Chapter 4
Adjuvants and Delivery Methods: Current and Novel

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Abstract Vaccination is the most appropriate method to control infectious diseases that threaten the aquaculture industry worldwide. Unfortunately, vaccines are usually not able to confer protection on their own, especially those vaccines based on recombinant antigens or inactivated pathogens. Therefore, the use of adjuvants or immunostimulants is often necessary to increase vaccine efficacy. Furthermore, an important additional problem that limits the entry of novel fish vaccines to the market is that many of the vaccines experimentally produced only work when injected (either intraperitoneally or intramuscularly). Therefore, the search for alternative methods of mass vaccine delivery (oral or immersion) should also be addressed in parallel. Unfortunately, it is probable that the search for a specific combination of antigen/adjuvant/delivery method has to be experimentally addressed for each pathogen/fish species, and only a few general conclusions can be drawn from each of these studies. In this chapter, we summarise previous studies performed with both traditional and new generation adjuvants as well as those studies that have explored methods for vaccine delivery alternative to injection.

Introduction

Disease prevention by vaccination is, on economic, environmental and ethical grounds, the most appropriate method for pathogen control currently available for the aquaculture production sector. Traditionally, vaccines comprise either live-attenuated, replicating pathogens or non-replicating, inactivated pathogens, or their subunits. In aquaculture, live vaccines are often not approved for safety reasons, and inactivated vaccines based on either killed pathogens or isolated non-replicating

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pathogen subunits are in many cases weakly immunogenic. Thus, adjuvants are required to ensure optimal immune responses and protection.

During the past, fish vaccines were made by a trial-and-error approach (conventional vaccine design) including pathogen identification, pathogen cultivation and vaccine formulation containing the complete inactivated pathogen, sometimes formulated in oil. Through using this strategy, vaccines based on whole inactivated extracellular bacterial pathogens were quite efficient – resulting in important reductions in mortalities and antibiotic usage in the aquaculture industry (Hastein et al. 2005). However, many of the economically important diseases as of today are due to intracellular pathogens, and for this type of pathogens, the production of effective vaccines has been quite challenging. Therefore, fish vaccine development strategies should be subjected to a rational vaccine design wherein a combination of a tailored adjuvant system with the most appropriate antigen(s) is used to create vaccines that may provide a more effective immune response against a specific pathogen with minimal side effects.

Additionally, injection vaccination is labour intensive, expensive and not feasible for early fish stages, even though it is precisely at these early stages when vaccination is often needed. Thus, exploring novel strategies for mass delivery (immersion or oral) of vaccines is an important field of research that still needs to be developed. In this sense, specific adjuvants may also contribute to increase the immunogenicity of vaccines delivered through these alternative routes.

One of the main limitations for the selection of an adequate antigen/adjuvant/delivery method is the fact that many aspects of fish immunology are still unknown, and we are far from close to understanding which are the exact immune correlates of natural or vaccine-mediated protection (Secombes 2008). Moreover, there are currently close to 22,000 different fish species, and most of them have their "immune peculiarities". Although the innate defence system of fish plays an important role in eradicating infectious agents, many pathogens resist innate defence mechanisms, and then an adaptive immune response, present for the first time in evolution in teleost fish, must come into play to fight these pathogens. The adaptive immune response is the basis for vaccinology and provides the vertebrate immune system with the ability to recognise and remember specific pathogens, to be able to mount stronger and faster responses each time this pathogen is encountered. In higher vertebrates, adaptive immunity to extracellular pathogens is generally mediated by humoral immune responses (antibodies), while immunity to intracellular pathogens (including viruses) often relies on cellular immune responses (cytotoxic T cells). In fish, and despite the fact that the main elements for an adaptive immune response are present in most species, the regulation of these elements greatly differs from mammalian systems and even among different species. Both immunoglobulin (Ig) or B cell receptor (BCR) and T cell receptor (TCR) genes are known among all lineages of gnathostomes (jawed vertebrates), but in fish Ig are expressed as only as three isotypes (IgM, IgD and IgT) with no isotype switching and with low affinity maturation (Hikima et al. 2011). Interestingly, there is a tight link between the innate and adaptive system that has not been much explored in fish immunology.

This link, governed by several innate receptors and signalling molecules such as cytokines and transcription factors, is key in the responses following activation by vaccine adjuvants, since recent advances in immunology have shown that the magnitude and specificity of the signals perceived by the innate immune cells following vaccination shape subsequent adaptive immune responses (Palm and Medzhitov 2009).

Principles of How Adjuvants Work

Adjuvants have traditionally been defined as helper substances that increase the magnitude of an adaptive response to a vaccine (potency) or ability to prevent infection and death (efficacy). But nowadays, scientists have acknowledged that adjuvants may become more important in the way they instruct or guide the type of adaptive response against a specific pathogen. Thus, adjuvants have been now defined as a group of structurally heterogeneous compounds able to modulate the intrinsic immunogenicity of an antigen (Guy 2007). They can be classed according to their chemical nature or physical properties; however, since even related compounds can have very different immunomodulating capacities, new classifications have focused on the immunological events they induce, even though for many of them the exact mechanism of action is unknown. At present, the classification of adjuvants that distinguishes between signal 1 facilitators and signal 2 facilitators has been widely accepted (Schijns 2001). According to this two-signal model, both the presentation of an antigen (signal 1) and the additional secondary signals (signal 2) are required for activation of specific T and B lymphocytes, which form the adaptive arm of the immune system (Ribeiro and Schijns 2010). The signal 1 facilitators influence the fate of the vaccine antigen in time, place and concentration, ultimately improving its immune availability, while signal 2 facilitators provide the co-stimulation signals during antigen recognition that will provide an adequate environment for the most adequate antigen-specific immune response.

Another important aspect of the immune response conferred by adjuvants is the fact that they mimic the recognition of microbes through the detection of conserved molecular patterns, designated as pathogen-associated microbial patterns (PAMPs) or microbe-mediated tissue damage through damage-associated molecular pattern molecules (DAMPs). These molecules activate pathogen recognition receptors (PRRs) that include Toll-like receptors (TLRs), C-type lectin receptors, NOD-like receptors, RIG-I-like receptors and peptidoglycan recognition proteins (PGRPs) that are predominantly found on cells of the innate immune system. Nowadays, this first recognition is considered critical in signal 2 induction and downstream activation of distinct T helper cell subsets; however, other authors make a distinction and refer to adjuvants that trigger PRRs as signal 0 adjuvants. In fact, most of the recent research on adjuvants has especially focused on different PRR ligands.

Signal 1 Adjuvants Used in Fish Vaccinology

To increase the immunogenicity of an antigen, a slow release is often achieved through the introduction of the antigen in the context of an emulsion. An emulsion is defined as a dispersion of a liquid, called the dispersed phase, and in a second liquid, called the continuous phase, with which the first one is not miscible. In vaccine formulations, these phases are water (often with added antigens) and oil. In order to stabilise the emulsions, surfactants are added. A surfactant is a compound containing a polar group that is hydrophilic and a non-polar group that is hydrophobic and often composed of a fatty acid residue. Surfactants can be defined by their hydrophilic-lipophilic balance (HLB) value which gives information on their relative affinity for both phases. According to the HLB value of the surfactant, different kinds of emulsions can be achieved (Aucouturier et al. 2001). Those having a low HLB value have a high affinity for oily phases and render W/O emulsions, whereas those with a high HLB value have a high affinity for the aqueous phase and render O/W emulsions, which are well tolerated but induce a shorter-term immune response. With certain specific surfactant systems, when the HLB value is intermediate, W/O/W emulsions can be achieved. In this case, the continuous phase is aqueous and the dispersed phase is oil. But inside the oil droplets, an entrapped aqueous phase with water-soluble antigens/suspended antigens is found. This type of emulsions has shown to generate long-term immune responses with various antigens.

Freund's Complete Adjuvant

Mineral oils are widely used in vaccine manufacturing processes and are a mixture of alkanes in the C_{15}–C_{40} range often obtained from non-vegetable sources. The most widely used and most effective adjuvant for experimental purposes has been Freund's complete adjuvant (FCA). FCA is composed of heat-killed mycobacteria and a mineral oil with surfactant (Opie and Freund 1937). Before injection, the antigen in an aqueous solution is mixed with the FCA producing a stable W/O emulsion. Immunisation with FCA and antigens results in strong Th1 and Th17 responses mostly via the MyD88 pathway. Unfortunately, the use of FCA has been associated with a variety of severe side effects including injection site granuloma; therefore, its use has been limited within animal research. Surprisingly, the use of FCA in fish has not always resulted in increase in immunogenicity or protection – as outlined below.

Pasteurellosis, caused by *Pasteurella piscicida*, also named *Photobacterium damselae* subsp. *piscicida* is one of the major diseases in many species of wild and farmed fish in Asia, the USA and Europe. In yellowtail (*Seriola quinqueradiata*), a susceptible species, vaccination against pasteurellosis has been assayed with a lipopolysaccharide (LPS)-mixed chloroform-killed bacterin that resulted in protection against challenge with the virulent bacterium. In this case, the inclusion of

FCA in the vaccine did not significantly enhance the protective effect (Kawakami et al. 1998).

Streptococcus iniae is a Gram-positive bacterium associated with disease in several commercial species including tilapia (*Oreochromis aureus* and *O. niloticus*), yellowtail, hybrid striped bass (*Morone saxatilis*), turbot (*Scophthalmus maximus*) and rainbow trout (*Oncorhynchus mykiss*). Vaccination of rainbow trout with a formalin-killed culture of *S. iniae* resulted in good protection against experimental challenge that was not significantly potentiated in the presence of FCA (Soltani et al. 2007).

Aeromonas salmonicida is the etiological agent for furunculosis. In a study in coho salmon (*Oncorhynchus kisutch*), formalin-killed *A. salmonicida* was interaperitoneally (i.p.) injected in the absence or presence of FCA. In this model, the best protection was found in the vaccine in which FCA was included with *A. salmonicida* compared to the antigen in saline. Interestingly, fish injected with FCA (without antigen) were partly protected even 90 days after challenge (Olivier et al. 1985). Thus, it seems that injection of inactivated *M. bovis* may induce innate defence mechanisms that may result a certain degree of protection to a heterologous pathogen, as shown by Kato et al. (2012) where Japanese flounder (*Paralichthys olivaceus*) were partially protected against nocardiosis with FCA exclusively. In a recent study, Zheng et al. (2012) compared naturally occurring adjuvants (astragalus polysaccharide and propolis) with FCA in a pentavalent vaccine. In that study, FCA outcompeted the other adjuvants despite the immunostimulant activities of the natural adjuvants.

Recently, a vaccine against *A. hydrophila* was prepared (LaPatra et al. 2010) using a bacterial lysate. The vaccine was administered i.p. in combination with FCA, and the efficacy of the vaccine was studied using a new challenge model optimised for rainbow trout in which *A. hydrophila* was injected into the dorsal sinus. The vaccine provided protection and this protection could be potentiated with FCA (LaPatra et al. 2010).

Flavobacterium psychrophilum is a widespread Gram-negative pathogen in freshwater causing rainbow trout fry syndrome (RTFS) and bacterial cold-water disease (BCWD) (Hogfors et al. 2008). In addition to rainbow trout, coho salmon is the most susceptible species together with other non-salmonid species which are also affected. Injection of a low molecular weight fraction emulsified in FCA resulted in an enhanced level of protection for rainbow trout (Hogfors et al. 2008).

Flavobacterium columnare is a Gram-negative bacterium responsible for columnaris disease. The disease was first described in 1917 in several warm-water fish species from the Mississippi river, and since has been isolated from freshwater fish species worldwide (Grabowski et al. 2004). Specific antibodies were found in tilapia plasma and mucus following i.p. injection of formalin-killed sonicated (disrupted cells with ultrasonic frequency) or whole cells of *F. columnare* in FCA within 2 weeks. After a secondary immunisation, the antibody response increased and remained elevated at 10 weeks post-immunisation. Antibodies were also observed in cutaneous mucus in fish i.p. immunised with formalin-killed sonicated cells in FCA 6 and 8 weeks post-immunisation (Grabowski et al. 2004).

Freund's Incomplete Adjuvant

Because of its high toxicity, the use of FCA has been widely replaced by Freund's incomplete adjuvant (FIA) that lacks the mycobacterial components of the emulsion, being therefore just a W/O emulsion. This adjuvant is still highly effective in vaccination with a significant reduction of toxicity; however, peritonitis is still a major side effect, as perfectly detailed for Atlantic cod (*Gadus morhua*) (Gjessing et al. 2012).

Edwardsiella tarda is a Gram-negative intracellular bacterium that can infect both marine and freshwater fish, including Japanese flounder. In order to develop effective vaccines against this pathogen, fish were i.p. injected with a vaccine containing a major antigenic protein of *E. tarda* in the absence or presence of FIA (Jiao et al. 2010a). Protection against experimental challenge achieved by the vaccine without adjuvant resulted in a relative percentage survival (RPS) of 34 % that was increased to 81 % in the presence of FIA. Moreover, vaccination with the oil-adjuvanted antigen stimulated the expression of a series of genes like complement component 3 (C3), major histocompatibility complex (MHC) class I and MHC class II, CD8α, CD40, Mx, interferon γ (IFN-γ), tumour necrosis factor α (TNF-α) and interleukin 6 (IL-6), whereas vaccination with the antigen alone resulted in increased expression of just IgM, MHC class I and class II and Mx (Jiao et al. 2010a).

Nocardia seriolae is a Gram-positive acid-fast bacterium that causes nocardiosis in cultured marine and freshwater fish in Taiwan, Japan and China. Although the disease results in considerable economic loss, no commercial vaccines are available. Recently, an oil-adjuvanted vaccine was developed and tested on protection against challenge with a virulent strain (Shimahara et al. 2010). Formalin-inactivated whole cell antigen was used as a vaccine with or without FIA; however, even though antibody levels increased, no protective effects were found.

Another Gram-positive bacterium that causes disease (lactococcosis) and mortality in rainbow trout is *Lactococcus garvieae*. In this case, the vaccine was prepared based on formalin-inactivated bacterin or bacterin suspended in FIA, fish were given i.p. injections and challenged by exposure to virulent bacteria 30, 75 and 125 days after vaccination (Kubilay et al. 2008). At 125 days after vaccination, the RPS in fish vaccinated with bacterin only was 54 %, whereas it was 85 % in fish vaccinated with bacterin and FIA.

Tenacibaculum maritimum is a marine bacterium that causes flexibacteriosis worldwide. In Australia, Atlantic salmon (*Salmo salar*) and rainbow trout are the most heavily affected species, and due to the lack of vaccines, so far the disease has been treated with trimethoprim and oxytetracycline with the subsequent negative impact on the environment (Van Gelderen et al. 2009). Salmon injected with formalin-inactivated bacteria mixed with FIA provided protection against challenge with *T. maritimum,* while the vaccine without the adjuvant could not provide sufficient protection against a moderate challenge of *T. maritimum*.

Infection with fungi oomycetes such as *Aphanomyces invadans* may cause heavy mortalities of fresh water and estuarine fish species as a result of granulomatous

inflammation. In catla (*Catla catla* Hamilton), a fungal extract combined with FIA showed to increase both the survival rate and the antibody response compared to non-adjuvanted vaccines (Saikia and Kamilya 2012).

Montanide

Mineral oil adjuvants registered under the trademark of Montanide by Seppic have been optimised in order to improve efficacy and stability of vaccine formulations and to reduce side effects. These adjuvants are based on either mineral oil, non-mineral oil or a mixture of both, as well as those made from specific surfactant chemistry using dianhydro-D-mannitol monooleate (e.g. Montanide ISA 720) and may be used to manufacture different type of emulsions, W/O, O/W or W/O/W, for use in both mammals and fish (Lawrence et al. 1997; Ravelo et al. 2006).

Philasterides dicentrarchi is a scuticociliate parasite that causes mortalities and significant economic losses in cultured turbot (Lamas et al. 2008). An important attempt to optimise a vaccine against this parasite was performed on the basis of antigenic dose, concentration of inactivating agent (formalin) and proportion of the adjuvant Montanide ISA763A (W/O, non-mineral oil) in the emulsion. The results of this study showed that a high concentration of antigen, 0.2 % formalin and 50 % adjuvant generated the longest time of survival after challenge 30 days after the second injection, and the highest levels of antibodies in the vaccinated fish (Lamas et al. 2008).

Pseudomonas plecoglossicida is a bacterium causing bacterial hemorrhagic ascites of cultured ayu (*Plecoglossus altivelis*). To develop a vaccine against the disease, formalin-killed *P. plecoglossicida* bacterin was emulsified with Montanide and injected i.p. The fish were challenged with an i.p injection of virulent *P. plecoglossicida* 22 and 52 days after vaccination (Ninomiya and Yamamoto 2001). The RPS of vaccinated fish was 17–58 % without adjuvant, 57–92 % with Montanide ISA711 and 65–86 % with Montanide ISA763A. Another study on the same disease and adjuvant (Montanide ISA 763A) concluded that there is a good correlation between antibody levels and protection against disease in a challenge test (Sitja-Bobadilla et al. 2008).

To study the efficacy of different adjuvants in Atlantic halibut (*Hippoglossus hippoglossus*), fish were injected i.p. with a model vaccine of human gamma globulin with either FCA or Montanide ISA711 as adjuvants (Bowden et al. 2003). Antibody responses and intraperitoneal adhesions were examined every month for up to 12 months. FCA produced the highest and fastest antibody response, since in the group injected with the Montanide adjuvant only 4 of 47 fish reached a titre of 1:1000 (on month 6) compared to 27 of 48 fish in the FCA group (after 2 months); however, FCA also induced the fastest intraperitoneal adhesions (Bowden et al. 2003).

In a recent study in carp (*Cyprinus carpio*), a recombinant S-layer protein of *A. hydrophila* was used to assess the ability to protect fish against six virulent isolates

of *A. hydrophila*. The recombinant S-layer protein of *A. hydrophila* was produced, diluted in phosphate buffered saline (PBS) and mixed with a Montanide adjuvant at a ratio of 30:70. Common carp were i.p. injected with the emulsion, and after 35 days, the fish were challenged with six different isolates of *A. hydrophila* (Poobalane et al. 2010). The RPS values varied between the different challenge isolates (40–75 %), but it was concluded that the S-layer protein together with Montanide adjuvant is a good candidate for an efficacious vaccine against this bacterium.

Furthermore, Montanide ISA-763 has also been used as an adjuvant in experimental bivalent vaccine for *L. garvieae* and *A. hydrophila* with high degree of efficacy in rainbow trout (Bastardo et al. 2012).

Other Mineral Oil Adjuvants

Moritella viscosa is the causative agent of winter ulcers in farmed fish like Atlantic salmon and Atlantic cod. Vaccination of Atlantic salmon against *M. viscosa* is performed with oil-adjuvanted polyvalent injection vaccines based on formalin-inactivated bacterial cultures, using an AJ-oil (Alphaject 5200) used in some vaccines commercialised by Pharmaq (Gudmundsdottir and Bjornsdottir 2007). However, a multivalent commercial salmon vaccine containing *M. viscosa* as one of five bacteria mixed in a mineral oil adjuvant (Alphaject 5200) did not protect turbot against challenge (Bjornsdottir et al. 2004), whereas moderate intra-abdominal adhesions were detected in vaccinated fish.

Other commercial oil-adjuvanted vaccines have been shown to give protection in Atlantic salmon against bacterial diseases like vibriosis, cold-water vibriosis and furunculosis for a long time. However, side effects and retardation in growth have been clearly demonstrated (Midtlyng and Lillehaug 1998; Midtlyng et al. 1996). Mutoloki and coworkers investigated the intraperitoneal lesions induced by an oil-adjuvanted vaccine against infection with *A. salmonicida* and *M. viscosa* in Atlantic salmon (Mutoloki et al. 2010). The cellular composition was typical of granulomas containing large macrophages, eosinophilic granular cells, lymphocytes and multinucleate cells.

Oil-adjuvanted vaccines are also used to control sea bass (*Dicentrarchus labrax*) against bacterial diseases like vibriosis and pasteurellosis. Sea bass is one of the most used fish species in the Mediterranean area, and suffers from infection by *V. anguillarum* and *Photobacterium damselae* subsp. *piscicida*. Oil-adjuvanted vaccines against these diseases have been prepared and injected i.p., but despite their effectiveness, granulomatous peritonitis was also recognised (Afonso et al. 2005).

The major bacterial disease of farmed Atlantic cod is classical vibriosis (Samuelsen et al. 2006). Cod vaccinated by injection with mineral oil-adjuvanted vaccines against both *V. anguillarum* and atypical *A. salmonicida* were very well protected against homologous challenges (Mikkelsen et al. 2004). In this model, even without adjuvant, the fish were protected against *V. anguillarum*, but not against atypical *A. salmonicida* challenge.

Signal 2 Facilitators and TLR Ligands as Adjuvants or Immunostimulants

In general, signal 2 facilitators do not influence the concentration and distribution of antigen between injection site and presentation site, but provide co-stimulatory signals during the antigen recognition phase, thus increasing the immune response or skewing it to provide the most suitable immune environment for the establishment of protection. This category of vaccine adjuvant has dominated the literature on vaccine research in the last decade, and includes "stranger" and "danger" molecules, as well as inflammatory cytokines.

"Stranger" and "danger" signals are recognised by innate receptors such as TLRs. Teleost fish species may possess close to twice the number of different TLRs compared to mammalian species, presumably due to an ancient genome duplication event. This may open up new possibilities adding signal 2 facilitators into fish vaccines. However, the existing polyvalent fish vaccines may already contain a high number of different PPR agonists that complicate a further improvement by using rational vaccine development. Several up-to-date reviews on immune relevant genes including TLR-like receptors in fish have been recently published (Palti 2011; Zhu et al. 2013), providing an excellent overview of the current knowledge on fish TLR. In general, those TLRs that, after ligand binding induce the production of IL-12, favour a Th1 response (TLR 3, 4, 5, 7, 8, 9 and 11) and may induce cross-presentation of antigens facilitating a cytotoxic T cell response under certain conditions (Manicassamy and Pulendran 2009). It should be mentioned that ligand binding to TLRs 3 and 4, 7 and 9 may also induce type I IFN responses via interferon regulating factors. Within this group of signal 2 facilitators, we have also included aluminium salts, as it has been recently discovered that these adjuvants directly interact with dendritic cells in a similar way to that of danger signals (Flach et al. 2011).

Aluminium-Containing Adjuvants

The adjuvant property of aluminium salts was discovered in 1926 (Glenny et al. 1926). Aluminium compounds (collectively termed as "alum"), especially aluminium phosphate and aluminium hydroxide, are some of the few adjuvants that have been allowed and considered safe to use in human vaccines. Aluminium adjuvants have been shown to induce Th2 responses almost exclusively (Jiao et al. 2010a), thus they have been used as adjuvants with great success, being particularly effective at promoting protective humoral immunity. However, alum is not optimally effective for diseases where cell-mediated immunity is required for protection. It was believed that alum activates NLRP3 inflammasome and induces necrotic cell deaths that release the danger signal "uric acid" (Coffman et al. 2010). However, it has been discovered very recently that being in a more crystalline form, alum binds

dendritic cell plasma membrane lipids with substantial force, independent of inflammasome and membrane proteins (Flach et al. 2011). The subsequent lipid sorting activates an abortive phagocytic response that leads to antigen uptake. Such activated dendritic cells, without further association with alum, show high affinity and stable binding with $CD4^+$ T cells via the adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function-associated antigen-1 (LFA-1). Despite its potential, only a few studies have used aluminium adjuvants in the optimisation of fish vaccines (see below).

A vaccine against *A. salmonicida* mixed with potassium aluminium sulphate (alum) as an adjuvant was tested in Atlantic salmon more than 15 years ago (Mulvey et al. 1995). Alum appeared to enhance the protection against challenge, but not significantly. In another study, an *Escherichia coli* mutant was used for vaccination against *Edwardsiella ictaluri*-induced enteric septicaemia of catfish (*Ictalurus punctatus*). Killed *E. coli* bacteria with or without alum were administered i.p to catfish, and the fish were challenged with virulent *E. ictaluri* bacteria (Tyler and Klesius 1994). Fish given *E. coli* in alum showed an enhanced survival (92 %) compared with the fish in which *E. coli* was administered alone (54 %) or fish given saline (56 %).

Recently, an aluminium hydroxide-adjuvanted *E. tarda* vaccine was prepared and injected i.p in Japanese flounder. After an experimental challenge, the RPS obtained was 69 % (Jiao et al. 2010a), higher than when the antigen alone was used (RPS = 34), but lower than that obtained with the FIA-coupled vaccine (RPS = 81).

Another experiment has been recently carried out by Fan et al. (2012), in which formalin-inactivated reddish body iridovirus (TRBIV) was mixed with alum and either injected or bath administered (prime-boost) in turbot. The resulting RPS calculated was 83.3 % and 90.5 %, respectively.

ß-Glucans

ß-glucans are known to stimulate the nonspecific (innate) immune response of both mammals and fish through the action of dectin-1 (Dalmo and Bogwald 2008; Robertsen 1999). A high number of different ß-glucans with varying molecular assembly (e.g. linear, branching by single residues, and/or ß1,3-ß1,6-branching networks) and thus molecular weight exist – often dependent on their source; but it is acknowledged that the ß-glucans possessing the ß1,3-D conformation are the biological active ones (Dalmo and Bogwald 2008).

DeBaulney and coworkers prepared an oral vaccine against vibriosis for use in turbot, and after feeding the vaccine for 5 days, the fish were challenged 28 days thereafter. Fish given the vaccine alone resulted in a RPS of 52 %, while a combination of the vaccine and ß-glucan gave a RPS on 61 %, higher protection levels, but not statistically different from the vaccine alone (DeBaulney et al. 1996). In 1998, an attempt to establish immunisation protocols to obtain the highest immune response against *V. damsela* was performed in Spain (Figueras et al. 1998). These

authors i.p. injected the O-antigen of *V. damsela* in combination with ß-glucan. As a correlate to vaccine efficacy, the phagocytic index of head kidney macrophages was evaluated. There was an enhancement of the phagocytic index in fish injected with ß-glucan at the same time or after the antigen injection when compared with fish injected with ß-glucan before the antigen. Similar results were obtained with regard to antibody titres (Figueras et al. 1998).

Yeast glucan (mainly a ß-1,3-D glucan) was included in a furunculosis vaccine based in a formalin-killed culture of *A. salmonicida* and *V. salmonicida* (Rørstad et al. 1993). The vaccine, either with or without ß-glucan, was injected i.p. and salmon challenged 3–46 weeks after vaccination. Vaccines supplemented with ß-glucan induced significantly higher protection against furunculosis than vaccines without this adjuvant (Rørstad et al. 1993), but ß-glucan alone did not result in protection. In another study, ß-glucan-adjuvanted vaccines against furunculosis seemed to give protection at an early time point after vaccination (6 weeks), but no protection was seen after 3 and 6 months (Midtlyng et al. 1996). As a side effect, the average weight of the ß-glucan-adjuvanted group was significantly lower compared to the controls, but this weight loss was even higher in fish given oil adjuvant (Midtlyng and Lillehaug 1998). In a further study performed in coho salmon, Nikl et al. evaluated the potentiating effect of seven substances in combination with a formalin-treated *A. salmonicida* bacterin (Nikl et al. 1991). Statistically significant improvement in survival over the group receiving bacterin alone was noted in fish groups that received ß-glucans like VitaStim-Taito and lentinan (Nikl et al. 1991).

Catla is one of the major Indian carp species often affected with *A. hydrophila*; thus a formalin-inactivated *A. hydrophila* vaccine was developed, and protection was studied in the absence and presence of ß-glucan (Kamilya et al. 2006). A reduction in mortality was found in the presence of ß-glucan compared to the vaccine itself, although the differences were not statistically significant (RPS of 67.7 % and 58.0 % with and without the adjuvant, respectively). In carp, a vaccine against *A. hydrophila* showed a higher antibody titre when ß-glucan was i.p. injected prior to vaccination, while bath and oral administration of ß-glucan before vaccination did not result in enhanced antibody response (Selvaraj et al. 2005). In a further study by Selvaraj and coworkers, carp were vaccinated against *A. hydrophila* with LPS from a virulent strain of the bacterium in the presence of different concentrations of ß-glucan and administered through various routes such as i.p, oral or bath (Selvaraj et al. 2006). The RPS was significantly higher in i.p.-injected groups even at the lowest concentration of ß-glucan, and fish given a mixture of LPS and ß-glucan orally obtained a higher RPS compared to controls. The administration of the LPS-glucan by bath did not result in increased survival, and antibodies were never detected in fish vaccinated either orally or by bath. However, no possible analysis of the contribution of β-glucan in the vaccine efficacy could be established because an obvious control group in this study was missing, namely, the protective effect of LPS without adjuvant (Selvaraj et al. 2006).

In another study, the i.p. injection of ß-glucan on days 1 and 3 followed by two i.p. immunisations of *E. ictaluri* on days 7 and 14 performed in channel catfish resulted in higher serum antibody levels relative to catfish receiving PBS instead of

ß-glucan before administration of *E. ictaluri* (Chen and Ainsworth 1992). Serum antibody levels were determined on day 7 (day 21) after the last immunisation, reaching antibody titres twofold higher in fish that had been treated with ß-glucan.

In order to investigate possible treatments against *A. hydrophila* in blue gourami, laminaran, a ß-1,3-D glucan, was injected i.p. in the absence and presence of formalin-killed *A. hydrophila* bacteria (Samuel et al. 1996). A single i.p. injection of 20 mg kg^{-1} laminaran alone was sufficient to protect the fish against infection by a virulent strain of *A. hydrophila* up until 29 days after injection in correlation with an increased phagocytic activity of head kidney phagocytes. Despite this, the addition of 20 mg kg^{-1} laminaran to a formalin-killed *A. hydrophila* did not significantly improve the protection (Samuel et al. 1996).

Recently, the potential immunostimulatory effect of orally administered β-glucan was investigated in combination with immersion vaccination against *Yersinia ruckeri* in rainbow trout (Skov et al. 2012). Although the β-glucan had no effect on survival in either unvaccinated or vaccinated fish, some immune effects due to β-glucan were observed in vaccinated fish. These effects included differences in plasma lysozyme activity, bacterial clearance and immune gene transcription in fish that were fed the β-glucan and unfed fish.

Saponins

Saponins are naturally occurring glycosides of squalenes or triterpenes that have been widely explored as adjuvants in different mammalian systems due to their capacity to stimulate both Th1 and Th2 responses (Sun et al. 2009). The most widely studied saponins are Quil A (saponin extracted from the cortex of the South American tree *Quillaja saponaria* Molina consisting on a mixture of more than 25 different saponin molecules and one out of three components of ISCOMs) and their derivatives; however, due to their high cytotoxicity and instability in aqueous phase, the use of different kinds of saponins is being explored.

In Japanese flounder, formalin-killed *E. tarda* cells were administered to fish by feeding in the absence or presence of curdlan (a ß1,3 glucan) or curdlan together with Quil A saponin. Although the incorporation of curdlan gave higher survival rates, only the group in which the vaccine was administered with both curdlan and Quil A showed a statistically significant increased survival (Ashida et al. 1999).

Poly I:C

Polyinosinic:polycytidylic acid (Poly I:C) is a double-stranded polyribonucleotide that mimics a viral infection and therefore has been widely used to induce type I IFN in many species including fish (Eaton 1990; Jensen et al. 2002; Plant et al. 2005). The number of residues of Poly I:C normally spans from 200 to 8 kb, but unfortunately, in most instances, the molecular weight of the Poly I:C used is not

listed in the various reports. This makes comparisons difficult – as the number of Poly I:C molecules added to the biological system differs from one study to another. This may be crucial when receptor-mediated and biological responses are addressed. IFNs are cytokines with a major role in the early defence against viral infections, and Poly I:C induces indeed a nonspecific antiviral response after its binding to TLR3 and the subsequent activation of intracellular signalling events inducing, e.g. type I IFNs. This nonspecific antiviral activity of Poly I:C has been tested in rainbow trout infected with infectious haematopoietic necrosis virus (IHNV) (Kim et al. 2009). Fish pre-injected with Poly I:C were protected against IHNV challenge 2 days later, and IHNV-specific antibodies were detected in survivors. The survivors showed a 100 % survival rate following re-challenge with IHNV both 21 and 49 days after the primary IHNV challenge (Kim et al. 2009), demonstrating the fact that fish were at an antiviral state during the initial infection by a virus, gave them an important advantage for posterior specific antibody production. A similar study was performed in the sevenband grouper *Epinephelus septemfasciatus* in which fish were immunised against the nodavirus red-spotted grouper nervous necrosis virus (RGNNV) (Nishizawa et al. 2009). Fish injected with Poly I:C intramuscularly (i.m.) and challenged i.m. with RGNNV 2 days post-injection showed more than 90 % survival rate. When surviving fish were re-challenged with RGNNV 3 weeks after the primary challenge, no mortalities were detected in the group that had been previously exposed to Poly I:C; probably because upon RGNNV challenge, the antibodies against the virus were higher in these fish. All survivors that were re-challenged with RGNNV showed even higher levels of specific antibodies. In addition, the RGNNV titres in brain tissues of the survivors in the Poly I:C-RGNNV-RGNNV group were all under the detection limit (Nishizawa et al. 2009). Following up this work, this research group conducted a field trial exploring the vaccine efficacy of a RGNNV vaccine followed by Poly I:C injection. The Poly I:C-adjuvanted vaccine showed a relatively high efficacy, but a one-shot Poly I:C injection in sevenband grouper 20 days after a natural RGNNV outbreak also induced a high survival rate (93.7 %) compared to non-treated fish (9.8 %) (Oh et al. 2012).

A prophylactic strategy using Poly I:C was also used by Takami and coworkers in Japanese flounder experimentally infected with viral haemorrhagic septicaemia virus (VHSV) (Takami et al. 2010). The survival rate in Japanese flounder pre-injected with Poly I:C before a VHSV challenge was 100 %, while all untreated fish died within 9 days. Survival rates of the fish given a secondary challenge with VHSV were 100 % in the Poly I:C-VHSV group (Poly I:C-VHSV-VHSV group), while non-immunised fish showed a 0 % survival.

Lipopeptides

Lipoproteins and lipopeptides have been found in a large number of microorganisms, the most prominent being mycobacteria and mycoplasms. These molecules have been found to exhibit both a strong inflammatory response and a long-lasting

adaptive immune response in mammals; however, very few studies have been performed on lipopeptides in fish. The adjuvant effect of polar glycopeptidolipids in experimental vaccines against *A. salmonicida* was investigated (Hoel and Lillehaug 1997), using polar glycopeptidolipids (pGPL-*Mc*) from *Mycobacterium chelonae*, one of three mycobacteria species that are fish-pathogenic. Twelve weeks after vaccination, the antibody response of fish given 0.25 mg kg^{-1} pGPL-*Mc* in combination with an *A. salmonicida* bacterin was significantly higher than that induced by a non-adjuvanted bacterin. Increased doses of pGPL-*Mc* suppressed the antibody response, while no significant side effects were observed in the peritoneal cavity after the use of this adjuvant (Hoel and Lillehaug 1997).

Flagellins

The structural protein of Gram-negative flagella is called flagellin. Flagella are composed of several monomeric flagellins assembled to a core region where the filaments then possess helical shape. Flagellin is a potent activator of a broad range of cell types within the innate and adaptive immune system, promoting cytokine production (Mizel and Bates 2010). Flagellin is known to induce immune responses via the TLR5 signalling resulting in a mixed Th1 and Th2 response, although it has also been reported that inflammasomes containing NLRC4/IPAF may bind cytosolically located flagellin (Coffman et al. 2010). During the last decade, the adjuvant effect of flagellin has widely been studied in vertebrates and, during the last couple of years, also in fish (Jiao et al. 2009, 2010b; Wilhelm et al. 2006).

Piscirickettsiosis is a severe disease reported in salmonids that has caused especially great problems for the Chilean aquaculture industry. In 1989, the bacterium *Piscirickettsia salmonis* was isolated from a moribund coho salmon and was found to be the etiological agent of this disease. The pathogen is a Gram-negative obligate intracellular bacterium. The disease has also been reported to affect Atlantic salmon, rainbow trout and other farmed salmonid species (Wilhelm et al. 2006). A recombinant subunit vaccine was developed in order to control the disease due to poor responses to antibiotic treatment. Three experimental formulations were prepared containing two or three recombinant proteins of the bacterium, and the formulations were emulsified with one volume of FIA (Wilhelm et al. 2006). The highest protective response was obtained with a vaccine formulation containing the subunit of the flagellum and chaperonins Hsp60 and Hsp70 of *P. salmonis*, suggesting that the use of more than one recombinant protein antigen is needed to obtain a good protective effect against this infectious bacterium.

Jiao and coworkers have been studying different vaccine concepts against *E. tarda* in the Japanese flounder to obtain effective protective formulations, based on both recombinant proteins and DNA vaccine constructs (Jiao et al. 2009, 2010b). The most promising vaccine concept was the one consisting in a chimaeric DNA vaccine coding for the *E. tarda* proteins Eta6 fused in-frame to FliC, the flagellin for *E. tarda*. Although they found that *E. tarda* FliC induced low protective immunity

by itself, it could function as a molecular adjuvant and potentiate the specific immune response induced by the *E. tarda* antigen Eta6. Fish immunised with pEta6 and FliC produced specific serum antibodies and exhibited enhanced expression of genes that are involved in both innate and adaptive immune responses (IL-1ß, IFN, Mx, CD8α, MHC-Iα, MHC-IIα, IgM) (Jiao et al. 2009, 2010b). Such upregulation following immunisation with flagellin has also been described by Hynes et al. (2011), where TNF-α, IL-6, IL-8 and IL-1ß were significantly upregulated compared to non-adjuvanted controls. In this study, however, there was no induction of specific antibody response against flagellin or the model antigen *Limulus polyphemus* hemolymph (LPH) in the Atlantic salmon.

Synthetic Oligodeoxynucleotides

Bacterial DNA and synthetic oligodeoxynucleotides (ODNs) expressing unmethylated CpG motifs trigger an immunostimulatory cascade that culminates in the maturation, differentiation and proliferation of multiple immune cells, including B and T lymphocytes, NK cells, monocytes, macrophages and dendritic cells. CpG motifs are approximately 20 times less common in mammalian than microbial DNA and therefore are recognised as a danger signal by cells that express TLR9. In mammals, it has been widely demonstrated that CpG ODNs function as adjuvants when co-administered with vaccines, being able to both accelerate and magnify the immune response (Bode et al. 2011). In fish, although many studies have been carried out on the immunomodulatory effects of CpGs (Carrington and Secombes 2007; Liu et al. 2010a, b; Rhodes et al. 2004), only a few studies have focused on the adjuvant effect of these molecules.

Chinook salmon (*O. tshawytscha*) reared in the Pacific Northwest of the United States often suffers from infection with *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (BKD). A study in which whole cell vaccines with or without CpG adjuvants were used revealed that both the vaccine alone or that with CpG provided protection against i.p. challenge with *R. salmoninarum* (Rhodes et al. 2004). However, a combination of a commercial *R. salmoninarum* vaccine (Renogen) with a CpG adjuvant significantly reduced the level of bacterial antigens in the kidney of naturally infected fish (Rhodes et al. 2004).

In rainbow trout, four groups were i.m. injected with a commercially available, a non-adjuvanted aqueous vaccine against furunculosis containing inactivated cultures of *A. salmonicida* (AquaVac Furovac 5) alone, or together with CpG ODN 1982, CpG ODNs 2133 or ODN2143. The fish were challenged by i.p. injection using a pathogenic *A. salmonicida* strain 7 weeks after injection. The only group that showed a significantly lower mortality compared to those injected with Furovac alone (mortality of 52 %) was the group injected with Furovac and the CpG ODN 2143 in which only 21 % of the fish died (Carrington and Secombes 2007).

The protective effect of CpG motifs was also studied by Liu and coworkers in turbot and Japanese flounder (Liu et al. 2010a, b). Sixteen different CpG ODNs

were synthesised and examined for the ability to inhibit bacterial dissemination in Japanese flounder blood. Four ODNs with the strongest inhibitory effects were selected, and a plasmid pCN6 was constructed containing the sequences of the four selected ODNs. Japanese flounder were injected i.m. with plasmids pCN6 and pCN3 (control) and PBS. Four weeks post-vaccination, the fish were challenged with *A. hydrophila* and mortality was monitored over a period of 20 days. Accumulated mortalities were 30, 66.7 and 63.3 % in pCN6-, pCN3-, and PBS-immunised flounder, respectively (Liu et al. 2010b). Fish were also vaccinated as above and challenged with *E. tarda* 4 weeks after vaccination, and the mortalities were 53.3, 90 and 93.3 %, respectively. Therefore, the pCN6 plasmid provided a nonspecific protection against both *A. hydrophila* and *E. tarda* infections. These nonspecific protective effects have also been observed in fish parasitic infections, since certain CpGs (e.g. CpG ODN 1668 and CpG ODN 2359) have also proved to be protective against *Miamiensis avidus* (Kang and Kim 2012). Following on, a salmonid alphavirus (SAV) vaccine containing antigen plus CpG and Poly I:C as adjuvants induced a significant production of neutralising antibodies and conferred some level of protection – as evaluated by percentage of SAV-positive fish compared to controls (Thim et al. 2012). The authors reported that the adjuvanted vaccines induced a prominent type I IFN expression, that is, a key factor in providing antiviral response.

To analyse the adjuvant effect of CpGs in turbot, fish were vaccinated with a *Vibrio harveyi* recombinant subunit vaccine, DegQ, in combination with a CpG that had previously been shown to provide anti-infectious effects in the host species after injection. Fish were vaccinated by i.p. injection including all the appropriate controls, and 28 days after vaccination, the fish were challenged with a virulent strain of *V. harveyi*, and accumulated mortalities were recorded (Liu et al. 2010a). The only vaccine formulation that induced a significant protection was DegQ in combination with this pCN5 CpG. The duration of the adjuvant effect was found to be at least 50 days.

One of the unique features of DNA vaccines is the ability to stimulate both cellular and humoral immune responses through the administration of a bacterial plasmid coding for a protective antigen (Weiner and Kennedy 1999). Plasmid DNA vaccines possess intrinsic immunostimulatory capacity due to the presence of CpG motifs in the bacterial plasmid backbone. Therefore, the inclusion of additional CpG motifs in the vaccine plasmid would provide higher intrinsic adjuvant activity, compared with the control plasmid, being this an easy method to increase the immunogenicity of DNA vaccines. Following this, Martinez-Alonso et al. (2011) explored the possibility of increasing the immunogenicity of a VHSV DNA vaccine though the introduction of several copies (either two or four) of a fragment containing multiple CpG sequences of known immunostimulatory effects into the DNA vaccine. The addition of these CpG motifs significantly increased the titre of neutralising antibodies in serum and increased the levels of transcription of several immune genes such as Mx or MHC-I, demonstrating for the first time that additional CpG motifs may be introduced in the plasmid to increase the immunogenicity of these DNA vaccines.

Cytokines

In the past years, a great number of cytokine genes have been identified in many fish species; however, despite the fact that the use of cytokines as adjuvants has been widely explored in mammals, not many studies have focused on the possible use of cytokine genes as vaccine adjuvants in fish (Wang and Secombes 2013). This may be due to the fact that for the majority of these molecules, many details concerning their immunological role are still lacking, and until we know what immune processes they are regulating, their use would be a mere trial and error process. In any case, some attempts to explore their potential have been made in some fish species.

Interferon regulatory factors (IRFs) form a large family of transcription factors. IRF-1 has been shown to have a role in cytokine signalling and host defence against pathogens. For example, IRF-1 is upregulated in response to virus infection in fish cells, inducing an antiviral state (Caipang et al. 2005). The potential use of IRF-1 as a vaccine adjuvant was thus investigated in Japanese flounder. The co-injection of IRF-1 plasmid with a DNA vaccine encoding the major capsid protein (MCP) gene of red sea bream iridovirus (RSIV) resulted in elevated serum neutralisation antibodies but was not significantly different from those in fish vaccinated with the DNA vaccine alone (Caipang et al. 2009). Despite the moderate effect in protection, IRF-1 was responsible for the upregulation of antiviral substances like nitric oxide (NO), IFN ß and IFN-inducible genes such as Mx.

IL-8 is a CXC chemokine produced by many cell types in mammals like macrophages, monocytes, epithelial cells, neutrophils and fibroblasts upon infection, or stimulated by cytokines like IL-1ß and TNF-α. In mammals, chemokines have been widely used as adjuvants in vaccines against viral infections, since not only they attract more cells to the site of inflammation but also regulate the immune functions of the recruited cells. In fish, IL-8 has been characterised in rainbow trout among other species, and its chemoattractant property is established (Harun et al. 2008). In this species, a vaccine plasmid coding for the glycoprotein gene of VHSV was co-injected with a plasmid coding for rainbow trout IL-8 to explore its potential adjuvant effect (Jimenez et al. 2006; Sanchez et al. 2007). When the plasmid coding of IL-8 (pIL-8+) was administered together with the VHSV vaccine, an increase of IL-1ß in the spleen was found together with a greater cellular infiltration at the site of inoculation. Furthermore, fish injected with pIL-8+ alone showed a significantly higher expression of TNF-α, IL-11, TGF-ß and IL-18 in the spleen (Jimenez et al. 2006). In a further study, the transcription of different inducible CC chemokines were studied in rainbow trout in response to both the VHSV DNA vaccine and/or pIL8+, demonstrating that when IL-8 is used as an adjuvant, the expression of other chemokines such as CK5A, CK6, CK7 and CK5B is also modulated (Sanchez et al. 2007). All these results showed that IL-8 was able to modulate the early immune response and could be a potential adjuvant in fish.

Although the administration of IL-1ß-derived peptides to rainbow trout by i.p. injection reduced the mortality of fish when exposed to VHSV 2 days after injection and induced leukocyte migration to the peritoneal cavity (Peddie et al. 2003), the

possible use of these peptides as adjuvants was not further explored. The role of IL-1ß as an adjuvant was investigated in carp after i.p. injection of killed *A. hydrophila* in the absence and presence of recombinant C-terminal peptide of carp IL-1ß. The agglutinating antibody titre obtained was significantly higher in the fish injected with killed bacteria plus recombinant IL-1ß peptide compared with killed bacteria alone 3 weeks after vaccination (Yin and Kwang 2000).

Immersion Delivery of Fish Vaccines

Immersion vaccination is the simplest method for vaccine delivery; however, it is not suitable for all antigens or for all farming situations. It can be performed using hyperosmotic infiltration (HI), direct immersion (DI) or spray. Vaccination by HI involves immersing the fish in solutions such as urea or sodium chloride for a short period of time followed by immersion in the vaccine. For vaccination by DI, fish are transferred to the vaccine for a certain period of time and then moved back to the holding tank (Plant and Lapatra 2011). This last method has been proven less stressful and equivalently effective; thus, HI is not commonly used. Although DI or spray of bacterins can provide significant levels of protection (Villumsen and Raida 2013), not many successful strategies to vaccinate against viruses through immersion have been reported. In 2008, Kai et al. performed a 20 min immersion of grouper with inactivated betanodavirus and obtained high protection levels when BEI was used to inactivate the virus (RPS > 75), but not when formalin was used (RPS = 39–43) (Kai and Chi 2008). Furthermore, the efficacy of formalin-inactivated vaccine could be significantly improved by nano-encapsulation (RPS = 85).

Some authors have used ultrasound to increase the uptake of vaccine antigens through immersion. Ultrasounds should open routes in the skin, thus facilitating the transdermal delivery of vaccines that will improve the effectiveness of vaccination by immersion (Navot et al. 2005). Delivering a *V. alginolyticus* bacterin with ultrasound resulted in similar protection levels that those obtained by injection vaccination, once the parameters for the application of ultrasound were optimised (Zhou et al. 2002). The delivery of a VHSV DNA vaccine in rainbow trout by immersion with short pulses of low-intensity ultrasound also provided some protection, although the levels were lower than those obtained after intramuscular injection (Fernandez-Alonso et al. 2001).

Novel Strategies for Oral Vaccination

From the practical point of view, oral vaccination is the most suitable strategy, because the vaccine would be delivered together with the feed to large groups of fish at the same time without stress. However, the main limitation is that not all animals eat the same amount of feed, thus the vaccine dose varies from fish to fish.

Traditionally, a lot of emphasis has been made on the fact that the antigen has to reach intact the second or third segment in order for the vaccine to be effective; however, although this might be true in most cases, a recent study has demonstrated that responsive immune cells are present all along the digestive tract in rainbow trout (Ballesteros et al. 2013). Furthermore, there is still a great lack of knowledge on how immune recognition and adaptive immune mechanisms are orchestrated in the digestive tract of different fish species that would most probably respond differently among species and according to the nature of the antigen delivered (Rombout et al. 2011). All these limitations have led sometimes to poor and inconsistent results when different strategies to orally deliver antigens to fish have been addressed.

Microparticles/Nanoparticles

Microparticles or nanoparticles offer a promising option to oil emulsions, and their beneficial use as carriers for vaccine delivery has been widely discussed (Sinyakov et al. 2006). An association of antigen(s) with microparticles can be achieved by covalent linkage or physical entrapment. Compared to the latter technique, where the antigen is non-covalently and physically incorporated in the interior of the microparticle, covalent coupling offers distinct advantages: lower amount of antigen is required; processing and presentation by antigen-presenting cells may be more efficient; a higher stability during storage is obtained and any excess of material can easily be regained. With the use of microparticles, even a very low dose of antigen can give rise to a robust humoral response. The structure and the properties of microparticles may change markedly with slight alterations in production conditions, but nanoparticles can be prepared in a physicochemically reproducible manner within narrow size limits. In addition to being vehicles for oral delivery of vaccines, the particles have also been suggested as potent adjuvants in mammalian systems (Cui and Mumper 2003). Therefore, all these encapsulation techniques could be catalogued as delivery methods, as well as signal 1 facilitator adjuvants.

PLGA Particles

Encapsulation of vaccines in biocompatible and biodegradable Poly(lactide-co-glycolide) (PLGA) polymers has been studied for over 20 years. Antigen is released from the microspheres by diffusion through matrix pores and by matrix degradation. Biodegradation rates can be regulated by alterations in polymer composition and molecular weights.

So far, a few studies have been carried out on fish with regard to uptake and degradation of PLGA particles and the immune response obtained. For the most part, these studies have focused on oral administration and have been performed in species such as Japanese flounder (Tian et al. 2008a; Tian and Yu 2011) or salmonids like rainbow trout (Adomako et al. 2012; Altun et al. 2010; Lavelle et al. 1997) or

Atlantic salmon (O'Donnell et al. 1996). In the case of Japanese flounder, a plasmid encoding the major capsid protein of lymphocystis disease virus (LCDV) was constructed and encapsulated in PLGA. Controls were naked plasmid vaccine and blank PLGA particles (Tian and Yu 2011). The fish were orally intubated, and 28 days post vaccination, the fish were challenged by intramuscular injection with LCDV. Vaccine effects were evaluated by observing the presence of lymphocystis nodules. The cumulative percentage of Japanese flounder with nodules after challenge was greatly reduced in the group receiving the plasmid coding for the LCDV protein in PLGA particles in the period of 15–120 days post-immunisation (Tian and Yu 2011). In addition, the levels of antibody in sera of fish vaccinated with PLGA microcapsules increased for up to 9 weeks; although from this point, it started to decrease (Tian et al. 2008a).

In rainbow trout, oral vaccination (as a feed additive) against lactococcosis was attempted with antigens encapsulated in PLGA particles (Altun et al. 2010). RPS of the PLGA vaccine amounted to 63 %, and booster vaccination with oral administration of the PLGA vaccine gave a RPS of more than 60 % 120 days after the first vaccination. Also in rainbow trout, HGG was microencapsulated in PLGA (Lavelle et al. 1997). Specific antibodies were detected in the intestinal mucus of fish fed the microencapsulated antigen after boosting with soluble HGG, but not in fish that were primed with the soluble antigen. The fate of orally administered HGG in Atlantic salmon was determined, demonstrating that 15 min after administration, the HGG-PLGA was found in the intestine as was the free HGG (O'Donnell et al. 1996). The results from this study indicate that orally delivered HGG-PLGA had higher levels and greater persistence of HGG systemically than free HGG.

A recent article appeared on parenteral immunisation of Indian major carp, rohu (*Labeo rohita*) with PLGA-encapsulated antigen (Behera et al. 2010). Outer membrane proteins (OMP) of *A. hydrophila* were encapsulated in PLGA microparticles and mixed with FIA in an emulsion or administered alone by i.p. injection in rohu. Twenty-one and 42 days after immunisation, the antibody titres were significantly higher in the PLGA-encapsulated antigen group containing FIA (Behera et al. 2010). A dose-dependent transient increase of antibody response following i.p injection of PLGA particles containing human gamma globulin (HGG) has been shown by Fredriksen and Grip (2012), where it was shown that microparticle carriers were superior compared to nanoparticles to induce antibody response. Furthermore, when the formulation of PLGA-entrapped HGG was performed with ß-glucan or oil, it resulted in a continuous increase of antibodies over time (up to day 120). Finally, feeding of rainbow trout with feed containing plasmid DNA encoding IHNV G protein induced slightly higher amount of neutralising antibodies against IHNV but no increased survival after experimental challenge with IHNV (Adomako et al. 2012).

ISCOMs

Immune-stimulating complexes (ISCOMs) were conceived to co-formulate antigen and adjuvant in a particle (Morein and Bengtsson 1999). ISCOMs represent an interesting approach to stimulate both the humoral and cell-mediated immune

response towards amphipathic antigens. A stable and non-covalently bound complex of Quil A with amphipathic antigens (approx. 40 nm diameter) in a molar ratio of approximately 1:1:1. ISCOMs produced through the patented Matrix™ technology by Isconova have been widely studied in combination with different veterinary vaccines and are currently incorporated in a number of commercialised animal vaccines. At this moment, Pharmaq is studying the introduction of these adjuvants in commercialised fish vaccines.

Alginate

Alginate is a copolymer of β-D-mannuronic acid and α-L-guluronic acid found in the cell wall of brown algae. It has been widely used to encapsulate antigens because it is cheap, has low toxicity and is adhesive to the mucosa (Wee and Gombotz 1998). Furthermore, the adjuvant effects of alginate have also been demonstrated in fish, since it has been shown to have effects on fish weight, innate immunity and disease resistance (Cheng et al. 2008; Yeh et al. 2008).

Concerning its use for encapsulating bacterial antigens, alginate microparticles with or without a *Vibrio anguillarum* bacterin were administered with feed to both carp and trout (Joosten et al. 1997). Although optimal responses were obtained in the different species with different alginate microspheres, mucus-IgM and mucosal plasma IgM cells were detected in both cases. On the other hand, the administration of *A. salmonicida* recombinant A-layer proteins in alginate beads delivered orally to carp did induce serum antibodies (Maurice et al. 2004). However, the encapsulation of *Flavobacterium columnare* bacterin did not induce the production of serum antibodies nor was able of conferring protection in Nile tilapia (Leal et al. 2010). Better results were obtained when the oral administration of bacterins in alginates is used as an oral booster after i.p. immunisation (Romalde et al. 2004). In this case, the oral vaccination alone provided some protection (RPS = 50), but when administered as a booster, the protection was significantly increased (RPS = 87), and longer protection periods were achieved in comparison to i.p. immunisation alone.

Concerning viral antigens, most studies have focused on the use of alginate to deliver DNA vaccines orally. Thus, a DNA vaccine against lymphocystis disease virus (LCDV) was delivered orally to Japanese flounder (Tian et al. 2008c). In this study, the antigen was detected in different tissues from day 10 to 90 post-vaccination, and serum antibodies were detected up to week 16. In a similar study, de las Heras et al. (2010) encapsulated a DNA vaccine against IPNV in alginate and also detected antigen expression and serum antibody production. In this case, IFN was also upregulated, and 80 % relative survival rates were obtained when fish were challenged 15 and 30 days after vaccine delivery.

Aeromonas salmonicida subsp. *salmonicida* bacterin was encapsulated in liposome-alginate particles (Eggset et al. 1995). Atlantic salmon were vaccinated by oral intubation into the stomach. As control, the fish were given liposome-alginate particles without antigen and non-encapsulated *A. salmonicida* bacterin by intubation. The fish were orally intubated each day for 2 days. Three weeks after, the fish were revaccinated by intubations on two successive days, and 7 weeks after the last

intubations, the fish were challenged by cohabitant salmon intraperitoneally injected with virulent *A. salmonicida* bacteria. Fish vaccinated by oral intubation with *A. salmonicida* bacterin in liposome-alginate showed moderately increased survival, and also increased anti-*A. salmonicida* antibody responses.

Chitosan

Chitosan is a mucopolysaccharide obtained from marine crustaceans with great potential for oral delivery of antigens (Rao and Sharma 1997). It has been used to deliver a DNA plasmid coding for a reporter gene (β-galactosidase) together with the feed (Ramos et al. 2005). β-Galactosidase expression could be observed in the stomach, spleen and gills, demonstrating the potential of this encapsulation method. Similar results were obtained in two other studies. Tian et al. (2008b) vaccinated with a DNA plasmid containing the major capsid protein (MCP) gene of lymphocystis disease virus (LCDV) encapsulated in chitosan and observed antigen expression in tissues up to day 90 post-vaccination and serum antibodies for up to 16 weeks post-vaccination. A DNA vaccine against the porin gene of *V. anguillarum* was also delivered after chitosan encapsulation to sea bass (Rajesh Kumar et al. 2008). In this case, although the antigen was detected in different tissues, only a moderate protection against experimental *V. anguillarum* infection was obtained.

Alternative Methods for Oral Delivery

Some other alternative methods to microencapsulation have been briefly explored by some authors for oral vaccine delivery in fish. For example, feeding young fish with brine shrimp (*Artemia* nauplii) used to bioaccumulate *Vibrio anguillarum* bacterin was studied as an oral vaccination method (Joosten et al. 1995). Although immunosuppression was encountered in younger fish, sea bream orally vaccinated showed significantly higher secondary responses compared with the control at days 57 or 69 post-immunisation. In a more recent experiment, formalin-killed *E. coli* expressing the *P. aeruginosa* exotoxin was fed to *Artemia* that were subsequently fed to zebrafish (*Danio rerio*). The fish were protected from *P. aeruginosa* challenge with 81 % of vaccinated fish surviving compared to 31 % of the controls (Lin et al. 2005). Furthermore, it is possible to genetically modify plants to express protective proteins that can be delivered directly in food. Although this method has not been widely explored in fish, it seems as an interesting area of research. When a fusion protein consisting in a gut adhesion molecule and a viral peptide was expressed from potato tubers and fed to carp, the adhesion molecule mediated the binding to and uptake from the gut, whereas the viral peptide induced a humoral immune response (Companjen et al. 2005). Alternatively, microalgae can also be

used to produce the antigen. For example, the *Renibacterium salmoninarum* protein 57 (p57) was expressed in the microalgae *Chlamydomonas reinhardtii* (Siripornadulsil et al. 2007). The delivery of the transformed algae either by immersion or in feed induced a specific antibody response. Whether these novel methods based on plant or microalgae are capable of conferring, real protection has still to be demonstrated.

Conclusive Remarks and Perspectives

The development of effective vaccines should be approached by combining the search for protective antigens together with the application of specific, and targeting, adjuvants that maximise the immunogenicity with a desired immune response. At the same time, the route chosen for immunisation has to be taken into account, because despite the fact that many details of immune regulation are still unknown in fish, it is clear that the site where the antigen is presented will strongly condition the immune response that is mounted. These vaccine-specific adjuvants should be able to trigger specific immunological processes, without producing a generalised response with strong side effects.

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References

Adomako M, St-Hilaire S, Zheng Y, Eley J, Marcum RD, Sealey W, Donahower BC, Lapatra S, Sheridan PP (2012) Oral DNA vaccination of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against infectious haematopoietic necrosis virus using PLGA [Poly(D,L-Lactic-Co-Glycolic Acid)] nanoparticles. J Fish Dis 35:203–214

Afonso A, Gomes S, da Silva J, Marques F, Henrique M (2005) Side effects in sea bass (*Dicentrarchus labrax* L.) due to intraperitoneal vaccination against vibriosis and pasteurellosis. Fish Shellfish Immunol 19:1–16

Altun S, Kubilay A, Ekici S, Didinen B, Diler O (2010) Oral vaccination against lactococcosis in rainbow trout (*Oncorhynchus mykiss*) using sodium alginate and poly(lactide-co-glycolide) carrier. J Fac Vet Med Univ Kafkas Kars 16:211–217

Ashida T, Okimasu E, Ui M, Heguri M, Oyama Y, Amemura A (1999) Protection of Japanese flounder *Paralichthys olivaceus* against experimental edwardsiellosis by formalin-killed *Edwardsiella tarda* in combination with oral administration of immunostimulants. Fisheries Sci 65:527–530

Aucouturier J, Dupuis L, Ganne V (2001) Adjuvants designed for veterinary and human vaccines. Vaccine 19:2666–2672

Ballesteros NA, Castro R, Abós B, Rodriguez Saint-Jean S, Perez-Prieto SI, Tafalla C (2013) 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 8(6):e66118

Bastardo A, Ravelo C, Castro N, Calheiros J, Romalde JL (2012) Effectiveness of bivalent vaccines against *Aeromonas hydrophila* and *Lactococcus garvieae* infections in rainbow trout *Oncorhynchus mykiss* (Walbaum). Fish Shellfish Immunol 32:756–761

Behera T, Nanda PK, Mohanty C, Mohapatra D, Swain P, Das BK, Routray P, Mishra BK, Sahoo SK (2010) Parenteral immunization of fish, *Labeo rohita* with Poly D, L-lactide-co-glycolic acid (PLGA) encapsulated antigen microparticles promotes innate and adaptive immune responses. Fish Shellfish Immunol 28:320–325

Bjornsdottir B, Gudmundsdottir S, Bambir SH, Magnadottir B, Gudmundsdottir BK (2004) Experimental infection of turbot, *Scophthalmus maximus* (L.), by Moritella viscosa, vaccination effort and vaccine-induced side-effects. J Fish Dis 27:645–655

Bode C, Zhao G, Steinhagen F, Kinjo T, Klinman DM (2011) CpG DNA as a vaccine adjuvant. Expert Rev Vaccines 10:499–511

Bowden TJ, Adamson K, MacLachlan P, Pert CC, Bricknell IR (2003) Long-term study of antibody response and injection-site effects of oil adjuvants in Atlantic halibut (*Hippoglossus hippoglossus* L). Fish Shellfish Immunol 14:363–369

Caipang CMA, Hirono I, Aoki T (2005) Induction of antiviral state in fish cells by Japanese flounder, *Paralichthys olivaceus*, interferon regulatory factor-1. Fish Shellfish Immunol 19:79–91

Caipang CM, Hirono I, Aoki T (2009) Modulation of the early immune response against viruses by a teleostean interferon regulatory factor-1 (IRF-1). Comp Biochem Physiol A Mol Integr Physiol 152:440–446

Carrington AC, Secombes CJ (2007) CpG oligodeoxynucleotides up-regulate antibacterial systems and induce protection against bacterial challenge in rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol 23(4):781–792

Chen D, Ainsworth AJ (1992) Glucan administration potentiates immune defence mechanisms of channel catfish, *Ictalurus punctatus* Rafinesque. J Fish Dis 15:295–304

Cheng AC, Chen YY, Chen JC (2008) Dietary administration of sodium alginate and kappa-carrageenan enhances the innate immune response of brown-marbled grouper *Epinephelus fuscoguttatus* and its resistance against *Vibrio alginolyticus*. Vet Immunol Immunopathol 121:206–215

Coffman RL, Sher A, Seder RA (2010) Vaccine adjuvants: putting innate immunity to work. Immunity 33:492–503

Companjen AR, Florack DE, Bastiaans JH, Matos CI, Bosch D, Rombout JH (2005) Development of a cost-effective oral vaccination method against viral disease in fish. Dev Biol (Basel) 121:143–150

Cui Z, Mumper RJ (2003) Microparticles and nanoparticles as delivery systems for DNA vaccines. Crit Rev Ther Drug Carrier Syst 20:103–137

Dalmo RA, Bogwald J (2008) Beta-glucans as conductors of immune symphonies. Fish Shellfish Immunol 25:384–396

de las Heras AI, Rodriguez Saint-Jean S, Perez-Prieto SI (2010) Immunogenic and protective effects of an oral DNA vaccine against infectious pancreatic necrosis virus in fish. Fish Shellfish Immunol 28:562–570

DeBaulney M, Quentel C, Fournier V, Lamour F, Le Gouvello R (1996) Effect of long-term oral administration of b-glucan as an immunostimulant or an adjuvant on some non-specific parameters of the immune response of turbot *Scophthalmus maximus*. Dis Aquat Organ 26:139–147

Eaton W (1990) Antiviral activity in four species of salmonids following exposure to polyinosinic:cytidylic acid. Dis Aquat Organ 9:193–198

Eggset G, McQueen Leifson R, Mikkelsen H, Hjelmeland K, Homme JM, Syvertsen C, Wathne E, Bøgwald J (1995) Oral vaccination of Atlantic salmon, *Salmo salar* L., against furunculosis with vaccines encapsulated in liposome/alginate particles. J Mar Biotechnol 3:196–199

Fan T, Hu X, Wang L, Geng X, Jiang G, Yang X, Yu M (2012) Development of an inactivated iridovirus vaccine against turbot viral reddish body syndrome. J Ocean Univ China 11:65–69

Fernandez-Alonso M, Rocha A, Coll JM (2001) DNA vaccination by immersion and ultrasound to trout viral haemorrhagic septicaemia virus. Vaccine 19:3067–3075

Figueras A, Santarem MM, Novoa B (1998) Influence of the sequence of administration of beta-glucans and a *Vibrio damsela* vaccine on the immune response of turbot (*Scophthalmus maximus* L.). Vet Immunol Immunopathol 64:59–68

Flach TL, Ng G, Hari A, Desrosiers MD, Zhang P, Ward SM, Seamone ME, Vilaysane A, Mucsi AD, Fong Y, Prenner E, Ling CC, Tschopp J, Muruve DA, Amrein MW, Shi Y (2011) Alum interaction with dendritic cell membrane lipids is essential for its adjuvanticity. Nat Med 17:479–487

Fredriksen BN, Grip J (2012) PLGA/PLA micro- and nanoparticle formulations serve as antigen depots and induce elevated humoral responses after immunization of Atlantic salmon (*Salmo salar* L.). Vaccine 30:656–667

Gjessing MC, Falk K, Weli SC, Koppang EO, Kvellestad A (2012) A sequential study of incomplete Freund's adjuvant-induced peritonitis in Atlantic cod. Fish Shellfish Immunol 32:141–150

Glenny A, Pope C, Waddington H, Wallace U (1926) Immunological notes. XXIII. The antigenic value of toxoid precipitated by potassium alum. J Pathol Bacteriol 29:31–40

Grabowski LD, LaPatra SE, Cain KD (2004) Systemic and mucosal antibody response in tilapia, *Oreochromis niloticus* (L.), following immunization with *Flavobacterium columnare*. J Fish Dis 27:573–581

Gudmundsdottir BK, Bjornsdottir B (2007) Vaccination against atypical furunculosis and winter ulcer disease of fish. Vaccine 25:5512–5523

Guy B (2007) The perfect mix: recent progress in adjuvant research. Nat Rev Microbiol 5:505–517

Harun NO, Zou J, Zhang YA, Nie P, Secombes CJ (2008) The biological effects of rainbow trout (*Oncorhynchus mykiss*) recombinant interleukin-8. Dev Comp Immunol 32:673–681

Hastein T, Gudding R, Evensen O (2005) Bacterial vaccines for fish – an update of the current situation worldwide. Dev Biol (Basel) 121:55–74

Hikima J, Jung TS, Aoki T (2011) Immunoglobulin genes and their transcriptional control in teleosts. Dev Comp Immunol 35:924–936

Hoel K, Lillehaug A (1997) Adjuvant activity of polar glycopeptidolipids from *Mycobacterium chelonae* in experimental vaccines against *Aeromonas salmonicida* in salmonid fish. Fish Shellfish Immunol 7:365–376

Hogfors E, Pullinen KR, Madetoja J, Wiklund T (2008) Immunization of rainbow trout, *Oncorhynchus mykiss* (Walbaum), with a low molecular mass fraction isolated from *Flavobacterium psychrophilum*. J Fish Dis 31:899–911

Hynes NA, Furnes C, Fredriksen BN, Winther T, Bogwald J, Larsen AN, Dalmo RA (2011) Immune response of Atlantic salmon to recombinant flagellin. Vaccine 29:7678–7687

Jensen I, Albuquerque A, Sommer AI, Robertsen B (2002) Effect of poly I:C on the expression of Mx proteins and resistance against infection by infectious salmon anaemia virus in Atlantic salmon. Fish Shellfish Immunol 13:311–326

Jiao XD, Zhang M, Hu YH, Sun L (2009) Construction and evaluation of DNA vaccines encoding *Edwardsiella tarda* antigens. Vaccine 27:5195–5202

Jiao XD, Cheng S, Hu YH, Sun L (2010a) Comparative study of the effects of aluminum adjuvants and Freund's incomplete adjuvant on the immune response to an *Edwardsiella tarda* major antigen. Vaccine 28:1832–1837

Jiao XD, Hu YH, Sun L (2010b) Dissection and localization of the immunostimulating domain of *Edwardsiella tarda* FliC. Vaccine 28:5635–5640

Jimenez N, Coll J, Salguero FJ, Tafalla C (2006) Co-injection of interleukin 8 with the glycoprotein gene from viral haemorrhagic septicemia virus (VHSV) modulates the cytokine response in rainbow trout (*Oncorhynchus mykiss*). Vaccine 24:5615–5626

Joosten PHM, Aviles-Trigueros M, Sorgeloos P, Rombout JHWM (1995) Oral vaccination of juvenile carp (*Cyprinus carpio*) and gilthead seabream (*Sparus aurata*) with bioencapsulated *Vibrio anguillarum* bacterin. Fish Shellfish Immunol 5:289–299

Joosten PHM, Tiemersma E, Threels A, Caumartin-Dhieux C, Rombout JHWM (1997) Oral vaccination of fish against *Vibrio anguillarum* using alginate microparticles. Fish Shellfish Immunol 7:471–485

Kai YH, Chi SC (2008) Efficacies of inactivated vaccines against betanodavirus in grouper larvae (*Epinephelus coioides*) by bath immunization. Vaccine 26:1450–1457

Kamilya D, Maiti T, Joardar S, Mal B (2006) Adjuvant effect of mushroom glucan and bovine lactoferrin upon *Aeromonas hydrophila* vaccination in catla, *Catla catla* (Hamilton). J Fish Dis 29:331–337

Kang Y, Kim K (2012) Effect of CpG-ODNs belonging to different classes on resistance of olive flounder (*Paralichthys olivaceus*) against viral hemorrhagic septicemia virus (VHSV) and *Miamiensis avidus* (Ciliata; Scuticociliata) infections. Aquaculture 324:39–43

Kato G, Kondo H, Aoki T, Hirono I (2012) Mycobacterium bovis BCG vaccine induces non-specific immune responses in Japanese flounder against *Nocardia seriolae*. Fish Shellfish Immunol 33:243–250

Kawakami H, Shinohara N, Sakai M (1998) The non-specific stimulation and adjuvant effects of *Vibrio anguillarum* bacterin, M-glucan, chitin and Freund's complete adjuvant against *Pasteurella piscicida* infection in yellowtail. Fish Pathol 33:287–292

Kim HJ, Oseko N, Nishizawa T, Yoshimizu M (2009) Protection of rainbow trout from infectious hematopoietic necrosis (IHN) by injection of infectious pancreatic necrosis virus (IPNV) or poly(I:C). Dis Aquat Organ 83:105–113

Kubilay A, Altun S, Ulukoy S, Ekici S, Diler O (2008) Immunization of rainbow trout (*Oncorhynchus mykiss*) against *Lactococcus garvieae* using vaccine mixtures. Isr J Aquacult Bamid 60:268–273

Lamas J, Sanmartin M, Parama A, Castro R, Cabaleiro S, de Ocenda M, Barja J, Leiro J (2008) Optimization of an inactivated vaccine against a scuticociliate parasite of turbot: effect of antigen, formalin and adjuvant concentration on antibody response and protection against the pathogen. Aquaculture 278:22–26

LaPatra SE, Plant KP, Alcorn S, Ostland V, Winton J (2010) An experimental vaccine against *Aeromonas hydrophila* can induce protection in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J Fish Dis 33:143–151

Lavelle EC, Jenkins PG, Harris JE (1997) Oral immunization of rainbow trout with antigen micro-encapsulated in poly(DL-lactide-co-glycolide) microparticles. Vaccine 15:1070–1078

Lawrence GW, Saul A, Giddy AJ, Kemp R, Pye D (1997) Phase I trial in humans of an oil-based adjuvant SEPPIC MONTANIDE ISA 720. Vaccine 15:176–178

Leal C, Carvalho-Castro G, Sacchetin P, Lopes C, Moraes A, Figueiredo HCP (2010) Oral and parental vaccines against *Flavobacterium columnare*: evaluation of humoral immune response by ELISA and in vivo efficiency in Nile tilapia (*Oreochromis niloticus*). Aquacult Int 18:657–666

Lin JH, Yu CC, Lin CC, Yang HL (2005) An oral delivery system for recombinant subunit vaccine to fish. Dev Biol (Basel) 121:175–180

Liu CS, Sun Y, Hu YH, Sun L (2010a) Identification and analysis of a CpG motif that protects turbot (*Scophthalmus maximus*) against bacterial challenge and enhances vaccine-induced specific immunity. Vaccine 28:4153–4161

Liu CS, Sun Y, Hu YH, Sun L (2010b) Identification and analysis of the immune effects of CpG motifs that protect Japanese flounder (*Paralichthys olivaceus*) against bacterial infection. Fish Shellfish Immunol 29:279–285

Manicassamy S, Pulendran B (2009) Modulation of adaptive immunity with Toll-like receptors. Semin Immunol 21:185–193

Martinez-Alonso S, Martinez-Lopez A, Estepa A, Cuesta A, Tafalla C (2011) The introduction of multi-copy CpG motifs into an antiviral DNA vaccine strongly up-regulates its immunogenicity in fish. Vaccine 29:1289–1296

Maurice S, Nussinovitch A, Jaffe N, Shoseyov O, Gertler A (2004) Oral immunization of *Carassius auratus* with modified recombinant A-layer proteins entrapped in alginate beads. Vaccine 23:450–459

Midtlyng P, Lillehaug A (1998) Growth of Atlantic salmon *Salmo salar* after intraperitoneal administration of vaccines containing adjuvants. Dis Aquat Org 32:91–97

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

Mikkelsen H, Schrøder M, Lund V (2004) Vibriosis and atypical furunculosis vaccines; efficacy, specificity and side effects in Atlantic cod, *Gadus morhua* L. Aquaculture 242:81–91

Mizel SB, Bates JT (2010) Flagellin as an adjuvant: cellular mechanisms and potential. J Immunol 185:5677–5682

Morein B, Bengtsson KL (1999) Immunomodulation by iscoms, immune stimulating complexes. Methods 19:94–102

Mulvey B, Landolt M, Busch R (1995) Effects of potassium aluminium sulphate (alum) used in an *Aeromonas salmonicida* bacterin in Atlantic salmon, *Salmo salar* L. J Fish Dis 18:495–506

Mutoloki S, Cooper GA, Marjara IS, Koop BF, Evensen O (2010) High gene expression of inflammatory markers and IL-17A correlates with severity of injection site reactions of Atlantic salmon vaccinated with oil-adjuvanted vaccines. BMC Genomics 11:336

Navot N, Kimmel E, Avtalion RR (2005) Immunisation of fish by bath immersion using ultrasound. Dev Biol (Basel) 121:135–142

Nikl L, Albright L, Evelyn T (1991) Influence of seven immunostimulants on the immune response of coho salmon to Aeromonas salmonicida. Dis Aquat Organ 12:7–12

Ninomiya K, Yamamoto M (2001) Efficacy of oil-adjuvanted vaccines for bacterial hemorrhagic ascites in ayu *Plecoglossus altivelis*. Fish Pathol 36:183–185

Nishizawa T, Takami I, Kokawa Y, Yoshimizu M (2009) Fish immunization using a synthetic double-stranded RNA Poly(I:C), an interferon inducer, offers protection against RGNNV, a fish nodavirus. Dis Aquat Organ 83:115–122

O'Donnell G, Reilly P, Davidson G, Ellis A (1996) The uptake of human gamma globulin incorporated into poly (D,L-lactide-co-glycolide) microparticles following oral intubation in Atlantic salmon, *Salmo salar* L. Fish Shellfish Immunol 6:507–520

Oh M, Takami I, Nishizawa T, Kim W, Kim C, Kim S, Park M (2012) Field tests of Poly I:C immunization with nervous necrosis virus (NNV) in sevenband grouper, *Epinephelus septemfasciatus* (Thunberg). J Fish Dis 35:187–191

Olivier G, Evelyn TP, Lallier R (1985) Immunity to *Aeromonas salmonicida* in coho salmon (*Oncorhynchus kisutch*) induced by modified Freund's complete adjuvant: its non-specific nature and the probable role of macrophages in the phenomenon. Dev Comp Immunol 9:419–432

Opie EL, Freund J (1937) An experimental study of protective inoculation with heat killed tubercle bacilli. J Exp Med 66:761–788

Palm NW, Medzhitov R (2009) Pattern recognition receptors and control of adaptive immunity. Immunol Rev 227:221–233

Palti Y (2011) Toll-like receptors in bony fish: from genomics to function. Dev Comp Immunol 35:1263–1272

Peddie S, McLauchlan PE, Ellis AE, Secombes CJ (2003) Effect of intraperitoneally administered IL-1 beta-derived peptides on resistance to viral haemorrhagic septicaemia in rainbow trout *Oncorhynchus mykiss*. Dis Aquat Organ 56:195–200

Plant KP, Lapatra SE (2011) Advances in fish vaccine delivery. Dev Comp Immunol 35:1256–1262

Plant KP, Harbottle H, Thune RL (2005) Poly I:C induces an antiviral state against Ictalurid Herpesvirus 1 and Mx1 transcription in the channel catfish (*Ictalurus punctatus*). Dev Comp Immunol 29:627–635

Poobalane S, Thompson KD, Ardo L, Verjan N, Han HJ, Jeney G, Hirono I, Aoki T, Adams A (2010) Production and efficacy of an *Aeromonas hydrophila* recombinant S-layer protein vaccine for fish. Vaccine 28:3540–3547

Rajesh Kumar S, Ishaq Ahmed VP, Parameswaran V, Sudhakaran R, Sarath Babu V, Sahul Hameed AS (2008) Potential use of chitosan nanoparticles for oral delivery of DNA vaccine in Asian sea bass (*Lates calcarifer*) to protect from *Vibrio* (*Listonella*) *anguillarum*. Fish Shellfish Immunol 25:47–56

Ramos EA, Relucio JL, Torres-Villanueva CA (2005) Gene expression in tilapia following oral delivery of chitosan-encapsulated plasmid DNA incorporated into fish feeds. Mar Biotechnol NY 7:89–94

Rao SB, Sharma CP (1997) Use of chitosan as a biomaterial: studies on its safety and hemostatic potential. J Biomed Mater Res 34:21–28

Ravelo C, Magarinos B, Herrero M, Costa L, Toranzo A, Romalde J (2006) Use of adjuvanted vaccines to lengthen the protection against lactococcosis in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 251:153–158

Rhodes LD, Rathbone CK, Corbett SC, Harrell LW, Strom MS (2004) Efficacy of cellular vaccines and genetic adjuvants against bacterial kidney disease in chinook salmon (*Oncorhynchus tshawytscha*). Fish Shellfish Immunol 16:461–474

Ribeiro C, Schijns VEJC (2010) Immunology of vaccine adjuvants. In: Davies G (ed) Vaccine adjuvants, methods in molecular biology. Springer Science+Business Media, New York, pp 1–14

Robertsen B (1999) Modulation of the non-specific defence of fish by structurally conserved microbial polymers. Fish Shellfish Immunol 9:269–290

Romalde JL, Luzardo-Alvarez A, Ravelo C, Toranzo A, Blanco-Wendez J (2004) Oral immunization using alginate microparticles as a useful strategy for booster vaccination against fish lactoccocosis. Aquaculture 236:119–129

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

Rørstad G, Aasjord P, Robertsen B (1993) Adjuvant effect of a yeast glucan in vaccines against furunculosis in Atlantic salmon (*Salmo salar* L.). Fish Shellfish Immunol 3:179–190

Saikia D, Kamilya D (2012) Immune responses and protection in catla (*Catla catla*) vaccinated against epizootic ulcerative syndrome. Fish Shellfish Immunol 32:353–359

Samuel M, Lam T, Sin Y (1996) Effect of laminaran [b(1,3)-D-glucan] on the protective immunity of blue gourami, *Trichogaster trichopterus* against *Aeromonas hydrophila*. Fish Shellfish Immunol 6:443–454

Samuelsen OB, Nerland AH, Jorgensen T, Schroder MB, Svasand T, Bergh O (2006) Viral and bacterial diseases of Atlantic cod *Gadus morhua*, their prophylaxis and treatment: a review. Dis Aquat Organ 71:239–254

Sanchez E, Coll J, Tafalla C (2007) Expression of inducible CC chemokines in rainbow trout (*Oncorhynchus mykiss*) in response to a viral haemorrhagic septicemia virus (VHSV) DNA vaccine and interleukin 8. Dev Comp Immunol 31:916–926

Schijns VE (2001) Induction and direction of immune responses by vaccine adjuvants. Crit Rev Immunol 21:75–85

Secombes C (2008) Will advances in fish immunology change vaccination strategies? Fish Shellfish Immunol 25:409–416

Selvaraj V, Sampath K, Sekar V (2005) Administration of yeast glucan enhances survival and some non-specific and specific immune parameters in carp (*Cyprinus carpio*) infected with *Aeromonas hydrophila*. Fish Shellfish Immunol 19:293–306

Selvaraj V, Sampath K, Sekar V (2006) Adjuvant and immunostimulatory effects of beta-glucan administration in combination with lipopolysaccharide enhances survival and some immune parameters in carp challenged with *Aeromonas hydrophila*. Vet Immunol Immunopathol 114:15–24

Shimahara Y, Huang Y-F, Tsai M-A, Wang P-C, Chen S-C (2010) Immune response of largemouth bass, *Micropterus salmoides*, to whole cells of different *Nocardia seriolae* strains. Fisheries Sci 76:489–494

Sinyakov MS, Dror M, Lublin-Tennenbaum T, Salzberg S, Margel S, Avtalion RR (2006) Nano- and microparticles as adjuvants in vaccine design: success and failure is related to host natural antibodies. Vaccine 24:6534–6541

Siripornadulsil S, Dabrowski K, Sayre R (2007) Microalgal vaccines. Adv Exp Med Biol 616:122–128

Sitja-Bobadilla A, Palenzuela O, Alvarez-Pellitero P (2008) Immune response of turbot, *Psetta maxima* (L.) (*Pisces: Teleostei*), to formalin-killed scuticociliates (*Ciliophora*) and adjuvanted formulations. Fish Shellfish Immunol 24:1–10

Skov J, Kania PW, Holten-Andersen L, Fouz B, Buchmann K (2012) Immunomodulatory effects of dietary beta-1,3-glucan from *Euglena gracilis* in rainbow trout (*Oncorhynchus mykiss*) immersion vaccinated against *Yersinia ruckeri*. Fish Shellfish Immunol 33:111–120

Soltani M, Alishahi M, Mirzargar S, Nikbakht G (2007) Vaccination of rainbow trout against *Streptococcus iniae* infection: comparison of different routes of administration and different vaccines. Iran J Fisheries Sci 7:129–140

Sun HX, Xie Y, Ye YP (2009) Advances in saponin-based adjuvants. Vaccine 27:1787–1796
Takami I, Kwon SR, Nishizawa T, Yoshimizu M (2010) Protection of Japanese flounder *Paralichthys olivaceus* from viral hemorrhagic septicemia (VHS) by Poly(I:C) immunization. Dis Aquat Organ 89:109–115
Thim HL, Iliev DB, Christie KE, Villoing S, McLoughlin MF, Strandskog G, Jorgensen JB (2012) Immunoprotective activity of a salmonid alphavirus vaccine: comparison of the immune responses induced by inactivated whole virus antigen formulations based on CpG class B oligonucleotides and poly I:C alone or combined with an oil adjuvant. Vaccine 30:4828–4834
Tian J, Yu J (2011) Poly(lactic-co-glycolic acid) nanoparticles as candidate DNA vaccine carrier for oral immunization of Japanese flounder (*Paralichthys olivaceus*) against lymphocystis disease virus. Fish Shellfish Immunol 30:109–117
Tian J, Sun X, Chen X, Yu J, Qu L, Wang L (2008a) The formulation and immunisation of oral poly(DL-lactide-co-glycolide) microcapsules containing a plasmid vaccine against lymphocystis disease virus in Japanese flounder (*Paralichthys olivaceus*). Int Immunopharmacol 8:900–908
Tian J, Yu J, Sun X (2008b) Chitosan microspheres as candidate plasmid vaccine carrier for oral immunisation of Japanese flounder (Paralichthys olivaceus). Vet Immunol Immunopathol 126:220–229
Tian JY, Sun XQ, Chen XG (2008c) Formation and oral administration of alginate microspheres loaded with pDNA coding for lymphocystis disease virus (LCDV) to Japanese flounder. Fish Shellfish Immunol 24:592–599
Tyler JW, Klesius PH (1994) Protection against enteric septicemia of catfish (*Ictalurus punctatus*) by immunization with the R-mutant, *Escherichia coli* (J5). Am J Vet Res 55:1256–1260
Van Gelderen R, Carson J, Nowak B (2009) Experimental vaccination of Atlantic salmon (*Salmo salar* L.) against marine flexibacteriosis. Aquaculture 288(1–2):7–13
Villumsen KR, Raida MK (2013) Long-lasting protection induced by bath vaccination against *Aeromonas salmonicida subsp. salmonicida* in rainbow trout. Fish Shellfish Immunol 35: 1649–1653
Wang T, Secombes CJ (2013) The cytokine networks of adaptive immunity in fish. Fish Shellfish Immunol 35:1703–1718
Wee S, Gombotz WR (1998) Protein release from alginate matrices. Adv Drug Deliv Rev 31:267–285
Weiner DB, Kennedy RC (1999) Genetic vaccines. Sci Am 281:50–57
Wilhelm V, Miquel A, Burzio LO, Rosemblatt M, Engel E, Valenzuela S, Parada G, Valenzuela PD (2006) A vaccine against the salmonid pathogen *Piscirickettsia salmonis* based on recombinant proteins. Vaccine 24:5083–5091
Yeh SP, Chang CA, Chang CY, Liu CH, Cheng W (2008) Dietary sodium alginate administration affects fingerling growth and resistance to *Streptococcus sp.* and iridovirus, and juvenile non-specific immune responses of the orange-spotted grouper, *Epinephelus coioides*. Fish Shellfish Immunol 25:19–27
Yin Z, Kwang J (2000) Carp interleukin-1 beta in the role of an immuno-adjuvant. Fish Shellfish Immunol 10:375–378
Zheng Z, Yingeng W, Qingyin W, Nannan D, Meijie L, Jiangbo Q, Bin L, Lan W (2012) Study on the immune enhancement of different immunoadjuvants used in the pentavalent vaccine for turbots. Fish Shellfish Immunol 32:391–395
Zhou YC, Wang J, Zhang B, Su YQ (2002) Ultrasonic immunization of sea bream, *Pagrus major* (Temminck & Schlegel), with a mixed vaccine against *Vibrio alginolyticus* and *V. anguillarum*. J Fish Dis 25:325–331
Zhu LY, Nie L, Zhu G, Xiang LX, Shao JZ (2013) Advances in research of fish immune-relevant genes: a comparative overview of innate and adaptive immunity in teleosts. Dev Comp Immunol 39:39–62