Ascencio-Valle F, et al. Oral delivery of live yeast *Debaryomyces hansenii* modulates the main innate immune parameters and the expression of immune-relevant genes in the gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol 2008;25(6):731–9.
[37] Song-Lin G, Jian-Jun F, Qiu-Hua Y, Rui-Zhang G, Yu W, Pan-Pan L. Immune effects of bathing European eels in live pathogenic bacteria, *Aeromonas hydrophila*. Aquaculture Res 2014;45(5):913–21.
[38] Song-Lin Guo. Immune effects of a bivalent expressed outer membrane protein to American eels (*Anguilla rostrota*). Fish Shellfish Immunol 2013;35(2):213–20.
[39] Esteve-Gassent MD, Fouz B, Amaro C. Efficacy of a bivalent vaccine against eel diseases caused by *Vibrio vulnificus* after its administration by four different routes. Fish Shellfish Immunol 2004;16(2):93–105.
[40] Lund V, Børdal S, Kjellsen O, Mikkelsen H, Schrøder MB. Comparison of antibody responses in Atlantic cod (*Gadus morhua* L.) to *Aeromonas salmonicida* and *Vibrio anguillarum*. Dev Comp Immunol 2006;30(12):1145–55.
[41] Tesh RB, Arroyo J, Travassos Da Rosa AP, Guzman H, Xiao SY, Monath TP. Efficacy of killed virus vaccine, live attenuated chimeric virus vaccine, and passive immunization for prevention of *West Nilevirus* encephalitis in hamster model. Emerg Infect Dis 2002;8(12):1392–7.
[42] Khushiramani R, Girisha SK, Karunasagar I, Karunasagar I. Cloning and expression of an outer membrane protein ompTS of *Aeromonas hydrophila* and study of immunogenicity in fish. Protein Expr Purif 2007;51(2):303–7.
[43] Hadidi S, Glenney GW, Welch TJ, Silverstein JT, Wiens GD. Spleen size predicts resistance of rainbow trout to *Flavobacterium psychrophilum* challenge. J Immunol 2008;180(6):4156–65.
[44] Gerwick L, Corley-Smith G, Cj B. Gene transcript changes in individual rainbow trout livers following an inflammatory stimulus. Fish Shellfish Immunol 2007;22(3):157–71.
[45] Randelli E, Buonocore F, Scapigliati G. Cell markers and determinants in fish immunology. Fish Shellfish Immunol 2008;25(4):326–40.
[46] Schmitz ML, Weber A, Roxlau T, Gaestelb M, Kracht M. Signal integration: crosstalk mechanisms and networks in the function of inflammatory cytokines. Biochim Biophys Acta: Mol Cell Res 2011;1813(12):2165–75.
[47] Zhang Z, Wu H, Xiao J, Wang Q, Liu Q, Zhang Y. Immune responses of zebrafish (*Danio rerio*) induced by bath-vaccination with a live attenuated *Vibrio anguillarum* vaccine candidate. Fish Shellfish Immunol 2012;33(1):36–41.
[48] Zhang DC, Shao YQ, Huang YQ, Jiang SG. Cloning, characterization and expression analysis of interleukin-10 from the zebrafish (*Danio rerion*). J Biochem Mol Biol 2005;(5):571–6.
[49] Hummelshoj L, Ryder LP, K. Poulsen L. The role of the interleukin-10 subfamily members in immunoglobulin production by human B cells. Scand J Immunol 2006;64(1):40–7.
[50] Zhang ZH, Wu HZ, Xiao JF, Wang QY, Liu Q, Zhang YX. Immune responses evoked by infection with *Vibrio anguillarum* in zebrafish bath-vaccinated with a live attenuated strain. Vet Immunol Immunopathol 2013;154:138–44.
[51] Raida MK. Association between Plasma Antibody Response and Protection in Rainbow Trout *Oncorhynchus mykiss* Immersion Vaccinated against *Yersinia ruckeri*. PLoS ONE 2011;6(6):e18832.
[52] Yang D, Liu Q, Ni C, Li S, Wu HZ, Wang QY. Gene expression profiling in live attenuated *Edwardsiella tarda* vaccine immunized and challenged zebrafish: insights into the basic mechanisms of protection seen in immunized fish. Dev Comp Immunol 2013;40(2):132–41.

Please cite this article in press as: Jiang X, et al. Immune effects of the vaccine of live attenuated *Aeromonas hydrophila* screened by rifampicin on common carp (*Cyprinus carpio* L). Vaccine (2016), http://dx.doi.org/10.1016/j.vaccine.2016.04.075