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Development and Visualization of Bioluminescent Virulent Aeromonas hydrophila in Live Catfish

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Development and Visualization of Bioluminescent Virulent *Aeromonas hydrophila* in Live Catfish

By

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Development and Visualization of Bioluminescent Virulent *Aeromonas hydrophila* in Live Catfish

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Virulent *Aeromonas hydrophila* (vAh) is an important emerging bacterial pathogen causing motile *Aeromonas* septicemia (MAS) in farmed catfish. Understanding the pathogenicity of the disease is essential for the development of preventive measures. In this study, we aimed to develop a bioluminescent virulent *A. hydrophila* (BvAh) strain to understand the pathogen-host interactions during infection.

To achieve this, a new bioluminescence expression plasmid, pAK*gfplux*3, was constructed and mobilized to vAh. Catfish were challenged with BvAh using immersion, injection, and adipose fin clip procedures, and bioluminescence signal was tracked in live catfish during infection.

We developed a novel BvAh strain for the first time, conducted imaging of BvAh in live fish, detected infection routes and attachment sites of the pathogen, and determined target organs, which provided new insights on the pathogenesis of vAh. MAS progressed better in fish when protection of skin was bypassed. Abraded skin seems to provide a potential portal of entry during vAh infection.

DEDICATION

Dedicated to my beautiful daughter Lora…

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CHAPTER I

INTRODUCTION

Aeromonas hydrophila is a Gram-negative facultatively-anaerobe rod in *Aeromonadaceae* (Janda and Abbott, 2010; Plumb and Hanson, 2011). *A. hydrophila* causes motile aeromonas septicemia (MAS) in catfish and other fish species including tilapia (Tellez-Bañuelos et al., 2010), goldfish (Harikrishnan et al., 2009; Irianto et al., 2003), common carp (Yin et al., 2009), and eel (Esteve et al., 1994). Other aeromonads such as *A. sobria* and *A. caviae* also cause disease in fish (Camus et al., 1998). Aeromonads are found in freshwater and brackish water, and they often act as opportunistic pathogens in vulnerable fish populations (Camus et al., 1998).

Channel catfish production is the most significant aquaculture industry in the U.S. Historically, *A. hydrophila* has not been considered a pathogen of major concern in catfish aquaculture. However, a highly virulent *Aeromonas hydrophila* (vAh) clade has been a causing motile aeromonas septicemia (MAS) epidemic in farmed catfish since 2009, causing significant production losses (Hossain et al., 2014). VAh has become one of the most significant catfish pathogens in commercial catfish farms in the southeastern United States (Hemstreet, 2010). The disease has spread to Arkansas and Mississippi (Pridgeon and Klesius 2011). Until 2014, disease outbreaks have caused an estimated annual loss of more than $12 million in catfish operations in the Southeastern United

States (Hossain et al., 2014). The disease impacts primarily food-size catfish, and mortality rates could reach up to 50–60% (Shariff, 1998).

Because of limited information on the pathogenesis and virulence factors of vAh, our lab initiated several research projects including sequencing of vAh genomes, study of histopathological changes in catfish after vAh challenge, and in-frame gene deletions targeting potential vAh virulence genes. However, no studies have systematically investigated the pathogen's invasion of catfish and target organs. We expect that monitoring vAh by noninvasive bioluminescence imaging (BLI) is a novel approach that will facilitate a better understanding of vAh pathogenesis in catfish. The research described is significant because this will be the first visual assessment of vAh infection in live catfish. The aquaculture industry has become the fastest growing agricultural industry in the United States. MAS caused by vAh is one of the most devastating diseases of catfish. Control of MAS by antibiotic treatment is costly and does not treat affected fish; it only prevents further spread because moribund fish do not eat medicated feed. In spite of the widely-appreciated magnitude of this problem, little progress has been made on the treatment of MAS economically and practically.

Taxonomy

Aeromonas is a member of the *Aeromonadaceae* family (Isonhood and Drake, 2002). Aeromonads are motile and have a single flagellum. There are 19 different Aeromonas species have been identified including *A. hydrophila*, *A. veronii*, *A. sobria*, *A. caviae*, *A. schubertii*, and non-motile *A. salmonicida.* Motile aeromonads can be adapted to environments that have different pH, salinity, and temperature (Cipriano et al., 1984).

Most *Aeromonas* strains grow at less than 5°C, so aeromonads can be problematic in refrigerated foods (Isonhood and Drake, 2002). Thirty-three *Aeromonas* spp. strains were classified as psychrophiles or mesophiles (Rouf and Rigney, 1971). Psychrophiles including six *A. hydrophila* and one *A. shigelloides* strains optimally grew at 15-20°C, maximum 40-45°C, and minimum 0-5°C (Rouf and Rigney, 1971). Optimum growth temperature for mesophiles is 35°C. Overall, the optimal temperature for growth is known as 28°C, but growth from 1 to 42°C has been observed (Isonhood and Drake, 2002).

Aeromonas spp. are facultatively anaerobic and oxidase and catalase positive (Hazen et al., 1978). A monotrichous polar flagellum makes *Aeromonas* motile. and they do not have a capsule (Post, 1987). *Aeromonas* spp. can produce lipase and do not require sodium for growth (Krieg and Manual, 1984).

Pathogenesis

Hemorrhage and septicemia are major characteristics of *Aeromonas* infection, and the hemolysin is a main factor in pathogenicity (Santos et al., 1988). Two forms of hemolysins (α and β) were reported for motile Aeromonads (Pollard et al., 1990). Beta-hemolysin, which is known as Asao toxin (Asao et al., 1984) or cytotoxic enterotoxin (Burke et al., 1981), causes incomplete lysis of erythrocytes. Although alpha-hemolysins probably have a minor role in *Aeromonas* infections, beta-hemolysins have been isolated from supernatant of human diarrhea (Santos et al., 1988). According to exoenzymatic studies, *Aeromonas* species produce gelatinase, amylase, protease, and DNase (Janda, 1985; Palumbo et al., 2001).

Internal lesions observed by light microscopy (LM) in catfish infected with vAh by immersion are hyperemia of abdominal organs, as well as petechia and ecchymosis scattered over mesenteric tissues. (Rasmussen-Ivey et al., 2016). While pathological lesions were detected in spleen and stomach at 1 h post-challenge (HPC), intestine, gills, kidney, and liver lesions were observed at 24 or 48 HPC by transmission electron microscopy (TEM) (Abdelhamed et al., 2017). Severe tissue destruction with multiple bacteria secreting outer membrane vesicles, especially in gills, spleen, and stomach were observed by transmission electron microscopy (TEM) (Abdelhamed et al., 2017). Degenerate bacteria were observed in the intestinal lumen and the phagosomes of phagocytic kidney cells. Degranulated eosinophilic granular cells and dendritic-like cells were observed in the necrotic intestinal epithelium (Abdelhamed et al., 2017).

Intraperitoneal (IP) injection is one of the main methods for comparing relative virulence of *A. hydrophila* isolates (Hossain et al., 2014) and for examining effect of prophylactic treatments on prevention of MAS (Zhang et al., 2014). IP injection is an effective and reproducible challenge model, but it is incongruous with the natural infection process. Another challenge method is immersion of fish in water containing vAh, which simulates infection under aquatic conditions, but results in low or no fish mortality even at very high doses of vAh (Xu et al., 2012). Immersion challenge after adipose fin clipping increased susceptibility of catfish to MAS, resulting in about 90% mortality within 48 h (Zhang et al., 2016).

Motile Aeromonas Septicemia has two forms that are acute hemorrhagic septicemia and chronic ulcerative syndrome (Cipriano et al., 1984; Huizinga et al., 1979).

Bacterial virulence and resistance/ condition of the fish population affect severity of MAS.

The acute form of the disease is characterized by fatal septicemia. Clinical signs are exophthalmia and fluid accumulation in the scale pockets (Faktorovich, 1969). Internally, liver presents pale or green, and kidneys may become enlarged. Liver and kidney lose their structure due to damage from bacterial toxins (Huizinga et al., 1979). Structural changes and necrosis of both organs are caused by the release of intracellular and extracellular products including hemolysin and protease from *A. hydrophila* (Abdelhamed et al., 2017).

The chronic form is the ulcerative form of disease (Faktorovich, 1969). Clinical lesions include ulcers in the dermal and epidermal layer of the skin, which becomes severely necrotic, focal hemorrhages, and inflammation (Huizinga et al., 1979). Internally, inflammation, swelling, and hemorrhage occur in the lower intestine (Faktorovich, 1969).

Treatment and prevention of Motile Aeromonas Septicemia

The most effective management strategy for MAS is avoiding infection from the hatchery. Handling, nutrition factors, transportation, and water quality increase the fish's susceptibility. Motile Aeromonas Septicemia is generally a stress-mediated disease. Stress factors such as increasing water temperature (Esch and Hazen, 1980) or decreasing dissolved oxygen concentration (Walters and Plumb, 1980) predispose infections.

Terramycin® (2.5-3.75 g/100 lb of fish per day for 10 days in feed) (Schnick et al., 1986), and Romet-30® (50 mg/kg of fish per day for 5 days) (Schnick et al., 1986)

can be used for treating MAS. Florfenicol (Aquaflor®) is an FDA-approved feed additive antibiotics, and these are currently available to use in USA (Pridgeon et al., 2011).

Bioluminescence

Bioluminescence is a process in which cells produce light through chemical reactions in bacteria, algae, fish, and insects (Kong et al., 2011). Bioluminescent bacteria have an ability to catalyze the oxidation of a long-chain aldehyde and reduced flavin mononucleotide ($FMNH_2$) for light production (Close et al., 2010). The luciferase binds to fatty acid aldehyde and $FMNH_2$ and oxidizes these molecules to generate light (Meighen, 1991). The overall reaction is as follows: $FMNH_2 + RCHO + O_2 \rightarrow FMH + H_2O + RCOOH + H_2O + h\nu_{490nm}$ (Meighen 1991).

IVIS Lumina XRMS (Caliper Life Sciences, Hopkinton, MA) is an instrument used for imaging luciferase light emission from fish and fish organs. With bioluminescence imaging (BLI), the same host animals can be used for data collection over the entire experiment, which reduces the number of animals used in studies and provides answers to the pathogenesis of bacteria (Ryan et al., 2011). BLI also decreases variability between time points (Contag et al., 1995; Contag et al., 1997; Contag et al., 1998). Employing bioluminescence in live animal models facilitates tracking of pathogens (Contag, Spilman et al. 1997) and indicates the physical location of pathogens (Siragusa et al., 1999), which will increase researchers' understanding of pathogenic processes (Contag et al., 1995). There are several studies using this bioluminescent technology to investigate a variety of physiologically relevant systems, including living cells (Willard et al., 2002), whole plants (Anderson et al., 2001), and rodents (Contag et al., 1995; Contag et al., 1997; Contag et al., 1998). The ability to track bioluminescent

pathogens in vivo through the GI tract has previously been successful in pigs, mice, and fish (Karsi et al., 2006; Willard et al., 2002).

CHAPTER II

DEVELOPMENT AND VISUALIZATION OF BIOLUMINESCENT VIRULENCE *A. HYDROPHILA* IN LIVE CATFISH

Abstract

Virulent *Aeromonas hydrophila* (vAh) is an important emerging bacterial pathogen, causing motile *Aeromonas* septicemia (MAS) in farmed catfish. Understanding the pathogenicity of the disease is essential for development of preventive measures. In this study, we aimed to develop a bioluminescent virulent *A. hydrophila* (BvAh) strain to understand the pathogen-host interactions during infection. To achieve this, a new bioluminescence expression plasmid, pAK*gfplux*3, was constructed and mobilized to vAh. Catfish were challenged with BvAh using immersion, injection, and adipose fin clip procedures, and bioluminescence signal was tracked in live catfish during infection.

We developed a novel BvAh strain for the first time, and we conducted imaging of BvAh in live fish, detected infection routes, and determined attachment sites and target organs of the pathogen. This study provided new insights into the pathogenesis of vAh. MAS progressed better in fish when protection of skin was bypassed. Abraded skin seems to provide a potential portal of entry during vAh infection.

Introduction

Motile *Aeromonas* Septicemia (MAS) caused by virulent *A. hydrophila* (vAh) is a high priority disease and causes considerable economic losses in the U.S. aquaculture

industry. Thus, there is an urgent need to develop therapeutics to prevent the current epidemic. The *rationale* of the proposed research is that understanding the pathogenic mechanisms of vAh is expected to lead to new knowledge of virulence mechanisms used by vAh, which in turn may lead to new therapeutics.

Channel catfish production is the most significant aquaculture industry in the United States. MAS caused by *A. hydrophila* is one of the most devastating diseases of catfish. Control of MAS by antibiotic treatment is costly, and antibiotics do not treat sick fish, but only prevent further spread because only healthy fish eat medicated feed. In spite of the widely-appreciated magnitude of this problem, little progress has been made on the treatment of MAS economically and practically. Understanding the pathogenicity of the disease is essential for the development of preventive measures.

In the current study, we aimed to develop a bioluminescent virulent *A. hydrophila* (BvAh) strain to understand pathogen-host interactions during infection. To achieve this, a new bioluminescence expression plasmid, pAK*gfplux*3, was constructed and mobilized to vAh. Catfish were challenged with BvAh by three different procedures (immersion, injection, and adipose fin clip), and bioluminescence signal was tracked in live catfish during infection.

Material and Methods

Expression of the *luxCDABE* operon in vAh

Our lab developed plasmids containing the *luxCDABE* operon of *Photorhabdus luminescens* (Karsi and Lawrence, 2007). pAK*gfplux*1 has ampicillin resistance for selection. Because the virulent *A. hydrophila* strain is resistant to ampicillin, we constructed a new plasmid named pAK*gfplux*3 to develop bioluminescent vAh, which

was used for BLI experiments. Bioluminescence was monitored using IVIS Lumina XRMS, Biotek Cytation 5, or Biotek Synergy H1.

Isolation of bioluminescence expression plasmid

Four bioluminescent colonies were inoculated into 2 ml brain-heart infusion (BHI) broth cultures with 6.25 µg/ml chloramphenicol and were grown for 16 h at 30 °C. Plasmids were isolated using a High-Speed Plasmid Mini Kit (IBI Scientific, Peosta, IA), and isolated plasmids were run on 1% agarose gels and imaged on a Bio-Rad ChemiDOC XRS+ (BioRAD, Hercules, CA).

Determination of optimal chloramphenicol concentration

Twelve bioluminescent vAh colonies were inoculated into 2 ml BHI broth and grown for 16 h at 30°C. BHI media contained 0 µg/ml, 5 µg/ml, 6.25 µg/ml, 7.5 µg/ml, 10 µg/ml, 12.5 µg/ml, 15 µg/ml, 25 µg/ml, 50 µg/ml, 75 µg/ml, and 100 µg/ml chloramphenicol. Ninety-five µl BHI with chloramphenicol and 5 µl broth culture were transferred to a 96 well plate, and bioluminescence and optical density measurements were conducted using Biotek Cytation 5. Also, bioluminescent images were taken using IVIS system and/or Cytation 5 at 0 h, 4 h, 8 h, 12 h, 16 h, and 20 h. In IVIS Lumina XRMS, bioluminescence was captured for 30 seconds at 30°C and quantified using Living Image Software. Bacterial concentration was determined for normalization of bioluminescence data at wavelength of 600 nm (OD_{600}) using a Biotek Cytation 5 or Biotek Synergy H1.

In a second experiment, chloramphenicol concentrations from 5 µg/ml to 15 µg/ml were prepared, and bioluminescent images were taken using IVIS system, and/or

Cytation 5 every 4 h until 20 h. OD measurements were also taken at these times for normalization, which was done by dividing total flux by $OD_{600,}$ readings of bioluminescence data.

In a third experiment, chloramphenicol concentrations from 0.5 µg/ml to 5.5 µg/ml were prepared, and bioluminescent images were taken using IVIS system, and/or Cytation 5 every 4 h until 20 h. OD measurements were also taken at these times for normalization of bioluminescence data.

Growth comparison between BvAh and vAh

Three bioluminescent vAh colonies were inoculated into 2 ml BHI broth with 6.25 µg/ml chloramphenicol; another 3 bioluminescent vAh colonies were inoculated into 2 ml BHI broth without antibiotic; and 3 non-bioluminescent vAh colonies were inoculated into 2 ml BHI broth. Bacteria were grown at 30°C for 16 h, and OD_{600} values were determined by Cytation 5. After the OD adjustment, fresh medium was inoculated with overnight cultures at 1:1000 dilution and grown as described above. OD measurements were taken with spectrophotometer every 4 h until 20 h.

Plasmid stability

Six bioluminescent and 6 non-bioluminescent vAh were inoculated into 2 ml BHI broth with 6.25 µg/ml chloramphenicol and grown for 16 h. Twenty µl culture was transferred to 980 µl BHI with chloramphenicol, and bioluminescence and OD were measured after 12 h. From this culture, 20 µl were transferred to fresh 980 µl BHI without chloramphenicol, and bioluminescence signals and OD were measured every 12 h for 4 days.

BLI of BvAh in live catfish

Eighteen specific-pathogen-free channel catfish were obtained from the fish hatchery at the College of Veterinary Medicine at Mississippi State University and stocked into three tanks (6 fish each tank). Fish were acclimated for one week under 12-h light cycle, and bacterial challenges were conducted at 30°C water temperature. The first group of fish was injected intraperitoneally with 4.57 x 10^6 CFU/ml BvAh; the second group was immersion exposed to 4.57 x 10^{10} CFU/ml water for 3 h; and the third group was immersion exposed to 4.57 x 10^{10} CFU/ml water for 1 h after removal of the adipose fin.

BLI was conducted at 1 h, 3 h, 6 h, 12 h, 18 h, 24 h, 36 h, and 72 h post-infection, or until fish died. Briefly, fish were anesthetized in 5 L water containing 200 mg/L tricaine methanesulfonate (MS222) for 2-3 minutes, and sedated fish were placed in the imaging chamber. Bioluminescence captured from both sides of the fish using an IVIS Lumina XRMS. Following imaging, fish were returned to well-aerated water for recovery. Bioluminescence was quantified from the fish images using the IVIS Lumina XRMS Living Image Software v 4.2.

Determination of BvAh in catfish organs

Forty-five specific-pathogen-free channel catfish were acclimatized for one week. Fish challenges were conducted as described above. At 6 h, 12 h, 18 h, and 24 h, three fish were euthanized at high dose MS-222 (350 mg/L), and posterior kidney, spleen, liver, and gills were collected aseptically. BLI was conducted as described above, and after BLI, posterior kidneys were homogenized in 1000 µL of PBS. After serial dilutions,

25 µl homogenate from 10^{-1}, 10^{-2}, and 10^{-3} were spread on BHI agar plates with chloramphenicol, and bioluminescence of the resulting colonies was confirmed.

Statistical analysis

The significance of the differences between means of treatments (organs and chloramphenicol concentrations) was established by one-way ANOVA procedure with Tukey's test in SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC).) The level of significance was set at $p<0.005$ in all analyses.

Results

Determination of optimal chloramphenicol concentration

The effect of chloramphenicol concentrations on growth of *A. hydrophila* carrying pAK*gfplux*3 was determined by growing bacteria in increasing concentrations of chloramphenicol. At 4 h, bacterial growth in medium without chloramphenicol was significantly different from that of in medium with chloramphenicol ($P < 0.05$). At 8 and 12 h, presence of chloramphenicol less than 12.5 µg/ml did not have a significant effect in bacterial growth. At16 h, bacterial growth was low only in the highest two chloramphenicol concentrations of 75 µg/ml and 100 µg/ml. At 20 and 24 h, all chloramphenicol concentrations except 100 µg/ml showed more bacterial density compared to no chloramphenicol group (Figure 1). In general, addition of chloramphenicol caused delayed growth.

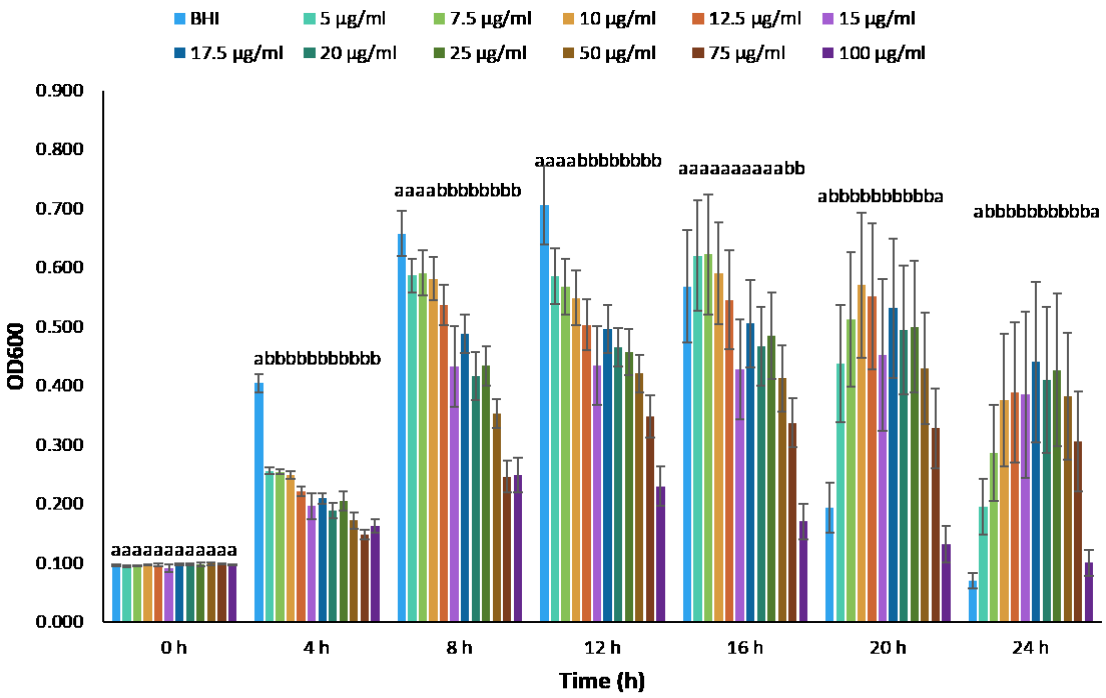

Figure 1 Bacterial growth at increasing chloramphenicol concentrations (0-100 µg/ml). Letters above the bars indicate statistical significance in each group.

In the second experiments where 0 to 15 µg/ml chloramphenicol used, a similar delayed growth has been observed in all chloramphenicol concentrations (Figure 2) ($P < 0.1082$). However, presence of chloramphenicol at all concentrations resulted in a higher bioluminescence production (Fig.3).

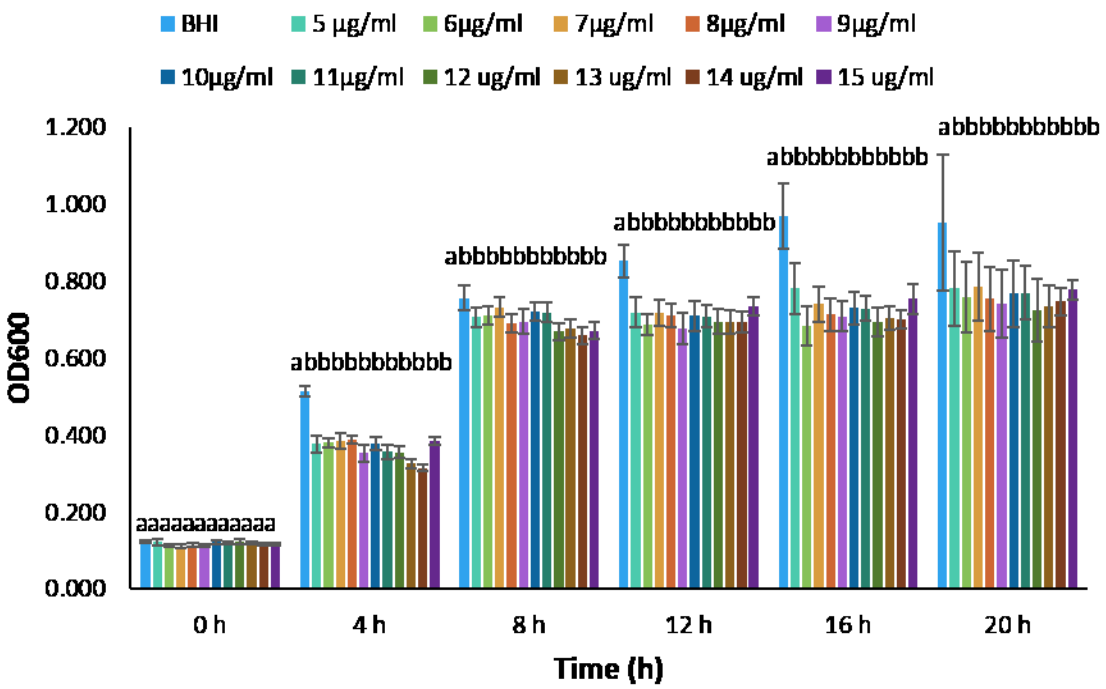

Figure 2 Bacterial growth at increasing chloramphenicol concentrations (0-15 µg/ml). Letters above the bars indicate statistical significance in each group.

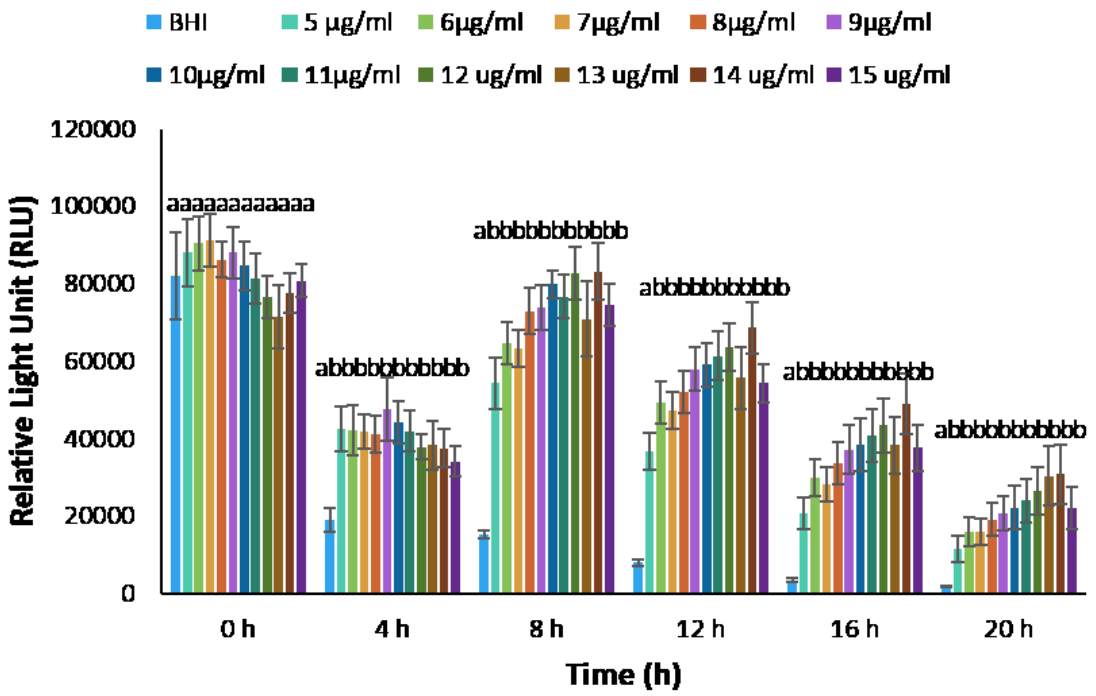

Figure 3 Bioluminescence at increasing chloramphenicol concentrations (0-15 µg/ml). Letters above the bars indicate statistical significance in each group.

In the third experiments where 0 to 5.5 µg/ml chloramphenicol used, results indicated that even very small amount of chloramphenicol causes delayed growth (Figure 4), but bioluminescence only increased at concentrations of 5.0 µg/ml and above (Figure 5).

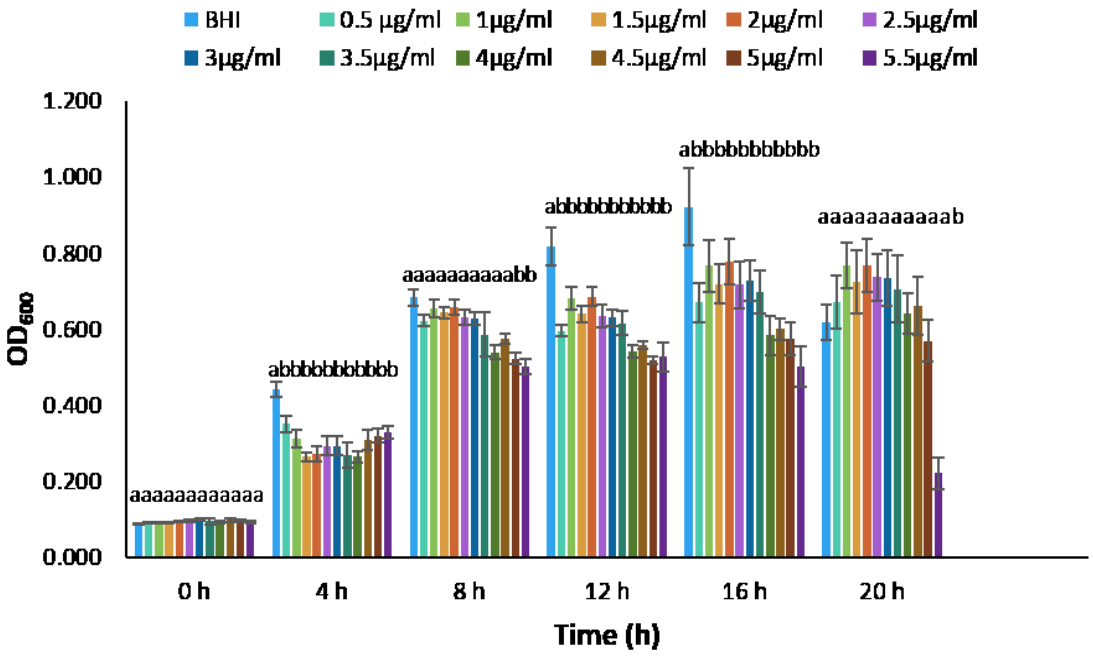

Figure 4 Bacterial growth at increasing chloramphenicol concentrations (0-5.5 µg/ml). Letters above the bars indicate statistical significance in each group.

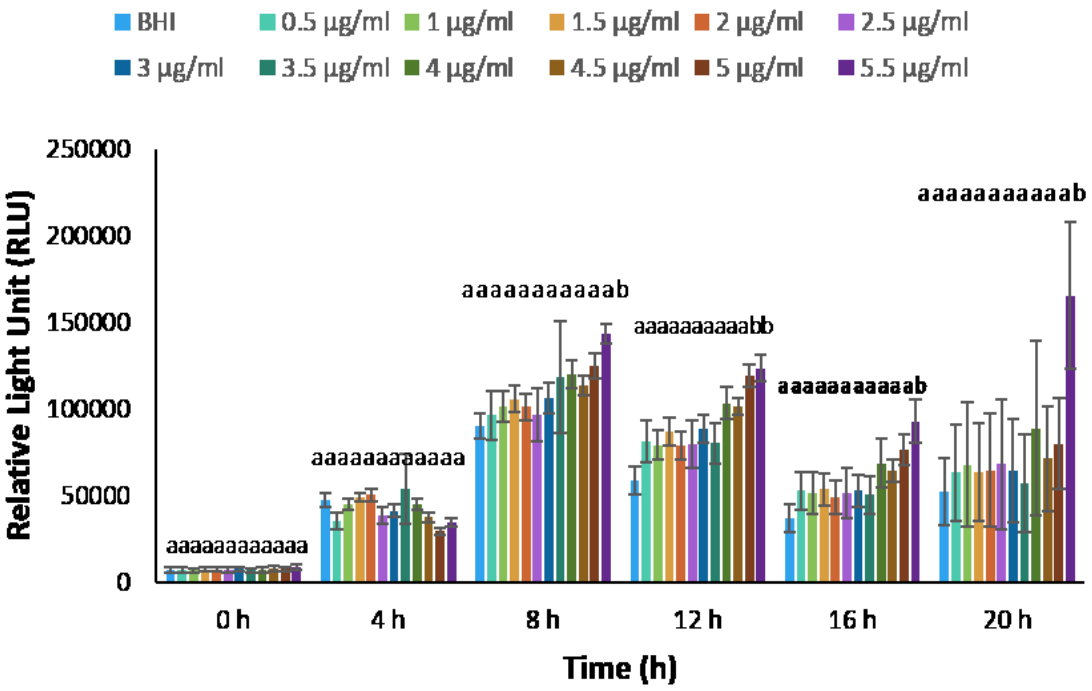

Figure 5 Bioluminescence at increasing chloramphenicol concentrations (0-5.5 µg/ml). Letters above the bars indicate statistical significance in each group.

Determination of CFU and Bioluminescence Relationship

The relationship between bacteria density (determined by serial dilution and plate counts) and bioluminescence (determined by photon emissions) appeared to be linear between 2.70 x 10^{10} CFUs and 2.70 x 10^{3} CFUs (Fig. 6). The minimum detectable number of *A. hydrophila* pAK*gfplux*3 in 96-well plate was less than 2,700 CFU/ml.

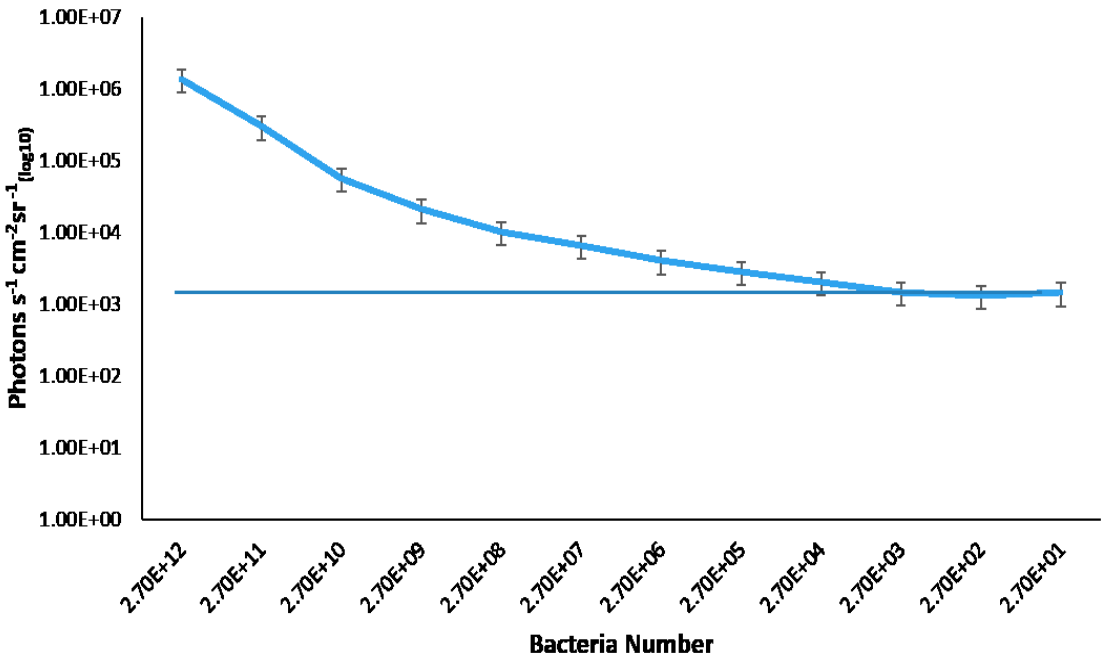

Figure 6 Correlation of luminescent signals (log_{10}) and number of bioluminescent *Aeromonas Hydrophila* (ML 09-119 pAK*gfplux*3).

Growth comparison between BvAh and vAh

The effect of pAK*gfplux*3 on the growth of virulent *A. hydrophila* was detected by growing vAh (ML-09-119) without chloramphenicol and BvAh (ML 09-119 pAK*gfplux*3) with and without chloramphenicol under the same conditions. Results indicated that the presence of pAK*gfplux*3 did not affect the growth of vAh. However, as shown above, chloramphenicol caused delayed growth in BvAh (Fig. 7) ($P < 0.0420$).

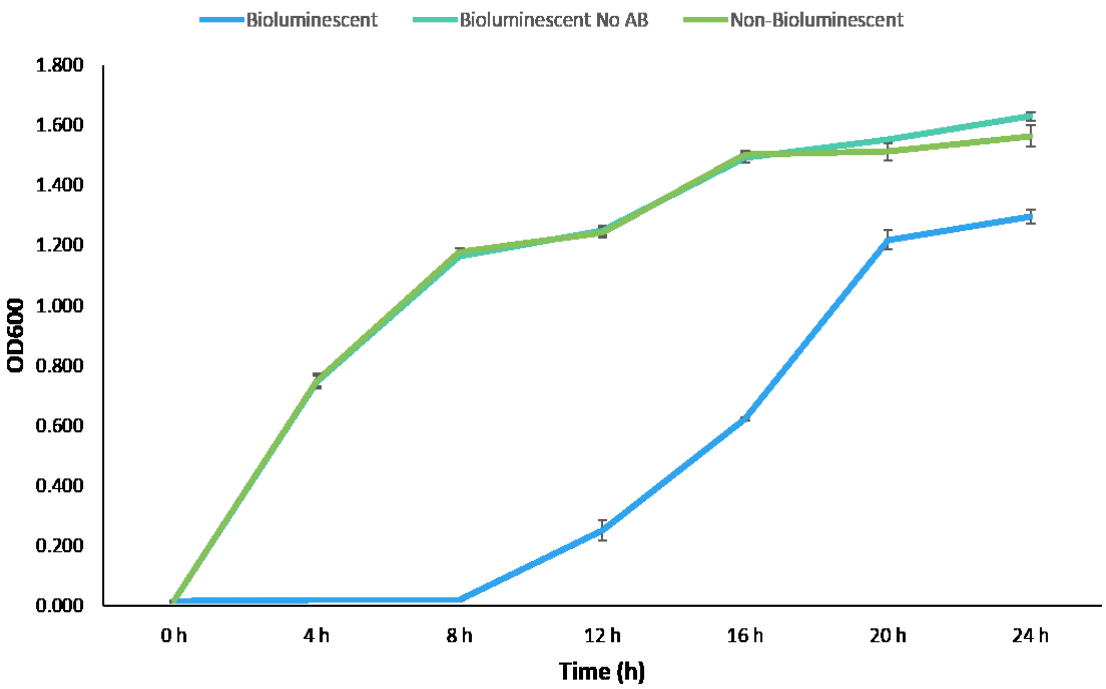

Figure 7 The effect of pAK*gfplux*3 on *A. hydrophila* growth kinetics. Bioluminescent strain was grown without and with chloramphenicol (6.25 µg/ml).

Plasmid stability

Stability of pAKgfp*lux3* was determined by cultivating ML 09-119 pAK*gfplux*3 in the absence of chloramphenicol for 96 h. Bacterial growth and bioluminescence were measured every 12 h. Results indicated that bacterial growth was similar at all time points except 0 h because at the start of the experiment, media contained 6.25 µg/ml chloramphenicol. (Figure 7). The bioluminescent signal decreased dramatically (Figure 8). The normalized RLU decreased 40% from at 12 h, while the decrease was 99.5% at 60 h. It appears that the half-life of pAK*gfplux*3 is about 16-18 h under the described conditions (Fig. 9).

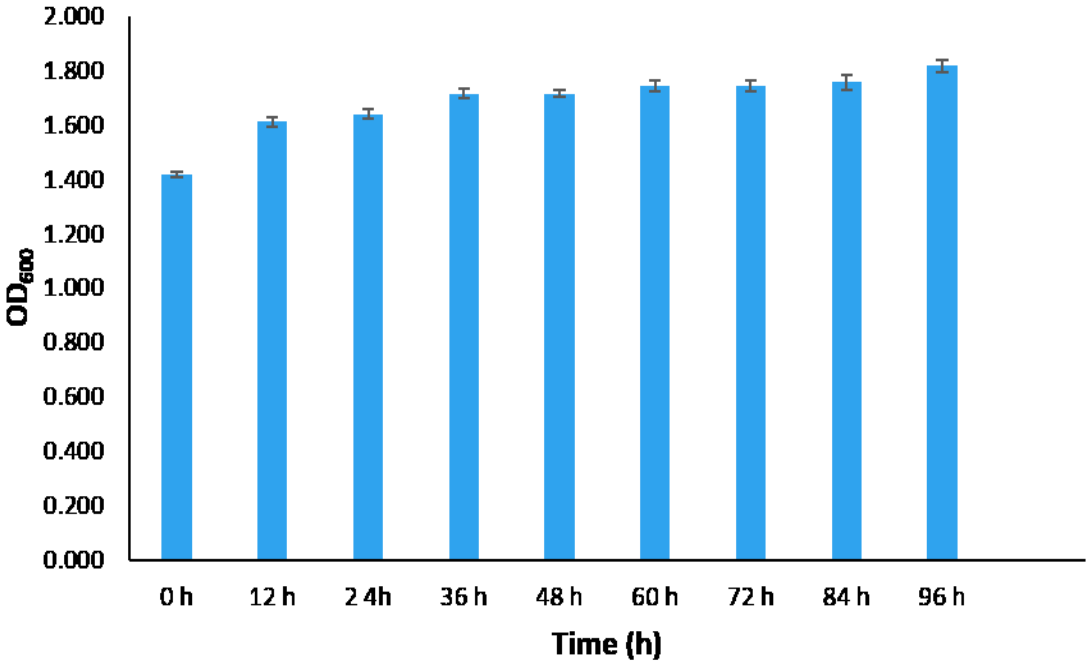

Figure 8 Growth of cultures during plasmid stability test. 0 h had the selective pressure of chloramphenicol, and later cultures were grown without chloramphenicol.

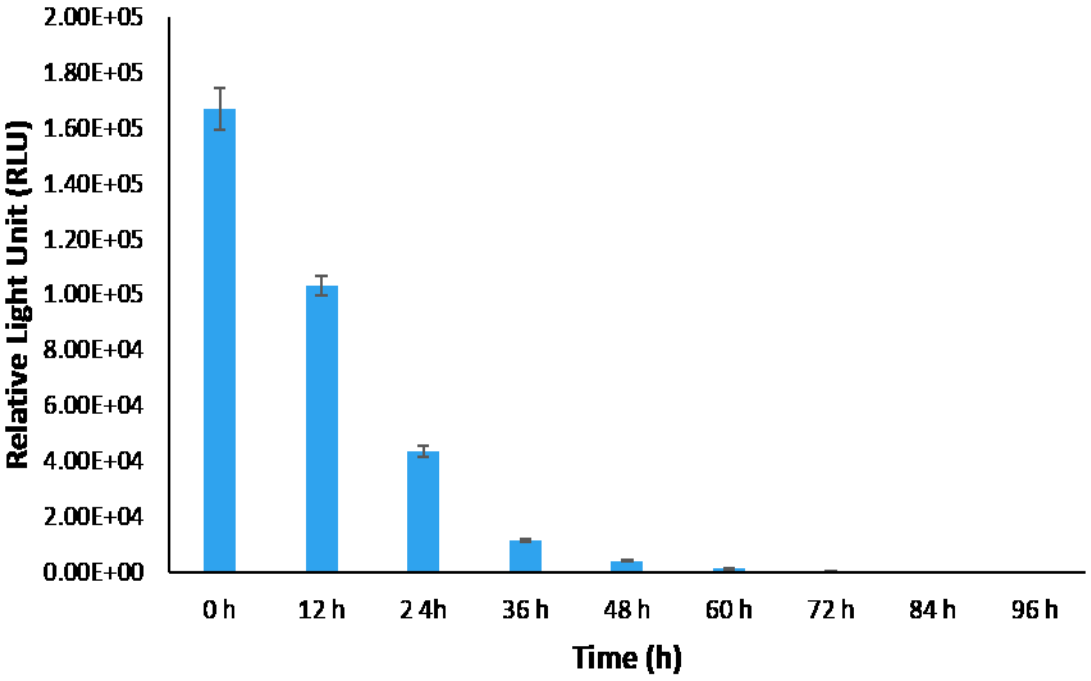

Figure 9 Bioluminescence of subcultures during plasmid stability test. 0 h had the selective pressure of chloramphenicol, and later sub-cultures were grown without chloramphenicol.

Monitoring in vivo bioluminescence in live fish

The bioluminescent imaging was used to monitor BvAh in live catfish. Bioluminescence was successfully detected from catfish that were IP injected, immersed, and adipose fin-clipped with immersion groups with ML 09-119 pAK*gfplux*3.

When catfish were injected with 4.57 x 10^6/fish ML 09-119 pAK*gfplux*3, in vivo bioluminescence was detectable in the whole abdominal area at 1 h following IP injection (Fig. 10) However, localized signals were not noticeable after 3 h. Total photon emissions at each sampling point in injected fish were calculated (Fig. 11). There was a

decrease in total light emission from fish until 6 h, followed by an increase between 6 to 12 h, and decrease again at 18 h.

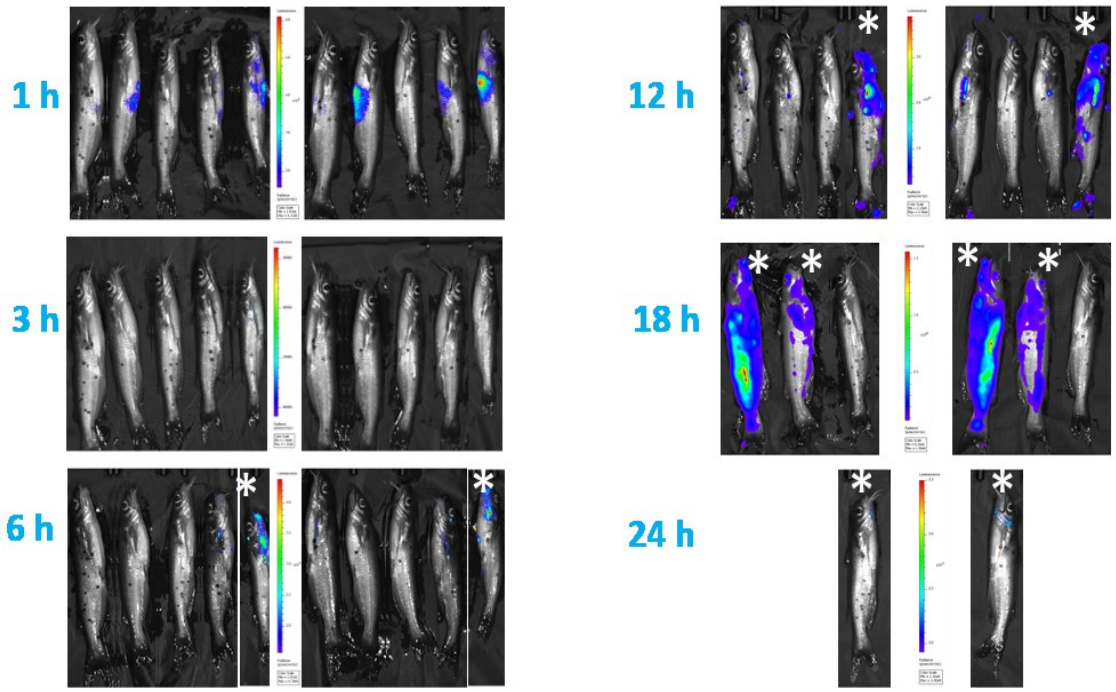

Figure 10 Detection of bioluminescence from IP injected catfish (4.57 x 10^6 CFU/ml) Imaging time after injection is indicated at the left, and dead fish are marked with a white star.

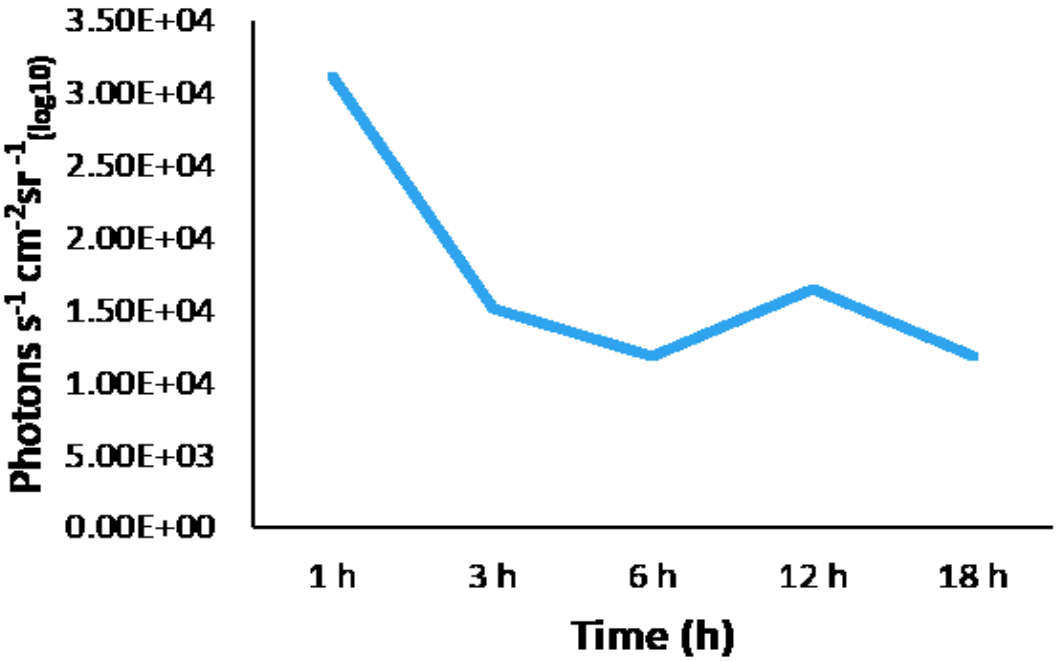

Figure 11 Total photon emissions from IP injected fish (4.57 x 10^6 CFU/ml).

In the immersion-exposed fish, bioluminescence was detected around barbels, but no signal was detected from the rest of the body. As disease progressed, patches of bioluminescence became visible around the eroded fin bases, fin epithelia, and around the nasal area (Figure 12). Total photon emissions showed a similar trend to the photon emissions from fish that were IP injected (Fig. 13).

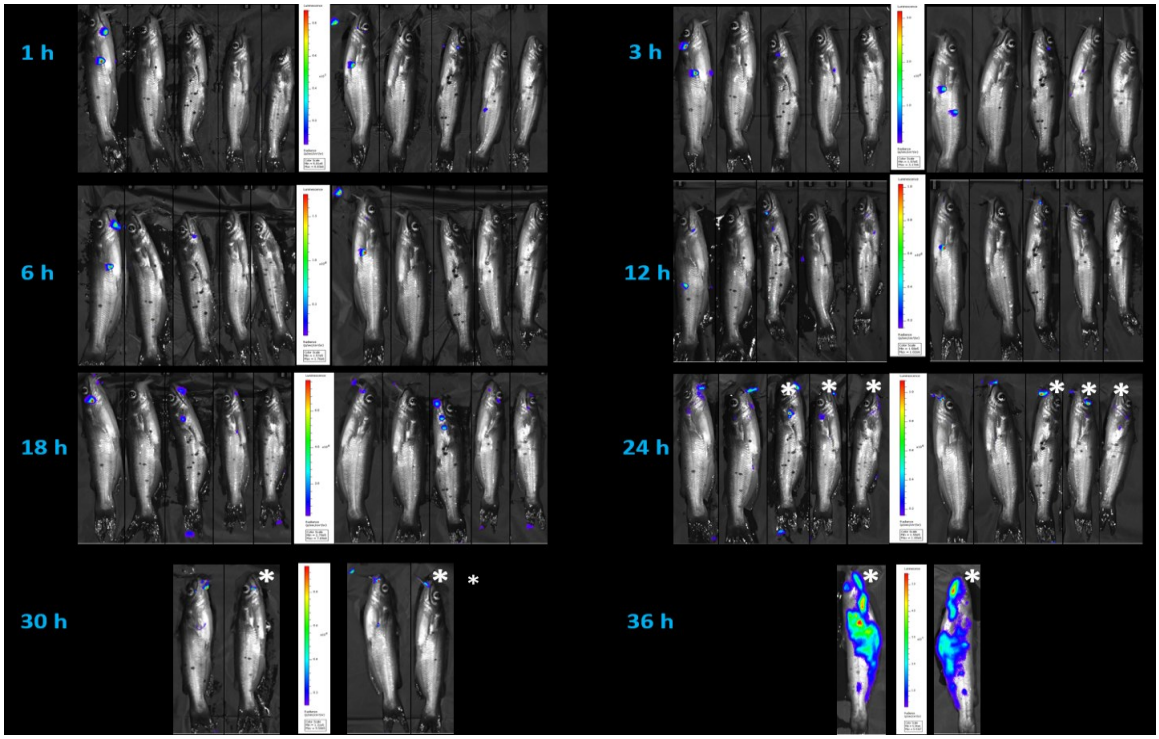

Figure 12 Detection of bioluminescence from catfish immersion challenged with BvAh (4.57×10^{10} CFU/ml in water for 3 h). Imaging time after injection is indicated at the left, and dead fish are marked with a white star.

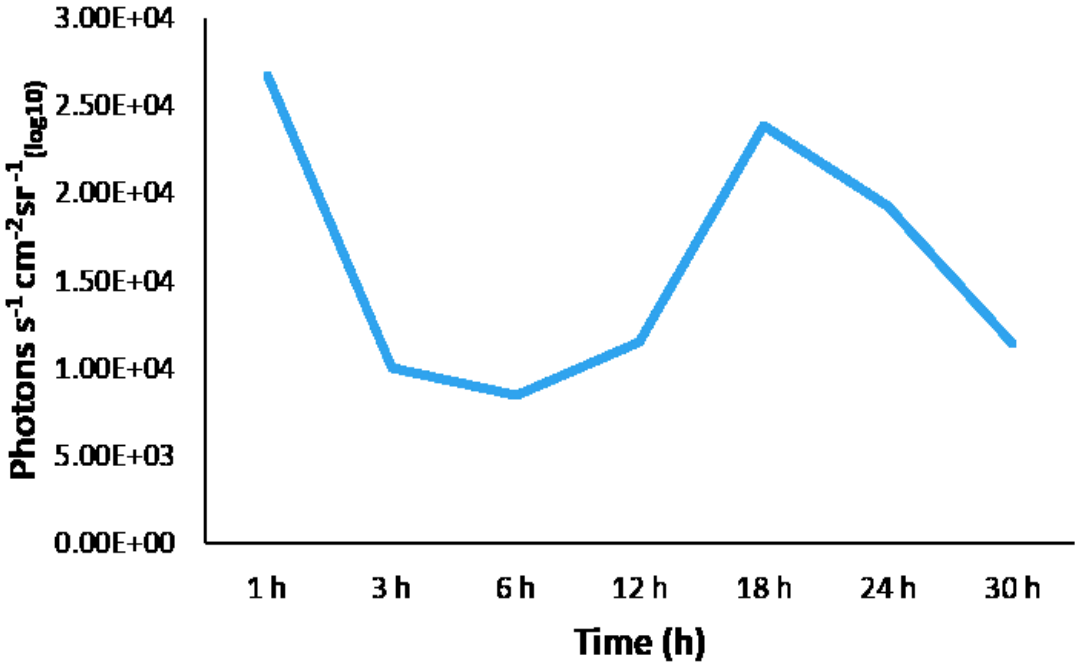

Figure 13 Total photon emissions from immersion challenged fish (4.57×10^{10} CFU/ml).

In the adipose fin clipped and immersion-exposed fish, bioluminescence was detected especially clipped fin area at early time points (1 h, 3 h, 6 h). At the same time, the signal was detected from barbels and eroded epithelia (Fig. 14). Total photon emissions decreased until 6 h and then increased (Fig. 15).

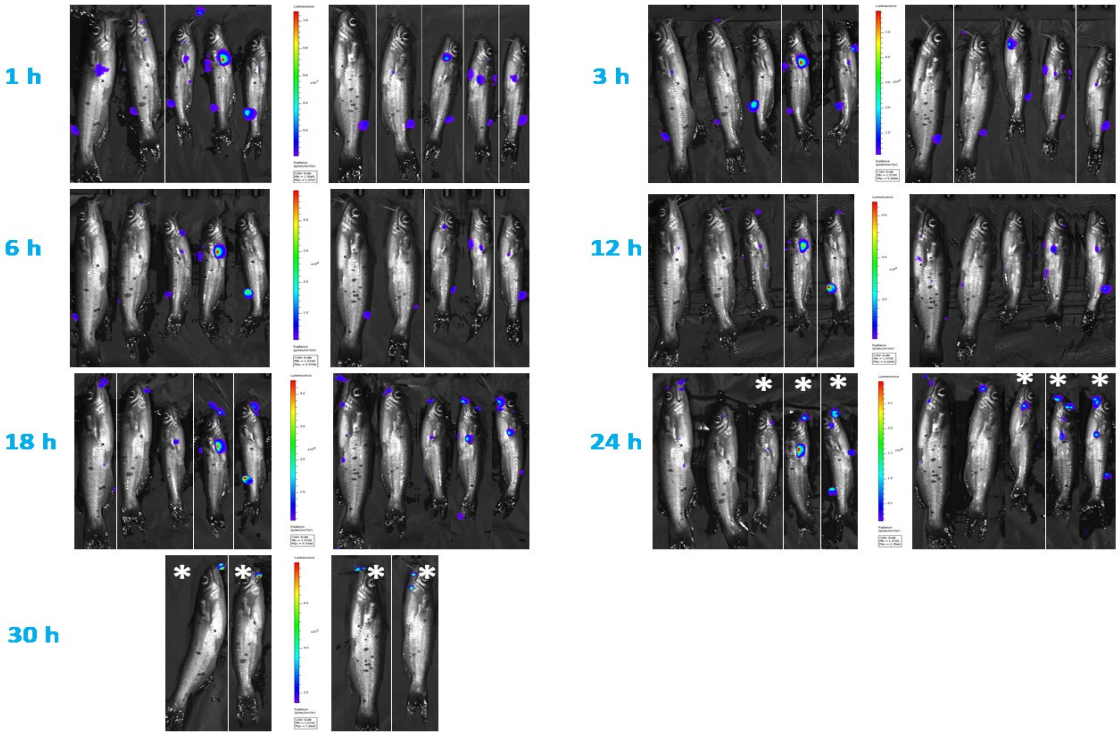

Figure 14 Detection of bioluminescence from catfish after adipose clipping and immersion challenge with BvAh (4.57 x 10^{10} CFU/ml in water for 1h). Imaging time after injection is indicated at the left, and dead fish are marked with a white star.

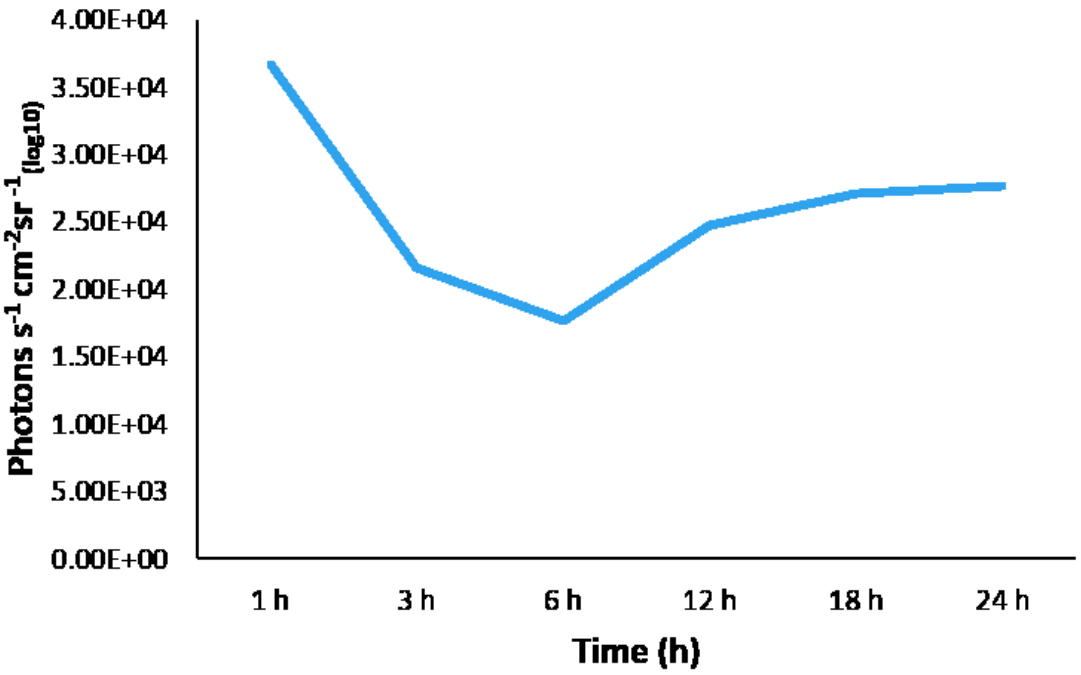

Figure 15 Total photon emissions from adipose fin clipped and immersion challenged fish (4.57×10^{10} CFU/ml).

Monitoring bioluminescence from internal organs

In injection group, bioluminescence was detected in stomach and intestine at early time points. At 12 h, the signal decreased but it increased at 18 h in all organs (Fig. 16).

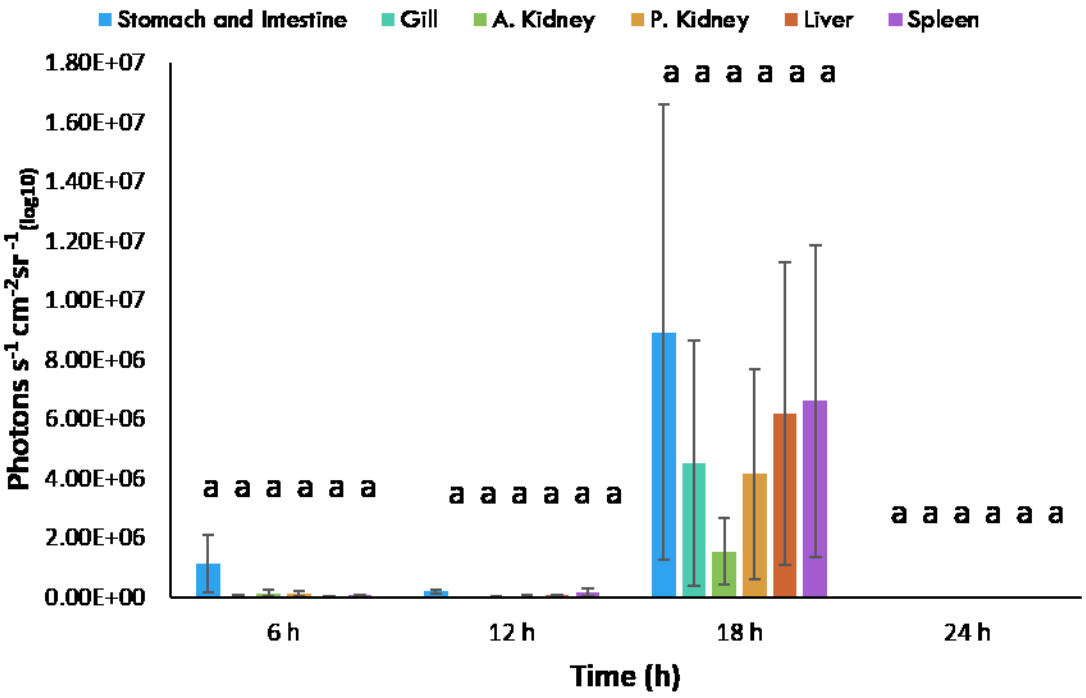

Figure 16 Detection of Ml-09-119 pAK*gfplux*3 in the internal organs of experimentally infected channel catfish.

In immersion group, photon emissions increased in gill at early time points. At 12 h, a reduction was detected in all organs (Fig. 17). There were no significant differences between photon emissions of different tissues at the same time point ($P > 0.05$).

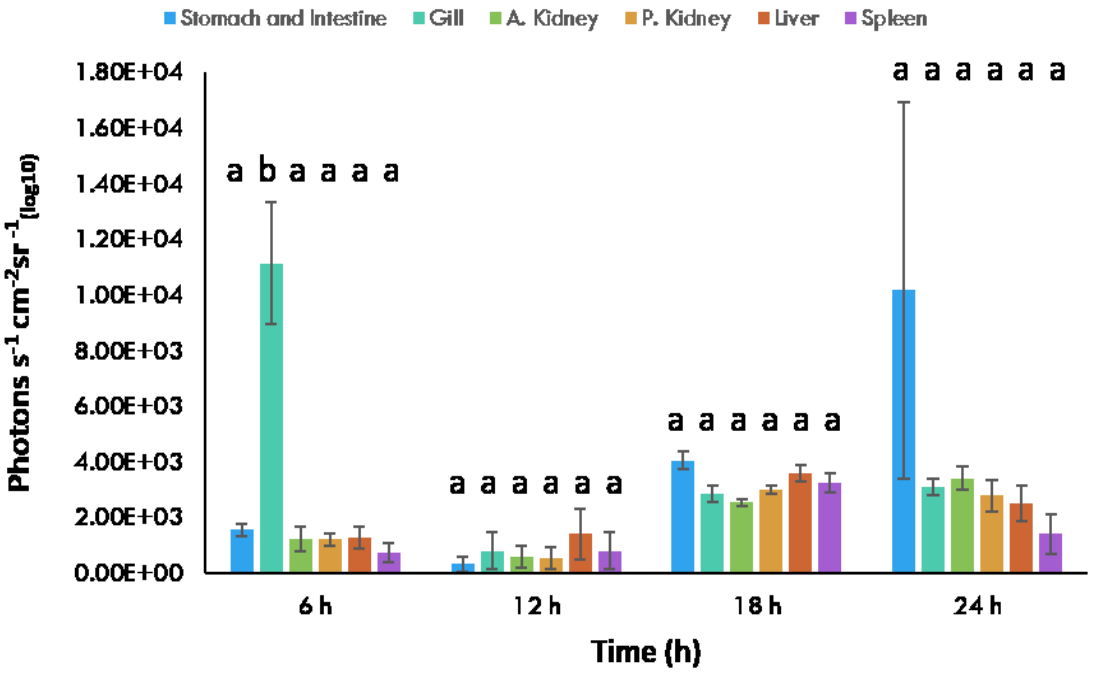

Figure 17 Detection of Ml-09-119 pAK*gfplux*3 in the internal organs of experimentally infected channel catfish

In the adipose fin-clipped group, high bioluminescence signal was detected in gills at the early time point of 6 h post-infection, and photon emission was significantly higher in gills when compared to other organs at the same time point ($P < 0.05$). Later, the bioluminescence signal decreased until 24 h (Fig. 18).

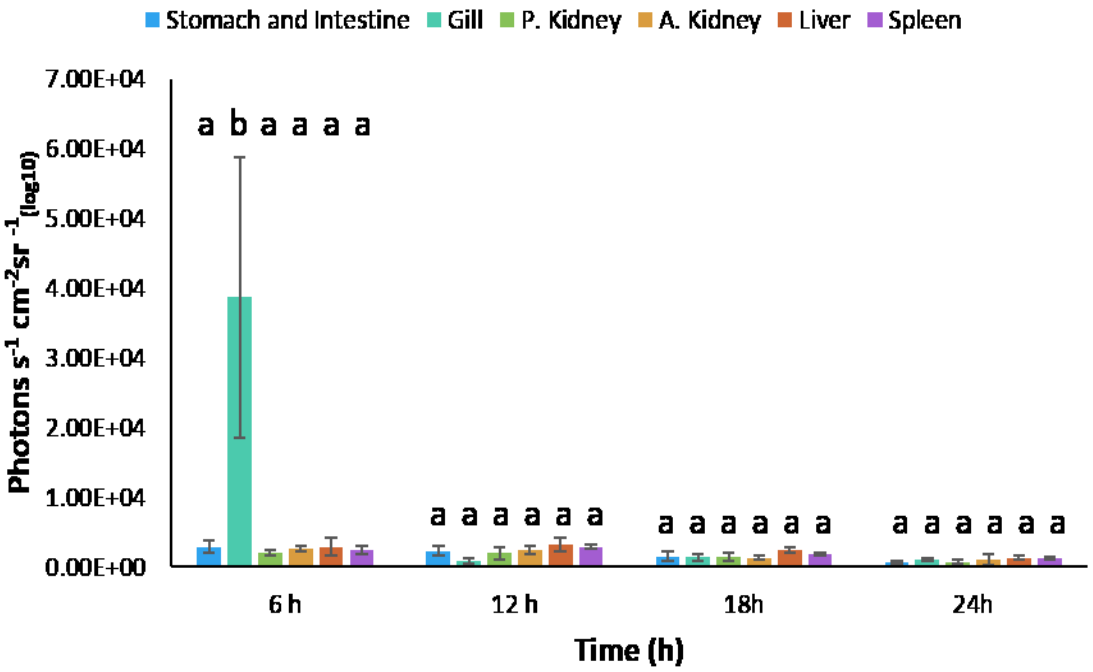

Figure 18 Detection of Ml-09-119 pAK*gfplux*3 in the internal organs of experimentally infected channel catfish.

BvAh colonies recovered from posterior kidney of infected fish were bioluminescent (Fig. 19, Fig. 20, Fig. 21). In addition, similar bacterial load was detected in posterior kidney at different time points following injection, immersion and adipose fin clipping.

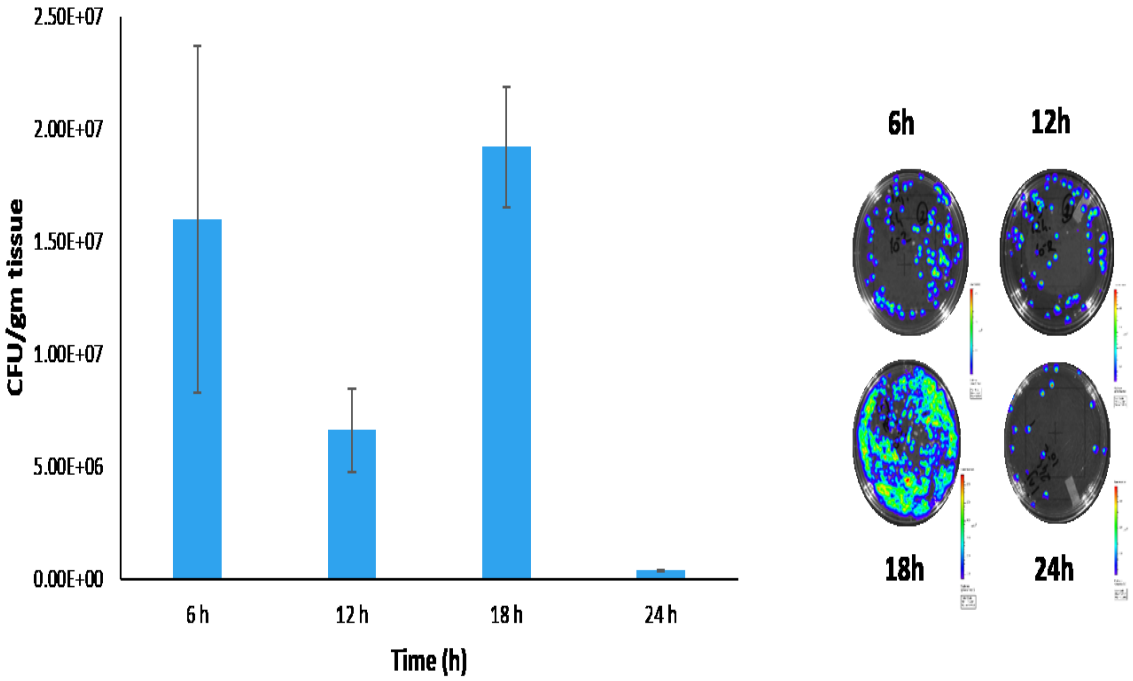

Figure 19 Bacterial load in posterior kidney and BvAh colonies from posterior kidney with injection challenge

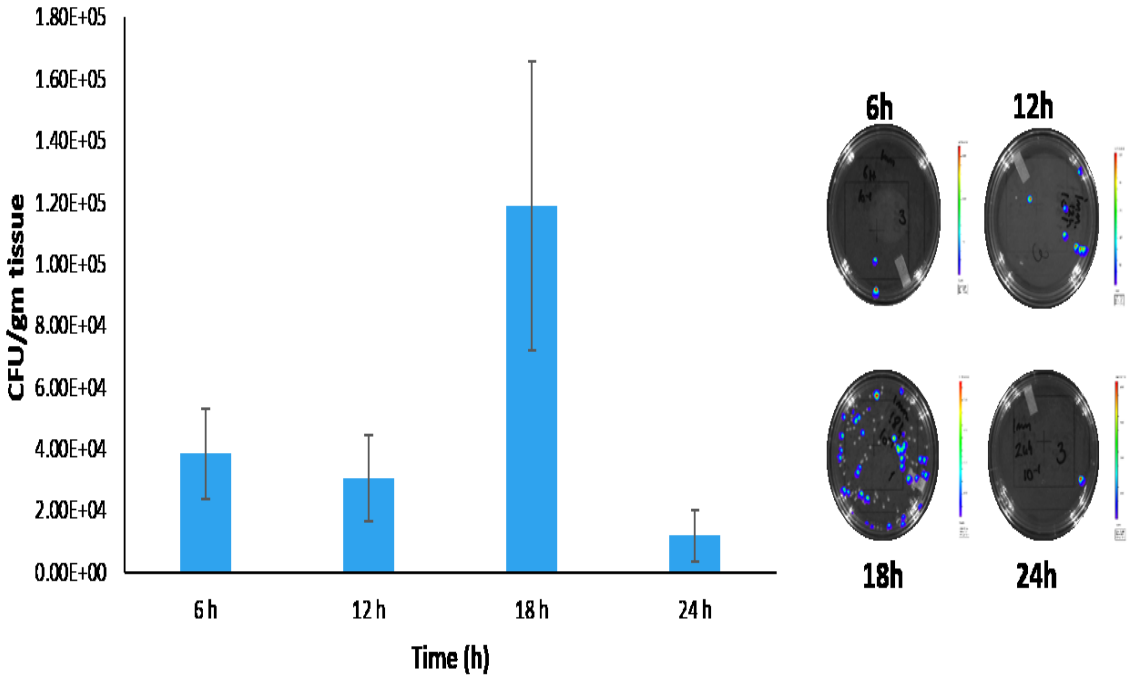

Figure 20 Bacterial load in posterior kidney and BvAh colonies from posterior kidney with immersion challenge

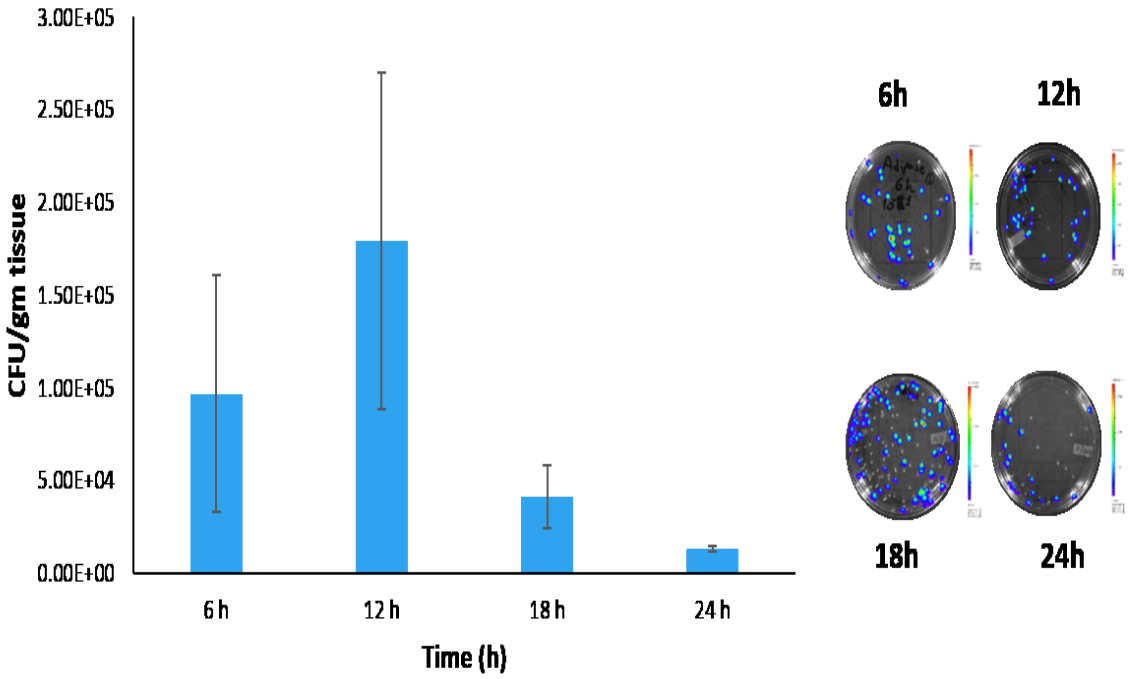

Figure 21 Bacterial load in posterior kidney and BvAh colonies from posterior kidney with adipose fin-clipped challenge

Discussion

The aim of our study was to develop a bioluminescent virulent *Aeromonas hydrophila* (BvAh) and understand more about its pathogenic mechanisms. A BvAh strain was developed successfully. Because vAh did not have any native plasmids, plasmid isolation yielded only the bioluminescence expression plasmid (pAK*gfplux*3). Later, we found out that this plasmid was not very stable in the vAh. Similar findings have been observed in *A. salmonicida* (Bartkova et al., 2017). In their work, pAK*gfplux*1 was used. This plasmid and pAK*gfplux*3 are exactly the same except that pAK*gfplux*3 has an additional chloramphenicol resistance gene because vAh are resistant to ampicillin.

Other studies indicated better stability of this broad host range plasmid in different species (Karsi et al., 2006).

Previous studies used intraperitoneal (IP) injection to determine the virulence of *A. hydrophila* (Pridgeon and Klesius, 2011; Zhang et al., 2014). In this study, we utilized injection, immersion, and adipose fin clipping-immersion methods. In the aquatic environment, immersion mimics natural infection (Ventura and Grizzle, 1988). According to other related studies, even small injuries can cause high susceptibility in fish (Bader et al., 2006).

In the current study, bioluminescence was tracked in live fish. Barbels, for the first time, were detected as an attachment site. In general, bacterial attachment is observed in the fish body where skin is damaged or epithelia is eroded (Bartkova et al., 2017). The adipose fin is not considered to have an essential function (Vander Haegen et al., 2005), but it functions as a precaudal flow sensor (Temple and Reimchen, 2008). It was found that clipping of adipose fin increased catfish susceptibility to vAh (Temple and Reimchen, 2008). This supports that integrity of fish skin is critical for fish health.

There were slight differences in bioluminescence patterns observed in different challenge models. BLI revealed that the infection progressed slower in immersion challenge compared to injection and adipose fin clip methods. vAh caused 90% mortality within 48 h post challenge in experimental infection models (Abdelhamed et al., 2017; Zhang et al., 2016). Similarly, approximately 90% mortality was observed in immersion challenge in the current study.

In previous studies, effect of *A. hydrophila* was revealed on the stomach in Nile tilapia (Yardimci and Aydin, 2011) and channel catfish (Abdelhamed et al., 2017) after

the infection. In the current study, high bioluminescence signal was tracked in the stomach in early time points with the injection challenge method, which is probably due to accumulation of bacteria around the blood vessels of gastric gland. Bacterial toxins could damage the endothelial lining of blood vessel, increasing permeability of interstitial tissue (Abdelhamed et al., 2017).

In a previous study, high numbers of necrotic phagocytic cells with many degenerated *A. salmonicida* and *A. hydrophila* were detected in the hematopoietic tissue of *Channa punctatus* (Ghosh and Homechaudhuri, 2012). In the current study, a high number of bacteria in anterior kidney, posterior kidney, liver, and spleen were detected at 18 h in injection and immersion challenges.

CHAPTER III

CONCLUSIONS

Virulent *A. hydrophila* (vAh) was able to express the bacterial lux genes located in pAK*gfplux*3 and emit strong bioluminescence under selective pressure by chloramphenicol. The plasmid is not very stable in vAh without selective pressure. Thus, a more stable bioluminescence expression system may be needed. Presence of pAK*gfplux*3 in vAh did not show any adverse effect on vAh growth. Fish challenges indicated that photons emitted by BvAh were able to pass through the fish tissues and were readily detectable.

There were slight differences in bioluminescence patterns observed in different challenge models. BLI revealed that the infection progressed slower in immersion challenge compared to injection and adipose fin clip methods. BLI indicated that BvAh was able to attach well to the fish body where skin integrity was broken. BLI showed increased bioluminescence in gills early in hours of immersion and fin clipping method. Bioluminescence also increased in GI track at later hours of immersion.

In conclusion, we developed a novel BvAh strain for the first time, and imaging of BvAh in live fish provided us with new insights on the pathogenesis of vAh. Infection progressed better in fish when protection of skin is bypassed. It is possible that amount of skin injuries in older fish is higher and thus the prevalence of MAS in larger fish.

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