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The adjuvant effect of low frequency ultrasound when applied with an inactivated *Aeromonas salmonicida* vaccine to rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Vaccine adjuvants are classified according to their properties of either inducing the persistence of antigens within the animal after immunisation and/or activation of the animal's immune response. The adjuvant effect of low intensity low frequency sonophoresis (LFS) was tested in rainbow trout using an *Aeromonas salmonicida* bacterin vaccine administered by immersion vaccination using LFS at 37 kHz. The adjuvant effect obtained with LFS was compared with that of normal immersion or intraperitoneal injection vaccination. Quantitative PCR was used to measure bacterial DNA in vaccinated fish up to 35 days post-vaccination, while RT-qPCR was used to assess gene expression during the early and late immune response post-vaccination. Results showed that antigen uptake in the gills was significantly higher in the group exposed to low intensity LFS compared to the other two vaccination groups 15 min post-vaccination, but this initially high uptake did not persist over the rest of the experiment. In the kidney, by comparison, the vast majority of the samples analysed did not show the presence or persistence of the bacterin. Showing that the route of vaccine uptake using the *A. salmonicida* bacterin, does not influence the persistence of the bacterin in the gills or the kidney. On the other hand, LFS induced a higher inflammatory response and T-helper cell activation, characterized by a significant up-regulation of interleukin-8 (IL-8), IL-1ß and CD-4, respectively. The expression of Ig-M, Ig-T and Ig-D was up-regulated in gills (being significant for Ig-M), but not in the spleen and kidney of the sonicated group. Conversely, Ig-M was up-regulated in the spleen of the non-sonicated groups, but not in the sonicated group. This highlights the ability of ultrasound to enhance mucosal immunity. It remains to be established whether the up-regulation of Ig-M in gills would be sufficient to offer protection in fish infected with *A. salmonicida*.

1. Introduction

The application of LFS on discrete sites of mammalian skin has been well documented in the literature as a safe method to increase the permeability of the skin to different substances [1,2]. Changes in the architecture of the skin as a result of LFS treatment, allows easier passage of target molecules through the skin [3]. More recently, LFS technology has been applied to fish, in order to enhance the permeability of their skin to facilitate vaccine uptake. Early studies of LFS vaccination in fish [4,5] resulted in an increased protection against viral haemorrhagic septicaemia (VHS) and *Vibrio* sp. using an intensity of 400 and 280 mW/cm^2, respectively. The technique has been shown to have significant advantages over normal immersion vaccination procedures although side effects have been reported. Despite the promising results only few studies have since

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been performed using LFS. Our recent results [6] describing the use of LFS at 37 kHz demonstrated that the gills, in comparison to skin, are very sensitive to sonication procedure due to the fragility of the epithelium surrounding the lamellae. Thus, lower intensities (57 mW/cm^2) are required when using the ultrasonic bath to reduce the side effects that occur during sonication, so as to increase gill permeability with minimum modification [6].

The term "immunological adjuvant" refers to compounds that are added to vaccines to enhance the immune response to the target antigen. Adjuvants, according to their properties have been classified in two categories, i.e. Type I and Type II facilitators [7]. Type I facilitators represent substances that improve the persistence or uptake of antigen in tissues, such as the use of oil emulsions, delivered by intra-peritoneal (IP) injection, resulting in a slow release of the antigen into the peritoneal cavity and which boosts antigen presentation and immunity [7]. Type II facilitators refer to compounds that directly enhance the immune response of the host, such as aluminium hydroxide or bacterial toxins [7–9]. When added to the vaccine, these provide an enhanced stimulus, triggering superior antigen presentation and a heightened adaptive immune response. The use of adjuvants in fish has been proven to confer protection with some pathogens, however, some injected adjuvants can cause side effects and reduced growth in the short term [10].

Aeromonas salmonicida is the causative agent of furunculosis, which persists globally and affects many species of fish, including salmonids [11]. The host response to the bacterium and effective vaccination has been well documented for *A. salmonicida* [12–14]. Protection against furunculosis is most successful when the vaccine is mixed with an adjuvant and administered by injection, with a positive correlation found between systemic antibody titres and levels of protection elicited by the vaccine [14]. Conversely, the administration of inactivated *A. salmonicida* without the use of an adjuvant can also produce elevated antibodies titres, but these are not necessarily correlated with protection [15]. Furthermore, it has recently been reported that immersion vaccination can fail to produce antibody titres in vaccinated fish that correlate with protection, although the vaccine still induces long term protection in the fish [16]. Thus, it should be considered that the protective response to the vaccine is not only be based on the production of systemic antibodies found in blood, but also on the mucosal protection found in fish gills [17,18].

In the current study, the adjuvant effects of low intensity LFS was examined in rainbow trout (*Oncorhynchus mykiss*), vaccinated with a low concentration of formalin-inactivated *A. salmonicida* administered by immersion with LFS exposure. Vaccination by immersion and IP injection were used in parallel as comparative routes of vaccine administration. The presence of bacterial DNA, and expression of the innate and adaptive immune genes in the gills and head kidney of vaccinated fish were assessed by quantitative PCR (qPCR) and RT-qPCR, respectively, and the level of systemic antibodies in the fish's plasma was assessed using an enzyme linked immunosorbant assay (ELISA).

2. Materials and methods

2.1. Fish husbandry

Juvenile rainbow trout (10–12 g) were obtained from a local farm in Trostadt, Germany. Fish were allocated into four 100 l tanks ($n = 42$ tank^{-1}) and acclimated to aquarium conditions for two weeks prior to the study. Water temperature was maintained at 16 ± 1 °C in a flow-through system. Animal experiments were performed in accordance with German animal protection legislation and were approved by the regional ethics committee (Application number G 0104/12).

2.2. Bacterin preparation

A. salmonicida subsp. *achromogenes* (DSMZ: 21281), were cultured on casein-peptone soymeal-peptone (CASO) agar plates for 48 h at 23 °C, before washing the bacteria from the plate with 0.02 M phosphate buffered saline (PBS) pH 7.3. The bacteria were inactivated by incubation in 0.4% formalin PBS at 4 °C overnight. The inactivation of the bacterin was checked by incubation on CASO agar plates for one week at 23 °C. Inactivated bacteria were washed twice with PBS and the concentration of the bacterin was determined by counting with an improved Neubauer chamber.

2.3. Vaccination of fish

Exposure of fish to the bacterin was performed using three different routes of administration. In the first group (DIP), fish were immersed in the bacterin at a final concentration of 1.6×10^7 cells/ml for one min. Fish in the second group (DIP + LFS) were sonicated as previously described by Cobo et al. [6], with sonication performed in five 30 s ON/OFF pulses at 65 mW/cm^2 at 37 kHz, and immediately after sonication, fish were exposed to the same dose of antigen as above for one min by immersion. The third group of fish (IP) was anesthetized with MS-222, and injected intraperitoneally with 50 μl PBS containing 5.0×10^7 cells of bacterin. The control group was performed similar to the DIP group, but bacterin was replaced by PBS.

2.4. Quantification of bacterial DNA in gills and kidney by qPCR

For each sampling point (i.e. 15 min, 6 h and 1, 5, 8 and 35 days post-vaccination (d.p.v.), seven fish per group were killed with an overdose of MS-222. For each analysis, weight of the samples were determined and 8 mg of tissue from the first and second gill arch was used, from which DNA was extracted using a DNeasy Blood & Tissue DNA extraction kit (Qiagen, Hilden, Germany). The concentration of DNA was determined using a NanoDrop ND-1000 (NanoDrop Technologies, Delaware, USA) and adjusted to 100 ng/μl. A standard curve was prepared from DNA extracted from seven dilutions of the bacterin (from 10^8 to 10^2 cells/ml).

A qPCR was performed to determine the amount of bacterial DNA in the tissues sampled using a specific set of primers [19], previously described by Skugor et al. [19]. The qPCR reaction was performed using 1.5 μl of DNA template, 6.25 μl of QuantiTect SYBR Green (Qiagen), 1.0 μl of each primer (Table 1) at a final concentration of 0.4 pmol/μl and 2.75 μl of RNase-free water. The reaction protocol consisted of 40 cycles, with denaturation at 94 °C for 15 s, annealing at 52 °C for 30 s and extension at 72 °C for 15 s performed in a Stratagene Mx 3005 cycler (La Jolla, CA, USA). Analysis of the DNA melting-curve and electrophoresis ensured that the desired amplicon was detected and that no secondary products were formed. The threshold point (Ct) value for duplicate samples was obtained using the Stratagene MxPro PCR software v3.00. The amount obtained in 8 mg of extracted tissue was multiplied by the total gills weight, assuming a homogenous uptake over all the gill tissue.

2.5. Gene expression analysis

At 6 h and 35 d.p.v., gills, head kidney and spleen were sampled from seven fish from each group at each time point and frozen in liquid nitrogen. The samples were kept at −80 °C until processing. The extraction of mRNA was performed using a PeqGold total RNA kit (PeqLab, Erlangen, Germany). Residual genomic DNA was removed using a PeqGold DNase I digestion kit I (PeqLab). The RNA integrity was checked for a subset of samples using a Bioanalyzer (Agilent technologies 2100 Bioanalyzer 6000 nanokit, Santa Clara, CA, USA).