Prospects and challenges of developing and commercializing immersion vaccines for aquaculture

Authors:

Ponnerassery S. Sudheesh[a]

Kenneth D. Cain[a*]

[a] Department of Fish and Wildlife Sciences and the Aquaculture Research Institute, College of Natural Resources, University of Idaho, Moscow, ID 83844-1136, USA

***Corresponding author:**
Kenneth D. Cain
+1-208-885-7608;
Fax: +1-208-885-9080
E-mail: kcain@uidaho.edu

Key words: Immersion vaccine, fish disease, immunity, live attenuated, aquaculture

Abstract

Vaccination promises to be the most sustainable means of preventing and managing disease outbreaks in aquaculture. Fish vaccines should be efficient, potent and safe, and should not have adverse effects on humans or the environment. Although vaccination for fish includes immersion and oral delivery routes, injection delivery is currently the most widely applied method industry wide. This is effective for highly valued fish species; however, injection vaccination is not practical for species of lower value or for diseases affecting fish at small sizes. Under these circumstances, oral and immersion delivery holds the greatest promise, but requires antigens to be efficiently taken up through mucosal surfaces. Oral delivery of vaccines in the feed is difficult due to challenges in delivering a consistent and adequate dosage, potential for degradation of antigens in the gastrointestinal tract, and risks of developing oral tolerance. Immersion delivery of killed antigens (bacterins) or live attenuated vaccines is more common and much less stressful than injection delivery. This method involves dipping or bathing fish in a vaccine solution for a period of time to allow antigen uptake across mucosal surfaces resulting in stimulation of both a mucosal and systemic immune response. This review discusses multiple delivery methods for vaccination of fish with primary emphasis on immersion delivery and the factors affecting efficacy such as dose, duration of protection, delivery time, size at first vaccination, booster regimes, and storage requirements. All of which must be optimized before a vaccine is commercialized. Such criteria have been evaluated for a recently developed live attenuated immersion vaccine, *Flavobacterium psychrophilum* 259-93-B.17 grown in iron limiting medium (called B.17-ILM) that protects trout and salmon against coldwater disease. This vaccine requires a booster immunization but protects rainbow trout for at least 24 weeks with a relative percent survival of 70%. A vaccine dose as low as ~ 10^5 cfu/mL has been shown to provide significant protection following pathogen challenge, and fish as small as 0.5 g can be administered the vaccine by immersion and protected from CWD. This review highlights aquaculture vaccines and emphasizes the potential to utilize live attenuated vaccines and mucosal vaccination (immersion) for aquaculture, but it is clear that a better understanding of antigen uptake mechanisms could aid in designing and optimizing future vaccines for fish. As newer developments in vaccine production and processing technologies, storage technologies and novel delivery strategies become available for aquaculture, it is likely that immersion vaccination will be the method of choice for most fish farmers.

1. Introduction

Aquaculture is the fastest growing food production sector globally. With a steady growth of 8-10% over the past three decades, aquaculture now supplies over half of the seafood used for human consumption [1]. Growth is likely to continue as human population and demand for healthy seafood products increase. Similar to other animal production systems, land, water, feed and energy are major inputs for aquaculture. Traditionally, aquaculture consisted of more extensive systems requiring less input or management. Aquatic species were grown at lower stocking densities in larger water bodies/ponds with minimal supplemental feeding. The need to improve production efficiency has led to the development of more intensive aquaculture systems such as net pens, off-shore cages, raceways and recirculating aquaculture systems (RAS). Intensive aquaculture typically raises high value species at high densities that are fed commercially available feed under highly controlled conditions. This level of intensification can lead to greater risk of disease outbreaks, which in turn affect production and result in substantial economic losses [2].

Infectious diseases, mainly caused by bacterial pathogens as well as viral and parasitic pathogens, are by far the most serious constraint to the sustainable expansion of aquaculture worldwide [3-6]. Prevention and control of disease outbreaks have become the biggest challenge for aquaculture. Diseases can be treated using chemicals and antibiotics, but such options are limited for fish farmers. The practice of using antibiotics to control bacterial diseases was predominant in the early stages of intensive aquaculture development. For example, during the beginning of development of industrial salmon aquaculture in Norway, as much as ~50,000 kg of antibiotics was used annually to control bacterial diseases [7]. Even recently, antibiotics have been extensively administered for disease control in the salmon industry in Chile [8] and in many other countries. It has been estimated that bacterial infections are responsible for 15–20 % loss of annual production in China, which accounts for more than 60% of global aquaculture production [1]. Extensive use of various classes of antibiotics, including sulfonamides, fluoroquinolones (e.g., ciprofloxacin), tetracyclines (e.g., oxytetracycline), and macrolides (e.g., erythromycin) is prevalent in Chinese aquaculture [9]. The lack of judicious use of antibiotics has led to public health and environmental concerns due to the potential development of antibiotic resistant bacteria and antibiotic residue in aquaculture products or the environment [10-14]. Hence, several countries have implemented strict regulations on the use of antibiotics for aquaculture. For example, only three antibiotics are approved for aquaculture in the United States and the Food and Drug Administration (FDA) now requires veterinary approval prior to their use.

In addition to antibiotics, fish farmers have adopted various disease control and prevention approaches including the use and application of various disinfectants and chemicals, establishment of best aquaculture practices, better environmental and feed management, strict biosecurity measures, and use of vaccines, probiotics and immunostimulants [7, 15-19]. Vaccination is proven as one of the most cost-effective, practical and environmental friendly methods currently available to prevent or minimize losses due to infectious disease in aquaculture [7]. In this review, we will highlight different delivery strategies for aquaculture vaccines, but will focus primarily on immersion delivery and work in the authors' laboratory on the development and challenges associated with commercializing a live attenuated vaccine for the industry.

2. Vaccination in aquaculture

The earliest report of experimental vaccination in fish was published in 1938, when protective immunity was demonstrated in carp immunized with *Aeromonas punctata* [20]. Later, oral vaccination of trout against *A. salmonicida* was reported in English [21] generating a wider interest in the field. However, fish vaccination was a neglected field for another three decades, mainly due to the availability of antimicrobial compounds after World War II. In an early study, injection vaccination of trout was shown to elicit a specific antibody response against *A. salmonicida* [22]. Concurrent developments in human vaccination and successful application of oral vaccination in the poultry industry sparked renewed interest in vaccination of fish [23-25]. Increasing public health and environmental concerns regarding the use of antibiotics in aquaculture continue to spark the search for effective alternatives to antibiotics and improved disease preventive methods such as vaccination. The first US licensed vaccines to be used in commercial aquaculture were against the gram negative bacterial pathogens *Yersinia ruckeri* and *Vibrio anguillarum* [26,27]. Currently, vaccines are available for more than 22 bacterial and 6 viral diseases in aquaculture [28].

3. Vaccine delivery methods

Administration of vaccines to fish is achieved by different methods, mainly by injecting into the intraperitoneal cavity or by immersing/dipping fish in a vaccine solution, but in some cases vaccines are delivered orally by incorporating into fish feeds (reviewed in [7, 28-30]). Other methods of vaccination with varying levels of commercial application include intramuscular injection of DNA vaccines [31], nasal vaccination [32,33] and hyper-osmotic pretreatment [34,35] before immersion vaccination.

3.1. Injection vaccination

Among different administration methods, injection vaccination is currently the most widely used method to vaccinate fish grown in commercial aquaculture facilities. The introduction and use of water-in-oil emulsion (w/o) injection vaccines in the 1990s helped the Norwegian salmon farming industry counter the problems of furunculosis and other diseases and has significantly improved production efficiencies [7]. Injection vaccination is highly efficacious and generally provides long-term protection [36]. Today, multivalent injection vaccines containing up to seven antigens are used in salmon farming to protect against four major bacterial diseases (furunculosis, vibriosis, cold-water vibriosis, and winter-ulcer) and two viral diseases (infectious pancreatic necrosis [IPN] and infectious salmon anemia [ISA]) [28]. Such multivalent vaccines have become a critical factor for the success of salmon farming in Norway and other countries [37]. Antigenic competition, interference between antigens and nonspecific immunosuppression can be a challenge for formulating and licensing such multivalent vaccines [38].

Major factors that determine the success of aquaculture vaccines are efficacy, potency and safety. Additionally, a vaccine should be cost-effective and easy to apply with minimal stress to the fish. Injection vaccination is a more practical method when vaccinating high value species such as Atlantic salmon. Fish greater than 20 g can be easily injected with a small volume of a multivalent vaccine to prevent major diseases. However, injection vaccination is a highly labor intensive method involving handling and anesthesia, and requires several vaccinators to work together to carry out the process at a facility. Recently, automated

vaccinating machines have been introduced that can lower labor costs and reduce stress to the fish [39,40], but such machines are not adapted to multiple fish species. More serious problems with injection vaccination are associated with the development of side effects and tissue damage around the injection site or formation of abdominal lesions [41]. Inflammation, granulomatous peritonitis, adhesion between body wall and internal organs, and pigmentation at the site of injection are commonly observed in injection vaccinated fish and may lead to increased stress, reduced growth, increased mortality and poor welfare [42-45]. The final product quality at commercial production facilities is very often downgraded to unacceptable levels because of poor flesh quality and melanization [42,46]. Moreover, injection vaccination cannot be used to vaccinate small size fish and if inflammation occurs in broodfish it could impact gonad development [41].

3.2. Oral vaccination

Orally feeding fish with antigens that have been top coated or mixed into the feed is a very attractive method of antigen delivery through the intestinal mucosa of fish [21,47]. It is a stress free method and a large number of fish can be mass vaccinated relatively easily. However, oral vaccination very often produces inconsistent results due to the instability and degradation of antigens while passing through the harsh and acidic environments of the foregut prior to absorption in the hindgut [48-51]. Lack of cost-effective and efficient methods to protect antigens and deliver them to the hindgut has prevented the widespread application of this vaccination method for aquaculture.

3.3. Immersion vaccines

Immersion vaccination is probably the simplest of three major vaccination methods. In general, fish are dipped or immersed in a defined dose of vaccine solution for a short period of time. In this review we will highlight the different types of bacterial immersion vaccines (bacterins) and further focus on the advantages and specific challenges of immersion vaccination in fish. In addition, challenges and considerations for cost-effectiveness, commercialization and licensing of immersion vaccines will be discussed. Currently, two forms of immersion vaccines are popular: killed bacterins and live attenuated vaccines.

Killed vaccines (*bacterins)*

Killed immersion vaccines were one of the earliest vaccines used in aquaculture and continue to be used widely. The first commercially licensed aquaculture vaccine was a formalin-killed immersion vaccine against *Y. ruckeri* [52]. Other formalin-killed immersion vaccines were developed to control vibriosis in salmon and trout and *A. salmonicida* infections in Atlantic salmon [53]. Currently, a range of killed vaccines are commercially available in various countries. In the US, there has also been an increase in the use of custom autogenous vaccines, which are typically killed vaccines developed from bacterial strains isolated from specific aquaculture facilities and used only at those sites. Killed immersion (and injection) vaccines are very important to the aquaculture industry globally, but more recently there has been increased interest in the development and use of live attenuated immersion vaccines.

3.4. Live attenuated immersion vaccines

There are several approaches used for the production of live attenuated immersion vaccines (reviewed in [54]). Attenuation can be achieved by generating avirulent mutated strains through genetic manipulations, sequentially growing the virulent strain in increasingly concentrated doses of specific antibiotics [55], or by simple serial passage

of virulent strains on laboratory media [56,57]. Early immersion vaccination trials on *O. mykiss* used both these approaches to generate live attenuated strains of *V. anguillarum* that provided protective immunity against *V. anguillarum* and were cross-protective against *A. salmonicida* [55]. However, most of the early experimental studies to develop live attenuated strains employed genetic manipulations to generate avirulent mutants [58-65]. Another method that has been utilized involves the induction of random mutation(s) by serially passaging bacteria in media containing increasing concentrations of the antibiotic rifampicin. This is an elegant strategy to generate live attenuated strains of bacteria that have been commercially developed for aquaculture and terrestrial animals [66]. This strategy has been successfully adopted in aquaculture to develop vaccines against pathogens such as *Edwardsiella ictaluri*, which causes enteric septicemia of catfish and *Flavobacterium columnare*, which causes columnaris disease in many warm water fish species. [63,67]. Two such vaccines currently licensed for commercial use are AQUAVAC-ESC® against enteric septicemia of catfish (ESC) and AQUAVAC-COL® against columnaris disease. Following a similar approach, our laboratory has developed a live attenuated vaccine strain of *F. psychrophilum* (259-93-B.17) that protects fish against bacterial coldwater disease (CWD) or rainbow trout fry syndrome (RTFS) as it is often referred to in Europe. CWD/RTFS is a devastating disease that primarily affects smaller life stages of salmonids and other species [68]. Efficacy of the 259-93-B.17 vaccine was recently enhanced by altering production conditions and growing this strain under iron limiting (ILM) conditions [69,70]. Further optimization of this vaccine is underway as part of the process to successfully commercialize and license in the US [70].

4. Strategies to enhance antigen uptake in immersion vaccination

Immersion vaccination is currently the most suitable method for mass vaccination of fish. Direct immersion (DI) is the most widely used form of immersion vaccination [71,72] and consists of fish being immersed in a defined dose of vaccine solution for a specific period of time (usually one to several minutes) and then transferred back to the rearing tank. Different variations of this method and other more novel methods such as ultrasound/immersion vaccination are described in the literature [47]. An important consideration for attempting these variations is the need to enhance antigen uptake during immersion exposure.

As the fish is immersed in the vaccine solution, all mucosal surfaces including skin, gills, nostrils, eyes, vent and intestinal surfaces are exposed to antigens in the vaccine solution. Unlike terrestrial animals, fish are continuously bathed in the surrounding water medium. Hence, they are in continuous contact with any non-pathogenic and potentially pathogenic organisms that may be present in the water. Mucosal surfaces of fish such as skin, gills and gut are the first site of interaction and clear portals of entry for pathogens [51,73-75]. Thus, the mode of contact between vaccine antigens and fish mucosal surfaces, and the immune response produced during immersion vaccination is similar to what occurs during exposure to a virulent pathogen [76-79]. In fact, antigen uptake during immersion vaccination is believed to occur mainly through the skin and gills of fish [80-84]. However, other studies have shown that antigen uptake mainly occurs through the intestine [85-88], and in some cases uptake occurs through the lateral line [89]. Bacteria have been recovered from gastrointestinal tracts of fish immediately following immersion in concentrated suspensions of a vaccine solution [90,91].

In a slightly modified method of immersion vaccination, called hyperosmotic infiltration (HI), antigens are infiltrated into the fish by using a hyperosmotic solution to enhance antigen uptake during immersion vaccination. For vaccination by HI, fish are briefly pre-treated with a hyperosmotic solution such as urea or sodium chloride before vaccination by a standard immersion method [89,92-95]. The hyperosmotic pretreatment is believed to stimulate the drinking and water intake in some fish [86,96]. These authors postulated that physiologically manipulating fish to stimulate chemoreceptors or the endogenous renin-angiotensin system that regulates drinking in fish may enhance antigen uptake during immersion vaccination. Literature on antigen uptake, immune response and protection following HI is contradictory [95,97-99], and at present this vaccination method is uncommon.

A recent immersion vaccination study in zebrafish using *Y. ruckerii* bacterin showed that antigen uptake is quick and varies with different life stages of the fish [100]. The bacterin was observed within 30 min of vaccination in scale pockets, skin, esophagus, intestine and fins of adult fish and within two hours in the spleen and at 24 h in liver and kidney.

5. Improving the efficacy of immersion vaccines

The exact mechanism of how a protective immune response is generated by immersion vaccination is still unclear. In contrast, injection vaccination in fish usually elicits an adaptive immune response measured by the presence of antigen specific antibodies in the circulation a few weeks later depending on water temperature. Such antibody molecules may function as antitoxins, anti-adhesions, and anti-invasions as well as activating the classical complement system [101-104]. Thus, injection vaccines are typically efficacious and generally provide long-term protection [36]. Immersion vaccination may stimulate mucosal immunity, but the role this plays in protection is not clear and there is a lack of reliable and standardized measures to determine specific as well as non-specific immunity at mucosal surfaces. Thus, optimizing immersion vaccines in a way that stimulates both a mucosal and systemic immune response has been constrained [105,106]. Measurement of serum antibody titers following immersion vaccination of rainbow trout with a live attenuated *F. psychrophilum* vaccine (259-93B.17 or B.17-ILM) induces a significant serum antibody response that generally (but not always) correlates with protection [69,70,107,108]. In the case of vaccines delivered by the immersion route, it is possible that adaptive immune response localized in different mucosal organs could play a more significant role. There is mounting evidence that mucosal immunity is important in fish and that mucosal immunoglobulins (Igs) are elicited following infection or vaccination. It may be important to have adequate stimulation of mucosal associated Igs to bind or neutralize antigens during pathogen invasion at such sites [51,109-121]. Our lab has found that secretory IgD and IgT gene expression levels were significantly upregulated in gill and intestinal mucosa of fish vaccinated with the B.17-ILM vaccine, both by immersion and anal intubation routes [107].

Unlike in mammals, there is still no clear evidence for a common mucosal immune system and mucosal homing receptors in fish [122]. Further research on mucosal immunity, especially homing receptors and transport and secretion of mucosal antibodies will shed more light on the protective mechanisms of immersion vaccination. The intricate mechanisms and molecules that coordinate the development of humoral and cell-mediated immunity

during immersion vaccination are poorly understood in fish. Induction of a variety of nonspecific molecules including antimicrobial peptides, surface defensins, proinflammatory cytokines (such as IL-1β, TNFα, and IL-8), chemokines and pattern recognition receptors, coupled with cell mediated immunity involving antigen presenting cells (APCs, monocytes macrophages, eosinophilic granulocytes, neutrophils, M-cells and dendritic-like cells) is essential for efficient protection achieved by immersion vaccination (Reviewed in [51,106,120, 123]).

A defined vaccine dose can be delivered into the fish when administering injection vaccines, whereas the actual dose absorbed into the fish during immersion vaccination is difficult to determine. Several factors related to the fish, environment (vaccination medium), the method of antigen delivery and the nature and composition of vaccine itself can affect the dose of vaccine actually exposed and absorbed into the fish mucosal surfaces [70,124,125]. Preliminary immersion vaccination trials in Coho salmon using the improved B.17- ILM vaccine produced high relative percent survival (RPS) values of 73% [69]. However, these fish were adipose fin clipped and immersed in the vaccine solution for 1 h. This may have contributed to antigen uptake, enhanced antibody responses, and strong protection. A commercial fish vaccine however, must be economical to produce and deliver using practical strategies that fit into the production schedule of an aquaculture facility. Ideally, the vaccine should produce the desired immune response and protect fish for a prolonged period with a minimum antigen dose delivered efficiently in a short period of time.

One of the most difficult tasks of optimizing live immersion vaccines is to determine the minimum dose that the fish should be exposed to in order to elicit an optimum immune response. Lack of standard methods to reliably measure the dose of vaccine exposed to and absorbed into the fish has led to empirical determination of doses and subjective assessments. However, it has been estimated that only 0.01-0.2% of the of the initial vaccine bath concentration is taken up by each fish [97]. They also noted that more antigens were absorbed through the head portion of the fish, and the uptake increased with increase in temperature, size of the fish, and use of an adjuvant.

Early reports of immersion vaccination of channel catfish with a live *E. ictaluri* isolate at a dose of 6 x $l0^5$ cfu/mL produced an RPS of > 90 % [126]. In another study, vaccination of different age groups of channel catfish (7-31 days post hatch) by a modified live *E. ictaluri* RE-33 vaccine for 2 or 10 min immersion at a dose of 5 x 10^5 to 5 x 10^6 cfu/mL produced RPS values ranging from 45.3 to 79.5% [127]. The efficacy of the same vaccine was found to vary significantly among different families of channel catfish when vaccinated at a dose of 1 x $l0^7$ cfu/mL with RPS values among families ranging from 67 to 100% [128].

In our studies, rainbow trout immersion vaccinated with the B.17-ILM vaccine for 3 min at doses of ~ 1 x 10^8 to 1 x 10^{10} cfu/mL were strongly protected against CWD out to at least 24 weeks with RPS values up to 70%, whereas, fish vaccinated with lower doses (~1 x 10^5 and 1 x 10^6 cfu/mL) were protected out to 12 weeks, but RPS values dropped to 34% and 36%, respectively by 24 weeks [70]. Studies on small (1.5-3.0 g) rainbow trout have shown that uptake of BSA-conjugated fluorescent latex microspheres from bath suspensions is logarithmically proportional to particle concentration in suspensions indicating the dose dependent nature of antigen uptake by immersion vaccinated fish [129].

How long the fish is exposed to antigens (vaccine delivery time) during immersion vaccination is important for practical field considerations as well as for efficacy of the vaccine. Commercial immersion vaccines typically dip a certain biomass of fish in the vaccine solution for 30-60 s [130,131]. Rainbow trout immersion vaccinated with B.17-ILM vaccine for 3, 6 and 30 min were significantly protected against CWD with comparable RPS values of 47%, 53% and 52%, respectively [70]; however, a 1.5 min exposure resulted in lower levels of protection. Earlier studies have shown that prolonged vaccination at lower doses can provide greater protection and reduce the stress on fish if it can be done with minimal handling in rearing troughs or tanks [98,129,132-134]. It has been demonstrated that antigen uptake is greater when fish are subjected to prolonged immersion in a low dose vaccine solution when compared to a brief dip in high dose vaccine [129]. Such techniques have been commonly used in the past even for killed bacterins [135], and continue to be used in the industry today either in the hatch house or on trucks during transport. Vaccination by single pass for 30 min may be more practical in hatcheries, where small fish held in tanks or raceways can be concentrated to a smaller area of the tank and vaccinated together. This will avoid subjecting fish to undue handling stress that happens while fish are vaccinated in multiple passes. This method of vaccination, in addition to reducing stress to fish during vaccination, can be more practical and cost-effective by reducing labor and time required for vaccine administration at the hatchery.

A critical consideration in the vaccination of fish is the minimum size at vaccination. Disease susceptibility and incidence varies with different life stages of fish, and early life stages are especially vulnerable to most infectious diseases. This is particularly true in the case of rainbow trout fry syndrome and certain viral diseases such as IPN. From a practical perspective, fish are considered immunocompetent once they are > 1 g in size; however, studies have suggested that long term duration of protection is achieved only when fish are > than 2 g [136]. Using our live attenuated bacterial vaccine, it has been shown that protection in rainbow trout against CWD can be achieved even when fish are vaccinated as early as 0.5 g size [70]. It has been shown that onset and development of both non-specific and humoral immunity and their coordinated actions involving non-specific molecules, specific antibody molecules and specialized immune cells in fish begins very early in life [137-140]. It is reported that a true functionally competent specific immune response involving immunological memory appears to develop in fish larvae of 20-30 mm in size or later [139]. Appearance of functional lymphocytes and other immune cells in the renal hematopoietic tissue shortly after hatch and the development of spleen by day 3 post-hatch in *Ictalurus punctatus* has been reported [141,142].

Channel catfish vaccinated with a live attenuated vaccine as early as 7- 12 day post hatch and as eyed eggs have been successfully protected against ESC and columnaris disease [127, 143-146]. Thus, it is likely that non-specific immune cells and maternally transferred immune molecules may be playing a key role in the protection of eggs and newly hatched larvae, and a fully functional adaptive response may play a more significant role in later life stages [139,147]. The onset of these processes may also vary among fish species.

6. Safety and regulatory issues of live attenuated immersion vaccines.

Efficacy, potency and safety are three major factors to consider when developing any vaccine. The vaccine should contain antigenic component(s) that are highly

immunogenic and have been demonstrated to provide protective immunity. It should be safe for the vaccinated fish as well as for humans without producing an infection. In addition, live vaccines should be traceable in the vaccinated fish population and in the environment. It is also desirable to be able to differentiate vaccinated fish from infected fish by serological or molecular means. Any commercial vaccine should be cost-effective and stable for long-term storage.

Live attenuated immersion vaccines are highly efficacious because they induce innate, cell-mediated, as well as systemic and mucosal antibody-mediated immunity [148,149]. Large scale production of live bacterial vaccines in industrial fermenters is possible and can be used to mass vaccinate fish by the immersion route relatively easily. However, the live vaccines are generally derived from pathogenic organisms by attenuation and can potentially survive and multiply in the host fish. Thus, the main safety concern of live vaccines is related to the instability and possible reversion to virulence, as has been reported for some live vaccines of terrestrial animals [150-152]. Although such reversion to virulence has not been reported for live vaccines used in aquatic animals, similar safety issues are a concern to regulatory agencies when developing and commercializing live vaccines for aquaculture. Adverse reactions in target animals, stability of the vaccine in experimental animals, extent of vaccine shedding, genetic stability and lot-to-lot consistency are some of the safety observations that must be demonstrated for regulatory approval [153]. Permitting and licensing for the production and use of commercial fish vaccines in the US is regulated by the United States Department of Agriculture (USDA)-Animal Plant Health Inspection Service's (APHIS) Center for Veterinary Biologics (CVB). Back passage studies should be carried out to show that a vaccine does not revert to virulence along with recovery and dose studies to show safety. In addition, risk analyses should be carried out to determine the potential of the vaccine strain for release to the environment, establishment in the environment and any possible harm to target and non-target organisms including humans.

7. Future challenges and research needs for commercialization of live immersion vaccines

Immersion vaccination of fish using live bacteria closely imitates the mode of natural infection by a pathogen, and therefore is a very effective method for inducing innate as well as the adaptive arms of the immune system. Typically, vaccinated fish are tested in the lab for efficacy and protective immunity by challenging against a particular pathogenic strain of the disease in question. Mortality and RPS after challenge with the virulent strain are considered the gold standard for measuring protective immunity in fish. Measurement of specific antibody titers using an ELISA assay is also important to assess development of a humoral immune response. However, developing, optimizing and commercializing a live immersion vaccine has its challenges and it is important to understand a range of issues related to identifying protective antigens, purity, safety, antigen dose, potency (efficacy) testing, scalability, storage, shelf life and packaging.

Considerations that must be addressed are many but it is important that protection be conferred to different strains of a pathogen with varying degrees of virulence. It is known that multiple bacterial strains can co-exist in fish farms complicating the etiology of the disease and the efficacy of the vaccine. The degree of protection offered by the vaccine during actual disease situations is related to the number of shared antigens and the degree of cross-protective immune response generated by those shared

antigens among the various strains. The cross-protective efficacy of the vaccine is especially important when vaccinating against diseases involving multiple virulent strains and related species causing similar diseases. Testing of cross protection to a variety of strain or serotypes is important. For example, trials using our B.17- ILM vaccine have demonstrated cross-protection to multiple different strains of *F. psychrophilum* and some similar pathogenic species within the *Flavobacteriaceae* family (Sudheesh and Cain, unpublished). It is nearly impossible to test the cross-protective efficacy against all virulent strains of a pathogen. It is believed that pathogen virulence continuously evolves and circumstantial evidence points to the increase in pathogenicity and emergence of more virulent strains of certain bacterial pathogens [154,155]. Maintaining the efficacy and meeting the label claims in the face of evolving pathogenic virulence can pose a challenge.

It is relatively easy to mass produce live bacterial vaccines in large fermenters starting from a small inoculum of the starting material. However, concentrating the bulk produced vaccine suspension to small quantities without losing the efficacy and potency is important and can potentially affect storage, marketability and profitability of immersion vaccines. Antigenic extracellular components that are released by the bacteria into the medium with some role in providing protection may be lost while concentrating immersion vaccines using industrial centrifuges and filters. The role and importance of extracellular antigens secreted by bacteria in providing protective immunity is well recognized [156].

Storage and distribution of a vaccine to remote farm locations without losing its efficacy and potency is critical for the successful commercialization of any fish vaccine. Live immersion vaccines are generally stored frozen. However, freeze drying may be a preferred choice for storage and distribution of live vaccines due to storage space and shipping demands [157].

A major factor limiting the commercial availability and development of aquaculture vaccines is the cost of optimizing and licensing such products. Due to the nature of aquaculture and variety of fish species reared, many fish diseases do not have a market large enough to attract investment from Aquatic Animal Health companies to fully license and commercialize a vaccine regardless of the efficacy observed in experimental trials.

Acknowledgement

We gratefully acknowledge the financial support provided by the Idaho Department of Commerce through the Idaho Global Entrepreneurial Mission (IGEM grant # 13587).

References:

1. FAO. The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all 2016; Rome. 200 pp.

2. Subasinghe RP, Bondad-Reantaso MG, McGladdery SE. Aquaculture development, health and wealth. In: Subasinghe RP, Bueno P, Phillips MJ, Hough C, McGladdery SE, Arthur JR, editors. Aquaculture in the Third Millennium. Technical Proceedings of the Conference on Aquaculture in the Third Millennium Bangkok: NACA and FAO 2001; p. 167–91.

3. Wei Q. Social and economic impacts of aquatic animal health problems in aquaculture in China, pp. 55–61. In: Primary aquatic animal health care in rural, small-scale, aquaculture development. Arthur JR, Phillips MJ, Subasinghe RP, Reantaso MB, MacRae IH, Eds. FAO Fisheries Tech Paper No. 406. 2002;Rome, Italy:FAO.

4. Walker PJ, Winton JR. Emerging viral diseases of fish and shrimp. Vet Res. 2010;41:51.

5. Jansen PA, Kristoffersen AB, Viljugrein H, Jimenez D, Aldrin M, Stien A. Sea lice as a density-dependent constraint to salmonid farming. Proc Royal Soc B: Biol Sci 2012; 279: 2330–2338.

6. Kibenge FS, Godoy MG, Fast M, Workenhe S, Kibenge MJ. Countermeasures against viral diseases of farmed fish. Antivir Res. 2012;95:257–81.

7. Sommerset I, Krossoy B, Biering E, Frost P. Vaccines for fish in aquaculture. Expert Rev Vaccines 2005;4(1):89-101.

8. Cabello FC. Antibiotics and aquaculture in Chile: implications for human and animal health. Rev Med. Chil 2004;132:1001–1006.

9. Mo WY, Chen Z, Leung HM, Leung AOW. Application of veterinary antibiotics in China's aquaculture industry and their potential human health risks. Environ Sci Pollut Res. DOI 10.1007/s11356-015-5607-z.

10. Miranda C, Zemelman R. Bacterial resistance to oxytetracycline in Chilean salmon farming. Aquaculture 2002;212:(1-4),31-47.

11. Cabello FC. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environ Microbiol 2006;8:1137–1144.

12. Sørum, H. Antimicrobial Drug Resistance in Fish Pathogens. In: Antimicrobial Resistance in Bacteria of Animal origin, Aarestrup F. Ed. ASM Press. 2006;Washington DC, USA..

13. Dang H, Zhang X, Song L, Chang Y, Yang G. Molecular determination of oxytetracycline-resistant bacteria and their resistance genes from mariculture environments of China. J. Appl. Microbiol. 2007;103:2580–2592.

14. Fernández-Alarcón C, Miranda CD, Singer RS, López Y, Rojas R, Bello H, Domínguez M, González-Rocha G. Detection of the floR Gene in a Diversity of Florfenicol Resistant Gram-Negative Bacilli from Freshwater Salmon Farms in Chile. Zoonoses Public Health. 2010;57:181-188.

15. Boyd CE. Environmental Best Management Practices for Aquaculture. Tucker CS, John A. Eds. 2008;p-73-90. Hargreaves© John Wiley & Sons, Inc. ISBN: 978-0-813-82027-9

16. Defoirdt T, Sorgeloos P, Bossier P. Alternatives to antibiotics for the control of

bacterial disease in aquaculture. Curr Opin Microbiol 2011;14:251–8.

17. Hine M, Adams S, Arthur JR, Bartley D, Bondad-Reantaso MG, Chávez C, Clausen JH, Dalsgaard A, Flegel T, Gudding R, Hallerman E, Hewitt C, Karunasagar I, Madsen H, Mohan CV, Murrell D, Perera R, Smith P, Subasinghe R, Phan PT, Wardle R. 2012. Improving biosecurity: a necessity for aquaculture sustainability, In Subasinghe RP, Arthur JR, Bartley DM, De Silva SS, Halwart M, Hishamunda N, Mohan CV, Sorgeloos P, Eds. Farming the Waters for People and Food. Proc Global Conf Aquaculture 2010; Phuket, Thailand. 22–25 September 2010. pp. 437–494. FAO, Rome and NACA, Bangkok.

18. Hasan MR, New MB, eds. On-farm feeding and feed management in aquaculture. FAO Fisheries and Aquaculture Tech Paper No. 583. 2013;Rome, FAO. 67 pp.

19. Newaj-Fyzul A, Austin B. Probiotics, immunostimulants, plant products and oral vaccines, and their role as feed supplements in the control of bacterial fish diseases. J Fish Dis 2015;38(11):937-55.

20. Snieszko S, Piotrowska W, Kocylowski B, Marek K. Badania bakteriologiczne iserogiczne nad bakteriami posocznicy karpi. Memoires de l'Institut d'Ichtyobiologie et Pisciculture de la Station de Pisciculture Experimentale a Mydlniki de l'Universite Jagiellonienne a Cracovie 1938;Nr38.

21. Duff DCB. The oral immunization of trout against *Bacterium salmonicida*. J Immunol 1942;44:87-94.

22. Krantz GE, Reddecliff JM, Heist CE. Development of antibodies against *Aeromonas salmonicida* in trout. J Immunol 1963;91:757-60.

23. Snieszko SF. Immunization of fishes: a review. J Wildl Dis 1970;6:24-30.

24. Garrison RL, Gould RW, O'Leary PJ, Fryer JL. Spray immunization of fish. US Patent No 4,223,014; 1980.

25. Horne MT, Tatner M, McDerment S, Agius C, Ward P. Vaccination of rainbow trout, Salmo gairdneri Richardson, at low temperatures and the long persistence of protection. J Fish Dis 1982;5:343-5.

26. Midtlyng P, Reitan L, Speilberg L. Experimental studies on the efficacy and side-effects of intraperitoneal vaccination of Atlantic salmon (*Salmo salar* L.) against furunculosis. Fish Shellfish Immunol 1996;6: 335–350.

27. Shao ZJ. Aquaculture pharmaceuticals and biologicals: current perspectives and future possibilities. Adv Drug Deliv Rev 2001;50: 229–243.

28. Brudeseth BE, Wiulsrød R, Fredriksen BN, Lindmo K, Løkling K-E, Bordevik M, Steine N, Klevan A, Gravningen K. Status and future perspectives of vaccines for industrialised fin-fish farming. Fish Shellfish Immunol 2013;14: 17–22.

29. Dhar AK, Manna SK, Thomas Allnutt FC. Viral vaccines for farmed finfish. Virusdisease. 2014;25(1): 1–17.

30. Embregts CW, Forlenza M. Oral vaccination of fish: Lessons from humans and veterinary species. Dev Comp Immunol 2016.

31. Lorenzen N, LaPatra SE. DNA vaccines for aquacultured fish. Rev sci 122 Gen impact final scientific report tech. Off int Epiz 2005;24: 201-213.

32. Tacchi L, Musharrafieh R, Larragoite ET, Crossey K, Erhardt EB, Martin SA,

LaPatra SE, Salinas I. Nasal immunity is an ancient arm of the mucosal immune system of vertebrates. Nat Commun 2014;5: 5205.

33. LaPatra S, Kao S, Erhardt EB, Salinas I. Evaluation of dual nasal delivery of infectious hematopoietic necrosis virus and enteric red mouth vaccines in rainbow trout (*Oncorhynchus mykiss*). Vaccine 2015;33:771-776.

34. Nakajima M, chikahata H. Efficiency of Oral and Hyperosmotic Vaccinations for Vibriosis in Ayu. Fish Pathol 1979;14, 9-13.

35. Huising MO, Guichelaar T, Hoek C, Verburg-van Kemenade BM, Flik G, Savelkoul HF, Rombout JH. Increased efficacy of immersion vaccination in fish with hyperosmotic pretreatment. Vaccine 2003;21(27-30):4178-93.

36. Aucouturier J, Dupuis L, Ganne V. Adjuvants designed for veterinary and human vaccines. Vaccine 2001;19:2666-72.

37. PHARMAQ. http://pharmaq.no/sfiles/1/23/2/file/vaccines_norway_2012-08_no.pdf. 2012.

38. Busch RA. Polyvalent vaccines in fish: the interactive effects of multiple antigens. Dev Biol Stand 1997;90:245–256.

39. Maskon. www.maskon.no/pages/vaksine.html. 2012.

40. Sharpe CS. Physiological stress responses to automated and hand vaccine injection procedures in yearling coho salmon. N Am J Aquacult 2007;69,180–184.

41. Midtlyng PJ, Reitan LJ, Speilberg L. Experimental studies on the efficacy and side-effects of intraperitoneal vaccination of Atlantic salmon (*Salmo salar* L.) against furunculosis. Fish Shellfish Immunol 1996;6(5): 335–350.

42. Poppe TT, Breck O. Pathology of Atlantic salmon Salmo salar intraperitoneally immunized with oil-adjuvanted vaccine: a case report. Dis Aquat Org 1997;29:219-226.

43. Koppang EO, Haugarvoll E, Hordvik I, Poppe TT, Bjerkås I. Granulomatous uveitis associated with vaccination in the Atlantic salmon. Vet Pathol 2004;41:122-130.

44. Koppang EO, Haugarvoll E, Hordvik I, Aune L, Poppe TT. Vaccine-associated granulomatous inflammation and melanin accumulation in Atlantic salmon, *Salmo salar* L., white muscle. J Fish Dis 2005;28:13-22.

45. Berg A, Rødseth OM, Tangerås A, Hansen T. Time of vaccination influences development of adhesions, growth and spinal deformities in Atlantic salmon *Salmo salar*. Dis Aquat Organ 2006;69:239-248.

46. Midtlyng PJ. Vaccinated fish welfare: protection versus side-effects. Dev Biol Stand 1997;90:371-9.

47. Plant KP, LaPatra SE. Advances in fish vaccine delivery. Dev Comp Immunol 2011;35:1256–62.

48. Rombout JHWM, Lamers CHJ, Helfrich MH, et al., Uptake and transport of intact macromolecules in the intestinal epithelium of carp (*Cyprinus carpio* L.). Cell Tiss Res 1985;239:519–30.

49. Quentel C, Vigneulle M. Antigen uptake and immune responses after oral vaccination. Dev Biol Stand 1997;90:69–78.

50. Rombout JHWM, van den Berg AA. Immunological importance of the second gut segment of carp. I. Uptake and processing of antigens by epithelial cells and macrophages. J Fish Biol 1989;35:13–22.

51. Rombout JHWM, Abelli L, Picchietti S, Scapigliati G, Kiron V. "Teleost intestinal immunology," Fish Shellfish Immunol 2011;31(5):616–626.

52. Plumb JA. Health maintenance and principal microbial diseases of cultured fishes. Iowa State University Press, 1999;328 p.

53. Evelyn TPT. A historical review of fish vaccinology. Dev Biol Stand 1997;90:3-12.

54. Shoemaker CA, Klesius PH, Evans JJ, Arias CR. Use of modified live vaccines in aquaculture J World Aquac Soc 2009;40:573–585.

55. Norqvist A, Hagstrom A, Wolf-Watz H. Protection of rainbow trout against vbriosis and furunculosis by the use of attenuated strains of *Vibrio anguillarum*. Appl Environ Microbiol 1989;55:1400–1405.

56. Daly JG, Griffiths SG, Kew AK, Moore AR, Olivier G. Characterization of attenuated *Renibacterium salmoninarum* strains and their use as live vaccines. Dis Aquat Organ 2001;44:121–126.

57. Itano T, Kawakami H, Kono T, Sakai M. Live vaccine trials against nocardiosis in yellowtail *Seriola quinqueradiata*. Aquaculture 2006;261:1175–1180.

58. Vaughan LM, Smith PR, Foster TJ. An aromatic-dependent mutant of the fish pathogen *Aeromonas salmonicida* is attenuated in fish and is effective as a live vaccine against the salmonid disease furunculosis. Infect Immun 1993;61:2172–2181.

59. Thornton JC, Garduno RA, Kay WW. The development of live vaccines for furunculosis lacking the A-layer and O-antigen of *Aeromonas salmonicida*. J Fish Dis 1994;17:194–204.

60. Lawrence ML, Cooper RK, Thune RL. Attenuation, persistence, and vaccine potential of an *Edwardsiella ictaluri purA* mutant. Infect Immun 1997;65:4642–4651.

61. Hernanz MC, Flano E, Lopez FP, Villena A, Anguita J, Cascon A, Sanchez SM, Razquin PB, Naharro G. Molecular characterization of the *Aeromonas hydrophila aroA* gene and potential use of an auxotrophic *aroA* mutant as a live attenuated vaccine. Infect Immun 1998;66:1813–1821.

62. Marsden MJ, Vaughan LM, Fitzpatrick RM, Foster TJ, Secombes CJ. Potency testing of a live genetically modified vaccine for salmonids. Vaccine 1998;16:1087–1094.

63. Klesius PH, Shoemaker CA. Development and use of modified live *Edwardsiella ictaluri* vaccine against enteric septicemia of catfish. In Schultz R, Ed. Adv Vet. Med, 1999; 41:523–537.Academic Press, San Diego, California, USA.

64. Thune RL, Fernandez DH, Battista JR. An *aroA* mutant of *Edwardsiella ictaluri* is safe and efficacious as a live, attenuated vaccine. J Aquat Anim Health 1999;11:358–372.

65. Locke JB, Vicknair MR, Ostland VE, Nizet V, Buchanan JT. Evaluation *of Streptococcus iniae* killed bacterin and live attenuated vaccines in hybrid striped bass through injection and bath immersion. Dis Aquat Organ 2010;89(2):117-23.

66. Linde K, Beer J, V. Bondarenko. Stable Salmonella live vaccine strains with two or more attenuating mutations and any desired level of attenuation. Vaccine 1990;8:278–282.

67. Shoemaker CA, Klesius PH, Evans JJ. Immunization of eyed channel catfish, *Ictalurus punctatus*, eggs with monovalent *Flavobacterium columnare* vaccine and bivalent *F. columnare* and *Edwardsiella ictaluri* vaccine. Vaccine 2007;25:1126–1131.

68. Nematollahi A, Decostere A, Pasmans F, Haesebrouck F. *Flavobacterium psychrophilum* infections in salmonid fish. J Fish Dis 2003;26:563-574.

69. Long A, Fehringer TR, Swain MA, LaFrentz BR, Call DR, Cain KD. Enhanced efficacy of an attenuated *Flavobacterium psychrophilum* strain cultured under iron-limited conditions, Fish Shellfish Immunol 2013;35:1477-1482.

70. Sudheesh PS, Cain KD. Optimization of efficacy of a live attenuated *Flavobacterium psychrophilum* immersion vaccine. Fish Shellfish Immunol 2016;56:169-80.

71. Gould RW, Antipa R, Amend. 1979. Immersion vaccination of sockeye salmon (*Oncorhynchus nerka*) with two pathogenic strains of *Vibrio anguillarum*. J. Fish. Res. Board Can. 36: 222-225

72. Amend, D.F., and R.W. Eshenour DE. Development and use of commercial fish vaccines. Salmonid 1980;4(3):8-12.

73. Brandtzaeg P. Basic mechanisms of mucosal immunity—a major adaptive defense system. Immunologist 1995;3(3)89–96.

74. Wilson JM, Laurent P. Fish gill morphology: inside out. J Exp Zool 2002;293(3)192–213.

75. Sansonetti PJ. To be or not to be a pathogen: that is the mucosally relevant question. Mucosal Immunol 2011;4(1)8–14.

76. Lobb CJ. Secretory immunity induced in catfish, *Ictalurus punctatus*, following bath immunization. Dev Comp Immunol 1987;11(4):727–38.

77. Lumsden JS, Ostland VE, Byrne PJ, Ferguson HW. Detection of a distinct gill-surface antibody response following horizontal infection and bath challenge of brook trout *Salvelinus fontinalis* with *Flavobacterium branchiophilum*, the causative agent of bacterial gill disease. Dis Aquat Organ 1993;16(1):21–27.

78. Gudding R, Lillehaug A, Evensen O. Recent developments in fish vaccinology. Vet Immunol Immunopathol 1999;72(1–2):203–212.

79. dos Santos NM, Taverne-Thiele JJ, Barnes AC, van Muiswinkel WB, Ellis AE, Rombout JH. The gill is a major organ for antibody secreting cell production following direct immersion of sea bass (*Dicentrarchus labrax*, L.) in a *Photobacterium damselae* ssp. *piscicida* bacterin: an ontogenetic study. Fish Shellfish Immunol 2001;11(1):65–74.

80. Tatner MF, Johnson CM, Horne MT. The tissue localization of *Aeromonas salmonicida* in rainbow trout, *Salmo gairdneri* Richardson, following three methods of administration. J Fish Biol 1984;25:95–108.

81. Nelson JS, Rohovec JS, Fryer JL. Tissue location of vibrio bacterin delivered by intraperitoneal injection, immersion and oral routes to *Salmo gairdneri*. Fish Pathol 1985;19:263-269.

82. Kawahara E, Kusuda R. Location of *Pasteurella piscicida* antigens in tissues of yellowtail *Seriola quinqueradiata* vaccinated by immersion. Nippon Suisan Gakkaishi—Bull Jap Soc Scient Fisher 1988;54:1101-1105.

83. Ototake M, Iwama GK, Nakanishi T. The uptake of bovine serum albumin by the skin of bath-immunised rainbow trout *Oncorhynchus mykiss*. Fish Shellfish Immunol 1996;6(5):321–33.

84. Kiryu I, Ototake M, Nakanishi T, Wakabayashi H. The uptake of fluorescent microspheres into the skin, fins and gills of rainbow trout during immersion. Fish Pathol 2000;35(1):41–48.

85. Tatner MF. The quantitative relationship between vaccine dilution, length of immersion time and antigen uptake, using a radioloabelled *Aeromonas salmonicida* bath in direct immersion experiments with rainbow trout, *Salmo gairdneri*. Aquaculture 1987;62:173–185.

86. Robohm RA, Koch RA. Evidence for oral ingestion as the principal route of antigen entry in bath-immunized fish. Fish Shellfish Immunol 1995;5(2):137–150.

87. Khimmakthong U, Deshmukh S, Chettri JK, Bojesen AM, Kania PW, et al. Tissue specific uptake of inactivated and live *Yersinia ruckeri* in rainbow trout (*Oncorhynchus mykiss*): Visualization by immunohistochemistry and in situ hybridization. Microbial Pathogenesis 2013;59–60:33–41.

88. Ohtani M, Villumsen KR, Strom HK, Raida MK. 3D Visualization of the Initial *Yersinia ruckeri* Infection Route in Rainbow Trout (*Oncorhynchus mykiss*) by Optical Projection Tomography. Plos One 2014;9.

89. Amend DF, Fender DC. Uptake of bovine serum albumin by rainbow trout from hyperosmotic solutions: a model for vaccinating fish. Science 1976;192:793-794.

90. Nelson JS, Rohovec JS, Fryer JL. Tissue location of vibno bacterin delivered by intraperitoneal injection, immersion and oral routes to Salmo gairdneri. Fish Pathol 1985;19:263-269

91. Kawahara E, Kusuda R. Location of *Pasteurella piscicida* Antigens in Tissues of Yellowtail *Seriola quinqueradiata* Vaccinated by Immersion. Nippon Suisan Gakkaishi 1988;54(7):1101-1105.

92. Antipa R, Amend DF. Immunization of Pacific salmon: comparison of intraperitoneal injection and hyperosmotic infiltration of *Vibrio anguillamm* and *Aeromonas salmonicida* bacterins. J Fish Res Board Can 1977;34:203-208.

93. Antipa R, Gould G, Amend DF. *Vibrio anguillarum* vaccination of sockeye salmon. *Oncorhynchus nerka* (Walbaum) by direct and hyperosmotic immersion. J Widl Dis 1980;3:161.165.

94. Aoki T, Kitao T, Fukudome M, Takahashi S, Egusa S. Modification of the hyperosmotic infiltration method of vaccination against vibriosis in cultured ayu, *Plecoglossus altivelis* Temminck and Schlegel. J Fish Dis 1984;7(2):149–156.

95. Huising MO, Guichelaar T, Hoek C, Verburg-Van Kemenade BML, Flik G, Savelkoul HFJ, Rombout J. Increased efficacy of immersion vaccination in fish with hyperosmotic pretreatment. Vaccine 2003;21:4178–4193.

96. Anderson DP, Roberson BS, Dixon OW. Induction of antibody-producing cells in rainbow trout, *Salmo gairdneri* Richardson—by flush exposure. J Fish Biol 1979a:15:317–322.

97. Tatner MF, Horne MT. Factors influencing the uptake of 14C-labelled *Vibrio anguillarum* vaccine in direct immersion experiments with rainbow trout, Salmo gairdneri Richardson. J Fish Biol 1983;22:585–591.

98. Fender DC, Amend DF. Hyperosmotic infiltration factors influencing uptake of bovine serum albumin by rainbow trout (*Salmo gairdneri*). J Fish Res Board Can 1978;35:871–874.

99. Thune R, Plumb J. Evaluation of hyperosmotic infiltration for the administration of antigen to channel catfish (*Ictalurus punctatus*). Aquaculture 1984;36:1−8.

100. Korbut R, Mehrdana F, Kania PW, Larsen MH, Frees D, Dalsgaard I, Jørgensen LG. Antigen Uptake during Different Life Stages of Zebrafish (*Danio rerio*) Using a GFPT aged *Yersinia ruckeri*. Plos One 2016. DOI:10.1371/journal.pone.0158968 July 12, 2016.

101. Ingram GA, Alexander JB. The lmmunoglobulin of brown trout, *Salmo trutta*, and its concentration in serum of antigen stimulated and non-stimulated fish. J Fish Biol 1979;14:249-260.

102. Olesen NJ, Jørgensen PEV. Detection of neutralizing antibody to Egtved virus in rainbow trout (*Salmo galrdnen*) by plaque neutralization test with complement addition. J appl Ichthyol 1986a;2:3341

103. Olesen NJ, Jørgensen PEV. Quantification of serum immunoglobulin in rainbow-trout *Salmo gairdneri* under various environmental conditions. Dis Aquat Organ. 1986b;1:183-189.

104. Ellis AE. Immunity to bacteria in fish. Fish Shellfish Immunol 1999;9:291–308.

105. Neutra MR, Kozlowski PA. Mucosal vaccines: the promise and the challenge. Nat Rev Immunol 2006;6(2):148–58.

106. Munang'andu HM, Mutoloki S, Evensen O. A review of the immunological mechanisms following mucosal vaccination of finfish. Front Immunol 2015;6:427.

107. Makesh M, Sudheesh PS, Cain KD. Systemic and mucosal immune response of rainbow trout to immunization by different routes. Fish Shellfish Immunol 2015;44(1):156-163.

108. Sudheesh PS, Zimmerman JK, Cain KD. Dietary effects on immunity, stress, and efficacy of two live attenuated *Flavobacterium psychrophilum* vaccine formulations. Aquaculture 2016;454:35–43.

109. Peleteiro MC, Richards RH. Identification of lymphocytes in the epidermis of the rainbow trout, *Salmo gairdneri* Richardson. J Fish Dis 1985;8(2):161–72.

110. Savan R, Aman A, Nakao M, et al. Discovery of a novel immunoglobulin heavy chain gene chimera from common carp (*Cyprinus carpio* L). Immunogenetics 2005a;57:458–463.

111. Savan R, Aman A, Sato K, et al. Discovery of a new class of immunoglobulin heavy chain from fugu. Eur J Immunol 2005b;35:3320–3331.

112. Hansen JD, Landis ED, Phillips RB. Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: implications for a distinctive B cell developmental pathway in teleost fish. Proc Natl Acad Sci U S A 2005;102(19):6919–24.

113. Hu YL, Xiang LX, Shao JZ. Identification and characterization of a novel immunoglobulin Z isotype in zebrafish: implications for a distinct B cell receptor in lower vertebrates. Mol Immunol 2010;47(4):738–46.

114. Zhang YA, Salinas I, Li J, Parra D, Bjork S, Xu Z, et al. IgT, a primitive immunoglobulin class specialized in

mucosal immunity. Nat Immunol 2010;11(9):827–U82.

115. Salinas I, Zhang YA, Sunyer JO. Mucosal immunoglobulins and B cells of teleost fish. Dev Comp Immunol 2011;35(12):1346–65.

116. Abos B, Castro R, Luque A, Gonzalez L, Tafalla C. Heterogenity of IgM cells in rainbow trout (*Oncorhynchus mykiss*) tissues. Fish Shellfish Immunol 2013;34(6):1636.

117. Abós B, Castro R, Pignatelli J, Luque A, González L, Tafalla C. Transcriptional heterogeneity of IgM(+) Cells in rainbow trout (*Oncorhynchus mykiss*) tissues. PLoS One 2013;8(12):e82737.

118. Ballesteros NA, Castro R, Abos B, Rodríguez Saint-Jean SS, Pérez-Prieto SI, Tafalla C. The pyloric caeca area is a major site for IgM(+) and IgT(+) B Cell recruitment in response to oral vaccination in rainbow trout. PLoS One 2013;8(6):e66118.

119. Xu Z, Parra D, Gómez D, Salinas I, Zhang YA, von Gersdorff Jørgensen L, et al. Teleost skin, an ancient mucosal surface that elicits gut-like immune responses. Proc Natl Acad Sci U S A 2013 110(32):13097–13102.

120. Castro R, Bromage E, Abós B, Pignatelli J, González Granja A, Luque A, et al. CCR7 is mainly expressed in teleost gills, where it defines an IgD(+) IgM(-) B lymphocyte subset. J Immunol 2014;192(3):1257–66.

121. Heinecke RD, Chettri JK, Buchmann K. Adaptive and innate immune molecules in developing rainbow trout, *Oncorhynchus mykiss* eggs and larvae: expression of genes and occurrence of effector molecules. Fish Shellfish Immunol 2014;38(1):25–33.

122. Bernard D, Sox A, Rigottier-Gois L, et al. Phenotypic and functional similarity of gut intraepithelial and systemic T cells in a teleost fish. J Immunol 2006;176:3942–3949.

123. Fuglem B, Jirillo E, Bjerkås I, Kiyono H, Nochi T, Yuki Y, et al. Antigen-sampling cells in the salmonid intestinal epithelium. Dev Comp Immunol 2010;34(7):768–74.

124. Bly JE, Quiniou SMA, Clem LW. Environmental effects in fish immune mechanisms. Dev Biol Stand 1997;90:33-43.

125. B. Magnadottir, Immunological control of fish diseases. Mar Biotechnol 2010;12:361-379.

126. Klesius PH, Shoemaker CA. Heterologous isolates challenge of channel catfish, *Ictalurus punctatus*, immune to *Edwardsiella ictaluri*. Aquaculture 1997;157:147–155.

127. Shoemaker CA, Klesius PH, Bricker JM. Efficacy of a modified live *Edwardsiella ictaluri* vaccine in channel catfish as young as seven days post hatch. Aquaculture 1999;176:189–193.

128. Wise DJ, Klesius PH, Shoemaker CA, Wolters WR. Vaccination of mixed and full-sib families of channel catfish *Ictalarus punctatus* against enteric septicemia of catfish with live attenuated *Edwardsiella ictaluri* isolate (RE-33). J World Aquacult Soc 2000;31:206-212.

129. Moore JD, Ototake M, Nakanishi T. Particulate antigen uptake during immersion immunization of fish: the effectiveness of prolonged exposure and the roles of skin and gill. Fish Shellfish Immunol 1998;8(6):393-408.

130. Adams A, Auchinachie N, Bundy A, Tatner MF, Horne MT. The potency of

adjuvanted injected vaccines in rainbow trout (*Salmo gairdneri* Richardson) and bath vaccines in Atlantic salmon (*Salmo salar* L.) against furunculosis. Aquaculture 1988;69:15-26.

131. Deshmukh S, Raida MK, Dalsgaard I, Chettri JK, Kania PW, Buchmann K. Comparative protection of two different commercial vaccines against *Yersinia ruckeri* serotype O1 and biotype 2 in rainbow trout (*Oncorhynchus mykiss*). Vet Immunol Immunopathol 2012;145: 379-385.

132. Tatner MF, Horne MT. The effects of vaccine dilution, length of immersion time, and booster vaccinations on the protection levels induced by direct immersion vaccination of brown trout, *Salmo trutta* with *Yersinia ruckeri* (ERM) vaccine, Aquaculture 1985;46:11-18.

133. Ototake M, Moore JD, Nakanishi T. The effectiveness of prolonged exposure on soluble antigen uptake during immersion immunization. Fish Pathol 1998;33:91–94.

134. Ototake M, Moore JD, Nakanishi T. Prolonged immersion improves the effectiveness of dilute vibrio vaccine for rainbow trout. Fish Pathol 1999;34:151–154.

135. Ellis AE. Fish Vaccination. Department of Agriculture and Fisheries for Scotland. Aquaculture Information Series 1989;No. 4.

136. Amend D, Johnson K. Current status and future needs of *Vibrio anguillarum* bacterins. In: Fish biologics: serodiagnostics and vaccines 1981.

137. Manning MJ, Grace MF. Developmental aspects of immunity and tolerance in fish, in: Roberts RJ. Ed, Microbial Diseases of Fish, Academic Press, 1982;pp.31-46.

138. Razquin BE, Castillo A, Lopez-Fierro P, Alvarez F, Zapata A, Villena A. Ontogeny of IgM-producing cells in the lymphoid organs of rainbow trout, *Salmo gardnieri* Richardson: an immunological and enzyme-histochemical study, J Fish Biol 1990;36:159-173.

139. Magnadottir B, Lange S, Gudmundsdottir S, Bogwald J, Dalmo RA. Ontogeny of humoral immune parameters in fish, Fish Shellfish Immunol 2005;19:429-439.

140. Zapata A, Diez B, Cejalvo T, Gutierrez-De Frias C, Cortes A. Ontogeny of the immune system of fish, Fish Shellfish Immunol 2006;20:126-136.

141. Warr GW. The adaptive immune system of fish. Dev Biol Stand 1997;90:15–21.

142. Petrie-Hanson L, A. Ainsworth J. Humoral immune response of channel catfish (*Ictalurus punctatus*) fry and fingerlings exposed to *Edwardsiella ictaluri*. Fish Shellfish Immunol 1999;9:579–589.

143. Wise DJ, Terhune J. The relationship between vaccine dose and efficacy in channel catfish *Ictalurus punctatus* vaccinated as fry with a live attenuated strain of *Edwardsiella ictaluri* (RE-33). J World Aquacult Soc 2001;32(19):177–183.

144. Klesius PH, Shoemaker CA, Evans JJ. *In ovo* methods for utilizing live *Edwardsiella ictaluri* against enteric septicemia in channel catfish. U.S. Patent 2002;No.6,153,202.

145. Shoemaker CA, Klesius PH, Evans JJ. *In ovo* method for utilizing the modified live *Edwardsiella ictaluri* vaccine against enteric septicemia in channel catfish. Aquaculture 2002;203:221–227.

146. Shoemaker CA, Klesius PH, Evans JJ. Immunization of eyed channel catfish, *Ictalurus punctatus*, eggs with monovalent *Flavobacterium columnare* vaccine and bivalent *F. columnare* and *Edwardsiella ictaluri* vaccine. Vaccine 2007;25:1126–1131.

147. Dalmo RA. Ontogeny of the fish immune system. Fish Shellfish Immunol 2005;19:395–396.

148. Marsden MJ, Vaughan LM, Foster TJ, Secombes CJ. A live (delta *aroA*) *Aeromonas salmonicida* vaccine for furunculosis preferentially stimulates Tcell responses relative to B-cell responses in rainbow trout (*Oncorhynchus mykiss*), Infect. Immun. 1996;64:3863-3869.

149. Clark TG, Cassidy-Hanley D. Recombinant subunit vaccines: potentials and constraints. Pages 153–163 in P. J. Midtlyng, editor, Progress in fish vaccinology. Dev Biol Stan 2005;121. Karger, Basel, Switzerland.

150. Zeman D, Neiger R, Nietfield J, Miskimins D, Libal M, Johnson D, et al. *Systemic Pasteurella haemolytica* infection as a rare sequel to avirulent live *Pasteurella haemolytica* vaccination in cattle. J Vet Diagn Invest 1993;5(4):555–9.

151. Hopkins BA, Olson LD. Comparison of live avirulent PM-1 and CU fowl cholera vaccines in turkeys. Avian Dis 1997;41(2):317–325.

152. Alexanderson S. Advantages and disadvantages of using live vaccines. Risks and control measures. Acta Vet Scand Suppl 2006;90:89–100.

153. Frey, J. Biological safety concepts of genetically modified live bacterial vaccines. Vaccine 2007;25:5598–5605.

154. Bakke T, Harris P. Diseases and parasites in wild Atlantic salmon (*Salmo salar*) populations. Can J Fish Aquat Sci 1998;55:247–266.

155. Pulkkinen K, Suomalainen L-R, Read AF, Ebert D, Rintamäki P, Valtonen ET. Intensive fish farming and the evolution of pathogen virulence: the case of columnaris disease in Finland. Proc R Soc B 2010;277:593–600.

156. Wiker HG, Harboe M, Nagai S. A localization index for distinction between extracellular and intracellular antigens of *Mycobacterium tuberculosis*. J GenMicrobiol 1991;137:875-884.

157. Adams GD. Lyophilization of vaccines. Methods Mol Med 1996;4:167–185

