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Using GFP as a biomarker to visualize the process of bacterial infection in black carp (*Mylopharyngodon piceus*)

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ABSTRACT

As one of the most critical pathogens, *Aeromonas hydrophila* (AH) can cause motile aeromonad septicemia (MAS) in freshwater fish. In recent decades, a myriad of studies had been done for bacterial infect fish. However, the mechanism of bacterial infects fish especially freshwater fish was scanty. This study was conducted for investigating the invasion pathway of *A. hydrophila* in vivo of black carp. We have performed *A. hydrophila* 4332 transformed with a plasmid encoding the green fluorescent protein (pGFPuv) ($AH^{4332GFPuv}$) in black carp. The $AH^{4332GFPuv}$ had similar growth properties and virulence as the wild-type strains under the simulated natural condition. In this study, black carp were divided into five groups: IM (challenged via immersion), IBD (increased stocking density), SAW (skin artificially wounded by scalpel), MR (mucus removed from the body surface), and C0 (control group). The number of $AH^{4332GFPuv}$ in gill, liver, spleen, intestine, mid kidney, head kidney, muscle, eye, brain, heart, and blood were examined after 72 h post-infection from all groups. Significantly high bacterial numbers were observed in the gills and intestine. The number of bacteria was significantly higher in IBD group than IM group. In conclusion, the gill, intestines, and injured skin are likely to be the primary infection routes.

1. Introduction

In China, Black carp is one of the "four famous domestic fishes" (Agustin et al., 1995). It is an important economic species for freshwater aquaculture in China. However, black carp is easily attacked by a large number of pathogenic microorganisms under natural conditions, especially *A. hydrophila*. (Noga, 2010; Roberts, 2012). It usually leads to extremely high mortality of black carp (Zhang et al., 2019; Liguo Liang and Xie, 2013) and caused severe economic losses for the black carp farming industry (Zhang et al., 2018).

A. hydrophila is a Gram-negative bacterium, which can cause a variety of diseases in aquaculture. The symptoms of *A. hydrophila* infection in fish are hemorrhage and necrosis in tail and skin rot. In other animals, it causes soft tissue wound infection and diarrhea (Rasmussen-Ivey et al., 2016; Lai et al., 2007; Janda et al., 2010). *A. hydrophila* contains multiple virulence factors (e.g., aerolysin, hemolysin) so that its pathogenesis is multifactorial (Rasmussen-Ivey et al., 2016; Xu et al., 2012; Allan and Stevenson, 1981). Besides, its occurrence and infection is usually followed by second infection of other major pathogens. Sometimes the environmental stress could be a factor, for instance, the deterioration of water quality (Allan and Stevenson, 1981; Baumgartner et al., 2017). However, the mechanism of *A. hydrophila* invasion in fish is currently unclear. (Jiang et al., 2016; Peatman et al., 2018; Alyahya et al., 2018). Here, we focused on pathogenesis and invasion pathways of *A. hydrophila* to black carp.

The visualization of pathogens entering the fish usually depends on microscopy, isolated culture, and radioactive tracer techniques (Brittain-long et al., 2010). However, the uncertainty and uncontrollability of these methods possibly lay effect on our experimental results. Conventional study on infection kinetics requires the quantification of the

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Table 1
Sequences of primers used in this study.

Primer	5′-3′	PCR Tm	Product length
Kan-F	CTTTTCGGGGAAATGTGGAAGATCCTTTGATCTTTTC	60℃	973bp
Kan-R	GTAAACTTGGTCTGACAGTTAGAAAAACTCATCGAG		
GFPuv-F	CACATTTCCCCGAAAAGTGCC	56℃	2371bp
GFPuv-R	GTATATATGAGTAAACTTGGTCTGACAG		

bacterial population from infected organs by culturing on antibiotic-containing plates. Radio-iodinated bacteria can be detected quantitatively, but it cannot discriminate either live or dead bacteria (Chu and Lu, 2008). With advances on bioluminescent labeling, fluorescent protein is universally used in many studies, including minimally invasive markers for tracking and quantifying single or multiple species (Jo, 2020; Kremers et al., 2011; Doi and Yanagawa, 2002). The green fluorescent protein (GFP), emits green or blue with UV excitation(Yang et al., 1996), is widely used (Niedenthal et al., 1996) and has the advantages of high detection sensitivity, strong stability, specificity, and low cytotoxicity (Patterson and Lippincott-Schwartz, 2002; Patterson et al., 2002). GFP was introduced as the endogenous fluorescent tag that can make the bacteria visible (Maffei et al., 2017; Weigele et al., 2017; Kaltwasser et al., 2002) for effectively track the path of pathogens invading fish (Qin et al., 2019; Oyarbide et al., 2015; Rekecki et al., 2012).

This study illustrates the usefulness of fluorescent proteins in tracking the *A. hydrophila* in black carp. Our purpose was to illustrate that *A. hydrophila* invade and replicate in black carp. By understanding the pathogenesis of MAS, we hope to develop new approaches to combat this disease.

2. Materials and methods

2.1. Ethics statement

All experiments with fish in this study were conducted following the guidelines on the care and use of animals for scientific purposes, set up by the Institutional Animal Care and Use Committee (IACUS) of Shanghai Ocean University, Shanghai, China. The IACUS approved this study within the project “Breeding of Black Carp” (approval number SHOU-16-014). The infection and dissection experiments were all performed under 3-aminobenzoic acid ethyl ester methanesulfonate (MS-222) (Sigma-Aldrich, USA) anesthesia to minimize the suffering of fish.

2.2. Experimental fish and bacteria

The healthy black carp (n = 500, weight: 23 ± 2 g) were obtained from the Binhai Base of Shanghai Ocean University in June 2019. The fish were kept in the circulating water system at Shanghai Ocean University. Water temperature was maintained at 28 ± 1 °C, and fish were fed a commercial pellet diet at 2% body weight per day. The fish were maintained under these conditions for two weeks before experimental. The *A. hydrophila* 4332 strain (AH^{4332}) was donated by Professor Chengping Lu from Nanjing Agricultural University, China.

2.3. Transformation of A. hydrophila with p-GFPuv-K

A 973 bp DNA fragment, containing a kanamycin-resistance gene marker (Kan^R) and, was obtained from pET-28a (Clontech, USA) via PCR amplification with primers Kan-F / Kan-R (Table 1). A 2371 bp DNA fragment, containing a *gfpuv* gene and *ori* (pBR322 origin), was obtained from p-GFPuv (Clontech, USA) by PCR using the primers GFPuv-F/GFPuv-R (Table 1). PCRs were performed using Ex Taq DNA polymerase (Takara, Tokyo, Japan). The kanamycin-resistance gene PCR product and the linearized PCR product of the p-GFPuv except the ampicillin-resistance gene were combined by EZ Fusion Cloning kit (Enzymonics, Korea), resulting in the pGFPuv-k.

Plasmids pGFPuv-k were transformed into AH^{4332} using a standard $CaCl_2$ transformation protocol (Loske et al., 2011). Plasmids pGFPuv-k has a kanamycin resistance marker and transformed *A. hydrophila* were plated out on Luria-Bertani (LB) containing kanamycin. Colonies that were resistant to kanamycin and fluoresced bright green under UV light were selected as $AH^{4332GFPuv}$.

2.4. Plasmid stability assay

A single colony of $AH^{4332GFPuv}$ was added to 10 mL of LB liquid medium and cultured at 28 °C for 16 h. This bacterial culture was continuously subcultured by reinoculating to another test tube containing 5 ml fresh LB daily for 7 d. The bacterial culture was sampled daily for 7 d to quantify the bacterial number by plate counting. Then, the green fluorescence state of $AH^{4332GFPuv}$ was observed under a UV lightbox, and the total number of bacterial colonies (green fluorescent and non-green fluorescent bacterial colonies) was counted to calculate the stability of the recombinant plasmid. Calculation formula: stability rate (%) = number of green fluorescent bacterial colonies / total number of bacterial colonies × 100.

2.5. Compare the growth curve of AH^{4332} and $AH^{4332GFPuv}$

Single colonies of AH^{4332} and $AH^{4332GFPuv}$ strains were picked and inoculated into LB liquid medium without or with kanamycin (50 μg/mL) and cultured overnight at 28 °C. Then, 100 μl of the bacterial solution was added to 10 ml of LB liquid medium and cultured for 24 h at 28 °C. The value of the OD_{600} of the bacterial solution was measured every 1 h, and the experiment repeated three times. Finally, draw the bacterial growth curve. At the same time, the two strains of bacteria were smeared on solid LB medium and incubated in a constant temperature box at 28 °C for 16 h to compare the colony morphology and growth rate of the two strains.

2.6. Compare LC50 of AH^{4332} and $AH^{4332GFPuv}$

To determine the lethal concentration 50 % (LC50) of the two strains, we selected black carp with a weight of 23 ± 2 g and divided them into 16 groups of 20 each. The two strains were resuspended in PBS and diluted (10^2, 10^3, 10^4, 10^5, 10^6, 10^7,10^8, 10^9 CFU/mL), and *A. hydrophila* 4332 and $AH^{4332GFPuv}$ strains were injected intraperitoneally (The infectious dose of each fish is: 4.4ul/g). After three days of monitoring, the lethality of different concentrations of the two strains was recorded, and the experiment repeated three times.

2.7. Study of infection kinetics of A. hydrophila in black carp

Single colonies of $AH^{4332GFPuv}$ were picked and inoculated into LB liquid medium containing Kan (50 μg/mL), and cultured at 28 °C with 200 rpm shaking overnight. The bacteria were resuspended in sterile PBS solution, and the final bacterial concentration was measured. Through preliminary experiments, we determined that the bacterial concentration in the infection experiment was 3.8 × 10^7 CFU/mL (infected black carp at this concentration have a specific clinical response and the survival time is not less than three days (Zhang et al., 2018)).

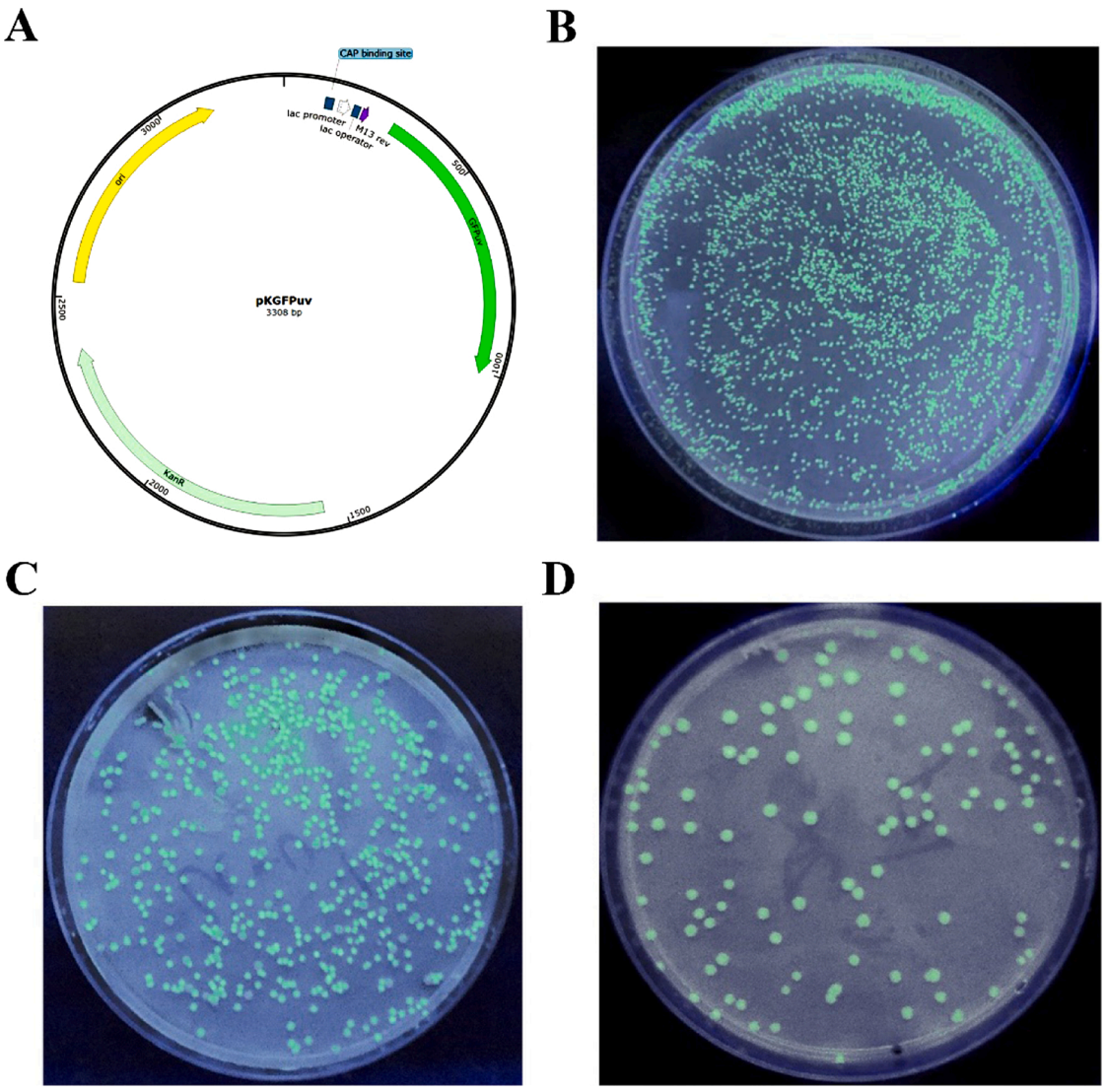

Fig. 1. Recombinant plasmid and screening of fluorescently labeled strains. (A) Map of plasmid structure. (B) DH5α containing p-GFPuv-K plasmid is fluorescent under UV light. (C) $AH^{4332GFPuv}$ has fluorescence under UV light. (D) The fluorescence of $AH^{4332GFPuv}$ on the tenth day of subculture.

In total, 240 black carp in the experiment and the control group were placed in five water tanks (160 L of water in each tank; The length, width and height of the water tank are 80 cm * 50 cm * 40 cm.) with a water circulation filter device and a water temperature constant device. The water temperature was controlled in the range of 25 ± 1 °C. IM (challenged via immersion), IBD (increased stocking density), SAW (skin artificially wounded by scalpel), MR (mucus removed from the body surface), and C0 (control group). The stocking density has doubled from 40 to 80. Before the bacterial bath, use a scalpel to cut 20 minor skin wounds (about 0.5 cm long, 2 mm deep under the skin) on the back of 40 black carp. The mucus of 40 black carp was removed from the head-to-tail direction was wiped off the surface mucus of the fish with a sterile paper towel before infection.

Subsequently, the experimental group (IM, IBD, SAW, and MR) were immersed in the bacterial solution at a concentration of 3.8×10^7 CFU/mL for 1 h, and the control group was in the diluted physiological saline (PBS). Four black carp were randomly selected from each group after 1, 4, 8, 12, 24, 48, 72 h. Furthermore, the gill, liver, spleen, intestine, mid-kidney, head kidney, muscle, eye, brain, heart, and blood were aseptically collected and weighed.

The tissue sample homogenized by adding 1 mL of sterile physiological saline, and then the tissue homogenate was separately diluted to a corresponding multiple (10, 10^2, 10^3, 10^4 times). After that, 100 μl of the diluted tissue homogenate was taken and cultured for 16 h at 28 °C in LB solid medium supplemented with Kan (50 μg/mL). Finally, the bacteria with green fluorescence were counted under a UV lamp. Each experiment repeated three times.

2.8. Statistical analysis

Three independent replicates performed for each data set. The growth curve was determined from data by GraphPad Prism 5. Values expressed as mean ± standard error.

3. Results

3.1. Constructed recombinant A. hydrophila: $AH^{4332GFPuv}$

First, a 973 bp DNA fragment, containing the kanamycin resistance gene was obtained using PCR. The PCR product was inserted into p-GFPuv, except the ampicillin-resistance gene, generated p-GFPuv-K (3308 bp in length). p-GFPuv-K was verified by sequencing. p-GFPuv-K transformed into *DH5α* strain for amplification culture (Fig. 1B). The p-GFPuv-K plasmid successfully transformed *A. hydrophila* strain 4332 into

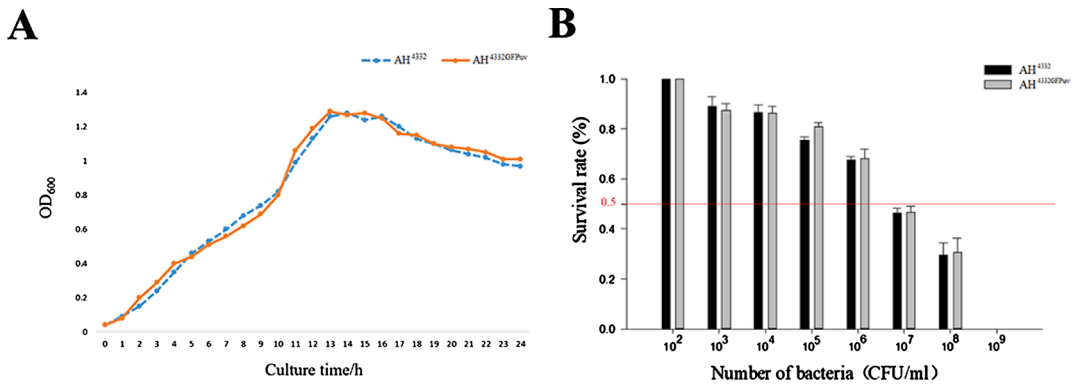

Fig. 2. Comparison of characteristics between AH^{4332GFPuv} and wild strains. (A) Growth curve of AH^{4332GFPuv} and wild strains, orange represents AH^{4332GFPuv}, and blue represents AH^{4332}. (B) LC_{50} of AH^{4332GFPuv} and wild strains, gray represents AH^{4332GFPuv}, black represents AH^{4332}, and the red line represents 50 % survival. Three fish were used per datum. Data were presented as mean ± SD.

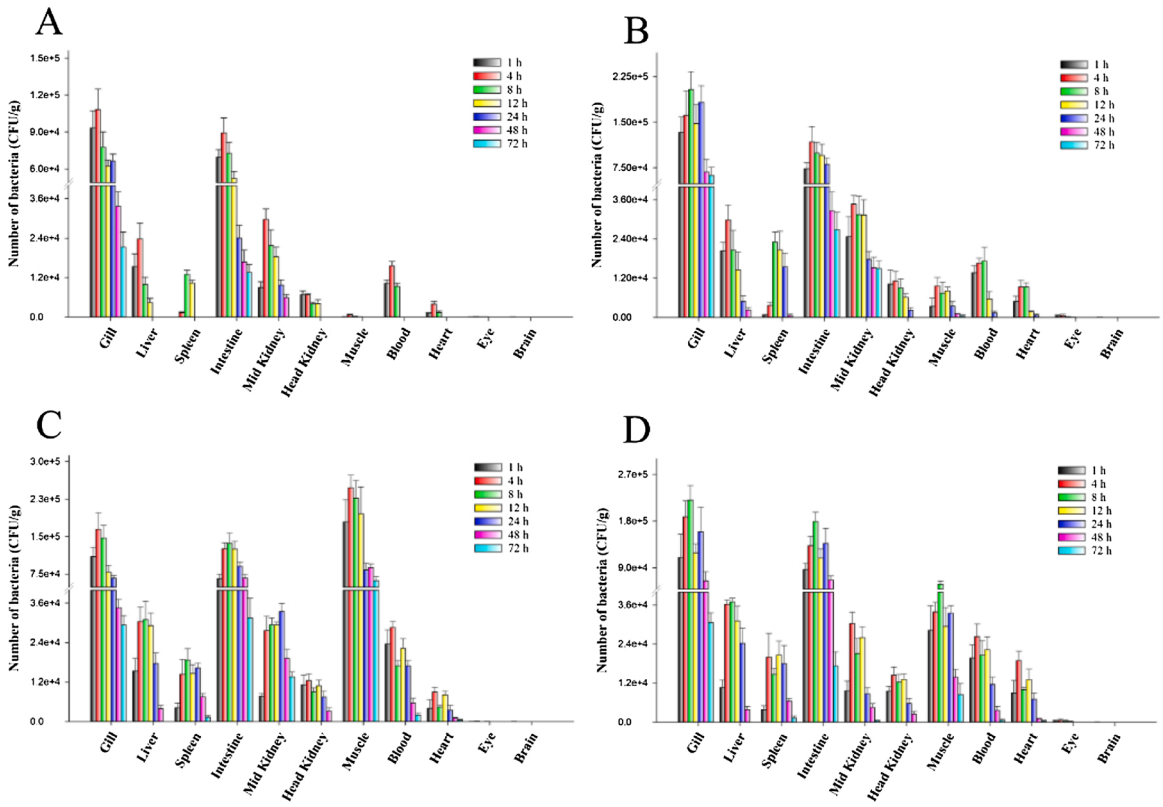

Fig. 3. The number of bacteria in various organs of the black carp after AH^{4332GFPuv} infection. (A) Uninjured fish. (B) Increased culture density. (C) Skin was wounded by a scalpel. (D) Body surface mucus was removed. Three fish were used per datum. Data were presented as mean ± SD.

AH^{4332GFPuv}, which fluoresced an intense green when illuminated with UV light (Fig. 1C). Finally, the AH^{4332GFPuv}, contained kanamycin-resistance and GFP tag, were constructed (Fig. 1A).

3.2. Characteristics of the AH^{4332GFPuv} strain

After culturing the AH^{4332GFPuv} strain in LB medium for ten days. After 16 h incubation, we found that all strains were fluorescent under UV light from LB solid medium containing *Kan* (Fig. 1D). The p-GFPuv-K was stable in AH^{4332GFPuv}. To compare the difference of growth rate between AH^{4332GFPuv} with wild strain, the growth curve of AH^{4332GFPuv} and wild strain were plotted (Fig. 2A). No significant difference were found in growth curves, logarithmic growth period and peak time (Fig. 2A). No significant difference was found in survival rate between the wild and AH4332^{GFPuv} induced black carp after three days. (Fig. 2B). The semi-lethal concentrations of AH^{4332GFPuv} is 4.8 × 10^7 CFU/mL.

3.3. Infection kinetics of A. hydrophila in black carp

Five groups (Group IM, IBD, SAW, MR, and C0) of black carp received different treatments. The number of AH^{4332GFPuv} in different tissues was shown in Fig. 3. No AH^{4332GFPuv} was found in the control group. The AH^{4332GFPuv} numbers were gradually decreased in all tested tissues within 72 h after infection. High bacteria numbers were noticed from all groups at 4–8 h post-challenge (Figs. 3 and 4A). In group IM, the numbers of AH^{4332GFPuv} were generally lower than in other infection groups. The total numbers of bacteria in group IBD was 1.89 times compare with group IM (Fig. 4B). High bacteria numbers were found in

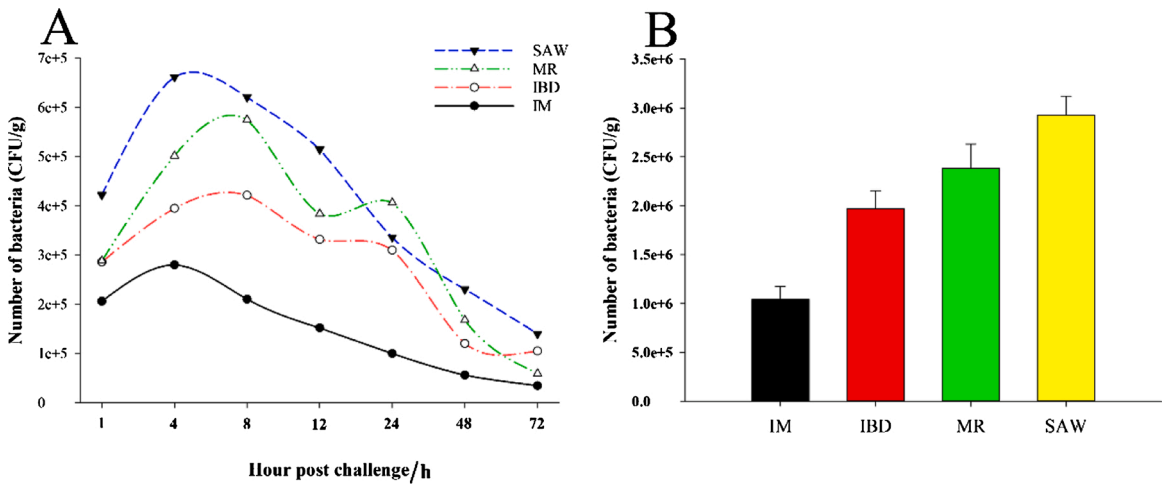

Fig. 4. (A) Number of bacteria at different time points in 4 groups of black carp after $AH^{4332GFPuv}$ infection. (B) Average total number of bacteria in each group of black carp after $AH^{4332GFPuv}$ infection. IM (challenged via immersion), IBD (increased breeding density), MR (mucus removed from the body surface), SAW (skin artificially wounded by scalpel). Three fish were used per datum. Data were presented as mean ± SD.

group IBD at 8 h post-challenge, similar result for group IM was in 4 h. In group SAW, the number of detected bacteria was significantly higher than IM, IBD, and MR (Fig. 4B). Besides, the number of target bacteria detected in the muscles of SAW is higher than that of other groups (Fig. 3C). The bacteria numbers from MR was lower than SAW but higher than IM and IBD (Fig. 4B). The bacteria number from muscle in MR was higher than IM and IBD (Fig. 3D). The bacteria number in SAW reached to peak at 8 h post-challenge, while the peak in group MR was found at 4 h post-challenge (Fig. 4A).

4. Discussion

Gram-negative bacteria are the most common microbial pathogens of fish (Souza, 2011). *A. hydrophila* is the most common reported gram-negative bacteria that can cause opportunistic infection in black carp(Zhang et al., 2018). Water with high nutrient levels can create *A. hydrophila* blooms that could be infectious through wounds or ingestion (Peatman et al., 2018; Saraceni et al., 2016; Gresham, 2014; Zhang et al., 2016). In this study, invasion pathways of *A. hydrophila* were studied in black carp using green fluorescent proteins. Green fluorescent protein has many characteristics that make it useful for bacteria localization. Its ability to fluoresce when fused to target polypeptides and there is no need to added exogenously substrates (Maffei et al., 2017; Phillips, 2001). Our results showed that wild and AH4332GFPuv strain had no significant differences in growth curves and LC_{50}, indicate this method is a very stable and useful tool for future studies.

The skin, gill, and intestine are the mucosal organs in fish. Fish mucus contains innate immune components, secreted by goblet cells that provide the primary defense against different pathogenic microbes and act as a barrier between fish and its immediate niche (Dash et al., 2018; Glover et al., 2013; Abdel-Shafi et al., 2019). *A. hydrophila* may escape from the host immune defense system to adhere and proliferation on the fish body surface when mucous deficiency (Benhamed et al., 2014). In our study, pathological wound or removal skin mucus could be promoted pathogen infection (Fig. 3C, D). The number of bacteria in gills and intestine was high in all groups (Fig. 3). One hour after *A. hydrophila* infected black carp, the number of target bacteria detected in the gill and intestine of all experimental groups was 8.38×10^5 CFU/g, which decreased by 53 times after 72 h at 1.57×10^4 CFU/g. Based on these results, we suggested that fish skin, gill, and intestine plays an essential role in resistance pathogens.

Except for mucosa-associated lymphoid tissues, the major lymphoid tissues in teleost fishes are the kidney, thymus, and spleen. An extensive network exists for the trapping of blood-borne substances, mainly in the kidney, spleen, and liver (Press and Evensen, 1999; Farrell, 2011). The kidney is an essential lymphoid tissue of fish and divide into the head kidney and the middle kidney (Hameed et al., 2006). The spleen is the central place for the production, storage, and maturation of fish red blood cells and neutrophils (He et al., 2001), which have chemotaxis, phagocytosis, and bactericidal action (Nathan, 2006). Studies have shown that a positive immune response is detected in the liver of *Tor putitora* after *A. hydrophila* infection (Kumar et al., 2017). In our study, *A. hydrophila* appears in the blood at 1 h post-infection, and the trend of bacterial growth in the blood is like that in the liver, spleen, kidney, head kidney, and heart (Fig. 3). The number of *A. hydrophila* in the kidney, spleen, and liver decreased to normal levels within 72 h. It indicates that the load of *A. hydrophila* was the tissue specific.

High-intensity aquaculture allows the greatest yields, space efficiency, monetary return and standardization of products (Lewandowski, 2017). It has been reported that an increase in the density of culture will lead to a decline in the innate immunity and metabolic capacity of the culture species, thereby increasing the probability of disease outbreaks (Yarahmadi et al., 2016; Costas et al., 2008; Vargas-Chacoff et al., 2014). In this study, the density of black carp was 11.50 g/L in IBD, 5.75 g/L was in IM. Our results are similar to previous studies (Das et al., 2014), the high density is conducive to the invasion of bacteria, which will increase the risk of disease and decrease the growth performance of black carp (Moniruzzaman, 2015).

In summary, we used GFP to detect the tissues persistence of *A. hydrophila* in infected black carp. The gill, intestines, and injured skin of the black carp are the main portals for *A. hydrophila* invasion. The skin and body surface mucus can effectively resist invasion by *A. hydrophila*. The kidney, liver, and spleen have the strong antibacterial ability. In addition, our research shows that blood may be one of the transmission mediators of pathogens. In the end, our results showed that the increase the cultural density will raise the possibility of fish disease. In conclusion, the visualizing methods using GFP-tagged pathogens can be used as a sound model system in vitro study of the interactions between *A. hydrophila* and its host. Because it can supply relative information about the pathogen localization, distribution after infection and the pathogen clearance in each targeted organ.

CRediT authorship contribution statement

Xueshu Zhang: Conceptualization, Methodology, Software, Validation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Investigation. **Xiaoyan Xu:** Data curation, Writing -

original draft, Writing - review & editing. **Yubang Shen:** Visualization, Investigation. **Lisen Li:** Visualization, Investigation. **Rongquan Wang:** Visualization, Investigation. **Jiale Li:** Supervision.

Declaration of Competing Interest

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.

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