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

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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.

1. I d c i

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

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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. Ma e ia$_l$ a d e h d

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 $22 \pm 2\,^{\circ}$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

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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

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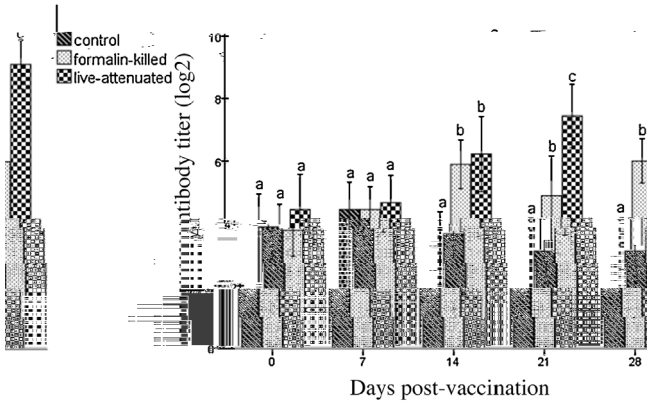

Fig. 3. Antibody titers in the serum of the vaccinated fish at 0, 7, 14, 21, and 28 dpv. Anti-XX1 antibody was determined by ELISA. Results are shown as log 2 antibody titer. Data are presented as mean ± SD ($n = 5$). The different letters (a, b and c) above the bars represent the significant difference 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.

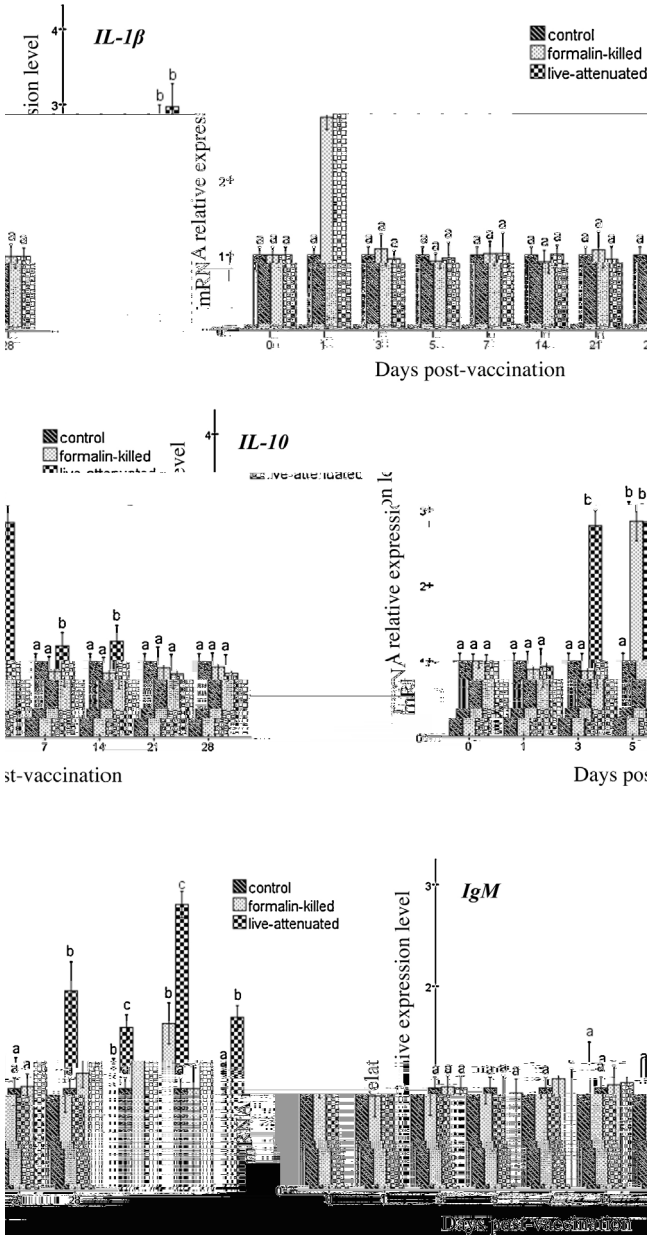

Fig. 4. The mRNA expressing patterns of immune-related gene *IL-1β*, *IL-10*, and *IgM* in liver of the vaccinated fish with the time extension. The mRNA level of each immune-related gene was normalized to that of *β-actin*, and the relative expressing levels were calculated compared to the control. 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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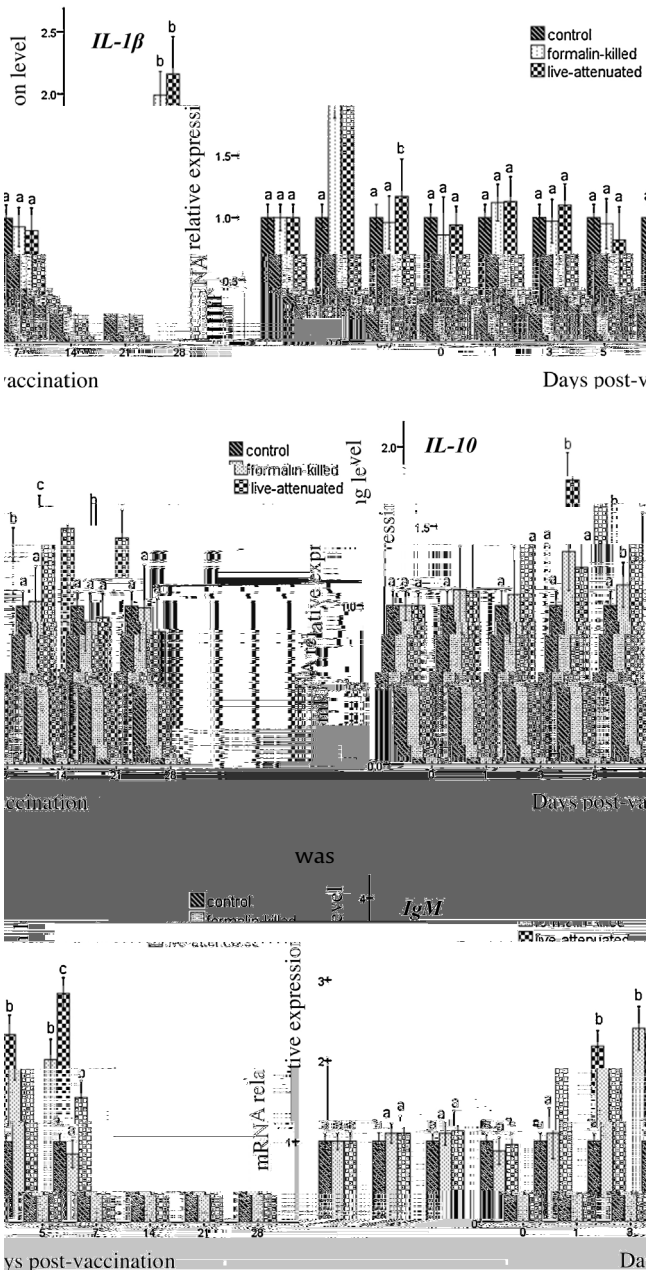

Fig. 5. mRNA expression levels of the immune-related gene *IL-1β*, *IL-10*, and *IgM* in spleen of the vaccinated fish at different time points. The mRNA level of each gene was normalized to that of *β-actin*, and the relative expression levels were calculated compared to the control. The values of data 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