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Membrane vesicles from *Piscirickettsia salmonis* induce protective immunity and reduce development of salmonid rickettsial septicemia in an adult zebrafish model

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ABSTRACT

Infections caused by the facultative intracellular bacterial pathogen *Piscirickettsia salmonis* remains an unsolved problem for the aquaculture as no efficient treatments have been developed. As a result, substantial amounts of antibiotic have been used to limit salmonid rickettsial septicemia (SRS) disease outbreaks. The antibiotic usage has not reduced the occurrence, but lead to an increase in resistant strains, underlining the need for new treatment strategies. *P. salmonis* produce membrane vesicles (MVs); small spherical structures know to contain a variety of bacterial components, including proteins, lipopolysaccharides (LPS), DNA and RNA. MVs mimics' in many aspects their mother cell, and has been reported as alternative vaccine candidates. Here, MVs from *P. salmonis* was isolated and evaluated as a vaccine candidate against SRS in an adult zebrafish infection model. When zebrafish was immunized with MVs they were protected from subsequent challenge with a lethal dose of *P. salmonis*. Histological analysis showed a reduced bacterial load upon challenge in the MV immunized group, and the mRNA expression levels of several immune related genes altered, including *mpeg1.1, tnfα, il1b, il10* and *il6*. The MVs induced the secretion of IgM upon immunization, indicating an immunogenic effect of the vesicles. Taken together, the data demonstrate a vaccine potential of MVs against *P. salmonis*.

1. Introduction

The Chilean salmon production is one of the largest aquaculture industries worldwide, with a production rate of 605.800 tons of Atlantic salmon in 2016 and a calculated exportation value of US$2.3 million [1,2]. The continuous expansion of the Chilean salmon industry has, however, not been without difficulties, as the introduction of new farming areas and species have led to the development of infectious diseases [3,4]. One of the most important pathogen found in seawater in Chile is the intracellular bacterial pathogen *Piscirickettsia salmonis*, the etiologic agent of salmonid rickettsial septicaemia (SRS), a chronic and often fatal disease in salmonid [5,6]. *P. salmonis* was isolated and characterized from Coho salmon (*Oncorhyncus kisutch*) in 1989 after a devastating epizootic in the Chilean aquaculture industry [5]. Since then, the bacteria have been recognized as an emerging problem with outbreaks of SRS reported across the world [7–9]. *P. salmonis* has been identified in salmon net-pens in Norway, Canada, Ireland and Scotland, but with a reduced virulence compared to the Chilean strains [10]. Continuous outbreaks of SRS have had a devastating impact on the Chilean aquaculture, with losses exceeding US$ 100 mill a year [11,12], despite the availability of several vaccine options on the marked [4].

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After the release of the first commercial vaccine against SRS in 1999, over 50% of the salmon in Chile was vaccinated against *P. salmonis*, but by 2003 the number had dropped to 17%, indicating that the initial vaccines provided unsatisfactory protection [11]. Nowadays, there are 33 different licensed vaccines against SRS available in Chile, where the majority are composed of *P. salmonis* pre-treated with either heat or formalin, known as bacterin based vaccines [4]. The use of bacterins for immunization of fish has provided substantial protection against a range of pathogens, including *Edwardsiella ictaluri*, *Flavobacterium columnare*, *Vibrio anguillarum* and *Yersinia ruckerii* [13,14]. There are, however, cases where the use of bacterins provides a limited protection against bacterial pathogens, which includes *P. salmonis* [10,13]. As a consequence, the Chilean aquaculture industry continues to use large amounts of antibiotics to control aquatic diseases, which in 2014 represented 70% of the total antibiotic use in the entire country [4]. The use of antibiotic treatment against SRS has, nonetheless, had little success in regards to disease control, but led to the emergence of antibiotic resistant strains of *P. salmonis* [15–17]. Thus, outbreaks of SRS are still an escalating problem for the aquaculture industry [4].

The development of new vaccines against *P. salmonis* is, however, challenging due to the intracellular nature of the bacteria. *P. salmonis* has been shown to infect, replicate and survive within macrophages as a part of its infection strategy. The infection process includes the formation of vacuoles within the host cells, enabling the bacterium to avoid the fish's primary immune defense [8,18–20]. Thus, vaccination against SRS depends on an activation of both the antibody- and cellular-mediated immune system to provide a sufficient protection [21]. Immunization activating both immune systems is, on the other hand, difficult as it require antigens to be represented through MHC receptors of specialized cells of the immune system [22]. Live attenuated vaccines have succeeded in activating both systems, as they in many ways mimic a natural infection upon immunization. There is, however, a risk of the attenuated bacterium reverting back to a virulent state, which can pose potential environmental, industrial and economical hazards [23]. This is particularly problematic for aquaculture, due to the potential release of a virulent strain into the fish's natural habitat [24]. An alternative would be a non-replicating version of the bacteria, like membrane vesicles (MVs), sharing many characteristics with live attenuated bacteria.

Membrane vesicles are 50–250 nm spherical structures, secreted from the surface of many bacteria during all stages of growth [25–27]. Bacterial MV secretion has been associated with several phenotypes including biofilm formation [28], bacterial survival [29], toxin delivery [30], cell-to-cell communication [31], and host-pathogen interactions [32]. Proteomic and biochemical characterization has revealed that the vesicles contain a variety of bacterial components, including proteins as well as lipopolysaccharides (LPS), DNA and RNA [33–36]. MVs have also been reported to contain several important immunogenic factors, such as toxins [37], chaperons [38], and active enzymes [34]. Together they represent several aspects of the bacteria, but in a non-replicative form. The mechanisms of the MV formation and their biological role have, however, yet to be clearly defined. Bacterial MVs have successfully been used for epidemic control against serogroup B meningococcal disease in Cuba, Norway, Brazil, and New Zealand [39–42]. MVs used in vaccination of fish have also been reported to provide protection against *Edwardsiella tarda* in olive flounder *(Paralichthys olivaceus)* [43], *Flavobacterium psychrophilum in* rainbow trout (*Oncorhynchus mykiss*) [44], and *Francisella noatunensis* in zebrafish *(Danio rerio)* [45]. MVs from *P. salmonis* have been shown to be internalized by fish cell cultures, express toxicity in adult zebrafish and contain several immunogenic proteins, such as TolC, GroEL and DnaK [46,47]. Thus, the main aim of this study was to evaluate the potential of MVs as a vaccine candidate against SRS using an adult zebrafish model.

2. Materials and methods

2.1. Bacteria, media and growth conditions

Cultivation of *P. salmonis* LF-89 (type-strain ATCC VR 1361) isolated from Coho salmon (*Oncorhyncus kisutch*) in Chile [5] were routinely grown at 20 °C on Eugon Chocolate Agar (ECA), containing 30.4 g/L BD Bacto TM Eugon Broth (Becton, Dickinson and Company), 15 g/L Agar Bacteriological (Thermo Fisher Scientific) and 5% bovine blood (Håtunalab AB) [48] or in EBFC containing BD Bacto TM Eugon Broth supplemented with 2 mM FeCl3 (Sigma-Aldrich) and 1% Casamino Acids (BD) with agitation (100 rpm) for 7–10 days. The bacterial stocks were frozen in autoclaved 10% skimmed milk (BD Difco) or in BD Bacto TM Eugon Broth supplemented with 20% glycerol (Sigma-Aldrich) and stored at −80 °C.

2.2. Isolation of membrane vesicles

10 mL of exponential-growth phase cultures of *P. salmonis* was used to inoculate 200 mL of EBFC. The cells were grown at 20 °C with agitation, and growth curves were measured by using optical density reading at 600 nm until the isolates reached late exponential-phase. OMVs were isolated as described [46]. Briefly, the bacterial cells were removed by centrifugation (10 min, 15 000 g, 4 °C), and the supernatant filtered sequentially through a 0.45- and 0.22 μm/pore filter in order to remove the remaining bacterial cells. The filtrate was then ultra-centrifuged sequentially at 125 000 g at 4 °C for 2 h and 125 000 g at 4 °C for 30 min to eliminate cell debris and aggregates. The MVs were resuspended in 100 μL 5 mM phosphate buffer (1:2 monobasic dihydrogen phosphate and dibasic monohydrogen phosphate) pH 6, and protein concentration determined by a Picodrop spectrophotometer (Picodrop Limited, UK). MV aliquots (10 μL) were spread onto ECA plates to check for sterility, and the remaining sample was stored at −80 °C until use.

2.3. Adult zebrafish rearing

10-11 months old male and female Zebrafish (*Danio rerio*) wild type strain AB was obtained from the model fish unit at the Norwegian University of Life Science. The fish were acclimatized to room temperature (20 ± 2 °C) two weeks prior to the experimental setup. The fish were fed every morning with brine shrimp (Scanbur AS) and SDS 400 Scientific Fish Food (Scanbur AS) in the afternoon. The water was provided by the model fish unit at the Norwegian University of Life Science and was supplemented with 0.55 g/L Instant Ocean sea salt, 0.053 g/L Sodium Bicarbonate and 0.015 g/L Calcium Chloride. The tanks were housed in a water-system with a controlled temperature (20 °C) and with a cycle consisting of 14 h of light and 10 h of darkness. The fish were closely monitored, and the animal's health recorded twice a day. Moribund fish that clearly showed deviant behavior and clinical symptoms not consistent with good animal welfare (greatly reduced level of activity, response to environment and appetite), were euthanized with an overdose of 250 mg/mL tricaine methanesulfonate (MS-222, Sigma Aldrich). Water parameters were monitored every third day using commercial test kits (TetraTest kit): pH, NO_2^-, NO_3^{2-}, NH_3/NH_4^+ and water hardness. All zebrafish experiment was approved by NARA (The Norwegian Animal Research Authority) and waste water decontaminated by chlorination and tested for sterility before disposal.