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Atlantic salmon bath challenged with *Moritella viscosa* – Pathogen invasion and host response

M. Løvoll [a,*], C.R. Wiik-Nielsen [a], H.S. Tunsjø [b], D. Colquhoun [a], T. Lunder [c], H. Sørum [b], S. Grove [a]

[a] National Veterinary Institute, Department of Animal Health, P.O. Box 750 Sentrum, 0106 Oslo, Norway
[b] Department of Food Safety and Infection Biology, Norwegian School of Veterinary Science, P.O. Box 8146 Dep, 0033 Oslo, Norway
[c] ScanVacc AS, 2150 Årnes, Norway

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ABSTRACT

The Gram-negative bacterium *Moritella viscosa* is considered to be the main causative agent of winter ulcer, a disease that primarily affects salmonid fish in sea water during cold periods. The disease is initially characterised by localised swelling of the skin followed by development of lesions. To gain more knowledge of the role of *M. viscosa* in the pathogenesis of winter ulcer, 159 Atlantic salmon (80–110 g) were exposed to a bath challenge dose of 7×10^5 cfu ml^{-1} for 1 h at 8.9 °C. The first mortalities were registered two days post-challenge and the mortality rate increased rapidly. Multi-organ samples were taken throughout the challenge for culture, immunohistochemistry and PCR analysis.
Using real-time PCR, *M. viscosa* DNA was first detected in the gills of all fish examined 2, 6 and 12 h after challenge. From day 2, the bacterium was detected in the muscle/skin, head kidney, spleen and liver. This was in correlation with positive cultured samples and confirmed systemic infection. The early and consistent detection of *M. viscosa* DNA in gill samples, and less or not in muscle/skin or intestine, could suggest gills as a port of entry for the bacterium. Immunohistochemical analysis using a polyclonal antiserum against *M. viscosa* demonstrated generalised staining in the lumen of blood vessels and some positive mononuclear cells. The antigens recognised by the antiserum may have originated from extracellular bacterial products and be part of a bacterial invasion strategy. To better understand the immune response in salmon to *M. viscosa* infection, the expression profiles of the immune genes IL1β, C3, ISG15 and CD83 were studied. Increased expression of IL1β and C3 was not induced until day 7, which may suggest that *M. viscosa* might utilize escape mechanisms to evade the host's immune system by suppressing relevant immune responses.

1. Introduction

Moritella viscosa is considered as the main aetiological agent of winter ulcer, a disease that primarily affects farmed salmonid fish in seawater during cold periods. Clinical winter ulcer disease is, at least during advanced outbreaks, generally easily diagnosed. Apart from extensive ulceration, gill pallor and fin rot, severe internal pathology may be observed. Fish exhibiting extensive ulceration may survive for long periods of time and the disease represents a considerable animal welfare problem [1]. Fish appear to recover when the seawater temperature rises above 10–12 °C or water salinity falls below 12–15‰. Winter ulcer is currently the main bacterial infection in Norwegian aquaculture and has not been eliminated either by vaccination, antibiotics, or management measures. Nearly half of all current prescriptions for antibiotics in Norwegian aquaculture relate to control of winter ulcer disease, despite the fact that antimicrobial therapy does not effectively control mortalities associated with this disease [2,3]. Even though mortality is generally less than 10% during an outbreak, winter ulcer is responsible for major economical losses due to subsequent downgrading of fillet quality at slaughter.

In cross-species challenge experiments using *M. viscosa* isolated from diseased Atlantic salmon (*Salmo salar*), turbot (*Scophthalmus maximus*) and cod (*Gadus morhua*) were found to be susceptible to infection, while Atlantic halibut (*Hippoglossus hippoglossus*) was more resistant [4,5]. In addition to strains isolated from Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*), *M. viscosa* has also been isolated from farmed cod [6] and wild lumpsucker (*Cyclopterus lumpus*) [7]. It was long considered that *M. viscosa* was a homogenous species, but significant strain heterogeneity, both genetically and antigenically, has been recently demonstrated between strains isolated from Atlantic salmon, rainbow trout and

* Corresponding author. Tel.: +47 23216336; fax: +47 23216302.
E-mail address: marie.lovoll@vetinst.no (M. Løvoll).

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Atlantic cod [8]. The only commercially available winter ulcer vaccines are developed for vaccination of salmonids [9,10]. Although the efficacy of multivalent vaccines containing whole cell *M. viscosa* antigens has been improved [11], protection against winter ulcer is still inferior to that conferred against other bacterial diseases in Atlantic salmon.

The pathogenesis of winter ulcer is in general poorly described. For knowledge-based development of vaccines and vaccine strategies, this is a considerable disadvantage. In the present study we have tried to elucidate some central questions, such as, where does the bacterium gain access to the fish? Does it spread once inside, to generate a systemic infection? How does the fish elaborate an immune response? To answer these questions we performed a *M. viscosa* bath challenge of Atlantic salmon. Tissue and blood were sampled for bacteriological culturing, and tissue samples were dissected to localise and quantify the distribution of *M. viscosa* using immunohistochemistry and a recently published real-time PCR quantification method [12]. To gain some knowledge of the immune response in infected fish, we studied transcription of relevant genes of the innate immune system; interleukin 1β (IL1β) (pro-inflammatory cytokine) [13], complement component 3 (C3) (acute phase protein) [14], surface marker Cluster of Differentiation 83 (CD83) (differentiated dendritic cells) [15] and the 15 kDa Interferon Stimulated Gene (ISG15) (interferon induced cytokine) [16], and followed their profiles throughout the experiment.

2. Material and methods

2.1. Fish and holding conditions

Unvaccinated Atlantic salmon (*S. salar* L.) (80–110 g), were obtained from Sørsmolt AS (Sannidal, Norway). The fish were transported to the Norwegian Institute for Water Research's aquaculture station (Solbergstrand, Norway) in a transportation tank containing 800 l freshwater. The fish were adapted to sea water in two separate 1400 l tanks prior to the challenge. Tank 1 contained 60 control fish, tank 2 contained 159 fish. The fish were kept in seawater (salinity 31–35‰) at 8.9 °C, with an oxygen content maintained stable at 8.2 mg l^{-1}. Water quality was monitored during the experiment using WTW type pH/Cond 340i and oxi 340i (Christian Berner, Oslo, Norway). The experiment was approved by the National Committee of Ethics as required by Norwegian law.

2.2. Bacteria and challenge procedure

M. viscosa (NVI 96/09/1016, recently passaged through Atlantic salmon) was cultured in Brain Heart Infusion Broth (BHIB) containing 2% NaCl at 9 °C for 48 h with shaking. Following reduction of the water volume in the challenge tank to approximately 500 l, bacteria were added to a final concentration of 7×10^5 cfu ml^{-1}. The final bacterial suspension was controlled by colony counting following serial dilution in 2% saline and plating of 100 μl aliquots (in duplicate) on blood agar containing 2% NaCl (BA 2%) and incubation for 48 h at 15 °C. During the challenge the water flow was stopped, and the oxygen saturation level was maintained at 8.2 mg l^{-1}. As the study of ulcer healing was a secondary objective of the trial, the rapid mortality rate was decelerated by increasing the water temperature from 8.9 to 13.4 °C on day 4 and reducing the salinity from 35 to 10‰ on day 8. Control fish (untreated) were maintained under identical conditions. All fish were fed to appetite for the duration of the trial.

2.3. Sampling and culturing

The challenged fish and fish in the control group were observed for four weeks. Dead fish were removed from the tanks daily and samples from live fish were taken 0, 2, 6 and 12 h post-challenge followed by 1, 2, 4 and 7 days post-challenge. Parallel samples of control fish were taken. All fish were anaesthetized by immersion in 0.005% benzocaine/water before handling. Blood samples for serology were centrifuged at 3000 × *g* for 15 min at 4 °C and plasma was stored at −20 °C. Blood samples for nucleic acid extraction were directly frozen at −20 °C. Sampled fish were examined bacteriologically for the presence of *M. viscosa*, and other bacterial species. Blood, kidney and ulcer inocula were plated onto BA 2%. The plates were initially examined following incubation at 15 °C for 48 h, and after further 5 days of incubation. *M. viscosa* colonies were presumptively identified by colony viscosity, colony pigmentation and typical hemolysis. Samples from ulcer, skin/muscle, gills, head kidney, spleen and liver were dissected and stored on RNA*later* (Ambion, Applied Biosystems, Foster City, CA) at 4 °C for 24 h, then −20 °C until nucleic acid extraction. Corresponding tissue samples for immunohistochemistry were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for two days, transferred to 70% ethanol, dehydrated and embedded in paraffin wax.

2.4. ELISA

The enzyme linked immunosorbent assay (ELISA) used for determination of antibody responses was performed as described previously [17], with minor modifications. Briefly, wells were coated with 100 μl of sonicated whole cells of *M. viscosa* type strain (NCIMB 13584) (5 μg protein ml^{-1}). Sample plasma (1:200) was incubated at 4 °C overnight, followed by incubation with a monoclonal antibody against rainbow trout immunoglobulin (4C10:3) (reactive with Atlantic salmon immunoglobulin) [18] for 60 min at room temperature. The plates were incubated with a sheep anti-mouse Ig conjugated to peroxidase (NA 931, Amersham, UK) before adding the substrate, tetramethylbenzidine (8622, Merck, Darmstadt, Germany). After 10 min, the reaction was stopped by adding 5 M H_2SO_4. The absorbance of duplicate sample wells was read at 450 nm. A pooled plasma sample from 24 Atlantic salmon hyper-immunized with *M. viscosa* was run as positive control on each plate. Results for each sample were calculated as an OD ratio: mean of OD_{sample}/mean of $OD_{positive\ control}$. A pooled plasma sample of 44 unvaccinated Atlantic salmon served as negative control.

2.5. Immunohistochemistry

Sections (2–3 μm) of paraffin embedded tissue samples from both infected and non-infected fish were mounted on poly-L-lysine coated glass microscope slides, dewaxed and rehydrated. The sections were incubated in 5% bovine serum albumin (BSA) in Tris–HCl, pH 7.6 for 20 min prior to incubation with rabbit anti-*M. viscosa* (see below) for 30 min, followed by biotinylated goat anti-rabbit (DAKO EO432) for 30 min. Streptavidin–alkaline–phosphatase complex (GE Healthcare RPN 1234 V1) and FastRed TR/naphthol AS-MX (Sigma–Aldrich) were used for visualization, and the sections were counterstained with Mayers hematoxylin (Merck). Between each incubation step, the sections were washed three times in Tris–HCl, pH 7.6. If not specifically stated, incubations were carried out in a humidity chamber at room temperature. Antiserum was raised in rabbit against *M. viscosa* strain NVI88/478 [19]. Briefly, bacterial cells cultured on blood agar containing 2% NaCl at 15 °C for 24 h were formalin killed and washed in PBS, pH 7.3 and adjusted to an optical density (OD525) of 0.65. Two ml of this suspension was injected intravenously at day 1, 4 and 7, while