Chapter 3
Development of Fish Vaccines: Focusing on Methods

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Abstract Sustainable aquaculture is not possible without disease prevention, and vaccination has become the single most important tool. There has been a dramatic reduction in the use of antibiotics in Norwegian salmon farming since the introduction of oil-based vaccines. Today, it is an industry standard in all salmon-producing countries, and we are seeing a similar approach being adopted in other countries producing high-value fish species, e.g. Japan. Fish can be vaccinated by immersion and via the oral route; however, the protection falls short using these methods compared to injection vaccination. Interesting new technologies have emerged over the last 5 years, particularly injection of a single dose of naked DNA into the fish muscle. New technologies are promising, but it is more likely that there will be improvements of existing vaccines than completely new technologies taking over the fish vaccination scene in the next 5–10 years.

Introduction

Sustainable development of aquaculture relies on disease prevention. We see an intensification of the production for several aquacultured fish species and, with this, an increased risk of disease and also spread of infectious diseases through transport and/or trade.

There is a profound and consistent positive attitude towards vaccines. Vaccines stimulate the immune system to help fight off diseases, and vaccination is of growing importance to control infectious diseases. This article summarises the development in fish vaccinology with focus on methods applied and discusses possibilities and limitations regarding the use of vaccination for control of infectious diseases in commercial fish farming.

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Immunological Basis

Fish possess, as mammals, a defence system, which enables them to survive and maintain their integrity in a hostile environment. The major lymphoid tissues in teleost fish are the (head) kidney, thymus, spleen and mucosa-associated lymphoid tissues (Press and Evensen 1999), including the skin (Xu et al. 2013), the gills (Haugarvoll et al. 2008) and the nostrils (Tacchi et al. 2014). An obvious difference from the mammalian immune system is that fish lack bone marrow and lymph nodes (Press and Evensen 1999).

Immunity and Vaccination of Fish

Vaccines aim at stimulating the adaptive immune system to mount a response against a pathogen or rather against defined structures of the pathogen, the immunogenic parts. Vaccination has been used as a prophylactic means in aquaculture for decades, and it has been estimated that ten percent of all cultured aquatic animals are lost because of infectious diseases alone, amounting to >10 billion USD in losses annually at global scale. High-value species like Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*) are vaccinated against a variety of diseases (Gudding et al. 1999). Administration of vaccines to aquatic animals represents obvious technical challenges different from what is encountered in terrestrial production systems. Modalities used are injection, immersion or oral delivery.

The Start

The history of fish vaccination dates back to an early publication by Duff in 1942 working on vaccinating against *Aeromonas salmonicida* infection in cutthroat trout (Duff 1942), and oral immunisation strategies were used to protect against the disease. This research and studies came out of the Pacific Biological Station in British Columbia, Canada. Many of the pioneers in the fields of fish vaccination were people with a scientific background that combined good theoretical knowledge with excellent understanding of practical fish farming. Many companies were involved at early stages, and Wildlife Vaccines (in Colorado) included Guy Tebbit, John Rohovec and Thomas Goodrich as main contributors (Gudding and Goodrich 2014). In 1976, this company was the first to manufacture and license a vaccine for fish. The second vaccine for fish was licensed by a subsidiary of Johnson & Johnson (Tavolek Inc.). Vaccine development for farmed fish gained momentum when Biomedical Research Laboratories in Bellevue outside Seattle, Washington, established their research and development programmes. Pioneers were Stephen Newman, Tony Novotny, Robert Busch and James Nelson. Their focus was production of bacterins, and Biomed Inc. was the first company to launch an oil-based

vaccine against furunculosis in Norway in 1992–3. Other companies were Aqua Health Inc. working out of Charlottetown, Canada (today part of Novartis), and Aquaculture Vaccines Ltd. in the UK (today Merck). All these companies and people played an important role in the early days of fish vaccination. In the mid-1980s, Apothekernes Laboratorium (AL; a Norwegian pharmaceutical company with focus on generic pharma) established their fish vaccine and therapeutics activities, and they acquired Biomed in the mid-1990s. AL's vaccine production started in Tromsø and later moved to Overhalla in mid-Norway, and they later changed name to ALPHARMA AS. PHARMAQ AS was later "created" through management buy-out in 2004, today with global presence. Yet another Norwegian company was Norbio, established in 1985 (in Bergen) and recruited scientists from the university in the region. Norbio was later sold to Intervet (1993), became part of Schering-Plough (2007), and in 2009 their vaccine division merged with the Merck group. Finally, Novartis is also involved in the fish vaccine business and acquired Aqua Health Inc. in 2000 on which they have later built their aquabusiness. The animal health business of Novartis was taken over by Elanco in 2014. Over the last decade, we have seen small companies growing in emerging markets, in Europe serving the Mediterranean region (Fatro Inc.), the Chilean salmon market (Centrovet Inc., Veterquimica) and also towards carp production in Israel (KoVax Inc.).

Current Vaccines in Different Markets

The majority of vaccines currently available for farmed fish are prepared by conventional methods, i.e. typically a suspension-based fermentation of bacteria or virus harvested from cell culture. Inactivation methods typically include the use of formalin or alkylating compounds, and downstream methods can include filtration ("washing"), concentration of antigens or purification of antigen preparations.

Vaccines available in the salmon markets (Norway, Faroe Islands, the UK, Ireland, Canada, the USA and Chile) are oil-adjuvanted, injectable vaccines and are provided by the main manufacturers (Centrovet, Elanco, Merck and PHARMAQ). Oil-based vaccines for injection are available for use in sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) in the Mediterranean countries against photobacteriosis (Sommerset et al. 2005; Hastein et al. 2005). Vaccines for use in the salmon markets are multivalent and can contain as many as seven different antigens. While salmon transferred to sea was previously vaccinated with a monovalent, oil-adjuvanted vaccine against pancreas disease (PD) in Norway; this antigen has now been included in a 7-valent vaccine (manufactured by MSD). In Canada (British Columbia), Atlantic salmon are vaccinated against infectious haematopoietic necrosis (IHN) with a plasmid (DNA) vaccine (intramuscularly) (Alonso and Leong 2013) in addition to vaccines given intraperitoneally.

An oil-adjuvanted vaccine against enteric septicaemia of catfish (*Pangasionodon hypophthalmus*) is licensed in Vietnam (by PHARMAQ AS), an inactivated vaccine based on whole cell preparations. Vaccines against *Streptococcus agalactiae*

infections in tilapia (*Oreochromis niloticus/mossambicus*) are available (from Merck) for use in several Asian countries and in Brazil. This vaccine is based on biotype 1 of *S. agalactiae*. There are also vaccines available against *Lactococcus garvieae* infections for use in rainbow trout in the Mediterranean region and for amberjack (*Seriola dumerili*) and yellowtail (*Seriola quinqueradiata*) in Japan, also combined with *Photobacterium damsela* sp. *piscicida* and *Vibrio anguillarum* (Sommerset et al. 2005; Hastein et al. 2005).

An inactivated, whole virus vaccine against red sea bream iridovirus infection (Nakajima et al. 1999) is available in Japan, combined with *Lactococcus garvieae* and *Vibrio anguillarum* (serotype J-O-3) (Kyoritsu Seiyaku Corp.). A monovalent iridovirus vaccine (against megalocytivirus) is available (from Merck) for use in Asian markets (tilapia). These vaccines are non-adjuvanted or oil adjuvanted.

Inactivated, immersion vaccines are available for vaccination against *Photobacterium damselae* sub-species *piscicida* in European sea bass and bream (from Merck). Similarly, an inactivated vibriosis vaccine is available for the same species (from Merck). An oral ERM (enteric redmouth) vaccine for use in trout is also available from the same supplier.

There is currently one commercial subunit (peptide; VP2) vaccine available for use against IPN in Norway (Merck) and one recombinant vaccine against infectious salmon anaemia (Centrovet) in Chile. The antigens are expressed in *Escherichia coli* (IPNV VP2 protein) or in *Saccharomyces cerevesiae* (ISAV HE and F proteins). Live attenuated vaccines against enteric septicaemia (*Edwardsiella ictaluri*) and *Flavobacterium columnare* infections of catfish (*Ictalurus punctatus*) are licensed in the USA (Klesius and Pridgeon 2014).

Vaccine preparations intended for injection are delivered at 25, 50 or 100 µl per fish per vaccine preparation, irrespective of fish size. The vaccine is delivered intraperitoneally and injected in the midline, approximately 1.5 cm cranial to the caudal fins. DNA vaccines are injected intramuscularly at 0.1 ml per fish. Live attenuated vaccines are delivered to small fish by immersion.

Development Trends

The development of fish vaccines goes along three major trends: (i) *Mode of delivery*, i.e. vaccination via mucosal surfaces (immersion or oral) or injected. (ii) *Nature of the antigen*, i.e. non-replicating or replicating vaccines, which are still the two poles of vaccine technology developed by Louis Pasteur more than 100 years ago (Pasteur 1880). This cover classical inactivated bacterial or viral vaccines. (iii) *Recombinant technologies* where purified or designed subunit, protein-based vaccines are used. Recombinant DNA-based technologies have taken this further where antigens are expressed in vector viruses, and in designed, attenuated virus and bacteria. Ultimately. one can use the animal to "produce" the antigens, via injection of plasmids encoding defined antigenic parts of the pathogen. The different trends will be discussed in more detail below, and advantages and disadvantages for the different delivery modes are summarised in Table 3.1.

Table 3.1 Summary of different routes of administration for vaccines for farmed finfish

Route of administration	Type of formulation/ modality	Advantages	Disadvantages
Injection (non-replicating; replicating vaccines)	Often comes with adjuvant; oil-based (water-in-oil, oil-in-water or w/o/w); aluminium salts; experimental liposomal vaccines	Most potent, little waste of vaccine Allows the use of adjuvants Cost-effective method for high-value species Mass vaccination is possible, but costly and time-/resource demanding	Stressful to the fish Impractical for fish <15 g (depending on species) Labour intensive and costly Injection-site reactions
Immersion (non-replicating; replicating vaccines)	Limited used in salmon Marine fish (smaller) more frequent use Live attenuated vaccines Vector vaccines	Large-scale application possible Moderate stress to the fish Moderately labour intensive Allows mass vaccination of immunocompetent fish High efficacy using live, attenuated vaccines	A large amount of vaccine is needed, can be cost prohibitive Low to moderate efficacy for inactivated vaccines, depending on antigen Inferior to injection delivery in terms of efficacy Cost prohibitive for large fish
Oral delivery	Top-dressing Incorporation into pellet (post extrusion) Formulation in PLGA; micromatrix systems (experimental)	Imposes no stress to the fish Moderate to high cost All fish sizes can be vaccinated when immunocompetent Usually safe – primes mucosal immunity (external surfaces) Used in combination with injection vaccines or immersion	Usually moderate to low efficacy when administered alone Better prospects as "boost" strategy Can be cost prohibitive for larger fish

w/o/w water-in-oil-in-water

Modalities: Mucosal Vaccines

Vibrio anguillarum and *Yersinia ruckeri* bacterins induce good protection following immersion vaccination of rainbow trout (Johnson and Amend 1983) and also Atlantic salmon and several marine fish species (Hastein et al. 2005). The protective antigen(s) are LPS for both pathogens (Croy and Amend 1977a), particularly the O-antigens (Welch and LaPatra 2016). The antigens are taken up across mucosal surfaces (gill, skin, stomach or gut) (Joosten et al. 1997) and likely induce local

immunity (mucosal) and/or a systemic immunity sufficient to protect the animal against lethal challenge. For other diseases, it has been difficult to obtain a sufficient level of protection using immersion or oral delivery, typical examples being furunculosis (*Aeromonas salmonicida*), pasteurellosis (*Photobacterium damselae* spp. *piscicida)* in sea bass and *Edwardsiella ictaluri* infections in catfish and pangasius. There are exemptions to the rule (Villumsen and Raida 2013; Thinh et al. 2009), but the use of mucosal vaccines against furunculosis and pasteurellosis are not frequently used under commercial conditions (Hastein et al. 2005). The explanation for mucosal vaccines falling short compared to injection vaccines are not understood in detail. The induction of an immune response after mucosal immunisation is dependent on either local responses (in the mucosa) or uptake of antigens from the external surfaces and/or the gut lumen and systemic distribution to head kidney and/or spleen. In higher vertebrates, proliferating and dead particulate antigens (as well as soluble antigens) are taken up through a specialised follicle-associated epithelium, the so-called M ("membrane") cells, and with subsequent transepithelial transport to underlying lymphoid tissue, the Peyer's patches (Brandtzaeg et al. 1987). Regular M cells are not found in fish, but cells with antigen-sampling capabilities have been identified in the gut epithelium of Atlantic salmon (Fuglem et al. 2010). Their involvement in particle uptake in association with oral vaccination is not known.

Despite the observation that vaccine efficacy in fish is limited after oral delivery, there are very few studies that address the uptake and transepithelial transport in enterocytes of soluble versus particulate antigens. The morphological or functional characterisation of enterocytes is also scant, yet there are indications for a regional specialisation of the gut epithelium with regard to uptake of macromolecules, and the hindgut enterocytes are considered important in this respect (Georgopoulou et al. 1985), and possibly sampling cells located between epithelial cells (Fuglem et al. 2010). Trinitrophenylated-lipopolysaccharides (TNP-LPS) and biologically active proteins like horseradish peroxidase are absorbed from the gut into the circulatory system (Doggett et al. 1993). Studies of uptake of particulate antigens in stomachless carp (*Cyprinus carpio*) using a bacterin of *Vibrio anguillarum* have shown that the bacteria are taken up by epithelial cells in the second gut segment and are later identified in intraepithelial macrophages (Rombout and van den Berg 1989). However, no attempts were made to distinguish between soluble (such as LPS) and particulate components of the antigen preparation, and thus no conclusion could be made with regard to the transport of particulate versus soluble antigens across the epithelial cells (Rombout and van den Berg 1989).

Nakanishi and coworkers (Nakanishi et al. 2002) explored some of the underlying factors that could possibly explain the importance of particle uptake across the dermis; this is for *Streptococcus iniae* vaccines in trout. They punctured the skin of the fish prior to vaccination allowing for percutaneous delivery when the fish were submerged in vaccine solutions (non-replicating) of α-haemolytic *Streptococcus sp.* It was shown that the puncture method facilitated uptake of antigens into the skin (and subcutaneous tissue), and the protection achieved was comparable to injection-vaccinated groups, while immersion gave no protection (Nakanishi et al. 2002). Skin puncture will result in a high number of particulate antigens being taken up for systemic distribution to lymphoid tissues, thereby inducing protective immune

responses. There will be no antigen uptake (to systemic distribution) in non-punctured fish, and thus the immune responses will be weak and non-protective. There are two important things to learn from this study. First, protection against *Streptococcus iniae* cannot easily be obtained through mucosal delivery. Second, protection against *S. iniae* is reliant on systemic distribution of particulate antigens likely involving the kidney/spleen in immune induction. This contrasts the protection obtained with bacterin preparations of *Vibrio* or *Yersinia* antigens delivered on mucosal surfaces, aligning with an understanding that protective antigens are LPS associated (Welch and LaPatra 2016; Croy and Amend 1977b) and likely soluble. Further to this, one can ask when local immunity versus systemic immunity would be required to obtain protection against mortality/disease?

It is known that the mucosa harbours a large community of commensal microbes, and the mucosal immune system deploys a wide variety of cells (locally) that creates a complex regulatory network with the aim to establish a balance between surveillance for pathogens and immunological tolerance (Chen and Cerutti 2010). Although there is limited knowledge of the interaction between the commensal flora and the immune cells in general and in fish in particular, one can anticipate that mucosal vaccine would have to leverage the functions of these immune cells and regulatory components. There is an asymmetric principle observed in mice where vaccines delivered parenterally induce strong systemic responses but fail to induce mucosal immunity (Belyakov et al. 1998). In contrast, mucosal vaccines elicit a local response and at the same time induce systemic immunity. These phenomena have not been studied to a very limited extent in fish (Zhang et al. 2010). In a recent study, we showed that salmon vaccinated against IPN had circulating antibodies and upregulated transcript levels of IgM mRNA in the kidney, while the hindgut was negative (Chen et al. 2015). When these fish were boosted orally with IPNV antigen, we saw upregulation of IgM mRNA in the kidney but also in the gut. These findings mirror the asymmetry described for immune responses in mice, and the practical importance of these findings needs to be explored in more detail in future studies.

The importance of the commensal flora for postnatal maturation of the immune system has not been studied to any detail in fish, but it is known to play an important role for postnatal maturation of mucosal immune systems in higher vertebrates (Artis 2008; Macpherson and Uhr 2004). It has been shown that intestinal IgA responses are directed against a minor proportion of the commensal microbial species (Chen and Cerutti 2010; Cerutti et al. 2011). It is further known that IgA immune responses in the intestine have a high threshold, and 10^8–10^9 bacteria are needed to elicit a response, below which there is no IgA production. Further, IgA responses lack the classical memory features seen with priming and boost, and another interesting finding is the attrition phenomenon observed for IgA responses, i.e. subsequent challenges with different antigens diminish the response to previous antigenic challenges. It thus seems as if IgA responses in the gut are programmed for strength control, and thereby IgA levels adapt to the microbiota of the gut. This is conceivable under a concept where high abundance of microbial species is more likely to breach the epithelial barrier. This brings an interesting concept to the recently discovered mucosal Ig in zebrafish and trout where gut bacteria were found covered with IgT (in trout)

under normal conditions (Zhang et al. 2010). There are several studies on the importance of delivery of antigens to different compartments in the gut, and long before IgT was described, it was shown that anal and to a lesser extent oral delivery of bacterins of *Yersinia ruckeri* will result in a high level of protection (Johnson and Amend 1983). Similarly, previous studies in other fish species have shown that bacterial antigens of *Vibrio anguillarum* were identified in the hindgut epithelium, but no transport to the circulation was observed (Tatner et al. 1984; Nelson et al. 1985). Interestingly, the LPS moiety of the bacterial cell is considered an important component of the protective antigens (Croy and Amend 1977b), and it is possible that induction of local immunity is sufficient to protect against lethal challenge with *V. anguillarum*.

The role of gut IgT (or skin) is still to be explored in more detail, and one role that should be subject of future research is the possibility that IgT plays a role in controlling the relative number of bacterial species in the gut and possibly also on the skin. Further studies are needed to understand these interactions in more detail.

Modalities: Injection Vaccines

Vaccination of Atlantic salmon against furunculosis and vibriosis/cold-water vibriosis with oil-adjuvanted vaccines results in the induction of long-lasting and protective immunity. The protection obtained is good and long-lasting, and salmon vaccinated with oil-adjuvanted vaccines against furunculosis will not result in clinical disease outbreaks after transfer to seawater (Gudding et al. 1999). Retention of antigens at the injection site is believed to be a prerequisite for long-term protection in fish, also known as the depot effect (Evensen et al. 2005). The antigens of a water-in-oil emulsion are located in the water droplets (mainly) and the distribution of antigens (schematically in Fig. 3.1). However, the

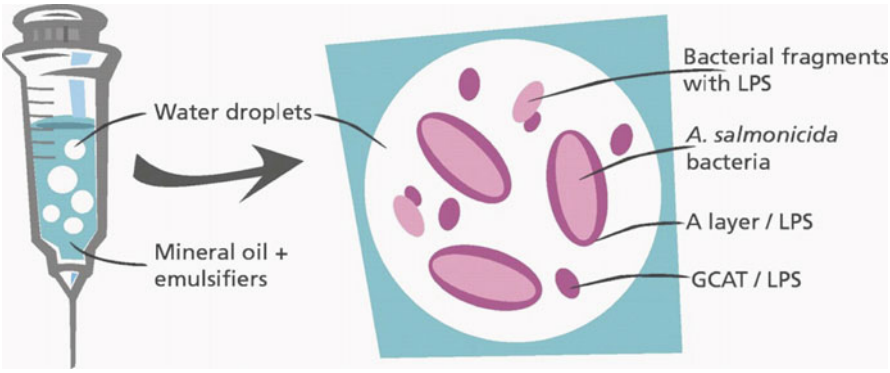

Fig. 3.1 Schematic presentation of a water-in-oil formulation. Water droplets are dispersed in a continuous oil phase, and antigens (here bacterial components) are found within the water droplet and at the interphase between water and oil, possibly also in the oil phase (Illustration by Ida Skaar)

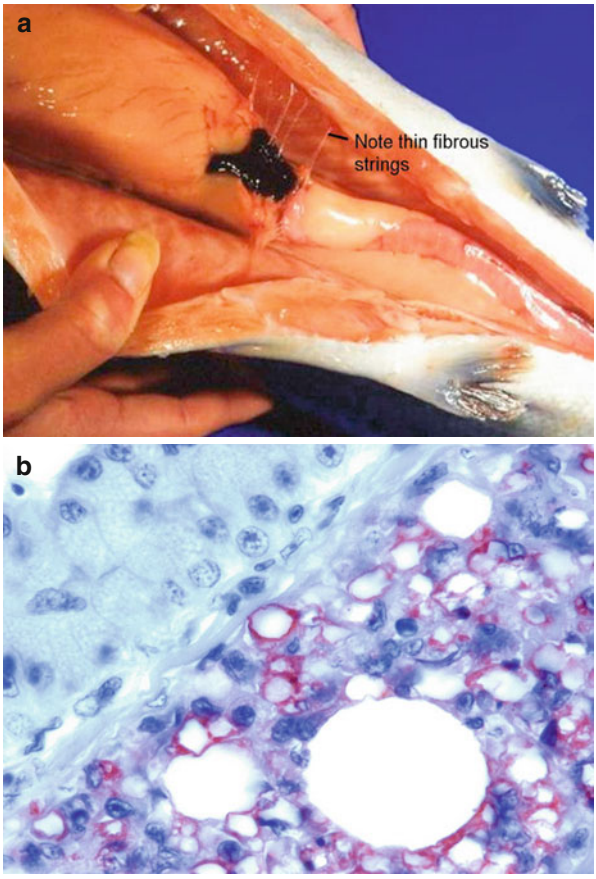

Fig. 3.2 (**a**) Mild intraperitoneal granulomas in Atlantic salmon following the use of oil-adjuvanted vaccines (photo by Trygve Poppe). (**b**) Immunohistochemical documentation of *Aeromonas salmonicida* LPS antigen (*red colour*) present in a peritoneal granuloma in Atlantic salmon vaccinated with oil-adjuvanted vaccine

drawback is that oil-adjuvanted vaccines result in the formation of visible injection-site lesions (Figs. 3.2a, b) that persist through to harvest size (Midtlyng 1997). Antigens (LPS) of *A. salmonicida* can be found as long as 18 months post vaccination using immunohistochemistry and in situ detection of antigens (Fig. 3.1b) (Evensen et al. 2005).

The side-effect may on some occasions also result in downgrading of fish at slaughter or after processing, and in fish with high side-effect scores, immune profiling indicates an autoimmune response (Mutoloki et al. 2010; Koppang et al. 2008). The intra-abdominal lesions are recognised grossly as melanisation and adhesions between internal organs or between the organs and the peritoneal wall (Mutoloki et al. 2004). Histomorphological examination shows the presence of granuloma with macrophages, epithelioid-like cells, and occasionally with formation of multinucleate giant cells and with varying numbers of lymphocytes and eosinophilic granular cells (EGC)/mast cells (Mutoloki et al. 2006, 2008).

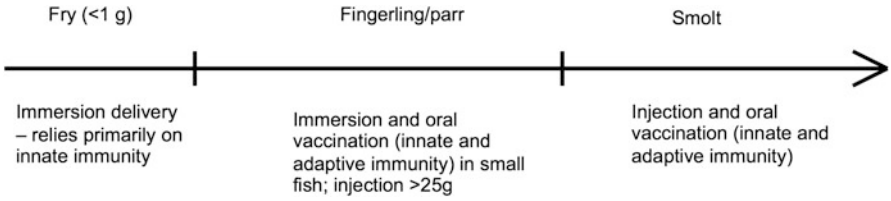

Fig. 3.3 Example from Atlantic salmon where size is given indicated relative to immunocompetence and vaccine modalities. In fry below 1 g, adaptive responses have not matured and any stimulation of the immune system would have to rely on an innate immune response. At a later stage and up to around 10 g, immersion or oral vaccination is preferred, while in larger fish, parenteral delivery is applied

Modalities and Fish Size

With intensification of fish farming, it has become prudent to protect the fish throughout the production cycle, including the early life cycle stages. Adaptive responses rely on partial or full immunocompetence, and the recommendation would be to postpone vaccination until the fish reach an age where they are able to mount an adaptive immune response. For salmonids, this will typically be around 0.5–1 g (Tatner and Horne 1983), while for other species, the animal can potentially have developed an ability to respond to vaccination at an even earlier stage, i.e. smaller sizes (Padros and Crespo 1996; Watts et al. 2003). Immersion and oral vaccines would have to meet these requirements, as would injection vaccines (Fig. 3.3).

Non-replicating or Nonlive Vaccines

The majority of fish vaccines intended for injection are inactivated, whole virus/bacteria vaccines, often prepared with an adjuvant like mineral or vegetable oil. Immersion vaccines are usually non-adjuvanted, and the majority of these vaccines are bacterin-based, while live vaccines, for injection or immersion, are used to a very limited extent. In line with the understanding that inactivated vaccines are B cell vaccines, i.e. elicit an antibody response, and that the oil formulation will function as a depot, injectable vaccines confer strong and long-lasting immunity towards infection with extracellular bacteria. Immersion vaccines will require repeated vaccinations to protect the fish throughout the production cycle.

Inactivated vaccines are less efficient against virus infections and diseases caused by intracellular bacteria. Examples are infectious pancreatic necrosis virus (IPNV), salmon pancreas disease virus (SPDV) and red sea bream iridovirus. Also it is difficult to obtain long-lasting immunity against salmon rickettsial syndrome (*Piscirickettsia salmonis* infections), while oral boost can strengthen the immune response to the pathogen (Tobar et al. 2015) and francisellosis (*Francisella noatunensis* infection). There is a need for alternative strategies to protect against intracellular

pathogens. Since the majority of fish is vaccinated by intraperitoneal injection, a future challenge in fish vaccinology is to develop vaccine formulations with lower injection-site reactions. Alternative delivery modalities are also needed, and vaccine efficacy must be improved when the antigen is delivered via mucosal surfaces.

Adjuvants and Principle of Action

The mode of action of adjuvants is in general poorly understood. Adjuvants aid in an early onset of immunity, long duration of effector responses, such as antibody formation or cytotoxic T cell activity, and make booster immunisations unnecessary (Singh and O'Hagan 2003). The mechanisms of adjuvanticity are complex and not fully understood. Adjuvants facilitate delivery of antigen (to the secondary lymphoid organs), which can be a time-dependent effect. Adjuvants provide a nonself microbial signal or a host-derived danger signal from stressed cells (Singh and O'Hagan 2003) and thereby increase the immune response to a given antigen and also prolong the immune responses, the latter being the depot effect. It is conceived as particularly important for fish for long-term immune protection (Evensen et al. 2005). Fish vaccines for parenteral delivery formulated with an adjuvant are typically a water-in-oil formulation with various emulsifiers and stabilisers added. Oils used are either of vegetable or mineral origin. Inactivated vaccines intended for immersion delivery come without an adjuvant and the same for live, attenuated vaccines and DNA vaccines.

Vaccines for salmonid fish, Atlantic salmon in particular, are for the most part administered parenterally and formulated with an adjuvant to enhance immunogenicity (and duration of immunity). Most vaccines currently available are non-replicating/nonliving vaccines. Non-replicating vaccines are preferred because of their safety in normal and even immunocompromised individuals, but they lack immunogenicity and require adjuvants being added.

Immunomodulation

Cytokines are involved in the regulation of immune response, both strength and profile. Cytokines can skew the immune response and will arm immune effector cells. T helper (Th) cells play a central role in the immune system by producing several cytokines that direct the immune responses into different categories of responses. Overall these are defined as Th1, Th2 and Th17, also in fish (Wang and Secombes 2013). Molecular tools allow in vitro production of cytokines that can be used as immunomodulators or stimulants in vaccine preparations. It should be noted though that cytokines serve in a fine-tuned network, and too high doses/concentrations can be deleterious to the host and promote disease rather than preventing it. Cytokines are also short-lived compounds, which can make it difficult to achieve prolonged effects.

Components added to vaccine preparations can also mimic pathogen "fingerprints" and interact with specific receptors of various cells, like toll-like receptors and cytosolic receptors recognising dsRNA compounds and triphosphate RNA sensors. Receptor ligands are potential compounds that can be used as immunomodulators in vaccines like dsRNA (poly I:C) (Kavaliauskis et al. 2015), CpG (Strandskog et al. 2011), and other synthetic compounds. The anticipation would be that we will see toll-like receptor ligands as immunomodulators in the vaccines before cytokines come into general use. These are particularly attractive for inactivated vaccines (Strandskog et al. 2011).

Molecular Technologies

Advances in molecular biology have provided many contributions to vaccine research particularly related to recombinant vaccine development (Kim et al. 2016). Techniques make it possible to knock out or insert genes in the pathogen genome; this can be used for study of virulence mechanisms (Kim et al. 2015) and pathogen components of importance for protective immunity. Techniques are used for development of vaccines candidates that carry certain advantages over conventional non-live or live vaccines, particularly when the pathogen is difficult to grow in culture (like piscine reovirus (Palacios et al. 2010) or piscine myocarditis virus (Haugland et al. 2011) of Atlantic salmon).

Recombinant Vaccines

New molecular technologies created expectations for new applications in recombinant vaccine technology; however, these were not fully met, and we have few recombinant vaccines for farmed fish and even so for warm-blooded animals or humans. The reasons are many. Recombinant, subunit vaccines have poor immunogenicity. For DNA vaccine technologies, we have seen that the dose needed to elicit an immune response is high (and thus there is a cost issue), and for many pathogens, we have seen poor efficacy (Evensen and Leong 2013), which again has discouraged many. Currently, one DNA vaccine is licensed for use in farmed fish against IHN in Atlantic salmon (for use in Canada (Alonso and Leong 2013).

Subunit Vaccines

Escherichia coli strains are used as competent cells for production of antigen at the end of the fermentation cycle. A classical example in fish vaccinology is the *E. coli*-based subunit vaccine against infectious pancreatic necrosis in Atlantic salmon,

licensed for the first time in 1995. *E. coli* is convenient for use, not the least because this bacterium is widely used in molecular biological work, and therefore many research laboratories have knowledge of the techniques and tools that are needed for proper expression of the transgene. Recombinant subunit vaccines have particular advantages if it is difficult to cultivate the disease-causing microorganism. This applies to some viruses and other micro-organisms. There is general agreement that recombinant subunit vaccines are safe for use but of inferior immunogenicity compared to inactivated, whole cell/virus vaccines (Webster and Laver 1966). Potent adjuvants are therefore needed to improve immunogenicity (see above).

Recombinant vaccines have also been produced in *Saccharomyces cerevisiae*, including a subunit vaccine against infectious salmon anaemia in Chile. Other vectors may also be used for production of recombinant of vaccines, like silkworms, cabbageworms, plants and insect cells. There are currently no commercial vaccines in the market based on any of these methods, but several experimental studies have been carried out, including IPN vaccines produced in cabbageworms (*Trichoplusia ni*) (Shivappa et al. 2005), plant-derived antigens expressing the capsid protein of nodavirus (Gomez-Casado et al. 2011), and the G-protein of viral haemorrhagic septicaemia virus has been produced in insect cells (Lorenzen and Olesen 1995; Lorenzen et al. 1993). All have been tested for their ability to induce immune responses and protective immunity and with variable results.

Plant-based vaccines are also attractive in the sense that they are potentially cheap to produce and can be maintained in well-defined environments. This is also referred to as molecular farming where whole plants or plant cells/tissues are cultured in vitro for the production of recombinant proteins (Schillberg et al. 2013). The system has been established as an economically viable alternative to mainstream production systems such as microbes and mammalian cells cultivated in large-scale bioreactors. Plants have several advantages compared with the traditional platforms for recombinant protein production; they are less expensive to maintain than cultured mammalian cells; they lack the undesirable components found in conventional systems, e.g. endotoxins in bacteria, and hyperglycosylated proteins produced by yeast, and there is no limit to the production scale and the cost of scaling up is low. This field is at an early stage for fish vaccines (Shin et al. 2013) but likely to develop in the near future.

Genetically Modified Vaccines

Live, attenuated vaccines generated through numerous in vitro passages have been used for many decades for vaccination of higher vertebrates. In vitro passaging results in accumulation of genome mutations that render the pathogen nonpathogenic, but the exact mechanisms of attenuation are usually not known. By the use of molecular techniques, it is possible to attenuate micro-organisms by removing/deleting specific genes or part of genes and thereby render the microorganism apathogenic. Live attenuated virus vaccine will replicate to a lower titre compared

to their pathogenic counterpart and will stimulate humoral (Munang'andu et al. 2013) and cellular immunity (Boudinot et al. 2001), although this is studied to a lesser extent in fish. They can also be used for induction of immunity at mucosal surfaces. Thus a broad immune response is one of the main benefits of live, attenuated vaccines as they are immunogenic and confer a high degree of protection against disease.

Reverse genetics is the method of choice for attenuated virus vaccines but of course not feasible for all virus species. There are examples where infectious haematopoietic necrosis virus and viral haemorrhagic septicaemia virus of the genus *Novirhabdovirus* have been rendered apathogenic by deleting the NV gene (Romero et al. 2008; Biacchesi et al. 2000; Thoulouze et al. 2004). These NV-knockout variants have then been used to immunise fish against IHN and VHS, and a high degree of protection has been obtained, for example, in Japanese flounder against VHS (Kim et al. 2011) and rainbow trout against IHN (Romero et al. 2008). Further avirulent variants of salmon pancreas disease virus (SPDV) made by reverse genetics have shown to give a high level of protection against sleeping disease in trout (Moriette et al. 2006). Mutations in the 3′-UTR of VHS virus have recently been used to develop a vaccine concept for use in Japanese flounder (Kim et al. 2016). The virus strain has attained some residual virulence in fry of flounder but induces a strong immunity.

Live attenuated bacterial vaccines can be made by recombinant technology where genes encoding specific enzymes are mutated or deleted. This can be enzymes required for production of certain amino acids and delta aromatic mutants (Δ*aroA*) of *Aeromonas salmonicida* have been used to vaccinate brown trout (*Salmo trutta*) against furunculosis with good protection (Vaughan et al. 1993; Marsden et al. 1996, 1998). The Δ*aroA* mutant proliferates in the kidney and is also retained for up to 12 weeks post vaccination (Grove et al. 2003). Similar findings have been obtained using an attenuated strain of *Edwardsiella ictaluri* against enteric septicaemia of catfish (Shoemaker et al. 2011).

Genetic stability is the key point when evaluating safety of live attenuated vaccines. The risk of "reversion to virulence" or "increase in virulence" is considered to be higher for vaccines attenuated by traditional methods (passage), than for vaccines attenuated by molecular methods. The reasoning is that vaccines attenuated by traditional passage will usually have point mutations in the genome, whereas vaccines generated by molecular techniques is better defined and can include "knock out" of the entire genes.

Vector Vaccines

A third type of recombinant vaccine is based on transfer of genes encoding one or more virulence factors and/or protective antigens of a pathogenic microorganism to a live avirulent microorganism, a vector. Vectors can be viruses and bacteria. A vector vaccine will stimulate a diverse immune response. Recombinant vector vaccines may stimulate humoral or cell-mediated immunity, which is usually not obtained for

inactivated vaccines. Vector vaccines have been tested to a very limited extent in finfish vaccinology. Immunisation with vector vaccines will also result in the development of an immune response against the vector/vector antigens. Pre-existing antibodies against the vector virus can neutralise or inhibit viral vector such that the immune response against the foreign antigens is reduced. Yet another type of vector vaccine is the replicon-based variant, where a gene of interest (GOI) has been cloned into an alphavirus-replicon, typically expressing the structural genes of the candidate alphavirus and the GOI (Vander Veen et al. 2012). A few studies have explored this potential for vaccines for farmed fish showing good level of protection using salmonid alphavirus-based replicons (Wolf et al. 2013, 2014). Additional studies have shown temperature sensitivity being associated with E2 protein expression (occurring only at temperatures between 10 and 15 C) is related to virion formation (Hikke et al. 2014) and would thus influence on immunity induced. Future studies should include other GOIs with the purpose to explore the applicability of this technology in general for finfish.

DNA Vaccines

DNA vaccination technology is rooted in gene therapy, the delivery of a therapeutic gene for expression in somatic tissue. It was shown relatively long ago that injection of naked plasmids into the muscle of mice can elicit an immune response (Ulmer et al. 1993). DNA vaccines will result in a transient expression of the gene of interest, and this is sufficient to evoke an immune response (Fig. 3.4).

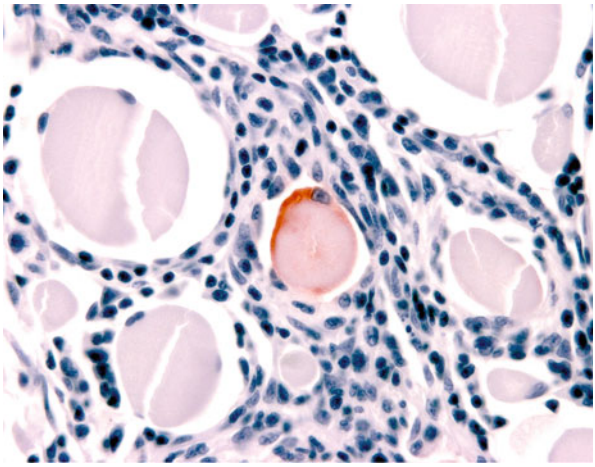

Fig. 3.4 Rainbow trout, skeletal muscle. Sample was collected 4 weeks post vaccination using a DNA vaccine encoding the G-protein of viral haematopoietic necrosis virus. Expression of the G-protein has been revealed by immunohistochemistry using a G-protein-specific rabbit serum (*red coloration*). There is a strong inflammatory response in the area of the muscle cell expressing the protein, dominated by lymphocytes

The efficacy of DNA vaccines is well documented for a number of fish pathogenic viruses and bacteria. More specifically, it has been demonstrated that DNA vaccines induce a strong and protective immunity to some viral infections in fish, particularly rhabdoviruses of rainbow trout and Atlantic salmon (Lorenzen and LaPatra 2005), for channel catfish herpesvirus infection (Nusbaum et al. 2002) and red sea bream iridovirus (Caipang et al. 2006). DNA vaccines also elicit protective immunity to bacterial kidney disease under experimental conditions (Gomez-Chiarri et al. 1996). DNA vaccines are, with a few exceptions (Ballesteros et al. 2012a, b, 2014), reliant on intramuscular injection for induction of protective immunity. For oral DNA vaccines, solid documentation of efficacy tested by in vivo challenge is meagre (Ballesteros et al. 2012b). The distribution to internal organs following i.m. vaccination has not been studied in detail, but it has been shown that a luciferase-encoding plasmid was distributed to internal organs and expression can be detected in organs shortly after administration (Romoren et al. 2004). Furthermore, luciferase expression in internal organs of fish has been observed over an extended period (up to 24 months) (Dijkstra et al. 2001). Cationic liposomes have been for delivery of DNA by the immersion route but have been met with severe toxicity problems (Romoren et al. 2002a, b, 2004). The mechanism of the acute toxicity is suggested to be an interaction between the cationic liposomes and anionic components of gill mucin, resulting in hypoxia and acute toxicity (Romoren et al. 2002b).

One important challenge for DNA vaccines is regulatory requirements and fish safety primarily related to genome integration. This applies to the vaccinated animal; the vaccine construct is not considered a GMO. Any such event is not likely to impact the health of the vaccinated animal, but such an event will have implications for food safety and the end user (Evensen and Leong 2013). Concerns have been raised as to production of anti-DNA antibodies in the vaccinated animal resulting in autoimmunity and also tumorigenicity, but studies so far lend very little support for this concern.

It has been demonstrated that retention and expression of antigens at the injection site appear for an extended time period, however, not beyond 4–5 weeks post vaccination (Lorenzen et al. 2005). The local reactions at the injection site are prominent and last for an extended period and longer than the actual antigen expression in situ. Strong inflammation and muscle cell degeneration and necrosis are seen at 3 and 12 weeks post vaccination (Lorenzen et al. 2005). There is currently one DNA vaccine for use in farmed fish against infectious haematopoietic necrosis virus in Atlantic salmon. This vaccine is licensed in Canada.

Marker Vaccines

Marker vaccines are used to distinguish between vaccinated and infected animals, also referred to as DIVA vaccine (differentiation between infected and vaccinated animals (Fu et al. 2011). Such vaccines are usually genetically modified, typically gene-deleted vaccines, or they lack an antigen against which the infected animal

will mount an immune response, while the infected animal will (van Oirschot 1999). All categories of recombinant vaccines may be used as marker vaccines. So far such marker vaccines (against a fish disease) have been tested in the lab (Enzmann et al. 1998) but are not available commercially.

Vaccines Against Parasitic Diseases

Parasites are causing major losses in aquaculture, worldwide, but there are currently no vaccines available for use in farmed fish. One reason is access to drugs for treatment and partly that it has proven difficult to vaccinate against parasites. Two main approaches have been explored for vaccination against parasites. These are based on live, attenuated parasites or they are based on subunit vaccines containing specific parasitic antigens or enteric origin and often produced by recombinant techniques, often referred to as concealed antigens (Wang and Nuttall 1999); the best studied is tick vaccine used in Australia against *Boophilus microplus* in cattle which is based on a protein, Bm86, as antigen (Jittapalapong et al. 2010; Nuttall et al. 2006; Willadsen and McKenna 1991). This protein is found in cell membranes in the intestine of the tick, and the host is not exposed to this protein when infested. The antigen is delivered as a subunit vaccine to the host, and blood feeding will result in circulating antibodies binding to the protein in the epithelial lining of the gut resulting in damage to intestinal functions of the parasite. The vaccine reduces losses and reduces the risk of other diseases transmitted by the parasite. A similar approach has been used for development of a sea lice vaccine in salmonids, *Lepeophtheirus salmonis* and *Caligus rogercresseyi* (Carpio et al. 2011). Studies report a significant reduction in the number of parasites per fish was observed at 24 days post challenge (Carpio et al. 2011), but such vaccines did not make it to field testing – at least not yet.

Future Directions

Multi enim sunt vocati, pauci vero electi "Many are asked to come, but only a few are chosen" (St. Matthews' Gospel, 22, 14). Oil-adjuvanted vaccines for fish are based on an "old technology", and while many studies have been carried out with an attempt to develop new and more advanced principles for immune induction, the light at the end of the tunnel is still dim. In humans, aluminium salts still remain the standard (Del Giudice et al. 2001), also an "old-fashioned" tool. There are few alternatives to oil adjuvants for many of the "difficult" fish pathogens. Many scientists and the industry have hopes for mucosal delivery systems, and major advancements have been done (Tobar et al. 2011, 2015), still with some limitations. Improvements of oil adjuvant delivery systems for fish have been seen over the last years, and reduced injection volume is a simple and effective way or reducing side-effect profiles. It is likely that we also for the future will make small steps rather than a giant

leap forward. My prediction would be that different modalities, prime-boost vaccination strategies and combination of modalities (injection prime and oral boost), where we have already seen the first products in the market (Tobar et al. 2015), will be the future also in fish vaccinology.

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