Review

Recent Development in Oral Vaccine Delivery Systems

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Oral delivery of vaccines is the least effective method of vaccinating fish. However, anal intubation of antigens is very effective in inducing high serum antibody levels and protection against vibriosis and enteric redmouth. It is generally considered that degradation of antigen in the stomach and anterior intestine prevents immunostimulation taking place and that if antigens could be protected during passage through the foregut effective immunization could be achieved.

Recent attempts to protect antigens have included the concomitant administration of antacids and antiproteases. Protective coating procedures have included encapsulation of particulated antigen with methacrylic acrylic acid polymers which resist acid but dissolve in the high pH of the intestine.

Incorporation of antigen into microparticles of poly lactide co-glycolide polymers protects against enzyme degradation and allows increased uptake of intact antigen.

Certain substances can enhance uptake of antigen by the intestine of fish and have adjuvant activity, such as cholera toxin β -subunit and Quil-A saponin.

Overcoming problems of palatability of oral vaccines delivered to fish fry has been achieved by incorporating vaccine into live food such as *Artemia*. However, delivery of antigen to very young fish, before they are fully immunocompetent can induce immune suppression.

Many of these methods have improved the level of antigen uptake by the intestine and the antibody response in fish and the prospects for improving the efficacy of oral vaccination appear optimistic.

Key words: vaccine, oral delivery, anal delivery, antacid, antiprotease, encapsulation, ECAMS, adjuvant

Introduction

Over the past decade the vaccination of Atlantic salmon smolts has become an integral part of the aquaculture industry and in Norway, Scotland and Ireland, virtually every individual fish is vaccinated at some time before transfer to sea water. Some 10 years ago the main vaccines used in salmon culture were against vibriosis and enteric redmouth (ERM). These vaccines work extremely well when administered by immersion. Just a decade ago (Egidius *et al.*, 1986), a new pathogen was recognised as causing important economic losses in Norwegian salmon farms, namely *Vibrio salmonicida*, the causative agent of cold water vibriosis. A vaccine against this disease was developed quickly but bath administra-

tion induced limited protection and injection delivery was promoted.

In Scotland, the major cause of loss in farmed salmon was from furunculosis, caused by the bacterium *Aeromonas salmonicida*, which also began to spread through Norwegian farms in the late 1980s. The widespread antibiotic resistance of this organism made the development of a vaccine imperative. A great deal of research indicated that immersion vaccination against furunculosis was ineffective, but injection of the vaccine in an oil adjuvant was highly effective. Under the enormous economic pressure from losses due to furunculosis, the industry quickly adopted an injection delivery programme with marked success. Over the last five years, injection vaccination of Atlantic salmon smolts has become routine and many commercial vaccines, especially in

Table 1. Protection against vibriosis in salmonids immunised by various routes

Vaccine preparation and route	% mortality		
	Lillehaug (1984)	Amend and Johnson (1981)	Johnson and Amend (1983)
Control	65	52	55
Intraperitoneal injection	0	0	—
Immersion	6	4	—
Anal (intubation)	—*	—	0
Oral (intubation)	16	—	15
Oral (in feed, for 15 days)	—	27	—
Oral "prills" (intubation)	29	—	—
Oral "acid resistant" (intubation)	44	—	—

* not tested.

Norway, are tri- or quadrivalent, i.e. contain a mixture of vaccines comprised of the classical *Vibrios*, *Y. ruckeri*, *V. salmonicida* and *A. salmonicida* in an oil emulsion. While most farms perform the injection vaccinations manually, automated injection machines have become available.

Injection vaccination has certain restrictions and costs. It is expensive in staff time, stressful to the fish and the practicalities impose a minimum size limit on the fish to be vaccinated which is about 15 g. Oil adjuvants also induce undesirable side effects such as visceral granulomas which may reduce carcass value. Thus, while injection is acceptable for Atlantic salmon smolts, the more convenient methods of immersion and oral administration are desirable and for other fish species where vaccination is required when they are smaller than 15 g these methods are essential.

For obvious reasons, oral delivery of vaccines is the most convenient but much experience with feeding vaccines simply mixed with feed has indicated that insufficient levels of protection are achieved by this method.

Oral delivery of ERM bacterins required in-feed delivery for at least several weeks and protection was of short duration (Ross and Klontz, 1965; Anderson and Ross, 1972). In the late 1970s it became clear that immersion delivery for ERM and *V. anguillarum* bacterins was superior to oral delivery in that it was more economical in vaccine dosage and gave higher and longer lasting protection (Amend and Johnson, 1981).

The reason why immersion was more effective than oral delivery was considered to be because the antigens were not absorbed as well through the gut compared with skin/gills and/or that the antigens

were digested in the gut. Evidence is quite strong for the protective antigens in both the ERM and *Vibrio* bacterins being lipopolysaccharide (LPS) (Kawai and Kusuda, 1983) and it is possible that this antigen has certain properties facilitating uptake by skin/gills. Nevertheless, the harsh digestive conditions in the anterior gut as a restricting factor for the efficacy of oral delivery was indicated by Johnson and Amend (1983) (Table 1) when they compared oral and anal delivery of *Vibrio* and ERM bacterins. Anal intubation provided an excellent level of protection which was equal to or better than the immersion method. The authors suggested that a means of protecting vaccines from inactivation by gastric fluids in the anterior intestine could lead to the development of effective oral delivery of vaccines to fish.

Since then a considerable amount of work has been done to improve antigen uptake by the gut of fish and to understand the mechanism of antigen absorption by the gut.

The Potential of Intestinal Antigen Stimulation-Oral Versus Anal Delivery

Rombout *et al.* (1986) demonstrated the remarkable potential for vaccines delivered to the hind intestine to stimulate high serum antibodies. Carp were immunised with a *V. anguillarum* bacterin by *i.m.* injection or by anal intubation (10^9 bacteria/fish). The serum antibody responses of these fish were very similar, peaking at a titre of about 256. However, in fish immunised by the oral route with the bacterin mixed with the feed and administered once or daily for 32 days, the serum antibody levels were barely detectable.

These antibody response data to *V. anguillarum* are corroborated by protection studies. Johnson and Amend (1983) found that anal intubation with *V. anguillarum* or *Yersinia ruckeri* bacterins gave a better protection than oral intubation. The level of protection was even greater than that after immersion and could be compared with that achieved by injection (Amend and Jonson, 1981). The principle of protecting antigens from degradation in the anterior intestine and methods of enhancing antigen uptake from the gastro-intestinal tract has been examined in a variety of ways. While much work is still to be done before oral delivery of vaccines to fish become economically feasible, the future prospects continue to look optimistic.

Neutralisation of Gastric Secretions

Antacids

In an attempt to improve intestinal uptake of intact proteins by neutralising gastric pH and removing the mucus lining of the gut epithelium, McLean *et al.* (1990) orally intubated coho salmon with recombinant bovine somatotropin, alone or mixed with NaHCO_3 and a detergent. The hormone was intubated once per week for seven weeks at a dose of $12.5 \mu\text{g/g}$ body weight. At the end of the treatment period the body weight was compared with that of untreated controls and with fish injected i.p. with $2.5 \mu\text{g}$ somatotropin/g body weight/week. The injected group had a significantly increased body weight compared with controls (saline intubated). While the group intubated with somatotropin alone showed a growth enhancement, the group intubated with the hormone in the presence of NaHCO_3 and detergent grew significantly faster than controls and matched that of the injected fish.

An important aspect of this study, though not in itself concerned with vaccines, is that the absorbance of intact protein molecules, retaining their biological activity, does occur in fish and the level of absorbance can be enhanced by neutralising gastric pH.

Antiproteases

The potential of soybean trypsin inhibitor (SBTI) to inhibit proteolytic degradation of proteins and increase intestinal uptake of intact protein was studied by McLean and Ash (1990). Horse-radish peroxidase (HRP) was orally intubated in rainbow trout with or without SBTI. The tissue concentrations of

HRP were significantly increased when the enzyme was delivered with SBTI.

Encapsulated Antigens

A variety of methods of coating antigens to protect them from degradation in the anterior intestine and release them in the posterior intestine to mimic anal intubation delivery has been attempted.

"Prills" and acid resistant films

Lillehaug (1989) explored the use of "prills", which are basically pellets (8–10 mg in weight) comprising lyophilised vaccine incorporated into a matrix of saturated long chain fatty acids, or granulated lyophilised vaccine coated with an acid-resistant acrylic film. Both preparations were designed to protect a *V. anguillarum* bacterin during passage through the anterior intestine and dissolve in the hind-intestine to release the vaccine. The preparations were delivered by oral intubation and the level of protection against a vibriosis challenge was compared with fish orally intubated with unprotected vaccine or immunised by immersion or injection.

Table 1 shows the percentage mortality of the different groups of fish alongside data from similar experiments reported by Amend and Johnson (1981) and Johnson and Amend (1983) involving delivery of *Vibrio* vaccine by various routes for comparison.

Although the results of Johnson and Amend (1983) comparing oral and anal deliveries indicated that protection of the vaccine against gastric digestion might improve efficiency of oral delivery, this was not achieved by the preparations tested by Lillehaug (1989). Indeed, the opposite effect was observed. Possibly the vaccine was not only protected from degradation but may also have been protected from being absorbed by the encapsulation procedures.

While prolonged feeding and relatively high doses of vaccine have been used in most studies on administering vaccines in the feed (Fryer *et al.*, 1978; Kawai and Kusuda, 1985; Amend and Johnson, 1981), the degree of protection achieved by a single intubation of a dose comparable to injection as reported by Lillehaug (1989) and Johnson and Amend (1983) (see Table 1) was relatively high.

Many studies (see below) have used oral intubation as an experimental method for investigating oral delivery of antigens and this, compared with mixing

the antigen with the feed may be a crucial factor for efficacy. Intubating the antigen may allow it to pass through the stomach relatively quickly, reducing the possible influence of gastric digestion, and reach the intestine in a more concentrated form to stimulate a greater immune response. Lillehaug (1989) suggested that efforts to develop a method in which a vaccine is given orally in a more concentrated form as a "vaccine pellet" may be more successful than encapsulating the vaccine to prevent digestion, at least when lipopolysaccharides (LPS) are the main antigens of importance as is the case with *V. anguillarum* vaccines (Kawai and Kusuda, 1983).

Enteric coated antigen microspheres (ECAMS)

A similar approach to protecting antigens against gastric digestion was studied by Piganelli *et al.* (1994). However, in this case, and developing upon the work of Lillehaug (1989), the protected antigen was delivered in the feed over a prolonged period (30 days) and an LPS antigen was compared with a protein antigen, the latter being more sensitive to denaturation in the gut.

ECAMS were prepared by soaking dextrose beads in aqueous solutions of antigen and then coating with a film of acrylic polymer. This created an acid resistant film which dissolved in the high pH of the intestine. The antigens chosen were trinitrophenylated-LPS (TNP-LPS) and TNP-keyhole limpet haemocyanin (TNP-KLH)

TNP-LPS was administered to coho salmon in ECAMS mixed with feed for 30 days at three different doses (10 µg, 10 ng and 1 pg per day). Other groups of fish were immunised by i.p. injection or immersion with 10 µg, 10 ng or 1 pg administered once. The serum antibody response in the immersion immunised fish was negligible. In the injected group, a dose-related antibody response was observed. In the case of the ECAM-groups, only those administered the high dose responded, but interestingly the peak titre was equal to that in fish immunised with the high dose delivered by injection.

The TNP-KLH was administered in ECAMS mixed with feed at doses of 100 µg, 5 µg and 0.5 µg daily, for 30 days. Other groups of fish were immunised by anal intubation of TNP-KLH in saline (100 µg, once) or by i.p. injection in saline (100 µg, once). Of the ECAM-groups, only the low dose induced a serum antibody response and the peak titre was equal to that induced by injection immunisation,

though it appeared later. Anal immunisation also induced peak serum antibody levels that were as high as i.p. immunisation.

These data once again demonstrate that anal immunisation can achieve a high serum antibody response and that the ECAMS system, given optimisation of the dose regime, is also capable of eliciting high serum responses by oral delivery of antigen mixed with the feed.

Poly lactide co-glycolide (PLG) microparticles

PLG is a biocompatible and biodegradable polymer and has been used in a wide variety of circumstances including pulsed delivery of pharmaceuticals in human medicine. Recently it has been used to entrap antigens within microparticles (less than 10 µm in diameter) and found to induce high serum antibody titres in mammals when either injected (Eldridge *et al.*, 1991) or when administered orally (Challacombe *et al.*, 1992). To date, little has been published on the application of PLG to immunisation of fish but current work suggests that intestinal uptake can be enhanced following oral intubation of antigen in PLG microspheres. Lavelle (1994) found that when human gamma-globulin (HGG) was orally intubated into rainbow trout, this antigen could be detected in the serum for up to six hours. However, using Western blotting analysis, only fragments of HGG were detected, indicating the antigen had been degraded in the intestine. On the other hand, HGG encapsulated in PLG could be detected in the serum for at least 24 h and it was estimated that over 60% of the antigen was still intact. The protection against enzymic degradation of antigens encapsulated in PLG, the ability of microparticles to be absorbed by the intestine and the timed release of the antigen (achieved by using PLG of differing physical properties which determine the rate at which the matrix of the polymer hydrolyses and disintegrates), offer exciting prospects for the delivery of oral vaccines in this medium.

Oral Adjuvants

Cholera toxin β -subunit, $Al(OH)_3$ and NH_4Cl

Most adjuvants are designed for injection but some have been demonstrated, in mammals, to enhance antigen uptake from the intestinal tract. Jenkins *et al.* (1994a) investigated antigen uptake and serum antibody responses when tilapia were im-

munised with HGG by injection, anal intubation or oral intubation. For all routes the HGG was administered in saline or mixed with cholera toxin β -subunit (CTB) aluminium hydroxide, or ammonium chloride.

The orally intubated fish were analysed for uptake of HGG into the plasma. Compared with HGG intubated in saline, only the CTB enhanced the plasma concentration of the antigen.

Serum antibody titres of all groups were monitored for 35 days following immunisation. Injection (i.p.) induced significantly higher titers than any other method, though none of the potential adjuvants enhanced the antibody response compared with antigen injected in saline. The antibody responses induced by oral and anal intubation were similar to each other. The peak titres induced by antigen administered with CBT and Al (OH)₃ were enhanced compared with antigen in saline while NH₄Cl had no effect compared with the latter.

It is noteworthy that in tilapia, the anal administration of the protein antigen, HGG, did not induce an antibody response that was enhanced compared with oral administration and these enteric routes induced significantly lower antibody titres as compared with injection immunisation. This contrasts with data reported by Rombout *et al.* (1986) and Piganelli *et al.* (1994) who immunised carp with ferritin and coho salmon with TNP-KLH (referred to above), respectively, and who reported that anal intubation induced equally high serum antibody titres as injection. This suggests that the superior antibody responses obtained from anal as compared with oral intubation may depend upon the species of fish and/or the antigen used.

Quil-A saponin

Quil-A saponin is a well known adjuvant in mammals and has been found to enhance immune responses when given with antigen by injection or orally. Jenkins *et al.* (1994b) investigated the effect on the antibody response of tilapia to HGG when this antigen was administered by i.p. injection or oral intubation with and without Quil-A saponin. Both systemic (serum) and local (bile and skin mucus) antibody responses were monitored. Injection induced the highest titres in all three samples. Oral intubation of HGG alone induced very low antibody titres while the presence of Quil-A significantly enhanced the titres in serum, bile and skin mucus.

Oral Delivery to Fish Larvae

Many fish species used in aquaculture have very small larval stages which require the feeding of live planktonic prey. The concept of using such live diets to deliver vaccines was investigated by Kawai *et al.* (1989). These workers incubated the rotifer *Brachionus plicatilis* with a *Vibrio* bacterin and then fed them to juvenile ayu for 8–14 days. Significant protection was achieved against vibriosis.

Campbell *et al.* (1993) investigated the potential of using *Artemia* (brine shrimp) nauplii as a vehicle for delivering *V. anguillarum* bacterins. The uptake of the bacterin by the nauplii was determined but the authors expressed some doubt as to whether it would be sufficient to induce immune protection in recipient fish.

Oral Boosting or Priming

Given the background information that oral delivery of vaccines had limited efficacy, some workers have investigated the potential of oral delivery to enhance the antibody response when given prior to or subsequent to injection vaccination and thereby improve efficacy of the latter.

Vinitnantharat and Plumb (1993) monitored the antibody response of channel catfish vaccinated by injection with an antigen extract of the bacterium *Edwardsiella tarda* and then fed antigen-impregnated feed every 5th or 10th day beginning 39 days after vaccinating by injection. Antibody titres in the control injected fish, not receiving the oral booster, peaked at day 20 and declined continuously thereafter. However, antibody titres in the orally boosted fish increased again.

The effect of oral priming juvenile carp and seabream (*Sparus aurata*) with a *V. anguillarum* vaccine incorporated into *Artemia* nauplii was investigated by Joosten *et al.* (1995). The bio-encapsulated antigen was fed to 58 day old carp and seabream for 2.5 h. Ten weeks later the fish received an i.m. injection of the bacterin and three weeks after this the fish were bled and assayed for serum anti-*Vibrio* antibody titres. These fish had significantly higher antibody levels compared with the control groups which did not receive the oral priming.

A further aspect of this work, with obvious importance, studied oral priming in carp younger than 58

days, namely 15 and 29 days old. An opposite effect of oral priming in these fish was observed in that the antibody titres following i.m. injection were significantly lower than the control groups not receiving the oral priming. This indicates that very young carp can develop immunological tolerance to intestinal exposure to antigens which lasts for at least 10 weeks; an important feature in developing protocols for oral priming of juvenile fish.

A Potential Problem for Oral Vaccination-Oral Hyposensitisation

In mammals, antigens in the diet can stimulate a local response in the gut mucosa and at the same time suppress a systemic response to the antigen when subsequently administered parenterally. This phenomenon is known as "oral hyposensitisation" (Mowat, 1987). The mechanism is still not fully understood and is dependent upon a complex set of factors including nature of the antigen (it is more readily induced by soluble T-dependent antigens), dosage, frequency of administration and age of the animal (Faria *et al.*, 1993). Generally speaking, high doses of antigen fed for long periods can induce suppression of the systemic response while low doses fed for shorter periods can prime or even stimulate a serum antibody response.

Some evidence for "oral hyposensitisation" in fish has been reported by Davidson *et al.* (1994). Rainbow trout were i.p. injected with HGG. At the same time, the fish were administered 2 mg HGG by oral or anal intubation. The intubation was repeated twice weekly for eight weeks. Treatment suppressed the serum antibody response compared with the control (saline intubated) group but the effect was dose dependent (20 mg and 0.2 mg HGG administered orally on each occasion, did not affect the antibody response).

When fish were administered HGG by oral intubation for five or 10 days prior to i.p. injection, no effect of the oral exposure was observed on the antibody response. Furthermore, the suppressive effect of oral delivery subsequent to i.p. injection of HGG was not observed with an *Aeromonas salmonicida* bacterin. Indeed, the data indicated that the serum antibody response was enhanced to this antigen by this treatment.

While under certain circumstances of high dose oral intubation of protein antigen, oral hyposensitisation

may be inducible in fish, the current experience suggests that it should not interfere with regimes of administration being sought in aquaculture.

Future Development in Oral Vaccination-Lectins and Live Vaccines

As described above, many studies have demonstrated that, using anal intubation, the hind-intestine of fish is sensitive to antigen stimulation and an important site of antigen uptake (Rombout *et al.*, 1989; Joosten *et al.*, 1995) resulting in high serum antibody titres that can equal the response achieved by injection immunisation. Thus, the major hurdles to overcome for achieving effective oral delivery of vaccines appear to be protecting them against degradation and improving uptake. Certain encapsulation procedures such as ECAMS (Piganelli *et al.*, 1994) show great promise in achieving this while procedures, e.g. prills (Lillehaug, 1989), have failed.

Lectins

PLG microspheres can protect antigens from enzymic degradation in the gut and are capable of being taken up by the intestinal mucosa in mammals (Eldridge *et al.*, 1989). One approach to increasing this uptake is to incorporate lectins into the microspheres which make them very adhesive to the epithelial cells (Shahin *et al.*, 1992; Santiago *et al.*, 1995).

Live vaccines

The ability of attenuated *Salmonella typhi* to invade, persist and proliferate in the human gut associated lymphoid tissues and induce a protective immune response, without causing clinical symptoms, is well known (Curtiss *et al.*, 1989). This observation has prompted many workers to introduce genes encoding antigens for other organisms into modified strains of *Salmonella*, using them as vectors for selective delivery of antigen via the intestine (Sadoff *et al.*, 1988).

A genetically attenuated strain of *A. salmonicida* (AroA mutant) has been produced by Vaughan *et al.* (1993) which has been shown to be avirulent, persist in trout tissues for up to 12 days post injection and induce strong protective immunity. It is feasible that this mutant could be used for oral administration and furthermore, genes from other fish pathogens, especially viruses, could be cloned into and expressed

by the AroA mutant thus acting as a vector for other vaccines.

Conclusions

There remains much research to be done to develop oral vaccines for fish but the potential of antigen delivery to the gut, as demonstrated by the high immune responses achieved by anal intubation, offers many advantages.

Oral delivery of vaccines can lead to higher antibody titres in skin mucus than are achieved with injection vaccination, e.g. carp immunised with a *V. anguillarum* bacterin (Rombout *et al.*, 1989). If protection against pathogens is based upon antibody responses in the mucosal surfaces of fish, oral delivery may be more efficacious than injection. More information concerning the local immune responses in the gut, skin and gills, induced by oral immunisation is needed to assess these potentials.

Optimisation of doses for oral delivery and duration of administration is required. In order to stimulate high systemic immune responses, means of protecting the antigen from degradation in the anterior gut and enhancing uptake in the hind-intestine, especially when antigen is given in the feed, appears to be of paramount importance. Some of the encapsulation processes already attempted show definite promise but many modifications to these processes still remain to be tested.

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