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# Progress, challenges and opportunities in fish vaccine development

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ABSTRACT

In 2014 the contribution of aquaculture to supply food for human consumption overtook wild-caught fish for the first time. Despite improvements in the aquaculture industry, it has been estimated that as much as 10% of all cultured aquatic animals are lost because of infectious diseases, amounting to > 10 billion USD in losses annually on a global scale.

Vaccination to prevent disease is used routinely in finfish aquaculture, especially for Atlantic salmon (*Salmo salar*), while in a limited capacity (or not at all) in many other fish species due to lack of vaccines, poor performance or cost. There has, nevertheless, been impressive progress in fish vaccine development over the last 4 decades with 24 licenced fish vaccines now commercially available for use in a variety of fish species. These comprise whole killed, peptide subunit, recombinant protein, DNA and live attenuated vaccines.

Challenges do, however, still exist as the majority of commercial vaccines are killed whole cell pathogen preparations administered by intraperitoneal injection. This may not be the optimal route to deliver some vaccines, but lack of effective adjuvants and basic knowledge on immune response has hindered progress in the development of mucosal vaccines. The cost of injecting fish may also be prohibitive in some countries leading to disease treatment (e.g. with antibiotics) rather than using preventative measures. It is important that these issues are addressed as the industry continues to grow globally.

Exciting opportunities exist for rapid development of fish vaccines in the future, with continued reduction in cost of technologies (e.g. of whole genome sequencing), regulations changing (e.g. DNA vaccines can now authorised in Europe), the introduction of novel antigen expression and delivery systems (such as virus-like particles, VLPs), development of novel adjuvants and advancements in the elucidation of basic mechanisms of mucosal immunity. Development of effective mucosal vaccines and optimisation of their delivery will facilitate novel vaccine development, and enable the aquaculture industries in LMIC to use vaccination routinely in the future. In addition, effective use of emergency (autogenous) vaccines will assist in tackling emerging disease challenges.

## 1. Introduction

Aquaculture contributes ~80 million Tonnes of aquatic animals with a value of US$ 232 billion and represents the fastest growing animal production sector in the world, with twenty-seven finfish species contributing 90 per cent of global aquatic animal production [1]. Atlantic salmon (*Salmo salar*) is listed as the number one fish species in terms of economic value while Carp species are top with regards to volume. Aquaculture is not only an important source of income, but contribution to food security and social development of many countries. The main constraint to aquaculture globally, however, is disease with an estimate that 10% of all cultured aquatic animals are lost because of infectious diseases, amounting to > 10 billion USD in losses annually on a global scale [2].

Vaccines are recognised as important tools for the prevention and control of fish diseases and are used routinely in aquaculture, especially for Atlantic salmon (*Salmo salar*), while in a limited capacity (or not at all) in other fish species due to lack of vaccines, poor performance or cost. Vaccination has been recognised as an essential route to the reduction in use of antibiotics within the aquaculture industry in the UK and Norway [3,4] although overuse or inappropriate use has been reported in various fish species in other regions of the world [5–7].

## 2. Progress in vaccine development

*History of fish vaccines:* Atlantic salmon and rainbow trout aquaculture in the UK, Norway and USA expanded in the 1980's, accompanied by a rapid increase in disease, particularly with bacterial pathogens such are *Vibrio* species, *Yersinia ruckeri* and *Aeromonas salmonicida*. As a consequence large quantities of antibiotics were used

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and concerns grew with regards to antibiotic resistance. This stimulated the development of fish vaccines and led to the first commercially available fish vaccines against Vibriosis, Enteric Red Mouth (ERM) and Furunculosis. The first vaccine for aquaculture was actually the ERM vaccine for salmonid fish licensed in 1976 in the USA [8]. Currently 19 major companies market fish vaccines globally and many small companies also exist [9].

*Commercial vaccines*: The number of commercial vaccines available for use in fish have expanded, from 2 in the 1980s to 24 currently [10,11], with one vaccine also available for lobsters [12]. Fish vaccines are available for a wide range of species [2,11,12], including Atlantic salmon, Rainbow trout, sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*), tilapia (*Oreochromis niloticus/mossambicus*), amberjack (*Seriola dumerili*) and yellowtail (*Seriola quinqueradiata*) in Japan, catfish (*Ictalurus punctatus*) and Vietnamese catfish (*Pangasionodon hypophthalmus*). Most are formalin killed whole cell vaccines although live attenuated vaccines are licensed in the USA for use in catfish [13]. A DNA vaccine against infectious haematopoietic necrosis (IHN) is licensed in Canada for use in Atlantic salmon [14] and a subunit vaccine (peptide; VP2) is used in Norway (against infectious pancreatic necrosis virus, IPNV) and a recombinant vaccine against infectious salmon anaemia virus, ISAV is used in Chile. Many Atlantic salmon vaccines are multivalent and there is a trend towards micro-dose application (*i.e.* 50ul versus 100ul). Although carp and tilapia are well established cultured species, there are few vaccines available for these species and the number of commercial vaccines available for trout has decreased, with the monovalent furunculosis vaccine being taken off the market.

There are a number of important considerations for the use of commercial vaccines in fish, including fish species, status of the immune system, production cycle and life history, when disease occurs, farming technology (handling, mechanisation etc), environment (*e.g.* temperature, salinity), stress factors, nutrition and cost benefits. Guidelines on the use of fish vaccines are provided by Responsible Use of Medicines in Agriculture Alliance [15]. The majority of commercial vaccines include adjuvants and are administered by intraperitoneal injection [11].

*Fish species and diseases where vaccines are needed for aquaculture*: Bacterial disease still presents major challenges for rainbow trout, carp, tilapia and catfish aquaculture. In addition, there are few effective vaccines against viral diseases and these pose significant problem in salmonid and marine finfish [16]. Parasite diseases, in particular the Ectoparasites, *Lepeophtheirus salmonis* (sealice) and *Paramoeba perurans* (which causes amoebic gill disease, AGD), currently represent significant disease threats for the Atlantic salmon industry and no commercial vaccines exist for these, nor for fungi or fungi-like organisms. In addition, there are no vaccines for opportunistic facultative parasites (*e.g. Saprolegnia* and *Aphanomyces*) that are problematic in salmonid aquaculture and tropical fish species (many freshwater and brackish species in the Asia-Pacific region and Australia), respectively.

## 3. Challenges in fish vaccine development

The most crucial step in developing an effective vaccine is identification of 'potentially' protective antigens and confirming their protective response in the host species against the pathogen of interest. The approach taken depends on pathogen type, fish species, administration method, availability of reagents, and whether a challenge model has been developed to efficacy test the vaccine candidates. Identifying protective antigens is not easy and requires a variety of approaches.

The majority of commercial fish vaccines are killed whole cell pathogens preparations and are administered by intraperitoneal injection. Using whole pathogens in a vaccine can pose problems if the pathogen species is very heterogeneous, when they are difficult or expensive to culture, if some epitopes immunosuppress, and in general for intracellular or complex pathogens (*e.g.* parasites). In addition, injection may not be the optimal route to deliver some vaccines and mucosal delivery may be required for effective protection, however, lack of adjuvants and insufficient basic knowledge has held back progress in the development of mucosal vaccines. The cost of injecting fish may also be prohibitive in some countries, with costs of hiring staff to inject fish and convenience playing a large role in available commercial fish vaccines in Low to Middle Income (LMIC) counties.

*Challenge models:* Testing the efficacy of vaccines requires standardised *in vivo* disease challenge models that closely mimic the natural exposure route to the pathogen. Although more difficult to control and standardise than injection challenge methods, bath and co-habitation challenges best fulfil the requirement of natural exposure. Pathogen load (measured by qPCR) and immunological markers of protection (analysed by immunohistochemistry and/or gene expression) can be used as proxies to test the efficacy of vaccines if no experimental disease challenge method is available or may not be reproducible; field trials can be performed is certain circumstances. For example, Rainbow Trout Fry Syndrome (RTFS) caused by the gram negative bacterium, *Flavobacterium psychrophilum*, is very difficult to induce experimentally by bath or co-habitation challenge unless scarification or stress is used [17,18]. Although intramuscular injection induces disease, his is not an appropriate challenge method to test a mucosal vaccine (*e.g.* dip immersion) administered to fry. Pre-treatment of fish with low levels of hydrogen peroxide is required for the challenge to succeed but the even with this treatment the level of infection is not sufficient to fully test vaccine efficacy [19–21] so field trials are planned.

*Fish Species and Diseases:* The diversity of fish species itself poses a challenge in vaccine development as we still do not fully understand the fish immune system and each fish species requires reagents/primers to elucidate host pathogen interactions. In addition, although injection is commonly used for Atlantic salmon, this may not be viable for some species *e.g.* tilapia and *Pangasius*.

*Administration methods:* Optimal methods of vaccine administration still need to be determined. It may be that some novel vaccines being develop *are* protective, but current administration methods and vaccination strategies are not appropriate for optimal efficacy (*e.g.* may need prime/booster vaccination). Fish have large mucosal surfaces (skin, gills, gut and nasal mucosae) and administration of vaccines via the mucosal route is also more practical and affordable for some sectors than injection. A limited number of mucosal (immersion and oral) vaccines are, however, commercially available. A number of challenges have hampered their development, including lack of correlates of protection, lack of optimisation of protective doses required, possibility of oral tolerance, the potential for denaturation of oral vaccines in the stomach, and the ability of antigens to cross mucosal barriers to gain access to antigen presenting cells (APCs) [22]. Antigen presenting cells (APCs) have been reported in the mucosal organs of fish and evidence suggests that fish APC's can be activated in a similar manner to mammalian APCs to enhance antigen uptake and presentation to the adaptive immune system [22].

*Adjuvants:* Adjuvants are included in injection vaccines but are limited for mucosal vaccination. Adjuvants are a group of structurally heterogeneous compounds that are capable of modulating the intrinsic immunogenicity of an antigen [23]. They have been classified according to the immune response they elicit, Signal 1 (presentation of antigen) or Signal 2 facilitators (additional secondary signals) [24]. Both are required for activation of specific T and B lymphocytes [25]. Adjuvants have improved over recent years and a range of effective products (*e.g.* Montanides, classified as Signal 1 adjuvants) are available from SEPPIC for use with injection vaccines for fish. Signal 2 adjuvants, such as beta glucans, alums, saponins, poly I:C, synthetic oligonucleotides, cytokines and flagellin, provide co-stimulatory signals during antigen recognition, recently reviewed by Dalmo, Bogwald and Tafalla [26]. Recent work using a recombinant flagellin from the salmonid pathogen *Yersinia ruckeri*, showed it to be a potent activator of inflammatory cytokines, acute phase proteins and antimicrobial peptides *in vitro* and to be a more inflammatory activator than other bacterial

PAMPs (LPS, peptidoglycan) [27]. *In vivo* studies revealed that it activated a variety of anti-microbial pathways with heightened expression of acute phase proteins, antimicrobial peptides and complement genes in multiple tissues. Trout liver in particular appeared responding to flagellin stimulation, with marked induction of IL-11, IL-23P19, IL-17C1, SAA, and cathelicidin-2. Overall this suggested that flagellin could be a potent immunostimulant and vaccine adjuvant for fish aquaculture [27]. Despite these exciting new findings there is currently a lack of effective commercial immersion adjuvants for use in fish.

*Immune response and markers of protection:* It is important to be able measure the responses induced by vaccination in teleost fish to enable the development of new and evaluate existing vaccines. The adaptive immune response has the main role in providing protection following vaccination, mediated by T and B lymphocytes. There are gaps in knowledge, however, especially with correlates of protection for mucosal vaccines [22; 47], although progress is being made on in this area. For example, protocols have been developed for isolation of GALT cells from salmonids and these have been shown to express a wide range of T-cell, B-cell and dendritic cell markers, and to be differentially responsive to a panel of PAMPS, cytokines and PHA [48]. Such information will assist in the development of oral vaccines.

*T cell response*: Efficient antigen recognition and presentation are required for effective vaccination. Vaccine antigens are usually presented through MHC-11 molecules to T cells, while DNA vaccination is mediated through the MHC-1 route, although it should be noted that both responses can be triggered simultaneously with one prevailing as the immune response develops, reviewed by Secombes and Belmonte [28]. This can be influenced with choice of adjuvant. Markers of protection for T cell responses still pose a challenge despite numerous studies on cytokine expression [28].

*B cell response*: With regards to B cell responses, correlation with antibody production does not necessarily infer a protective response; passive immunisation can be used to demonstrate the protective effect of antibodies [28]. In fish, in contrast to mammals, there appears to be no increase in antibody level following booster vaccination and it is thought that more cells are actually produced during the memory response rather than an increase in antibody level. Numerous publications report increases in specific IgM following injection vaccinations, while IgT has been reported following immersion vaccination is some fish species. For example, immersion vaccination of rainbow trout with an RTFS vaccine resulted in an increase in IgT producing cells in the kidney [21]. Munang'andu, Mutoloki and Evensen [29] suggested that there is compartmentalisation in the physiological distribution of IgT and IgM in some mucosal organs, as demonstrated in the gills where IgT is mainly found on exterior surfaces of the gill lamellae [30,31] suggesting that this isotype may act as gatekeeper at pathogen portals of entry. In contrast, as IgM is mainly found in arterioles [30,31], Munang'andu, Mutoloki and Evensen [29] suggested that its role is a secondary defence strategy that would kick in when IgT on mucosal surfaces fails to prevent the penetration of pathogens into the systemic environment.

## 4. Opportunities and future directions

The genomes of a variety of fish species are now available. In addition, with the reduction in cost of technologies, such as the whole genome sequencing, the genomes for specific pathogens are regularly reported [32], enabling targeted vaccine design for heterogenous species. For examples, Ngo and co-authors [33] characterised over 300 *Flavobacterium psychrophilum* species, mainly form the UK, and produced an effective tivalent whole cell vaccine. These results are also extremely important from an epidemiological point of view [32]. Sometimes it may not be possible to develop a whole cell vaccine as some epitopes may be immunosuppressive and therefore specific potential protective antigens need to be identified and vaccines produced, thereby eliminating unwanted epitopes.

Identification of specific potential protective antigens:

Reverse vaccinology is a genome based approach widely used to identify potential vaccine candidates for development of protein subunit vaccines. Protein sequences from the pathogen are screened and then potential vaccine candidates selected using software programmes. They may be selected on the basis of being known vaccine candidates for other pathogens, highly immunogenic proteins or other criteria. Recombinant subunit vaccines (or DNA vaccines) are then produced and efficacy tested *in vivo*. DNA vaccines are now authorised for use in Europe [49] and so many more will likely be developed in the future.

*mRNA vaccines*: As an alternative to DNA vaccines, mRNA vaccination is now being used in clinical medicine. The technology was first introduced in 1990 but as there were concerns over stability, high innate immunogenicity and inefficient *in vivo* delivery it was not pursued at that time. Stability and delivery methods have been improved and the vaccines can be produced quickly and inexpensively [34]. Such vaccines have been used particularly for prophylactic and therapeutic applications in cancer, with clinical trials of mRNA vaccines for infectious disease still in their infancy. They have been shown to be safe in clinical trials for HIV, but although the vaccines elicit antigen specific $CD4^+$ and $CD8^+$ T cell immune responses, there was no reduction of viral load. Vaccination against rabies virus demonstrated that efficacy was highly dependent on the dose and route of administration, with needle free administration superior to direct injection, reviewed by Pardi and co-authors [34].

*Vaccine delivery using nanoparticles:* Nano materials ($< 1000$ nm) such as virus-like particles (VLPs), liposomes, immunostimulating complexes (ISCOMs), polymeric, and non-degradable nanospheres have been reported to have potential as delivery vehicles for vaccine antigens; these stabilise vaccine antigens as well as acting as adjuvants [35]. They can drive the immune response in various directions and may be important for induction of protective responses. Such delivery systems are suitable for mucosal delivery of vaccines. Nanoparticles serve both as antigen delivery vehicles and to allow a sustained release of antigens, thus reducing the need for booster vaccinations [22,36–39]. Among the polymeric systems, poly D,L-lactide-co-glycolic acid (PLGA) nanoparticles have been widely used for the controlled delivery of peptides, synthetic proteins, and nucleic acids in humans [40] and have been tested in fish for the delivery of oral vaccines [36–39]. More recently VLPs have been tested in fish. These are considered a novel vaccine platform because they are not infectious and they induce neutralizing antibodies. Chien, Wu and Li [41] demonstrated that orange-spotted grouper NNV (OSGNNV) VLPs have potential as oral vaccines in grouper. The recombinant capsid proteins were produced in *Escherichia coli* and cell-free self-assembled into VLPs. *Pichia pastoris* can be used as an alternative expression system to *E.coli* and has been shown to be a good vehicle for oral antigen delivery. It can be used in non-encapsulated form for older fish or in bio-encapsulated form for larval fish, while the yeast itself acts as an adjuvant [42].

*Prime versus prime booster vaccination:* Injection vaccines administered with adjuvants are generally only injected once, eliciting a long lived response (up to one year). Immersion vaccination performed without adjuvant, on the other hand, will be short lived and a booster vaccination will normally be required. Fish vaccines so far, have not, provided sterile immunity and perhaps stimulating both mucosal and systemic immunity is required to achieve this [22]. This could be achieved by an early immersion vaccination then IP booster vaccination, or IP booster vaccination followed by oral booster vaccination.

*Live attenuated vaccines:* The first vaccine to protect humans against smallpox was a live vaccine in the 1770s [43]. Such vaccines have the advantage that they stimulate both humoral and cellular activity, but due to safety concerns they are not allowed to be used in European aquaculture. This is because there is a risk that live attenuated isolates may revert to virulent forms. Traditionally attenuation was achieved by passaging the pathogen *in vitro* multiple times leading random

mutations. Molecular methods can now be used to target specific genes and produce genetically modified the pathogens, but these are classified as GMOs and regulations for their use are very strict in aquaculture, more so in fact that traditional live vaccines [9]. It has been reported that defined genetic modifications permit better control and safety than random mutations in live bacterial vaccines [44], so perhaps these types of vaccine will be permitted in the future.

*Autogenous vaccines*: It is expensive and time consuming to develop commercial vaccines and there are a huge variety of fish species being cultured that are susceptible to a various diseases. It is therefore not realistic to develop licenced vaccines against all fish pathogens, and this may in fact not be possible for some particular pathogens due to a variety of reasons. Emergency, or autogenous vaccines, can be a useful alternative. For example, cleanerfish (*e.g.* Ballan wrasse, *Labrus bergylta* and lumpsucker, *Cyclopterus lumpus*) that are being used to control sealice in Norway and the UK are affected by disease. The pathogens isolated from Ballan wrasse appear to vary from site to site and autogenous vaccine against bacterial pathogens have been effective in controlling at least some of these diseases, such as atypical *Aeromonas salmonicida* when administered by injection [45]. Nevertheless, diseases are still problematic in fry and there is a lack of knowledge of when this species becomes immunocompetent and can be successfully vaccinated by immersion vaccination, if at all. Recently, it has been reported that natural IgM is at very high levels in the intestine of Ballan wrasse and it was speculated that this may be a compensatory mechanism in stomach-less fish [46].

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