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Effect of vaccination against yersiniosis on the relative percent survival, bactericidal and lysozyme response of Atlantic salmon, *Salmo salar*

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ABSTRACT

The bacterium *Yersinia ruckeri* serovar O1b causes yersiniosis in Atlantic salmon, *Salmo salar*, in the southern hemisphere. Despite vaccination this disease has resulted in significant hatchery losses in the Tasmanian Atlantic salmon aquaculture industry. A poor response to vaccination in juveniles, 1–5 g, has lead to the investigation of the suitability of the current formalin killed whole-cell vaccine Yersinivac-B. In this study trypsin was added to the Yersinivac-B to expose the bacteria's protective O-antigen to make the vaccine more immunogenic. At six weeks post vaccination, the effect of Yersinivac-B and the novel trypsinated Yersinivac-B vaccine on body mucus lysozyme and mucus and serum bactericidal activity of fish was determined over a 48 h period following challenge with *Y. ruckeri*. Body and gill mucus lysozyme and mucus and serum bactericidal activity was also determined in surviving fish at 10 weeks post *Y. ruckeri* challenge. Following the challenge period of 14 days the trypsinated Yersinivac-B fish demonstrated a significantly higher percent survival compared to the Yersinivac-B and control unvaccinated fish. Body mucus lysozyme concentration was also significantly elevated at 8 h post challenge in the trypsinated Yersinivac-B fish compared to controls. This variable however appears unlikely to play a significant role in protection as positive bactericidal activity was not found in the mucus of any fish following challenge. Bactericidal activity was not observed in the serum or mucus of any challenge survivors. At 8 h post challenge the trypsinated Yersinivac-B fish demonstrated the highest serum bactericidal activity. However, the unvaccinated control fish also displayed positive serum bactericidal activity despite being unlikely to have been previously exposed to *Y. ruckeri*. A significantly higher gill mucus lysozyme concentration in control survivors compared to vaccinated fish suggests that this response may be important in the protection of unvaccinated fish against yersiniosis. This research has highlighted the potential use of trypsin to increase the efficacy of Yersinivac-B. It has also contributed to better understanding of the role of humoral immune responses during a *Y. ruckeri* challenge.

1. Introduction

The Gram-negative bacterium *Y. ruckeri* is the causative agent of enteric redmouth (ERM) disease predominantly in rainbow trout, *Oncorhynchus mykiss*, in the northern hemisphere and is the cause of yersiniosis predominately in Atlantic salmon, *Salmo salar*, in southern hemisphere (Carson and Wilson, 2008). Yersiniosis caused the mortality of half a million juvenile fish within six months during 2007 in a single Tasmanian hatchery despite vaccination. The poor level of protection has lead to investigations to assess vaccination strategies and host response in juvenile fish vaccinated with a conventional formalin inactivated whole-cell bacterin.

In fish, first contact with pathogens is via the epithelial surfaces of the skin, gills and alimentary tract. A layer of mucus secreted by the mucous cells plays an integral role in the trapping and sloughing of pathogens and contains humoral immune parameters such as lysozyme, complement and antibodies (Narvaez et al., 2010). Previous studies have shown that the gills may be an important portal of entry for *Y. ruckeri* (Zapata et al., 1987; Torroba et al., 1993; McIntosh et al., 2000; Tobback et al., 2009). Other sites of entry however including the skin and gut however may also be important for this bacterium (Busch and Lingg, 1975; Valtonen et al., 1992). Lysozyme is an important innate immune response in fish (Magnadóttir, 2006). Lysozyme can be found in body surface secretions including skin and gill mucus, as well as in the intestinal tract and blood (Saurabh and Sahoo, 2008). Lysozyme is particularly effective against gram positive bacteria where is splits the β (1–4) linkages between N-acetylmuramic acid and N-acetylglucosamine in the cell walls (Saurabh and Sahoo, 2008). Lysozyme can also be effective against gram negative bacteria once the inner petidoglycan layer has been exposed via the action of complement and/or other enzymes (Saurabh and Sahoo, 2008). In fish, spontaneous bactericidal activity is also considered one of the major defence mechanisms in the early stages of microbial infections

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(Hollebecq et al., 1995). Furthermore, bactericidal activity has been found to be a reliable biological marker of resistance against furunculosis (Hollebecq et al., 1995).

Most Tasmanian Atlantic salmon are currently vaccinated with Yersinivac-B, a formalin killed whole-cell vaccine by bath immersion at 1 g, followed by a booster at 5 g. However, the industry goal is a single vaccination that affords protection over 18 months. The gills are an efficient uptake system during immersion vaccination and this type of vaccination best imitates the natural route of infection (Raida and Buchmann, 2008). Understanding how the immune system functions after vaccination against yersiniosis is important for improved protection, husbandry and health management of the species (Whyte, 2007). Experimental fish vaccines can be improved through the addition of the protease trypsin, which can help expose the bacteria's protective O-antigen in order to make the vaccine more efficient (Egidius and Andersen, 1979). Therefore, with a view to improving the current vaccine, as well as the understanding of yersiniosis in Atlantic salmon, the main aims of this study were to assess a novel trypsinated vaccine and determine the role of mucosal lysozyme in both vaccinated and non-vaccinated fish. Identification of a possible biomarker of effective vaccination might lead to an improved vaccine and increased industry sustainability and fish welfare, as fewer challenge trials that sacrifice large amounts of fish would need to be conducted in the future (Marsden et al., 1996). Therefore bactericidal activity in both serum and mucus was also assessed as a possible indicator of effective vaccination.

2. Materials and methods

2.1. Fish

S. salar, of approximately 2 g were provided by Salmon Enterprises of Tasmania (SALTAS). The *Y. ruckeri*-free status of the fish was both assessed and confirmed before challenge from a sample of fish by culturing kidney samples on blood agar plates and standard PCR (Carson et al., 1998) using DNA isolated from spleen and whole blood in an attempt to isolate and identify *Y. ruckeri*. All fish were held in three flow-through fresh water holding tanks of 3000 L at approximately 15 °C. Other water quality parameters including pH (7.2–7.6), ammonia (<0.25 mg L^{-1}), nitrite (<0.25 mg L^{-1}), nitrate (<0.5 mg L^{-1}) were monitored every second day. Fish were fed daily to satiation with a commercial diet (Skretting, Cambridge, Tasmania) and starved 24 h prior to sampling. All animals used for this study were subject to practices reviewed and approved by the University of Tasmania, Animal Ethics Committee (Animal Ethics Number A0016729).

2.2. Vaccination

Once an average weight of 5 g had been achieved fish were vaccinated against *Y. ruckeri*. Commercial vaccines against ERM have been reported to offer the highest protection in fish 5 g or more (Håstein et al., 2005). Therefore the fish were vaccinated once they had achieved an average weight of 5 g at 4 weeks post initial transfer to the holding tanks. Vaccination was achieved by reducing the water level of each holding tank to 500 L and adding 1 L of one of two vaccines; either Yersinivac-B, a commercially available vaccine for yersiniosis, or a trypsinated version of Yersinivac-B. The water volume of the holding tank containing control fish was similarly reduced; however, no vaccine or alternative chemical was added. During the vaccination period of 1 h a small submersible pump was used to recirculate the water and to ensure adequate mixing of the vaccine. At 1 h post vaccine administration the holding tanks were refilled with water from a pre-heated sump to dilute the vaccine out, as per the manufacturer's instructions. The Yersinivac-B vaccine was a killed whole-cell preparation that was inactivated with 0.5% formalin to 1×10^{10} cells mL^{-1}. The trypsinated version of the Yersinivac-B vaccine was prepared following the method of Egidius and Andersen (1979). Both vaccines were prepared by the Fish Health Unit, Department of Primary Industries, Parks, Water and Environment, Tasmania.

2.3. Challenge

At six weeks post vaccination fish (n = 30) from each treatment were randomly transferred to an infection room where they were challenged with *Y. ruckeri* O1b, strain TCFB 2282 at 4.3×10^{6} colony forming units (CFU) mL^{-1} for 1 h in air saturated 20 L buckets with 15 °C fresh water. Having initially estimated the challenge dose using turbidity measurements, viable counts were determined by the Miles and Misra method (Miles et al., 1938) using TSA plates incubated for 48 h at 25 °C. The challenged fish were then rinsed in fresh water and transferred into 200 L recirculating tanks with the same water quality as the respective holding tanks. There were 4 tanks per each of the three treatments. Moribund fish and any mortalities were removed from the tanks daily and used to calculate relative percent survival (RPS) using the formula RPS = (1 − (% mortality/% control mortality)) × 100 (Amend, 1981). At 14 days the challenge was terminated. Survivors were transferred to 400 L recirculating tanks and fed to satiation daily with a commercial Skretting diet until they were sampled at 10 weeks post challenge. The average weights of the fish from the six week post vaccination challenge were: control = 14.82 g, Yersinivac-B = 13.96 g and trypsinated Yersinivac-B vaccine = 12.70 g. The average weights of the survivors were: control = 16.75 g, Yersinivac-B = 17.95 g and trypsinated Yersinivac-B vaccine = 13.12 g.

2.4. Isolation of *Y. ruckeri* in fish kidney following challenge

After challenge anterior kidney samples from fish mortalities were cultured on blood agar plates to re-isolate *Y. ruckeri*. Standard PCR using *Y. ruckeri*-specific 16S ribosomal RNA gene primers (Carson et al., 1998) was used to confirm that colonies obtained on the blood agar were *Y. ruckeri* and only then were the mortalities considered to be due to *Y. ruckeri*.

2.5. Sampling

Vaccinated (Yersinivac-B and trypsinated Yersinivac-B) and non-vaccinated control fish (n = 6) were sampled at 0, 8, 24, and 48 h post challenge. At each sampling period fish were anaesthetised with clove oil (1 mL in 15 L of water). Blood obtained from the caudal vein using a 1 mL syringe and 25 gauge needle was allowed to clot for 24 h at 4 °C prior to centrifugation for 10 min at 1000 × g at the same temperature. The serum was then stored at −80 °C. Body mucus was collected by gently scraping the fish with the blunt side of a scalpel blade into a Petri dish. It was then mixed with approximately 500 μL of phosphate buffer saline (PBS: 0.85 g L^{-1} Na_2HPO_4, 0.25 g L^{-1} KH_2PO_4, 8.5 g L^{-1} NaCl pH 7.2), transferred to a microcentrifuge tube and immediately placed on ice. Following centrifugation at 3000 × g at 4 °C for 30 min, the supernatant of the mucus was stored at −80 °C. Survivors (Yersinivac-B and trypsinated Yersinivac-B, n = 12 and non-vaccinated control, n = 8) were sampled at ten weeks post challenge. Additionally, survivors were sampled for gill mucus that was obtained by gently removing the mucus with a sterile cotton swab. The swab was then transferred into a microcentrifuge tube and placed immediately on ice prior to storage at −80 °C. For later analysis, the gill mucus was removed from the swab by adding 400 μL of PBS and vortexing for 10 s, followed by squeezing the swab against the side of the tube (Vincent et al., 2006). Due to limited volumes of serum, lysozyme was only measured in the mucus of the fish.