Development in fish vaccinology with focus on delivery methodologies, adjuvants and formulations

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Development in fish vaccinology with focus on delivery methodologies, adjuvants and formulations

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Abstract. Sustainable development in aquaculture is equivalent to disease prevention, and vaccination has become the single most important tool. There has been a dramatic reduction in use of antibiotics in Norwegian salmon farming since the introduction of oil-based vaccines. Today, it is an industry standard in all salmon-producing countries, and we are seeing a similar approach being adopted in Japan for farming high value species. Fish can be vaccinated by immersion and the oral route, however, the protection falls short compared to injection vaccination. Interesting new technologies have emerged over the last 5 years, particularly injection of a single dose of naked DNA into the fish muscle. Nevertheless, the prospect of having a commercial product on the market within 5 years is meagre. New technologies are promising but it is more likely there will be improvements of existing vaccines than completely new technologies taking over the fish vaccination scene in the next 5-10 years.

Keywords. Fish vaccination – Delivery methods – Adjuvants – New technologies.

Développement de la vaccinologie des poissons focalisée sur les méthodologies d'administration, les adjuvants et les formulations

Résumé. *Le développement durable en aquaculture équivaut à la prévention des maladies, et la vaccination est devenue l'outil le plus important. Il y a eu une réduction spectaculaire de l'utilisation d'antibiotiques dans l'élevage de saumon en Norvège depuis l'introduction de vaccins basés sur des huiles. De nos jours, ceci constitue une norme d'industrie dans tous les pays producteurs de saumon, et nous voyons qu'une approche semblable est en train d'être adoptée au Japon pour l'élevage d'espèces à haute valeur. Les poissons peuvent être vaccinés par immersion, cependant pour la voie orale, la protection est moindre que pour la vaccination par injection. De nouvelles technologies intéressantes sont apparues lors des 5 dernières années, en particulier l'injection d'un seule dose d'ADN nu dans le muscle du poisson. Toutefois, il y a peu de chances de disposer d'un produit commercial sur le marché dans les 5 ans. Les nouvelles technologies sont prometteuses, mais il est probable que, dans le domaine de la vaccination des poissons, sur les 5-10 prochaines années, on verra des améliorations de vaccins existants plutôt que des technologies totalement nouvelles.*

Mots-clés. *Vaccination des poissons – Méthodes d'administration – Adjuvants – Nouvelles technologies.*

I – Introduction

Sustainable development of aquaculture relies on disease prevention. With an intensification of operations, the risk of disease occurrence and spread of infectious diseases increases. There is a profound and consistent general positive attitude towards vaccines. Vaccines stimulate the immune system to help fight off diseases and the application of these methods to control infectious diseases is growing in importance. Perfecting the use of adjuvants and delivery systems is needed to meet the demand for vaccines in order to ensure the safe supply of healthy fish products. This article summarises the recent development in fish vaccinology and discusses possibilities and limitations regarding the use of vaccination for the control of infectious diseases in commercial fish farming.

Fish possess, as mammals, a defence system which enables them to survive and maintain their integrity in a hostile environment. The major lymphoid tissues in teleost fish are the (head) kidney, thymus, spleen and mucosa-associated lymphoid tissues, including the skin and gills (Press and Evensen, 1999), and obvious differences from the mammalian immune system are that fish lack a bone marrow and lymph nodes. The protective mechanisms are directed against foreign matter, including pathogens and malignant cells, and comprise a number of innate and adaptive reactions.

II – Immunity and vaccination of fish

Vaccines aim at stimulating the adaptive immune system to mount a response against a pathogen or rather against defined structures of the pathogen, the immunogenic parts. Vaccination has been used as a prophylactic means for decades and it has been estimated that ten percent of all cultured aquatic animals are lost because of infectious diseases alone, amounting to 8-10 billion USD in loss annually on a global basis. High value species like Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*) are today vaccinated against a wide range of diseases (Gudding *et al.*, 1999), and we are seeing the early start of vaccination in yellowtail farming in Japan. Vaccine administration of aquatic animals poses obvious technical problems, not encountered in other animals. The vaccines are either delivered by an intraperitoneal injection, by immersion, where animals are placed in a vaccine solution, or by oral administration. The advantages and disadvantages for the different routes of delivery are summarised in Table 1.

Table 1. Summary of different routes of administration for vaccines of farmed fin fish

Route of administration	Type of formulation/delivery method	Advantages	Disadvantages
Injection	• Oil-based (water-in-oil, oil-in-water or w/o/w) • Liposomes (experimental)	• Most potent with little waste of vaccine • Allows the use of adjuvants • Cost effective method for high value species • Mass vaccination is possible	• Stressful • Impractical for fish <15 g • Labour intensive • Injection-site reactions • Immune response (level of protection)
Immersion (inactivated and live vaccines)	• Used to a limited extent (mainly in marine fish species) • Live attenuated vaccines • Vector vaccines	• Large scale application • Moderate stress to the fish • Easy – allows mass vaccination of immunocompetent fish • High efficacy using live, attenuated vaccines	• Large amount of vaccine is needed • Low efficacy for inactivated vaccines • Inferior to injection routes in terms of efficacy • Cost prohibitive for large fish
Oral delivery	• Top-dressing • Formulation in PLG (experimental)	• Imposes no stress on the fish • Moderate cost • All fish sizes can be vaccinated when immunocompetent • Usually safe – primes mucosal immunity (external surfaces)	• Usually low efficacy • Can be cost prohibitive for larger fish

III – Vaccination strategies

The choice of delivery method, or combinations thereof, is of crucial importance for obtaining good protection. Further to this, if there is a need to protect the fish at an early stage of the life cycle, immunocompetence has to be considered and, in general, the recommendation would be to wait for the fish to reach an age where it is able to mount an appropriate immune response. For salmonids, this will typically be around 0.5-1 g (Tatner and Horne, 1983), while for other species the animal can potentially have developed an ability to respond to vaccination at an even earlier stage (Padros and Crespo, 1996; Watts *et al.*, 2003).

The assumption is that to protect fish prior to the development of full immunocompetence, one would have to rely on innate immunity or innate immune responses, since it is known that these responses mature prior to the animal being fully immunocompetent (Lam *et al.*, 2004), i.e. being able to respond by an adaptive immune response.

IV – Adjuvants and principle of action

The mode of action of adjuvants, in general, is poorly understood. It is known that the formation of a depot at the site of inoculation is a typical trait of many of the adjuvants resulting in slow release of the antigens and the presentation of antigen to immunocompetent cells (Audibert and Lise, 1993). This is a typical feature of oil-based adjuvants and most likely plays a key role in induction of immunity for many of the fish vaccines currently available for use in different aquaculture markets.

Vaccines for salmonid fish, Atlantic salmon in particular, are for the most part administered parenterally and formulated with an adjuvant to enhance immunogenicity. All vaccines currently available are inactivated (non-replicating). Non-replicating vaccines are preferred because of their safety in normal and immunocompromised vaccinates, but they inherently lack immunogenicity and require vaccine adjuvants. Adjuvants are necessary to activate and direct the innate and adaptive immune responses to the rather poorly immunogenic vaccine antigens, the aim being to develop vaccines with more predictable efficacy.

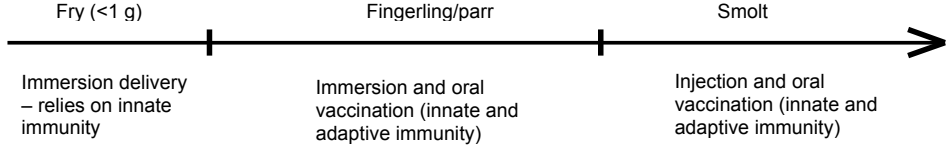

Fig. 1. Size of Atlantic salmon relative to development of immunocompetence. In fry below 1 g, adaptive responses have not matured and any stimulation of the immune system would have to rely on an innate immune response. At a later stage and up to around 10 g, immersion or oral vaccination is to be preferred, while in larger fish parenteral delivery can be applied.

Adjuvants aid in an early onset of immunity, long duration of effector responses such as antibody formation or cytotoxic T cell activity, and avoidance of booster immunizations (Singh and O'Hagan, 2003). The mechanisms of adjuvanticity are complex. They facilitate delivery of antigen (to the secondary lymphoid organs) for a sufficient period of time, and adjuvants provide either a non-self microbial signal or a host-derived danger signal from stressed cells (Singh and O'Hagan, 2003). In conclusion, adjuvants aid (adjuvare in Latin means "help") the immune response to a given antigen and also increase and prolong the immune responses.

V – Injection vaccination

1. Oil-adjuvant vaccines

Vaccination of Atlantic salmon against furunculosis and vibriosis/cold-water vibriosis with oil-adjuvant vaccines results in the induction of long-lasting and protective immunity. In terms of level of protection, use of oil-adjuvant vaccines against furunculosis will not result in clinical disease outbreaks after transfer to sea water (Gudding *et al.*, 1999). However, the draw-back is that oil-adjuvant vaccines result in the formation of visible injection-site lesions (Figs 2a and b) that persist through to harvest size (Midtlyng *et al.*, 1996). This may on some occasions also result in down-grading of fish at slaughter or after processing. The intra-abdominal lesions are recognised grossly as melanisation and adhesions between internal organs or between the organs and the peritoneal wall (Mutoloki *et al.*, 2004). Histomorphological examination reveals granuloma-formation (granulomata) comprising macrophages, epithelioid-like cells, occasionally with formation of multinucleate giant cells and with varying numbers of lymphocytes and eosinophilic granular cells (EGC)/mast cells (Mutoloki *et al.*, 2002, 2006).

Fig. 2a. Mild intraperitoneal granulomas in Atlantic salmon following use of oil-adjuvant vaccines (photo by Trygve Poppe).

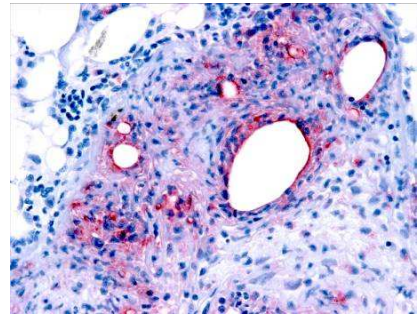

Fig. 2b. Immunohistochemical examination of *Aeromonas salmonicida* LPS antigen (red colour) of peritoneal granuloma in Atlantic salmon vaccinated with oil-adjuvanted vaccine.

Retention of antigens at the injection site is believed to be a prerequisite for long-term protection, also known as the depot effect. The antigens of a water-in-oil emulsion are located in the water droplets (mainly) and the distribution of antigens is given schematically in Fig. 3. Retention of LPS and A-layer protein of *Aeromonas salmonicida* sp*. salmonicida* (*A. salmonicida*) in head kidney and of lipopolysaccharide (LPS) in spleen has been observed 16 weeks following intraperitoneal injection of mono- and trivalent killed vaccines with Al_2PO_4 adjuvant (Press *et al.*, 1996). Høie *et al.* (1996) demonstrated the presence of *A. salmonicida* 16S rDNA at 16 weeks following intraperitoneal injection of formalin-killed bacterin. Further to this, it has been shown that antigens (LPS) of *A. salmonicida* can be found as long as 18 months post vaccination using immunohistochemistry and *in situ* detection of antigens (Fig. 2b and Evensen *et al.,* 2005).

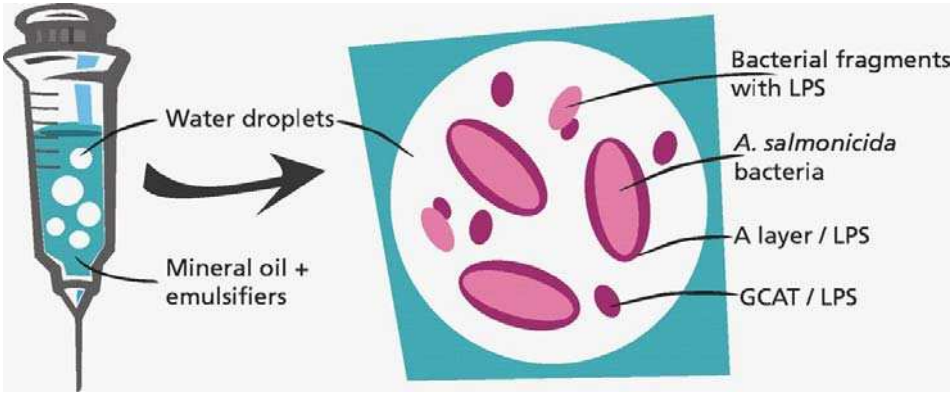

Fig. 3. Schematic presentation of a water-in-oil formulation. Water droplets are dispersed in a continuous oil phase and bacterial components are found within the water droplet and at the interphase between water and oil, possibly also in the oil phase on some occasions.

2. DNA vaccines

Gene therapy can be defined as the delivery of a therapeutic gene for expression in somatic tissue. There has been a rapid development in the field of gene therapy and DNA vaccination since expression of foreign genes *in vivo* was demonstrated. Subsequently, it was known that injection of naked plasmids into the muscle could also elicit an immune response (Tang *et al.*, 1996). DNA vaccines do not need the gene to be permanently expressed as a transient expression of the gene is sufficient for evoking the immune response.

It has been demonstrated that DNA vaccination induces a strong and protective immunity to some viral infections in fish, particularly the rhabdoviruses infecting rainbow trout and Atlantic salmon, and also for channel catfish herpesvirus infection (Nusbaum *et al.*, 2002). There is also documentation that a DNA vaccine elicits protective immunity to BKD (*Renibacterium salmoninarum*; Gómez-Chiarri *et al.*, 1996). The challenge, as regards DNA vaccination, is that so far, with a few exceptions (Fernández-Alonso *et al.*, 2001), induction of protective immunity has been reliant on intramuscular injection. Immersion is a delivery route offering many advantages compared to conventional ways of administration. Use of cationic liposomes as a delivery system for DNA by the immersion route has met with severe toxicity problems. The mechanism of the acute toxicity is suggested to be an interaction between the cationic liposomes and anionic components of gill mucin. The consequence is hypoxia and this is most likely the cause of acute toxicity observed in rainbow trout fry (Romøren *et al.*, 2002).

The safety of DNA vaccines for use in fish is more of a concern than their efficacy. Safety issues are related to integration into chromosomal DNA, pathological processes at the site of injection (Fig. 4), distribution to internal organs and longevity of retention of foreign DNA in these organs. Issues related to tumourigenicity will probably raise public concern and potentially also with the regulatory bodies.

It has been demonstrated that retention and expression of antigens at the injection site appear for an extended time period, however not beyond 4-5 weeks post vaccination (Lorenzen *et al.*, 1999). The local reactions at the site of injection are prominent and last for an extended period and (much) longer than the actual antigen expression, as detected by immunohistochemistry. Strong inflammation, muscle cell destruction and granuloma formations are evident at 3 and 12 weeks post vaccination (Lorenzen *et al.*, 1999; own studies as shown in Fig. 4).

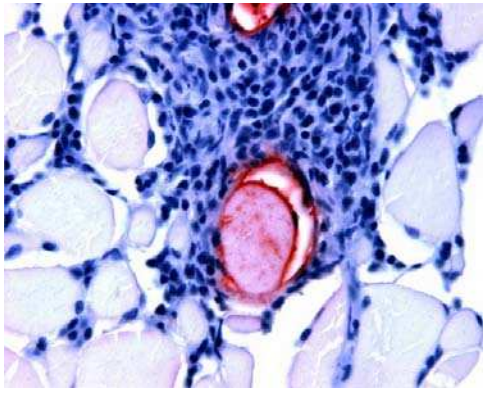

Fig. 4. Rainbow trout muscle tissue. Sample was collected 3 weeks post vaccination with a DNA vaccine coding for the G-protein of viral hemorrhagic septicaemia virus. Expression of the G protein has been revealed by immunohistochemistry using a G-protein specific monoclonal antibody (red coloration). Note strong inflammatory responses centered around the muscle cell expressing the protein. The cells are dominated by lymphocytes at this stage.

The distribution to internal organs following i.m. vaccination has not been studied in any detail, but we have shown that a luciferase encoding gene will be distributed to internal organs and that expression takes place in these organs shortly after administration (Romøren *et al.* 2004). Further to this, expression of luciferase in internal organs has been observed over an extended period by others (up to 24 months; Dijkstra *et al.*, 2001).

VI – Live attenuated vaccines

1. AroA mutants

The use of oil adjuvant vaccines has proven to be efficient in conferring protection against furunculosis, as mentioned earlier. However, the use of oil-adjuvants has been associated with severe side-effects at the site of injection on a few occasions (Poppe and Breck, 1997) and the use of live, attenuated strains of *A. salmonicida* in future furunculosis vaccines could overcome this problem.

Delta aromatic mutants (Δ*aroA*) of *Aeromonas salmonicida* have been used to vaccinate brown trout (*Salmo trutta*) against furunculosis and with good protection (Vaughan *et al.*, 1993). The Δ*aroA* mutant was shown to proliferate in the kidney for up to 12 days, but the prolonged retention of antigen was not assessed. The data obtained showed that there is potential for a commercially viable live vaccine to be produced against furunculosis. Similar findings have been obtained using an attenuated strain of *Edwardsiella ictaluri* against enteric septicaemia of catfish (Klesius and Shoemaker, 1999).

As an attempt to study the long-term retention of antigens following vaccination by the intraperitoneal route with aroA mutants of *Aeromonas salmonicida*, we have recently shown that LPS antigens can be detected up to 12 weeks post vaccination (Grove *et al.*, 2003). Interestingly, when examining head kidney samples for 16S rDNA (ribosomal DNA) of *A. salmonicida* using PCR, the aroA group was positive at 4 weeks but negative when examined at 12 weeks post vaccination (Grove *et al.*, 2003). This was in contrast to a group injected with an oil-adjuvant vaccine that was still positive at 12 weeks.

Methods have also been developed whereby a reverse genetic system has been used to generate recombinant IHN virus from cloned cDNA. Variants of IHNV (rendered non-pathogenic) have been used to vaccinate rainbow trout and it has been shown that high level protection can be obtained (Thoulouze *et al.*, 2003).

VII – What are the reasons that immersion vaccination falls short for many antigens/pathogens?

It is well known that for antigens like *Vibrio anguillarum* and *Yersinia ruckeri* it is possible to obtain protective immunity following immersion vaccination of rainbow trout (Harrell *et al.*, 1975; Johnson and Amend, 1983). The protective antigens, probably LPS for both antigens (Croy and Amend, 1977), are likely taken up across mucosal surfaces (gill, stomach or gut) and induce a local immunity and/or a systemic immunity sufficient to protect the animal against lethal challenge. For other diseases, it has not been possible to obtain a sufficient level of protection using immersion delivery of antigens, typically examples being furunculosis (*Aeromonas salmonicida*) and pasteurellosis (*Photobacterium damselae* spp. *piscicida)* in sea bass.

The reasons for lack of efficacy have not been studied in any detail, but a recent publication has explored certain aspects (Nakanishi *et al.*, 2002). In this publication, the skin of the fish was punctured using a puncture instrument which allowed percutaneous administration by immersion of antigens. The authors used a *Streptococcus iniae* model in rainbow trout and fish were vaccinated with α-haemolytic *Streptococcus* vaccine by different methods, followed 2 weeks later by intra-peritoneal challenge with *S. iniae*. It was shown that the puncture method facilitated uptake of antigens into the skin (and underlying tissue) and the protection achieved was comparable to injection vaccinated groups, while immersion gave no protection (Nakanishi *et al.*, 2002). The explanation given was that the puncture method will result in a higher number of particulate antigens being taken up by fish and delivered to the lymphoid tissues, which will result in induction of protective immune responses. Immersion without skin puncture will not result in any uptake of particulate antigens and thus the immune responses will be weak and non-protective.

VIII – Oral delivery

1. Inactivated vaccines

Oral administration of antigens has obvious advantages by reducing the amount of labour and also expense, and most importantly it reduces the stress incurred by immunization. Unfortunately, there is a general experience that the protection after oral vaccination falls short compared to those attained after injection or immersion. The induction of a local or systemic immune response after oral immunization is dependent on uptake of antigens from the gut lumen, and in higher vertebrates, proliferating and dead particulate antigens (as well as soluble antigens) are taken up through a specialized follicle-associated epithelium, the so-called M ("membrane") cells, and with subsequent transepithelial transport to underlying lymphoid tissue, the Peyer's patches (Brandtzaeg *et al.*, 1987).

Despite the observation that vaccine efficacy in fish is so limited after oral delivery, there are very few studies that address the uptake and transepithelial transport in enterocytes of soluble versus particulate antigens. The morphological or functional characterization of enterocytes is also scant, yet there are indications for a regional specialization of the gut epithelium with regard to uptake of macromolecules, and the hindgut enterocytes are considered important in this respect (Georgopoulou *et al.*, 1985). Macromolecules like trinitrophenylated-lipopolysaccharides (TNP-LPS) and biologically active proteins like horse-radish peroxidase are absorbed from the gut into the circulatory system (Doggett *et al.*, 1993). With regard to uptake of particulate antigens, studies in the stomach-less carp (*Cyprinius carpio*) using a bacterin of

Vibrio anguillarum have shown that the bacteria are taken up by epithelial cells in the second gut segment and are later identified in intraepithelial macrophages (Rombout and van den Berg, 1989). However, no attempts were made to distinguish between soluble (such as LPS) and particulate components of the antigen preparation, and thus no conclusion could be made with regard to the transport of particulate versus soluble antigens across the epithelial cells (Rombout and van den Berg, 1989).

Even so, previous studies have shown that after anal and to a lesser extent after oral delivery of bacterins of the Gram-negative bacterium *Yersinia ruckeri*, a high level of protection was attained (Johnson and Amend, 1983). Similarly, previous studies in other fish species have shown that bacterial antigens of *Vibrio anguillarum* were identified in the hindgut epithelium but no transport to the circulation was observed (Tatner *et al.*, 1984; Nelson *et al.*, 1985). Interestingly, the LPS moiety of the cell is considered to be an important component of the protective antigens (Croy and Amend, 1977) and it is possible that induction of local immunity is sufficient to protect against lethal challenge with *V. anguillarum*.

Despite all this, there is a general experience that protection after oral vaccination falls short compared to those attained after injection or immersion (Harrell *et al.*, 1975). It is my belief that there is a need to learn more about local uptake of soluble and particulate antigens over the gut epithelium and also about the induction of local immune responses versus systemic responses, and their importance in protection.

2. Vector vaccines

A new principle has also been explored whereby genetically modified *E. coli* expressing the exotoxin A of *Pseudomonas aeruginosa* have been fed to live Artemia brine shrimps. The Artemia has been subsequently fed to zebra fish (*Danio rerio*). After allowing a period for immunity to develop, zebra fish were challenged with *P. aeruginosa* and vaccinated fish demonstrated good protection against lethal challenge (Yang *et al.*, 2003). However, the potential application of these methods needs to be explored in more detail.

3. Future directions

Multi enim sunt vocati, pauci vero electi - "Many are asked to come, but only a few are chosen" (St. Matthews' Gospel, 22, 14). Despite the fact that oil adjuvant vaccines are based on old technology and that many studies have been carried out (and are on-going) in an attempt to develop new and more advanced principles for immune induction in fish, there does not seem to be light at the end of the tunnel for many of the new delivery systems at this stage. In humans, aluminium salts still remain the standard (Giudice *et al.*, 2002) but there are currently few other alternatives to oil adjuvants for fish vaccines delivered by the parenteral route for the foreseeable future. Consequently, improvements of oil adjuvant delivery systems for fish are likely to emerge, not least with improved safety profiles.

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