Infection routes of Aeromonas salmonicida in rainbow trout monitored in vivo by real-time bioluminescence imaging

Bartkova, Simona; Kokotovic, Branko; Dalsgaard, Inger

Published in:
Journal of Fish Diseases

Link to article, DOI:
10.1111/jfd.12491

Publication date:
2017

Document Version
Publisher's PDF, also known as Version of record

Journal of Fish Diseases 2017, **40**, 73–82 doi:10.1111/jfd.12491

Infection routes of *Aeromonas salmonicida* in rainbow trout monitored *in vivo* by real-time bioluminescence imaging

S Bartkova, B Kokotovic and I Dalsgaard

Section for Bacteriology and Pathology, National Veterinary Institute, Technical University of Denmark, Frederiksberg C, Denmark

Abstract

Recent development of imaging tools has facilitated studies of pathogen infections *in vivo* in real time. This trend can be exemplified by advances in bioluminescence imaging (BLI), an approach that helps to visualize dissemination of pathogens within the same animal over several time points. Here, we employ bacterial BLI for examining routes of entry and spread of *Aeromonas salmonicida* susbp. *salmonicida* in rainbow trout. A virulent Danish *A. salmonicida* strain was tagged with pAK*gfplux*1, a dual-labelled plasmid vector containing the mutated *gfpmut3a* gene from *Aequorea victoria* and the *luxCDABE* genes from the bacterium *Photorhabdus luminescens*. The resulting *A. salmonicida* transformant exhibited growth properties and virulence identical to the wild-type *A. salmonicida*, which made it suitable for an experimental infection, mimicking natural conditions. Fish were infected with pAK*gfplux*1 tagged *A. salmonicida* via immersion bath. Colonization and subsequent tissue dissemination was followed over a 24-h period using the IVIS spectrum imaging workstation. Results suggest the pathogen's colonization sites are the dorsal and pectoral fin and the gills, followed by a progression through the internal organs and an ensuing exit via the anal opening. This study provides a tool for visualizing colonization of *A. salmonicida* and other bacterial pathogens in fish.

Correspondence *S Bartkova, Section for Bacteriology and Pathology, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C, Denmark (e-mail: sibar@vet.dtu.dk)*

Keywords: Aeromonas salmonicida, bioluminescence imaging, furunculosis, *luxCDABE* genes, rainbow trout.

Introduction

Aeromonas salmonicida susbp. *salmonicida*, the causative agent of the disease furunculosis, is one of the major bacterial pathogens in aquaculture throughout the world. Furunculosis is a septicemic infection that was first described in Denmark in freshwater rainbow trout (*Oncorhynchus mykiss*) farms during the 1950s (Rasmussen 1964). Currently, furunculosis causes the greatest problems in Danish rainbow trout production in sea water, where outbreaks occur during stress-associated periods with elevated temperatures around 20 °C in July and August (Larsen & Mellergaard 1981; Dalsgaard & Madsen 2000; Pedersen *et al.* 2008). This indicates a possibility of *A. salmonicida* being spread from freshwater into the sea via rainbow trout carriers (Dalsgaard & Madsen 2000). However, attempts to isolate bacteria from these presumed carrier fish have not been successful so far (Dalsgaard & Madsen 2000). Consequently, discerning the sites of entry and dissemination pattern of *A. salmonicida* within fish could be an important factor for optimizing future sampling procedures and detection of the bacterium.

Given the advances in the field of *in vivo* imaging in recent years, real-time monitoring of pathogens with various fluorescence and luciferase protein reporters has emerged as a promising strategy for following the pathogens' dissemination

Journal of Fish Diseases

within their hosts (Contag *et al.* 1995; Rocchetta *et al.* 2001; Karsi, Menanteau-Ledouble & Lawrence 2006; Karsi & Lawrence 2007; Zinn *et al.* 2008; Menanteau-Ledouble, Karsi & Lawrence 2011). In comparison with fluorescence reporters, luciferase proteins that exhibit bioluminescence show higher sensitivity, lower toxicity and faster response to changing environments (Burns *et al.* 2001; Troy *et al.* 2004). Moreover, there is scarcely any autoluminescence (bioluminescence background noise) emitted by animal tissues, as opposed to other light sources where the background disturbance emitted by tissues usually affects the light-emitting reporters' sensitivity and overall applicability (Troy *et al.* 2004).

Bioluminescence imaging (BLI) has been applied for monitoring *A. salmonicida* in dead Atlantic salmon (*Salmo salar*) (Ferguson *et al.* 1998). However, requirement for an exogenous addition of aldehyde for BLI visualization and incorporation of luciferase genes into *A. salmonicida* significantly lowered the virulence of the bacterium (Ferguson *et al.* 1998). First application of BLI for real-time monitoring of bacteria in live fish was reported by Karsi *et al.* (2006) who used the method to investigate dissemination of *Edwardsiella ictaluri* inside channel catfish (*Ictalurus punctatus*). Recently, Méndez & Guijarro (2013) used BLI to trace dissemination of *Yersinia ruckeri* in rainbow trout. Both studies used a bacterial luciferase operon from *Photorhabdus luminescens* consisting of five genes (*luxCDABE*) encoding the luciferase and fatty acid reductase enzyme complex, enabling emission of luminescence without the addition of any cofactors or exogenous substrates (Meighen 1993; Burns *et al.* 2001; Troy *et al.* 2004).

The aim of this study was to explore the routes of entry and subsequent tissue dissemination of *A. salmonicida* using *in vivo* imaging and luciferase coding operon (Karsi *et al.* 2006; Méndez & Guijarro 2013). In short, a virulent *A. salmonicida* was tagged with a dual-labelled reporter plasmid containing a mutated green fluorescence protein (GFP) gene and the BLI coding genes (*luxCDABE*) under the same *lacZ* promoter that requires only oxygen for constitutive expression of both protein reporters (Karsi & Lawrence 2007). Expression of GFP provided a marker for verifying the presence of plasmid in the tagged bacterium and proper function of the *lacZ* promoter, while expression of BLI provided a marker for *in vivo* monitoring of a bacterium in the host after experimental infection.

Materials and methods

Bacterial strains and plasmid transfer

Aeromonas salmonicida subsp. *salmonicida* 090710-1/23 (further referred to as *A. salmonicida* WT) is a virulent strain from a disease outbreak in Denmark. This strain has been used several times for various infection experiments in our and other Danish laboratories (e.g. Chettri *et al.* 2015). *Escherichia coli* B/K 12 (Addgene) is a donor strain that has an incorporated plasmid vector pAK*gfplux*1 consisting of the plasmid pBBR1MCS4 with an inserted: (i) *gfpmut3a* mutant gene from *Aequorea Victoria*, (ii) *luxCDABE* operon from *P. luminescens* and (iii) an ampicillin resistance gene. The pAK*gfplux*1 plasmid was first isolated from the *E. coli* B/K 12 donor strain by QIAprep Spin Miniprep Kit (Qiagen), according to the manufactures instructions. The plasmid was then transformed into *A. salmonicida* WT by conjugal mating using a natural kanamycin-resistant *E. coli* strain SM10 λpir (Biomedal) as described by Karsi & Lawrence (2007).

In detail, natural kanamycin-resistant SM10 λpir was transformed with pAK*gfplux*1 plasmid carrying ampicillin resistance by electroporation using a Gene Pulser instrument (Bio-Rad) at 25 μFD, 200 Ω, 1.8 kV and with a time constant (tau value) of 5 ms. SM10 λpir ampicillin and kanamycin-resistant colonies were grown overnight in 2 mL of Luria–Bertani (LB) medium (Difco) at 37 °C with shaking at 225 rpm. The recipient was grown separately for 48 h in veal infusion broth (VIB) (Difco) at 20 °C. Subsequently, 750 μL of the donor *E. coli* SM10 λpir and 1.5 mL of recipient culture were centrifuged separately at 8700 ***g*** for 2 min and the supernatant was removed. Collected bacteria were then washed by resuspending the pellets in 750 μL of brain–heart infusion (BHI) (Difco). Washing procedure was repeated three times to remove antibiotics used during broth culture. In 1.5-mL centrifuge tubes, 50 μL of donor and 100 μL of recipient washed cells were mixed and bacteria were centrifuged as previously. Supernatant was poured off, and donor and host mixture was suspended in 5–10 μL of BHI.

Punched and sterilized 0.45-μM filters (Life GE Healthcare Life Sciences) were placed on blood agar plates [Columbia agar base (Oxoid) with 5% calf blood] without antibiotics, and 5 μL of bacterial mixture was transferred on the filter as a spot. Plates were returned to incubator set to suitable temperature for the recipient (20 °C) and conjugation continued for 48 h. At the end of the conjugation period, filters were dropped into sterile 1.5-mL Eppendorf tubes and bacteria were washed away by adding 750 μL of VIB with 100 μg mL^{-1} ampicillin (Sigma-Aldrich). Ten microlitres of bacterial suspension was mixed with 990 μL of ddH_2O, and 25 μL of the mixture was spread onto selective blood agar plates containing 100 μg mL^{-1} ampicillin and incubated for 48 h at 20 °C. Incubation temperature 20 °C ensured optimal growth of *A. salmonicida* and ampicillin selected for *A. salmonicida* with incorporated pAK*gfplux*1 (further referred to as *A. salmonicida gfplux*). *A. salmonicida gfplux* colonies showing the strongest fluorescence under an Axio imager M1 (Zeiss) were transferred into 5 mL VIB and incubated for 48 h at 20 °C. The bacteria were subcultivated two times under these conditions and then two times using blood agar plates without ampicillin, from which a single *A. salmonicida gfplux* colony was selected for storage in glycerol stocks (600 μL of 50% glycerol with 900 μL of the 48-h grown bacterial culture in VIB) at −80 °C until further use.

Experimental fish

Fertilized eggs of rainbow trout from Fousing Trout Farm were brought to our institute, where disinfection, hatching and rearing were carried out under pathogen-free conditions. Fish were held at 10 ± 1 °C in 180-L tanks containing a flow-through system with non-chlorinated tap water and air supply. Fish were fed dry commercial feed (Inicio Plus; BioMar A/S) at 1% of biomass per day. Average weight and length of rainbow trout used for all challenges were 8.8 ± 2.7 g and 9.3 ± 1.7 cm. Experimental infections were carried out in accordance with the accepted guidelines for the care and use of laboratory animals in research and with regulations set forward by the Danish Ministry of Justice and Animal Protection committees by Danish Animal Experiments Inspectorate permit number 2013-15-2934-00976.

Sensitivity of IVIS for detecting *Aeromonas salmonicida gfplux*

To determine the threshold detection limit for visualization of *A. salmonicida gfplux* using an IVIS spectrum imaging workstation (PerkinElmer) with an exposure time of 30 s, four separate two-fold serial dilutions in 0.9% saline solution ranging from 8×10^4 to 2×10^2 colony-forming units (CFU) mL^{-1} of the bacterium grown in VIB for 48 h at 20 °C were made in a black 96-well microtiter plate (Thermo Scientific) using a volume of 0.1 mL per well. To measure background noise (autoluminescence), aliquots of 0.1 mL 0.9% saline solution were also added to four wells as controls. The plate was scanned for 30 s. Relative intensity of luminescence emission for each well was estimated by IVIS software and represented with a pseudo-colour scale of counts s^{-1}. Each dilution series was then cultivated on blood agar plates to confirm CFU mL^{-1}, and after 48-h growth at 20 °C, all plates were visualized using an Axio imager M1 to observe fluorescence emitted from the bacterial colonies. Mean autoluminescence was subtracted from luminescence values of all *A. salmonicida gfplux* wells, and correlation between CFU mL^{-1} and relative intensity of luminescence emission was determined.

Plasmid stability and effect of transformation on bacterial growth *in vitro*

Plasmid stability was previously investigated by Karsi *et al.* (2006) and Karsi & Lawrence (2007). Results from these studies suggested that the broad host range vector employed in this study can be transferred and stably maintained in Gram-negative bacteria.

To test whether the introduction of plasmid affected growth properties of the wild-type strain, bacterial growth of *A. salmonicida* WT and *A. salmonicida gfplux* was compared (i) indirectly using procedure by Karsi *et al.* (2006) for measuring optical density of bacterial culture and (ii) directly by plating serial dilutions onto blood agar to obtain CFU mL^{-1}. Readings of OD_{625} were analysed by a Student's paired *t*-test using the Microsoft Excel statistical package. After the 48-h period, a serial dilution was made from each bacterial culture and plated on blood agar to determine CFU mL^{-1} and fluorescence emitted from

the bacterial colonies was observed using an Axio imager M1. Determined CFU mL^{-1} was compared by a Student's *t*-test analysis using Microsoft Excel. The experiment was repeated three times.

Effect of transformation on *in vivo* virulence

In vivo virulence of *A. salmonicida* WT and *A. salmonicida gfplux* was compared by determination of CFU required to infect 50% of the fish population. Bacteria were grown in VIB for 48 h at 20 °C. Subsequently, ten-fold serial dilutions of bacteria, containing 10^8–10^4 CFU mL^{-1}, were made in 0.9% saline solution. Each dilution was then used for inoculation of six fish. Each fish was inoculated intraperitoneally with 0.1 mL of the corresponding dilution, so the infection doses administrated ranged from 10^7 to 10^3 CFU. One control group with six fish was injected with 0.1-mL sterile veal infusion broth, and one control group with six fish was left uninjected. Fish were held at 10 ± 1 °C in 10-L tanks containing a flow-through system with non-chlorinated tap water and air supply. Fish were observed several times per day and moribund fish were killed by immersion in 250 mg L^{-1} of 3-aminobenzoic acid ethyl ester (MS-222) (Sigma-Aldrich) until it was certain that swimming and gill movement had ceased. After 2 weeks, the experiment was terminated. In order to confirm bacterial infection and to estimate plasmid stability during infection of the host, spleen, kidney and brain specimens were cultivated on blood agar and LB agar containing 100 μg mL^{-1} ampicillin. Fluorescence emission in the isolated bacteria was observed using an Axio imager M1. Calculation of ID_{50} was done according to Reed & Muench (1938), and Student's *t*-test in Microsoft Excel was used to assess significance.

Experimental set-up for real-time monitoring *Aeromonas salmonicida gfplux in vivo*

Two fish were experimentally infected with *A. salmonicida gfplux* by separate immersion in two well-aerated 19 ± 1 °C 10-L tanks containing three litres of 5×10^7 CFU mL^{-1} *A. salmonicida gfplux* for 2 h. For visualization, each fish was anesthetized with MS-222, immersed twice in distilled water and dipped on a paper towel before finally being laid on a Tissue Culture Dish (Greiner Bio-One) and scanned in the IVIS for 30 s to estimate relative intensity of luminescence emission (counts s^{-1}). After scanning, each fish was placed in a new well-aerated 10-L tank containing three litres of distilled water for recovery. Visualization was performed at the following time points: 2, 4, 6 and 24 h after immersion in the infection bath. Fish were killed before the last visualization time point (24 h). Scanning in the IVIS was done on the whole fish and for the last scanning also on fish that were cut open to expose internal organs. Finally, for bacteriology examination, kidney and spleen samples were taken from each fish and streaked on blood agar that was incubated for 48 h at 20 °C. Subsequently, all plates were examined in an Axio imager M1 microscope for fluorescence emission. One non-infected (control) fish was also scanned in the IVIS as whole and cut open for monitoring of autoluminescence (counts s^{-1}) emitted from different external as well as internal areas. Obtained autoluminescence was subtracted from all luminescence readings of infected fish in order to acquire the correct luminescence (counts s^{-1}) for each scanning, which could then be correlated to a CFU count and be represented with a pseudocolour scale. The experiment was repeated three times using one fish per tank and two times using two fish per tank. A total of fourteen infected fish and five non-infected control fish were examined.

Results

Detection limit of *Aeromonas salmonicida gfplux*

Visualization and measurement of the *A. salmonicida gfplux* twofold dilutions showed that for the exposure time of 30 s, the threshold detection limit for visualization of *A. salmonicida gfplux* appears to be 4×10^4 CFU mL^{-1}. Correlation between CFU mL^{-1} and measured relative intensity of luminescence emissions was linear ($R^2 = 0.977$) over the range of 8×10^4–2×10^2 CFU mL^{-1} (Fig. 1), indicating luminescence emission should present the accurate CFU values for a given sample. Average per cent of *A. salmonicida gfplux* colonies emitting fluorescence on a blood agar plate was 95% (data not shown).

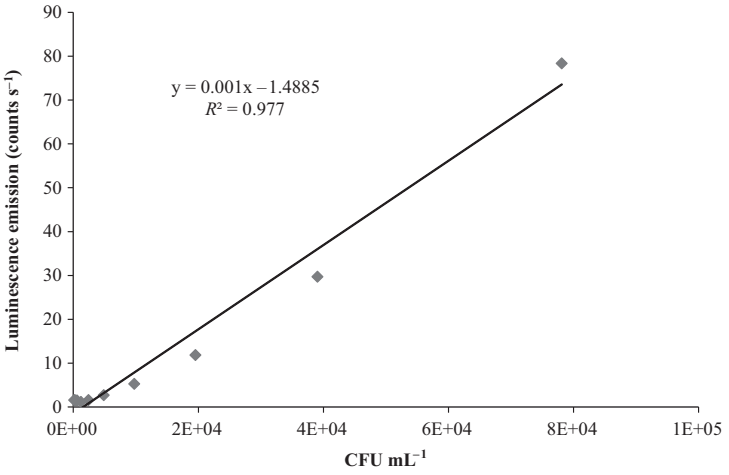

Figure 1 Correlation between measured relative intensity of luminescence emission (counts s^{-1}) and CFU mL^{-1} for *Aeromonas salmonicida gfplux* serial dilutions ranging from 8×10^4 to 2×10^2 CFU mL^{-1} after being scanned for 30 s in an IVIS spectrum imaging workstation. Correlation between CFU mL^{-1} and relative intensity of luminescence emission for *A. salmonicida gfplux* was determined to be linear ($R^2 = 0.977$) over the range of 8×10^4–2×10^2 CFU mL^{-1}. The correlation coefficient, slope and intercept of the linear regression curve are shown.

Effect of transformation on bacterial growth *in vitro*

No significant difference was observed between *A. salmonicida* WT and *A. salmonicida gfplux* growth as measured by the indirect method of OD_{625} when analysed with a Student's paired *t*-test ($P > 0.05$). Bacterial CFU mL^{-1} after 48 h of growth at 20 °C for each experiment was analysed by a Student's *t*-test and showed no significant difference ($P > 0.05$) in all experimental repeats (Table 1). Average per cent of *A. salmonicida gfplux* colonies emitting fluorescence on a blood agar plate in all experimental repeats was 96%, while no fluorescence emission was observed in any *A. salmonicida* WT colonies (data not shown).

Effect of transformation on *in vivo* virulence

The ID_{50} value for *A. salmonicida* WT was 5×10^5 CFU and for *A. salmonicida gfplux* 6×10^5 CFU. Insertion of pAK*gfplux*1 into *A. salmonicida* WT did not seem to affect *in vivo* virulence of the bacterium. *A. salmonicida gfplux* colonies were re-isolated from all three sampled organs, that is kidney, spleen and brain in killed fish. The number of colonies emitting fluorescence on blood agar plates gradually decreased over the course of the experiment, and after 10 days, fluorescence emission could not be detected any longer (Table 2). Colonies grown on LB agar with ampicillin showed consistently strong fluorescence, but their number rapidly decreased. From day 5 of the experiment, only few colonies grew on LB agar, and after the day 8, no growth was recorded (Table 2).

Table 1 Bacterial CFU mL^{-1} comparison of wild-type *Aeromonas salmonicida* WT and *A. salmonicida gfplux* after 48-h growth at 20 °C, including *P*-values of a Student's *t*-test, for three experimental repeats

Experiment	Strain	$\log_{10}$ CFU mL^{-1} ± SD	*P*-value
1	*A. salmonicida* WT	8.85 ± 0.08	0.972
	A. salmonicida gfplux	8.85 ± 0.10	
2	*A. salmonicida* WT	8.51 ± 0.12	0.393
	A. salmonicida gfplux	8.45 ± 0.06	
3	*A. salmonicida* WT	8.69 ± 0.06	0.105
	A. salmonicida gfplux	8.80 ± 0.10	

Experimental infection for real-time monitoring *Aeromonas salmonicida gfplux in vivo*

For the experimental infection, luminescence signal from *A. salmonicida gfplux* was observed in overall twelve of the fourteen examined fish following a 2-h immersion time (Table 3). At the 2-h time point, a luminescence signal was detected on eight of the twelve positive fish. Bacteria were visualized on the following sites: the dorsal, pectoral, caudal and anal fin, anal opening, gills, oral and nasal cavity and eyes (Table 3; Fig. 2).

For the 4- and 6-h time points, luminescence emission could be seen in three fish (Table 3). In two fish, the gills where observed luminescence was found at the 2-h time point were still emitting luminescence at the 4-h time point. Luminescence was also observed in the body organ area in

Table 2 Per cent *Aeromonas salmonicida gfplux* colonies emitting fluorescence on blood agar plates and Luria–Bertani (LB) plates with 100 μg mL^{-1} ampicillin. Colonies were re-isolated on agar plates from the kidney, spleen and brain in fish during the ID_{50} experiment where fish were injected with different dilutions of *A. salmonicida gfplux* culture

Blood agar plates		LB plates with 100 μg mL^{-1} ampicillin	
Colonies emitting fluorescence (%)[a]	Period (day)	Colonies emitting fluorescence[a]	Period (day)
30–60	3–4	100%	3–4
10–30	5–8	100%[b]	5–8
<10	9–10	NA[c]	9–10
0	11–14	NA[c]	11–14

[a]Average per cent of fluorescence emitting colonies on a plate, isolated from each organ, that is kidney, spleen and the brain.
[b]There were very few colonies on the LB plates compared to the number of colonies on the respective blood agar plates.
[c]There was no growth on the plates.

one fish at the 6-h time point, which was presumed to be emitted from the inside of the fish.

After 24 h, luminescence was found in four of the total of twelve positive fish (Table 3). Among the ten fish where the luminescent bacteria were found, in three fish the luminescence signal was strong enough to be seen through the skin in whole fish (Table 3; Fig. 3). In one of the three fish, the luminescence signal was located around the anal opening and after being cut open; the signal was still located around that area and the lower intestine (Fig. 3). The seven remaining fish needed to be cut open in order to be able to detect a luminescence signal. In all ten positive fish at the 24-h time point, the signal was located in the intestine (Table 3). In one fish, the signal was also located in the stomach (Table 3; Fig. 3). In two fish, the signal seemed to be located both in the intestine and in spleen. Bacteriological examination of kidney and spleen samples was positive for *A. salmonicida gfplux* in all fourteen fish. However, very faint or no fluorescence emission was observed from these colonies (data not shown). In all five non-infected control fish, no bacteria were isolated and measured autoluminescence emission was scarce compared to measured luminescence emission from infected fish (negative data not shown).

Discussion

Experimental infections with *A. salmonicida gfplux* provided an indication of potentially important

Table 3 Overview of results from the experimental infection. A total of 14 fish infected with *Aeromonas salmonicida gfplux* were scanned for 30 s in an IVIS spectrum imaging workstation for detection of luminescence emission at four time points: 2, 4, 6 and 24 h post-infection in five independent experiments. Fish from the same experiment are grouped together and given an identification number. All areas of the fish where luminescence was observed at least once are displayed on the left. Observed luminescence signal is presented with an 'X'. No luminescence signal was observed from fish 1.1 and 2.3

		Fish #													
Time point (h)	Area	1.1	2.1	1.2	2.2	1.3	2.3	1.4a	1.4b	2.4a	2.4b	1.5a	1.5b	2.5a	2.5b
2	Caudal fin		X												
	Anal fin											X			
	Dorsal fin		X		X			X				X	X	X	
	Pectoral fin		X	X	X								X		
	Anal opening							X							
	Gills		X		X			X	X						
	Nasal cavity		X										X		
	Oral cavity		X										X		
	Eyes		X												
4	Gills				X				X						
6	Inside of fish					X									
24 uncut	Anal opening							X							
	Inside of fish					X							X		X
24 organs	Stomach					X									
	Upper intestine				X	X				X			X	X	
	Middle intestine			X	X	X				X	X	X	X	X	X
	Lower intestine			X		X		X		X			X		X
	Spleen			X[a]	X[a]										

[a]In a few fish, organ structure at the 24-h time point was deteriorated, making it difficult to distinguish which organ the luminescence signal was coming from; however, it was believed that in two fish the luminescence signal could be emitted from the spleen.

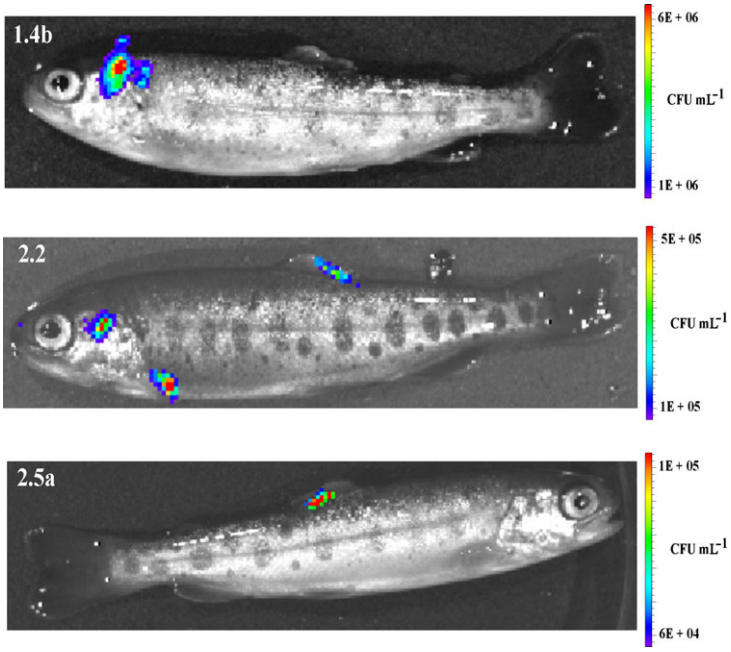

Figure 2 Three bioluminescence imaging illustrations from the 2-h time point of the experimental infection of rainbow trout with *Aeromonas salmonicida gfplux*. Illustrations show *A. salmonicida gfplux* colonization of the dorsal and pectoral fin and gills of the following three fish from Table 3: 1.4b, 2.2 and 2.5a.

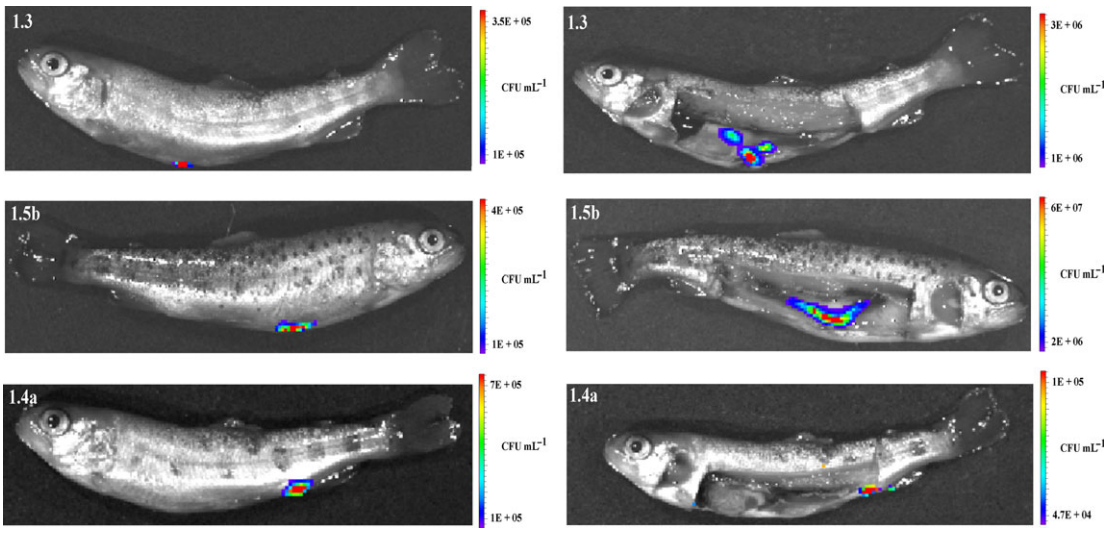

Figure 3 Six bioluminescence imaging illustrations from the 24-h time point of the experimental infection of rainbow trout with *Aeromonas salmonicida gfplux*. After 24 h, fish were killed and visualized in the IVIS as whole fish and were then cut open. In uncut fish 1.3, luminescence signal was observed in the body organ area and when cut open, the signal was observed in the stomach and upper, middle and lower intestine. In uncut fish 1.5b, luminescence signal was observed in the body organ area and when cut open, the signal was observed in the upper, middle and lower intestine. In uncut fish 1.4a, luminescence signal was observed around the anal opening and when cut open, the signal was observed around the anal opening and in the lower intestine.

colonization sites of *A. salmonicida*. However, colonization and dissemination of *A. salmonicida gfplux* in fish could only be visualized in twelve of the fourteen experimentally infected fish, and after 24 h, bacteria were only visualized in the digestive system, while bacteria were re-isolated from the spleen and kidney in all fourteen fish after 24 h. The lack of visualization in other organs could be due to the bacterial amount being below the threshold detection limit and/or a lack of plasmid stability. The later scenario is supported by the fact that the re-isolated bacteria on blood agar plates either emitted very little or no luminescence at all. Previous studies using plasmids with the

same luciferase coding operon as in this study did not report on any difficulties regarding plasmid stability within fish (Karsi *et al.* 2006; Menanteau-Ledouble *et al.* 2011; Méndez & Guijarro 2013). It is not possible to explain the reason(s) for the observed instability of the plasmid within fish in this study.

When comparing the threshold detection limit of BLI to previous studies, the limit of 4×10^4 CFU mL^{-1} in this study is higher than the 10^3 CFU mL^{-1} as reported by both Karsi *et al.* (2006) and Méndez & Guijarro (2013). The luminescence signal is proportional to exposure time, that is the duration a sample is scanned for (Caliper Life Sciences). In this study, fish were scanned for 30 s as oppose to 1 min in the previous studies (Karsi *et al.* 2006; Méndez & Guijarro 2013). We have chosen a shorter exposure time taking into the consideration: (i) uncertainty of the anaesthetic effectiveness over longer durations than 30 s, (ii) minimizing chance of obtaining false positives and (iii) the overall well-being of the fish.

In this study, fins were suggested as one of the key colonization sites of *A. salmonicida*. This results is in agreement with the findings by Hiney, Kilmartin & Smith (1994), who used ELISA to detect *A. salmonicida* in presmolt Atlantic salmon with stress-inducible furunculosis infections. Fins were also found as major colonization sites in other fish pathogens (Martinez, Casado & Enriquez 2004; Harmache *et al.* 2006; Menanteau-Ledouble *et al.* 2011). One reason why fins and especially the dorsal fin seem to be an important attachment site could be related to bite wounds (Jobling, Jørgensen & Christiansen 1993). Consistently, Svendsen & Bøgwald (1997) found *A. salmonicida* infected salmon with artificial wounds exhibiting higher mortality than infected salmon with no wounds. In contrast, fish used in our study did not have any injuries around the fins during the experiment.

No luminescence emission from the skin was observed in this study, and the role of skin as possible colonization site of *A. salmonicida* found in previous studies by Cipriano *et al.* (1992, 1994), Svendsen & Bøgwald (1997), Ferguson *et al.* (1998) could not be confirmed. Still, given the relatively high threshold limit of luminescence detection, an attachment of a low number of bacteria to the skin cannot be ruled out. An alternative explanation is that teleost (bony) fish feature variation in their immune system (Svendsen, Dalmo & Bøgwald 1999), including varying mucosal activity against pathogens (Dickerson 2009). Rainbow trout could thus have a better mucosal protection against pathogens compared to many other farmed fish species. This hypothesis is supported by other studies where rainbow trout showed the highest degree of resistance against furunculosis compared to other farmed fish species (Cipriano & Heartwell 1986).

A strong BLI signal was also seen around the gills, indicating that this might be an important colonization site as well. This finding is consistent with both Tatner, Johnson & Horne (1984) who investigated *A. salmonicida* infection in rainbow trout and with Svendsen *et al.* (1999) who studied *A. salmonicida* infection in Atlantic salmon. The gills also seem to be an important colonization site for other bacterial fish pathogens like *Y. ruckeri*, demonstrated by Ohtani *et al.* (2014) who observed infection of the gill epithelial cells as early as one minute post-infection.

Our study also indicates the oral and nasal cavity and the eyes might be colonization sites for *A. salmonicida*. These three sites are all 'open', that is lacking the primary barrier of the skin as a defence against pathogens (Roberts & Ellis 2012). The mouth has also been proposed as a possible entry route for *A. salmonicida* by Svendsen & Bøgwald (1997). Moreover, all three sites were seen to be probable colonization sites for *Novirhabdovirus* in a bioluminescence experiment conducted on juvenile trout (Harmache *et al.* 2006). Finally, Karsi *et al.* (2006) reported that bioluminescent *E. ictaluri* became visible around the eye and mouth area during early disease progression.

At the 4- and 6-h time points, luminescence was detected in only three of the infected fish, which could, at least in part, be due to the relatively low sensitivity of the method. In two fish, the gills that were found positive at the 2-h time point were still positive. A luminescence emission signal was observed at the 6-h time point at the location of the digestive system in one fish, which had not been detected at the 2- and 4-h time point. Due to the limitation of only being able to acquire two-dimensional images by this method, it was not possible to ascertain whether the luminescence signal came from the inside or the outside of the fish. Although it is likely that the signal came from inside the fish, because the fish had been transferred to clean water after the 2-h

infection immersion time and it would be expected that the bacteria can progress into the fish after a few hours as seen in the study with *Y. ruckeri* (Méndez & Guijarro 2013). In support of this, the luminescence signal intensified at the same location from the 6-h time point to the 24-h time point and when the abdominal area was opened revealing the internal organs, a luminescence signal was observed in the intestine (Table 3).

At the final 24-h time point, bacteria were seen by imaging in the digestive system and in two fish, also in the spleen. Bacteria were re-isolated from spleen and kidney in all fourteen fish. The reason why bacteria in the kidney and spleen were not detected by imaging could be that the bacteria had lost the pAK*gfplux*1 plasmid, as supported by the lack of fluorescence emission by the re-isolated colonies on blood agar plates, or the bacterial amount was below the threshold detection limit. Dissemination of *A. salmonicida* in the two organs is in agreement with Svendsen *et al.* (1999) who found *A. salmonicida* in blood already after 2 h post-infection and thereafter in kidney and a strong correlation between bacterial amounts in blood and gill tissue samples (Svendsen *et al.* 1999). It is known that bacteria spread from gills to the blood (Dickerson 2009), which is then filtered by the kidney and spleen (Tatner *et al.* 1984; Hadidi *et al.* 2008).

The imaging results at the 24-h time point revealed consistently signal from organs after dissection, indicating dissemination of *A. salmonicida* in fish through the digestive system and that this can happen after 24 h. This scenario is similar to the gut dissemination pattern seen in the study with *Y. ruckeri* (Méndez & Guijarro 2013). Radiolabelled *A. salmonicida* have also previously been found in tissue of the gut (Svendsen *et al.* 1999). In four fish, we did not find any luminescent bacteria by 24 h. However, at the 2-h time point, bacteria were seen in three of these fish, indicating that the initial bacteria that entered the digestive system could have passed through the fish in less than 24 h. This is in agreement with research on digestive responses to feed pellets in rainbow trout, where gastric evacuation was seen after only 4–6 h (Windell *et al.* 1969). Further investigations are needed to shed more light on the role of digestive system in dissemination of *A. salmonicida*.

In summary, our results indicate that the dorsal and pectoral fin and gills are important colonization sites for *A. salmonicida* in rainbow trout. Novel information regarding *A. salmonicida* tissue dissemination pattern was also revealed, including the possible significant role of the digestive system. The bioluminescence-based *A. salmonicida gfplux* model used in the present study provides a valuable tool for *in vivo* real-time imaging of *A. salmonicida* and studying host–pathogen interaction.

Acknowledgements

This work was supported by The Danish Council for Strategic Research under ProFish project (Grant no. DSF: 11-116252) and the National Veterinary Institute (DTU). The authors would like to thank all ProFish partners. Mark Lee Lawrence and Simon Menanteau-Ledouble are thanked for their advice on plasmid incorporation and bioluminescence experiments with fish. The authors are also grateful to Martin Weiss Nielsen, Mette Boye, Lisbeth Schade Hansen and Anastasia Isbrand at the National Veterinary Institute (DTU) for their technical support, as well as Kamilla Ellerman Jensen and Petra Hamerlik at the Danish Cancer Society for their collaboration and introduction to the IVIS spectrum imaging workstation.

References

Burns S.M., Joh D., Francis K.P., Shortliffe L.D., Gruber C.A., Contag P.R. & Contag C.H. (2001) Revealing the spatiotemporal patterns of bacterial infectious diseases using bioluminescent pathogens and whole body imaging. *Contributions to Microbiology* **9**, 71–88.

Caliper Life Sciences. *Living Image® Software User's Manual version 4.2*. URL: http://www2.udel.edu/ctcr/sites/udel.edu.ctcr/files/Living_Image_4-2_User_Guide.pdf (last accessed 4 March 2015)

Chettri J.K., Skov J., Jaafar R.M., Krossøy B., Kania P.W., Dalsgaard I. & Buchmann K. (2015) Comparative evaluation of infection methods and environmental factors on challenge success: *Aeromonas salmonicida* infection in vaccinated rainbow trout. *Fish & Shellfish Immunology* **44**, 485–495.

Cipriano R.C. & Heartwell C.M. (1986) Susceptibility of salmonids to furunculosis: differences between serum and mucus responses against *Aeromonas salmonicida*. *Transactions of the American Fisheries Society* **115**, 83–88.

Cipriano R.C., Ford L.A., Teska J.D. & Hale L.H. (1992) Detection of *Aeromonas salmonicida* in the mucus of salmonid fishes. *Journal of Aquatic Animal Health* **4**, 114–118.

Cipriano R.C., Ford L.A., Schachte J.H. & Petrie C. (1994) Evaluation of mucus as a valid site to isolate *Aeromonas*

salmonicida among asymptomatic populations of lake trout (*Salvelinus namaycush*). *Biomedical Letters* **49**, 229–233.

Contag C.H., Contag P.R., Mullins J.I., Spilman S.D., Stevenson D.K. & Benaron D.A. (1995) Photonic detection of bacterial pathogens in living hosts. *Molecular Microbiology* **18**, 593–603.

Dalsgaard I. & Madsen L. (2000) Bacterial pathogens in rainbow trout, *Oncorhynchus mykiss* (Walbaum), reared at Danish freshwater farms. *Journal of Fish Diseases* **23**, 199–209.

Dickerson H.W. (2009) The biology of teleost mucosal immunity. In: *Fish Defenses, Volume 2: Pathogens, Parasites and Predators* (ed. by Z. Giacomo, C. Perrière, A. Mathis & B.G. Kapoor), pp. 2–42. CRC Press, Published by Science Publishers, Enfield, NH, USA.

Ferguson Y., Bricknell I.R., Glover L.A., MacGregor D.M. & Prosser J.I. (1998) Colonisation and transmission of lux-marked and wild-type *Aeromonas salmonicida* strains in Atlantic salmon (*Salmo salar* L.). *Fems Microbiology Ecology* **27**, 251–260.

Hadidi S., Glenney G.W., Welch T.J., Silverstein J.T. & Wiens G.D. (2008) Spleen size predicts resistance of rainbow trout to *Flavobacterium psychrophilum* challenge. *Journal of Immunology* **180**, 4156–4165.

Harmache A., LeBerre M., Droineau S., Giovannini M. & Brémont M. (2006) Bioluminescence imaging of live infected salmonids reveals that the fin bases are the major portal of entry for *Novirhabdovirus*. *Journal of Virology* **8**, 3655–3659.

Hiney M.P., Kilmartin J.J. & Smith P.R. (1994) Detection of *Aeromonas salmonicida* in atlantic salmon with asymptomatic furunculosis infections. *Diseases of Aquatic Organisms* **19**, 161–167.

Jobling M., Jørgensen E.H. & Christiansen J.S. (1993) Growth performance of salmonids exposed to different flow regimes. In: *Fish Farming Technology. Proceedings of the First International Conference of Fish Farming* (ed. by H. Reinertsen, L.A. Dahle, L. Jørgensen & K. Tvinnereim), pp. 301–302. A.A. Balkema, Rotterdam, Netherlands.

Karsi A. & Lawrence M.L. (2007) Broad host range fluorescence and bioluminescence expression vectors for Gram-negative bacteria. *Plasmid* **57**, 286–295.

Karsi A., Menanteau-Ledouble S. & Lawrence M.L. (2006) Development of bioluminescent *Edwardsiella ictaluri* for noninvasive disease monitoring. *FEMS Microbiology Letters* **260**, 216–223.

Larsen J.L. & Mellergaard S. (1981) Microbiological and hygienic problems in marine aquaculture: furunculosis and vibriosis in rainbow trout (*Salmo gairdneri*). *Bulletin of the European Association of Fish Pathologists* **1**, 29–32.

Martinez J.L., Casado A. & Enriquez R. (2004) Experimental infection of *Flavobacterium psychrophilum* in fins of Atlantic salmon *Salmo salar* revealed by scanning electron microscopy. *Diseases of Aquatic Organisms* **59**, 79–84.

Meighen E.A. (1993) Bacterial bioluminescence – organization, regulation, and application of the lux genes. *FASEB Journal* **7**, 1016–1022.

Menanteau-Ledouble S., Karsi A. & Lawrence M.L. (2011) Importance of skin abrasion as a primary site of adhesion for *Edwardsiella ictaluri* and impact on invasion and systematic infection in channel catfish *Ictalurus punctatus*. *Veterinary Microbiology* **148**, 425–430.

Méndez J. & Guijarro J.A. (2013) In vivo monitoring of *Yersinia ruckeri* in fish tissues: progression and virulence gene expression. *Environmental Microbiology Reports* **5**, 179–185.

Ohtani M., Villumsen K.R., Strøm H.K. & Raida M.K. (2014) 3D visualization of the initial *Yersinia ruckeri* infection route in rainbow trout (*Oncorhynchus mykiss*) by optical projection tomography. *PLoS One* **9**, e89672.

Pedersen K., Skall H.F., Lassen-Nielsen A.M., Nielsen T.F., Henriksen N.H. & Olesen N.J. (2008) Surveillance of health status on eight marine rainbow trout, *Oncorhynchus mykiss* (Walbaum), farms in Denmark in 2006. *Journal of Fish Diseases* **31**, 659–667.

Rasmussen C.J. (1964) Furunkulose hos damørreder (Furunculosis in farmed trout). *Scandinavian Journal of Veterinary Science* **16**, 463–472 (in Danish).

Reed L.J. & Muench H.H. (1938) A simple method of estimating fifty percent endpoints. *American Journal of Hygiene* **27**, 493–497.

Roberts R.J. & Ellis A.E. (2012) The anatomy and physiology of teleosts. In: *Fish Pathology* (ed. by R.J. Roberts), pp. 17–62. Wiley-Blackwell, UK.

Rocchetta H.L., Boylan C.J., Foley J.W., Iversen P.W., Letourneau D.L., McMillian C.L., Contag P.R., Jenkins D.E. & Parr T.R. (2001) Validation of a noninvasive, real-time imaging technology using bioluminescent *Escherichia coli* in the neutropenic mouse thigh model of infection. *Antimicrobial Agents and Chemotherapy* **45**, 129–137.

Svendsen Y.S. & Bøgwald J. (1997) Influence of artificial wound and non-intact mucus layer on mortality of Atlantic salmon (*Salmo salar* L.) following a bath challenge with *Vibrio anguillarum* and *Aeromonas salmonicida*. *Fish and Shellfish Immunology* **4**, 317–325.

Svendsen Y.S., Dalmo R.A. & Bøgwald J. (1999) Tissue localization of *Aeromonas salmonicida* in Atlantic salmon, *Salmo salar* L., following experimental challenge. *Journal of Fish Diseases* **22**, 125–131.

Tatner M.F., Johnson C.M. & Horne M.T. (1984) The tissue localization of *Aeromonas salmonicida* in rainbow trout, *Salmo gairdneri* Richardson, following three methods of administration. *Journal of Fish Biology* **25**, 95–108.

Troy T., Jekic-McMullen D., Sambucetti L. & Rice B. (2004) Quantitative comparison of the sensitivity of detection of fluorescent and bioluminescent reporters in animal models. *Molecular Imaging* **3**, 9–33.

Windell J.T., Norris D.O., Kitchell J.F. & Norris J.S. (1969) Digestive response of rainbow trout, *Salmo gairdneri*, to pellet diets. *Journal of the Fisheries Research Board of Canada* **26**, 1801–1812.

Zinn K.R., Chaudhuri T.R., Szafran A.A., O′Quinn D., Weaver C., Dugger K., Lamar D., Kesterson R.A., Wang X. & Frank S.J. (2008) Noninvasive bioluminescence imaging in small animals. *Institute for Laboratory Animal Research Journal* **49**, 103–115.

Received: 1 March 2016
Revision received: 30 March 2016
Accepted: 30 March 2016

