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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

Link back to DTU Orbit

Citation (APA):
https://doi.org/10.1111/jfd.12491

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

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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* subsp. *salmonicida* in rainbow trout. A virulent Danish *A. salmonicida* strain was tagged with pAKgfplux1, 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 pAKgfplux1 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.

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

Introduction

*Aeromonas salmonicida* subsp. *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.
within their hosts (Contag et al. 1995; Rochetta 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 pAKgfp-lux1 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 pAKgfp-lux1 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 pAKgfp-lux1 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 real 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 microliters of bacterial suspension was mixed with 990 µL of ddH₂O, 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 pAKgflux1 (further referred to as *A. salmonicida* gflux). *A. salmonicida* gflux 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* gflux 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* gflux**

To determine the threshold detection limit for visualization of *A. salmonicida* gflux 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 × 10⁴ to 2 × 10² 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* gflux 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* gflux 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 OD₆₂₅ 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 10⁸–10⁴ 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 10⁷ to 10³ 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 ID₅₀ 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 × 10⁷ 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 × 10⁴ CFU mL⁻¹. Correlation between CFU mL⁻¹ and measured relative intensity of luminescence emissions was linear (R² = 0.977) over the range of 8 × 10⁴–2 × 10⁷ 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).
Effect of transformation on bacterial growth

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$^{-1}$ and relative intensity of luminescence emission for *A. salmonicida* gfplux was determined to be linear ($R^2 = 0.977$) over the range of $8 \times 10^4$ – $2 \times 10^5$ CFU mL$^{-1}$. The correlation coefficient, slope and intercept of the linear regression curve are shown.

Effect of transformation on in vivo virulence

The ID$_{50}$ value for *A. salmonicida* WT was $5 \times 10^5$ CFU and for *A. salmonicida* gfplux $6 \times 10^5$ CFU. Insertion of pAKgfplux1 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).

Experimental infection for real-time monitoring

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

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**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 $8 \times 10^4$ to $2 \times 10^5$ 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 $8 \times 10^4$ – $2 \times 10^5$ CFU mL$^{-1}$. The correlation coefficient, slope and intercept of the linear regression curve are shown.

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**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.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Strain</th>
<th>log$_{10}$ CFU mL$^{-1}$ ± SD</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>A. salmonicida</em> WT</td>
<td>8.85 ± 0.08</td>
<td>0.972</td>
</tr>
<tr>
<td></td>
<td><em>A. salmonicida</em> gfplux</td>
<td>8.85 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>A. salmonicida</em> WT</td>
<td>8.51 ± 0.12</td>
<td>0.393</td>
</tr>
<tr>
<td></td>
<td><em>A. salmonicida</em> gfplux</td>
<td>8.45 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>A. salmonicida</em> WT</td>
<td>8.69 ± 0.06</td>
<td>0.105</td>
</tr>
<tr>
<td></td>
<td><em>A. salmonicida</em> gfplux</td>
<td>8.80 ± 0.10</td>
<td></td>
</tr>
</tbody>
</table>
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).

### Table 2

<table>
<thead>
<tr>
<th>Blood agar plates</th>
<th>Colonies emitting fluorescence (%)a</th>
<th>Period (day)</th>
<th>Colonies emitting fluorescencea</th>
<th>Period (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30–60</td>
<td>3–4</td>
<td>100%</td>
<td>3–4</td>
<td></td>
</tr>
<tr>
<td>10–30</td>
<td>5–8</td>
<td>100%</td>
<td>5–8</td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>9–10</td>
<td>NA</td>
<td>9–10</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>11–14</td>
<td>NA</td>
<td>11–14</td>
<td></td>
</tr>
</tbody>
</table>

aAverage per cent of fluorescence emitting colonies on a plate, isolated from each organ, that is kidney, spleen and the brain.

bThere were very few colonies on the LB plates compared to the number of colonies on the respective blood agar plates.

cThere was no growth on the plates.

### 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

<table>
<thead>
<tr>
<th>Fish #</th>
<th>Time point (h)</th>
<th>Area</th>
<th>1.1</th>
<th>1.2</th>
<th>1.3</th>
<th>1.4a</th>
<th>1.4b</th>
<th>2.1</th>
<th>2.2</th>
<th>2.3</th>
<th>2.4a</th>
<th>2.4b</th>
<th>1.5a</th>
<th>1.5b</th>
<th>2.5a</th>
<th>2.5b</th>
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<tbody>
<tr>
<td>2</td>
<td>2</td>
<td>Caudal fin</td>
<td>X</td>
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<td></td>
<td>Anal fin</td>
<td></td>
<td>X</td>
<td>X</td>
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<td></td>
<td></td>
<td>Dorsal fin</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>X</td>
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<td></td>
<td></td>
<td>Pectoral fin</td>
<td>X</td>
<td>X</td>
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<td></td>
<td></td>
<td>Anal opening</td>
<td>X</td>
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<td>X</td>
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<td>Gills</td>
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<td></td>
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<td>Nasal cavity</td>
<td>X</td>
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<td>Oral cavity</td>
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<td>4</td>
<td>6</td>
<td>Gills</td>
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<td>Inside of fish</td>
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<td>24</td>
<td>24 uncut</td>
<td>Anal opening</td>
<td>X</td>
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<td>X</td>
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aIn 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.
colonization sites of *A. salmonicida*. However, colon-
ization 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

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**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.
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 × 10⁴ CFU mL⁻¹ in this study is higher than the 10³ 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 (Martínez, Casado & Enríquez 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 (Johling, 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 pAKgfplux 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). Radio-labelled *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 Istrand 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.

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