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Final report

The overall goal of the proposed study was to develop an ultrasound-mediated delivery protocol for non-invasive, mass vaccination of Atlantic salmon against infectious salmon anemia (ISA) using a whole-killed virus vaccine. Because of delays in obtaining Atlantic salmon fry of the approporate size, this project was initiated using rainbow trout as an appropriate surrogate model.

Initial studies were done using fluorescently labeled polystyrene nanospheres visualized with scanning-confocal microscopy in order to determine an optimal protocol for ultrasound-based vaccine delivery. The delivery protocol is designed for vaccinating fish of 1-2 grams body weight, the appropriate size and developmental stage for vaccination of salmon against ISA. Based on these studies, fish are immersed in a 15 cm. column of fresh water and exposed to 2 watts/cm^2 continuous-wave ultrasound for 30 seconds at 1 megaHertz. Fish are then transferred to fresh water containing the vaccine and kept at 10 ^{O}C for 3 hours. This protocol was used for all subsequent vaccine deliveries.

The second study utilized the ISA vaccine, and was designed to determine in greater detail the efficiency of penetration of this particular vaccine within the fish skin. Two hundred juvenile trout, average weight of one gram, were obtained from Clear Springs Foods, Inc. (Buhl, Idaho) and acclimated in fresh water for ten days in our Aquaculture Research Facility at C.O.M.B. This acclimation period was critical in order to allow the outer skin layers of the fish to renew after being damaged by mechanical stresses involved with shipping and handling. After acclimation, three groups of 30 fish each were utilized: 1) fish immersed in ISA vaccine without ultrasound treatment; 2) fish immersed in ISA vaccine after ultrasound treatment; and 3) control, untreated fish. Vaccine treated fish were immersed in whole, formalin-killed ISA virus (provided by our commercial partners, Maine BioTek) at a concentration of 10^6 pfu/ml. Five fish from each group were sampled at 0, 2 and 24 hours post-treatment, and biopsies taken from the caudal fin for subsequent analyses as described below. No mortalities occurred. Caudal fin biopsies were processed according to standard protocols for immunohistochemistry at the light and electron microscopy levels, the two methods employed to analyse ultrasound treatment effects on vaccine penetration and uptake in the skin. Processing of samples for this work was contracted out to the Integrated Imaging Center at Johns Hopkins University.

Morphometric analyses of the effects of the ultrasound treatment on the fish skin, in comparison to mechanical damage due to handling, was determined using routine electron microscopy (EM). The expected mechanical damages to the dermal layers of the ultrasound-treated fish skin biopsies were obsereved. Intercellular space widening

between cells of the upper dermal layers were seen at 2 hours post treatment, with sloughing of the outer cell layer occurring by 24 hours post-treatment. In addition, putative viral particles were identified using EM in the upper dermal layers of ultrasound-treated skin biopsies. Tannic acid staining of skin sections lent further support to the identification of viral particles in the dermal layers using EM. We were unable to obtain absolutely definitive identification of IHV in treated skin sections. Attempts to identify viral particles in treated fish skin biopsies were stymied by lack of an appropriate antibody. Using the IHV antibody provided by our commercial partner, attempts were made at immunocytochemistry on semi-thin sections and immunogold staining of ultrathin sections for EM, to no avail. Although numerous protocol modifications were tried for these methods, and the antibody was further purified, this antibody was unable to detect viral particles in the skin biopsies. As discussed in the previous reports, this is most likely due to the antibody, and not because of procedural mistakes or a complete absence of the virus. Because the antibody is a monoclonal, it is unlikely to be effective in detecting viral particles at low concentrations in highly processed tissues. In fact, successful use of this particular antibody has been restricted to diagnostic ELISA assays and immunocytochemistry of cultured cells that overexpress the ISA virus. In spite of the availability of several commercial sources of ISA virus antibody, an appropriate alternative antibody was not obtained. Although we have not been able to demonstrate definitively that the viral particles are present in the skin of treated fish, we do believe the viral particles are penetrating the dermal layers, based on the results from the EM analyses and previous work using similarly-sized particles, as detailed in our original proposal.

Studies were also conducted which unfortunantly raise doubts as to whether the proposed mode of vaccination will elicit an immune response using a whole, formalin-killed ISA vaccine. In collaboration with a commercial trout grower, a whole, formalin-killed vaccine for IHNV was administered to Rainbow trout fingerlings via several routes: intraperitoneal (IP) injection, intramuscular (IM) injection, immersion, or immersion coupled with ultrasound treatment. Although IP-injected fish were able to mount an immune response, none of the other treatment groups produced antibodies to the whole killed virus. The fact that IM-injected fish were not successfully vaccinated indicates that the route of entry for this, and possibly other whole killed vaccines, is important. Because such large particles are likely to be shed with the natural sloughing of the fish skin, we suspect that the presence of whole killed viral particles in the skin is not likely to elicit an immune response. It should be noted that vaccination of salmon for ISA is done around the time of smoltification via IP injection. In addition, the whole killed ISA vaccine used in the present study was only recently developed, and has not been well-characterized in terms of efficacy.

These issues have made us turn our attention toward alternative vaccines, which we plan to test in conjunction with our ultrasound-mediated delivery method. One alternative is a commercially-available recombinant ISA vaccine, available from MicroTek International. This vaccine has been shown to confer immunity in laboratory challenge tests. Significant advantages to recombinant vaccines include small particle size, ease of preparation and handling, and engineering of adjuvant-like epitopes into the vaccine to

elicit robust immune responses at the cellular as well as humoral level. Another alternative currently being pursued is a DNA vaccine encapsulated in cationic nanoparticles. These nanoparticles are presently being produced by a start-up company and the manner in which they are produced may not be described at this time. A number of month past, we carried out preliminary challenges using such a viral DNA vaccine. We compared naked DNA (positive control) to the DNA incorporated into the nanoparticles, by IM injection. Significant differences in protection were not found between the two preparations, indicating that these particles are indeed suitable for DNA vaccination in fish. These nanoparticles are inert and safe, and have been used in a number of mammalian animal models for pre-clinical gene therapy studies. In this past reporting period, we have recruited a recent post-doctoral graduate with significant experience in fish vaccination. It is anticipated that alternative sources of funding will be available to enable this researcher to explore these alternatives an apply them to this project.