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Uptake and processing of biofilm and free-cell vaccines of *Aeromonas hydrophila* in Indian major carps and common carp following oral vaccination — antigen localization by a monoclonal antibody

I. S. Azad, K. M. Shankar*, C. V. Mohan, B. Kalita

Fish Pathology and Biotechnology Laboratory, Department of Aquaculture, University of Agricultural Sciences, College of Fisheries, Mangalore-575002, India

ABSTRACT: Uptake and processing of biofilm (BF) and free-cell (FC) vaccines of *Aeromonas hydrophila* were studied in the Indian major carps catla *Catla catla*, and rohu *Labeo rohita* and in the common carp *Cyprinus carpio* following a single dose oral vaccination of 10^{11} CFU g^{-1} fish. Fish were sampled at 0.5, 1, 3, 6, 12, 24 h and at 2, 3, 5 and 10 d following vaccination and antigen localization was studied in the gut, kidney and spleen employing monoclonal antibody based immunofluorescence and immunoperoxidase. The general pattern of antigen localization was similar in catla, rohu and common carp. Initially, both the BF and FC antigens were localized in the gut lumen, followed by their uptake by intraepithelial vacuoles and macrophages. Antigen administered orally was also seen in the spleen and kidney. Both BF and FC antigens were detected in the gut lumen of carp within 30 min following oral delivery. However, BF antigen remained in the lumen of the hindgut for 48 h compared to 6 h in the case of FC antigen. In the early stages, BF antigen was localized in the gut epithelial vacuoles while FC antigen was associated with the small macrophages of the hindgut. Antigen localization in spleen and kidney was observed at 3 h and persisted even up to 10 d following oral delivery. In general, there was a distinct difference between BF and FC vaccines in the duration of retention and quantity of uptake in the gut, kidney and spleen.

KEY WORDS: Biofilm vaccine · Uptake and processing · Indian major carps · Aeromonas hydrophila · Monoclonal antibody

INTRODUCTION

Oral delivery of a vaccine, which is time and labour saving and avoids stress caused by manipulation of the fish, is generally considered the most suitable method for vaccination of fish of all sizes (Quentel & Vigneulle 1997). Although the results of a first attempt at oral vaccination of cutthroat trout against furunculosis were highly encouraging (Duff 1942), later efforts were either unsuccessful (Post 1966, Schachte 1978) or produced variable results (Fryer et al. 1976, 1978, Amend & Johnson 1981). One of the suggested reasons for these poor and variable responses following oral vaccination was the destruction of antigenic epitopes in the stomach and foregut of the fish before they reached the immune responsive areas of the hindgut (Rombout et al. 1985). Strategies for improvement of oral vaccination in terms of antigen protection are oral or anal intubation of antigens (Davina et al. 1982, Johnson & Amend 1983, Rombout et al. 1986) and the use of encapsulated antigen microspheres (Piganelli et al. 1994, Polk et al. 1994, Dalmo et al. 1995). Johnson & Amend (1983) described a much better protection of salmonids against vibriosis and yersiniosis after anal intubation as compared with oral and bath vaccination.

*Corresponding author. E-mail: kmshankar@eudoramail.com

We have developed a biofilm of *Aeromonas hydrophila* for oral vaccination of Indian major carps which has produced better humoral and protective responses compared to the free-cell (FC) vaccine of this pathogen (Azad et al. 1997). The glycocalyx of the biofilm (BF) vaccine is believed to protect the antigen against destruction in the gut, thus facilitating its transport in intact condition to the immune responsive areas (Azad et al. 1999). However, this concept needs further investigation with respect to antigen retention in the gut lumen, and the uptake and processing mechanisms of the BF vaccine.

Detailed accounts of particulate antigen uptake and processing in the gut of common carp following oral vaccination have been previously provided (Rombout & Berg 1989, Rombout et al. 1989). Antigen localization studies in other cultured fish species following oral or anal intubation of antigens are also available (Davina et al. 1982, Gergopolou & Vernier 1986, Press et al. 1996). Oral vaccination has great potential in Indian aquaculture. However, there is little information on the uptake and processing of antigens following oral vaccination of the widely cultured Indian major carps.

The present work employs a monoclonal antibody to analyse the retention, mode of uptake and processing of BF and FC antigens of *Aeromonas hydrophila* in Indian major carps following oral vaccination. Common carp was used as a model for comparison.

MATERIALS AND METHODS

Preparation of vaccines. BF and FC vaccines of *Aeromonas hydrophila* (SAh 93) were prepared and incorporated in feed according to Azad et al. (1997). Briefly, the isolate was grown on chitin flakes suspended in 0.225% Tryptone soya broth (TSB) and BF was harvested intact with chitin and heat inactivated at 90°C for 40 min. FC vaccine was prepared by growing the isolate in 1.5% TSB for 24 h, harvested and inactivated at 60°C for 40 min. Inactivated BF and FC vaccine preparations were then mixed with cooked and cooled ingredients of feed, pellitized and sun dried.

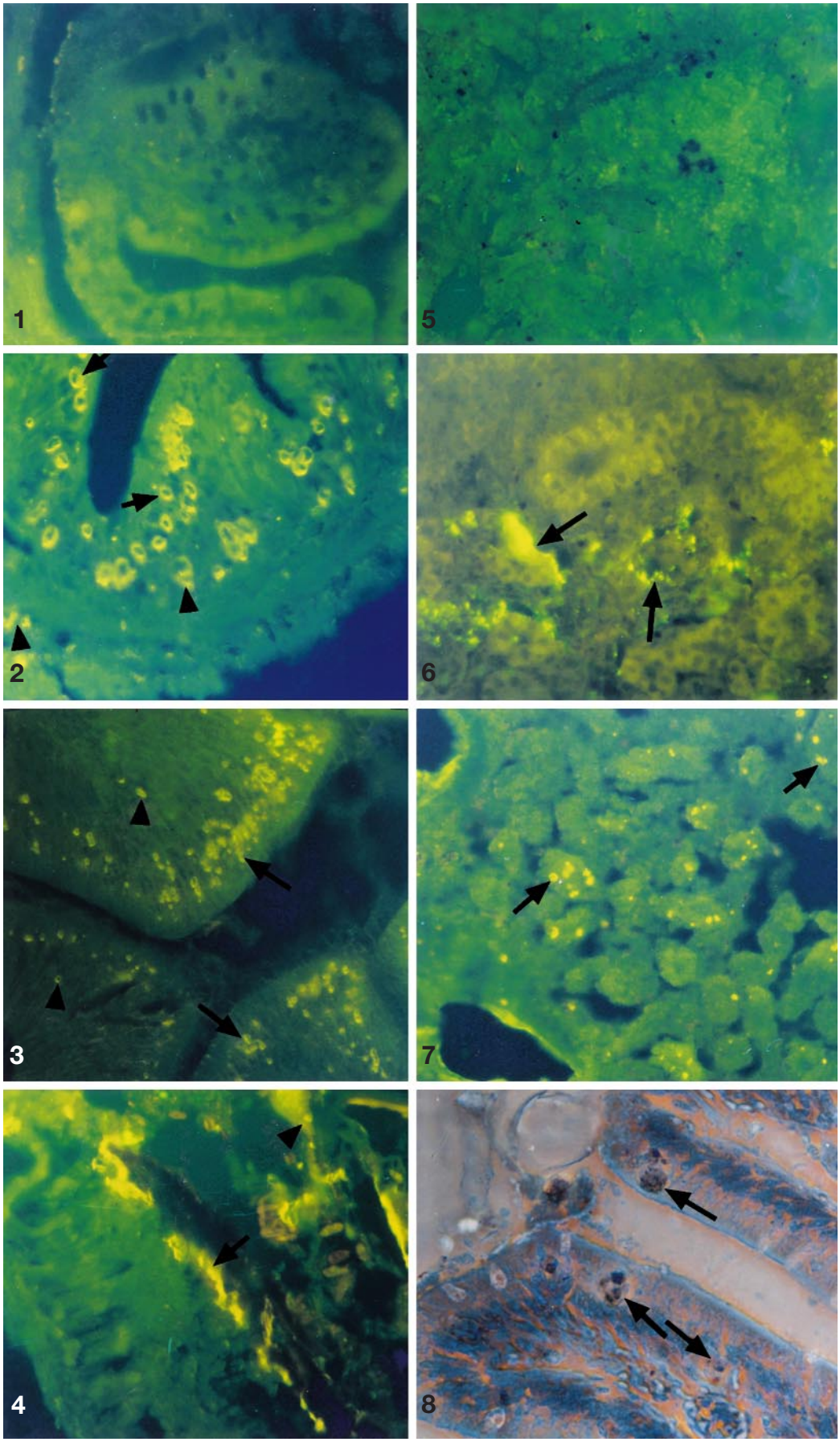
Oral vaccination. One hundred each of fingerlings of catla *Catla catla* (6.0 ± 0.4 g), rohu *Labeo rohita* (5.8 ± 0.91 g) and common carp *Cyprinus carpio* (5.3 ± 1.03 g) were fed with a BF incorporated diet at 10^{11} CFU g^{-1} fish following a 24 h starvation period. A similar number of fingerlings from each of the 3 species were fed with FC-incorporated feed at the same dose. A control group was maintained with normal feed without vaccine. The feed was given in feed trays suspended in 1 m^3 cement tubs. Feed provided was consumed by fish in 1 h, after which time trays were removed and 75% of the water in the tub was replaced by new water.

Antigen localization. Fish were sampled (n = 5) at 0.5, 1, 3, 6, 12, and 24 h and also at 2, 3, 5, and 10 d post feeding. The fish were fixed in 10% neutral buffered formalin. Kidney, spleen and gut were processed in an automatic tissue processor (Citadel-1000). The gut was divided into anterior (first 30% of the total length) and posterior portions and processed separately. Processed tissues were wax embedded and 5 µm sections cut using a microtome. The sections were transfered onto clean and egg white (10% emulsion in phosphate-buffered salines, PBS) coated slides. The sections were heat fixed (50°C overnight). Tissue sections were dewaxed in xylene, hydrated in graded alcohol and blotted lightly to remove excess moisture.

Immunofluorescence. Hydrated tissue sections, were flooded with anti-*Aeromonas hydrophila* (SAh 93) monoclonal antibody (MAb) for 2 h. The MAb was produced at the Department of Aquaculture, according to Shankar et al. (1994). The MAb was an IgG1 isotype reacting with the lipopolysaccharide (LPS) of the pathogen. After washing with PBS, sections were treated with FITC-labelled goat anti-mouse IgG (Bangalore Genei Ltd, India) for 1 h. The sections were thoroughly rinsed with PBS, air-dried and then coverslipped using glycerol as the mountant containing propylgallate as an antifadant. Immunostained sections were observed with an Olympus fluorescent microscope.

Immunoperoxidase. Hydrated sections were incubated with 0.3% H_2O_2 in methanol for 10 min to block the endogenous peroxidase activity. Sections were then washed briefly 3× with 0.01 M PBS (pH 7.2), tipped to remove moisture and incubated for 2 h with 2 to 3 drops of anti-*Aeromonas hydrophila* MAb. Slides

Figs. 1 to 7. Antigen localization in catla *Catla catla* fed with biofilm (BF) and free-cell (FC) vaccines of *Aeromonas hydrophila* (10^{11} CFU g^{-1} fish) employing monoclonal antibody-based immunofluorescence. Fig. 1. Hindgut microvilli of catla fed with control diet. 400×. Fig. 2. Large vacuoles (arrows) and macrophages (arrowheads) in hindgut showing presence of BF antigen at 3 h post feeding. 400×. Fig. 3. Immunopositive small vacuoles (arrows) and small intraepithelial macrophages (arrowheads) with FC antigen at 3 h post feeding. 400×. Fig. 4. Gut lining (arrows) and lumen (arrowheads) with BF antigen at 6 h post feeding. 400×. Fig. 5. Kidney of catla fed with control diet. 200×. Fig. 6. Intertubular (arrows) localization of BF in kidney at 12 h post feeding. 400×. Fig. 7. Intratubular (arrows) lodging of FC antigen in kidney at 12 h post feeding. 220×. Fig. 8. Antigen localization in rohu *Labeo rohita* by monoclonal antibody-based immunoperoxidase. Large macrophages (arrows) retaining BF at 72 h post feeding. 440×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were rinsed with PBS and incubated at room temperature for 1 h with 1:1000 horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Bangalore Genei Ltd, India). The sections were rinsed with PBS and incubated for 20 min in substrate containing 0.05% diamino benzidine (Sigma, USA) in PBS, pH 7.6, 0.01 and 0.3% H_2O_2 w/v $COCl_2$. Sections were rinsed with PBS, stained with haemalum for 15 min and dipped 2 to 3× in 0.02% NH_4OH (w/v). The sections were dehydrated in graded levels of ethanol, cleared in xylene and mounted using DPX mountant and observed under Olympus microscope. Suitable negative controls with unvaccinated fish and without antibody were used for comparison and confirmation of immunoreaction.

In the above observations, from each fish at each sampling point, 6 slides per organ (3 each for immunofluorescence and immunoperoxidase) were prepared and from each slide 3 fields were observed. The immunorectivity recorded is the average of these observations as there was no conspicuous difference between the fields within a slide and between the slides.

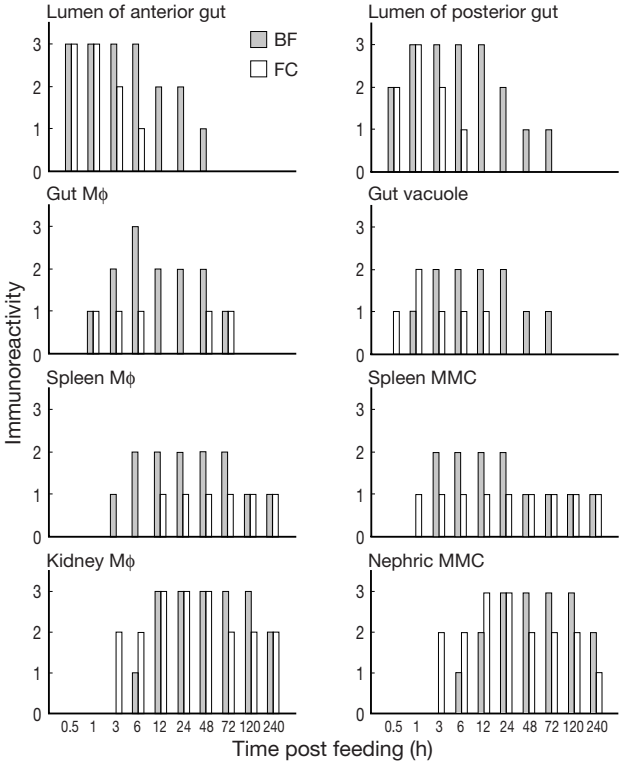

Fig. 9. Antigen localization in the various organs/sites of catla, rohu and common carp. 1: Moderate immunoreactivity, 2: Intense immunoreactivity, 3: High immunoreactivity. BF: biofilm vaccine; FC: free-cell vaccine; Mϕ: macrophages; MMC: melano-macrophage centres

RESULTS

In general, pattern of antigen localisation was similar in catla, rohu and common carp and is shown in Figs. 1 to 8. A comprehensive trend of antigen localisation in the various organs/sites is given in Fig. 9.

Lumen of the gut

Both FC and BF antigens were detected within 30 min post feeding in the lumen of the both the anterior and posterior gut (Figs. 1 & 2). BF could be detected in considerable quantity for as long as 48 h, while FC antigen could only be seen clearly up to 6 h. Faint immunoreactivity with the BF antigen in the hindgut of catla and rohu could be seen up to 72 h post feeding, while it was totally absent in the case of the FC antigen. In common carp, the BF antigen could be detected only up to 24 h, while FC antigen could be detected only up to 6 h.

Gut vacuoles and macrophages

Antigen was seen in vacuoles of epithelial cells from the posterior gut of catla, rohu and common carp 1 h post feeding with BF, while FC antigen could be seen within 30 min in catla and rohu and within 1 h in common carp (Figs. 3 & 4). BF was clearly observed in intraepithelial vacuoles in all species up to 24 h. In common carp, BF was retained up to 72 h, but at a lower intensity in catla and rohu. In contrast, FC was observed for only 6 h in catla and rohu and up to 24 h in common carp. BF and FC antigens could be located in the gut macrophages 1 h post feeding in all 3 species. BF was retained clearly up to 48 h in catla and rohu and up to 72 h in common carp. There was a marked difference in the quantity of uptake of FC and BF antigen by the macrophages (Figs. 3, 4 & 8).

Spleen

Solitary macrophages were immunopositive at 3 and 12 h in FC- and BF-fed catla, respectively. Macrophages retained the antigens even 10 d after feeding. Ellipsoids and melanomacrophage centres (MMC) showed positivity for both BF and FC from 3 h to 10 d following oral delivery. Similar trends were noticed in rohu and common carp.

Kidney

Pronounced and strong immunopositivity was noticed in the kidney of all the species fed with BF and FC. Solitary macrophages and the MMC of catla showed the presence of FC as early as 3 h following feeding, whereas BF localization was clear only at 6 h.

The BF was associated with the intertubular spaces, mostly with the MMC while the FC with only intratubular cells (Figs. 5 to 7). Kidney localization of BF was more intense than that of FC from 72 h to 10 d following oral delivery. Similar trends were noticed in rohu and common carp.

DISCUSSION

It has been demonstrated by immunohistochemistry that the posterior gut of agastric and gastric, herbivorous or carnivorous fish is able to absorb intact bioactive proteins and particulate elements such as bacterin from the lumen (Quentel & Vigneulle 1997). Intact macromolecules that reach the posterior segment of the gut, under normal physiological conditions, are absorbed in considerable amounts and transfered either to intracellular spaces or to macrophages (Rombout et al. 1985). In Indian major carps, the pattern of antigen uptake and processing in the gut was similar to that described above in common carp (Rombout et al. 1985) and in other fish species (Nelson et al. 1985, Gergopolou & Vernier 1986). Both the BF and FC antigens were initially seen along the lumen of fore, and hindgut, followed by localization in the vacuoles of epithelial cells and gut macrophages. The antigens could also be detected in the solitary macrophages and MMC of secondary lymphoid organs such as kidney and spleen.

The sequence of antigen uptake and processing was similar with both the BF and FC antigens. Both these antigens could be located from 30 min post-feeding in the lumen of both fore- and hindgut of the carps. However, at 3 h post feeding the intensity of immunopositivity in the lumen was higher with BF than with FC. After 6 h, FC could not be traced in lumen, whereas BF could be traced up to 48 h, which suggests faster clearance of FC than BF from the gut. This indicates longer retention of BF in the gut lumen than of FC. In the BF vaccine the cells are encased in glycocalyx material which renders protection against digestive degradation in the foregut. This might help to ensure longer retention of antigen in an intact form in the gut lumen for subsequent absorption.

The absorption process of antigens from the gut lumen is rapid beginning 1 h post anal intubation in carp (Rombout et al. 1986) and 30 min after oral intubation in rosy barb (Davina et al. 1982), turbot and seabass (Vigneulle & Baudin Laurencin 1991). After absorption, the sequential location of antigenic determinants of *Vibrio anguillarum* were first detected in vacuoles of cytoplasm of epithelial cells; then they reached intraepithelial macrophages.

A similar pattern of localization of antigen has been observed here with BF and FC vaccines. Immunostained macrophages were first noticed at 1 h following administration of FC, whereas large stationary macrophages were positive for BF. However, judging by the intensity of immunoreactivity, the absorption was higher with BF than with FC indicating a large quantity of antigen. Overall antigen could be localised for up to 72 h post feeding. Antigen uptake has been observed 2 to 6 d post delivery in various experiments (Davina et al. 1982, Tatner et al. 1984, Rombout et al. 1985, Rombout & Van Den Berg 1989).

Further, there was a difference in the location of FC and BF antigens, the former being located within intraepithelial macrophages and the latter within bigger vacuoles of epithelial cells. This difference in localization appears to be due to the difference in the size of the 2 particulate antigens; BF antigens are usually in large flocs while those of FCs are small. Further, longer retention of BF cells inside host cells is probably due to protection provided by the glycocalyx coat. A similar differential absorption based on particle size has been documented in carp where intact ferritin was found to localize in the vacuoles, whereas the degraded ferritin reached the lamina propria and then the blood stream of carp (Rombout et al. 1989). A similar differential uptake of molecules based on their size has also been demonstrated in rainbow trout (Gergopolou et al. 1985).

The overall quantity of antigen available is greater with BF because of the longer retention of BF flocs and higher uptake at all levels, and this appears to contribute to overall higher serum titers and greater protection. Enhanced serum agglutination titers and protection recorded following oral vaccination with the BF vaccine in our earlier studies (Azad et al. 1997, 1999) bear a direct relationship with antigen uptake and processing.

Oral bacterin, in the present study, was associated with the MMC of spleen and kidney. The role of the kidney and spleen in processing oral antigens has been demonstrated in Indian major carp in this study by observing intense localization of BF and FC with the MMC. Peroral intubation of *Aeromonas salmonicida* resulted in the detection of specific antibody secreting cells (ASC) in cell suspensions from both head kidney and intestinal mucosa of rainbow trout (Davidson et al. 1993). However, increased protection in Indian major carps with the biofilm vaccine may also be due to gut-associated lymphoid tissue (GALT) as demonstrated in carp (Rombout et al. 1993). The role of GALT in Indian major carps needs further study.

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LITERATURE CITED

Amend DF Johnson KA (1981) Current status and future needs of *Vibrio anguillarum* bacterin. Dev Biol Stand 49: 403–417

Azad IS, Shankar KM, Mohan CV (1997) Evaluation of an *Aeromonas hydrophila* biofilm for oral vaccination of carps. In: Flegel TW, McRae IH (eds) Diseases in Asian aquaculture III. Fish Health Section, Asian Fisheries Society, Manila, p 181–186

Azad IS, Shankar KM, Mohan CV, Kalita B (1999) Biofilm vaccine of *Aeromonas hydrophila*—standardisation of dose and duration for oral vaccination of carps. Fish Shellfish Immunol 9:519–528

Dalmo RA, Leifson RM, Bogwald J (1995) Microspheres as antigen carriers: studies on intestinal absorption and tissue localization of polysterene microspheres in Atlantic salmon, *Salmo salar* L. J Fish Dis 18:87–91

Davidson GA, Ellis AE, Secombes CJ (1993) Route of immunization influences the generation of antibody secreting cells in the gut of rainbow trout (*Onchorynchus mykiss*). Dev Comp Immunol 17:373–376

Davina JHM, Parmentier HK, Timmermans LPM (1982) Effect of oral administration of vibrio bacterin on the intestine of cyprinid fish. Dev Comp Immunol 2:157–166

Duff DCB (1942) The oral immunization of trout against *Bacteri salmonicida*. J Immunol 44:87–94

Fryer JL, Rohovec JS, Tebbit GL, Michael JS, Pilcher KS (1976) Vaccination for control of infectious diseases in Pacific salmon. Fish Pathol 10:155–164

Fryer JL, Rohovec JS, Garrison RL (1978) Immunization of salmonids for control of vibriosis. Mar Fish Rev 40:20–23

Gergopoulou U, Vernier JM (1986) Local immunological response in the posterior intestinal segment of the rainbow trout after oral administration of macromolecules. Dev Comp Immunol 10:529–537

Gergopoulou U, Sire MF, Vernier JM (1985) Macromolecular absorption of proteins by epithelial cells of the posterior intestinal segment and their intracellular digestion in the rainbow trout ultrastructural and biochemical study. Biol Cell 53:269–282

Johnson KA, Amend DF (1983) Efficacy of *Vibrio anguillarum* and *Yersinia ruckeri* bacterin applied by oral and anal intubation of salmonids. J Fish Dis 6:473–476

Nelson JS, Rohovec JS, Fryer JL (1985) Tissue localization of vibrio bacterin delivered by intraperitoneal injection, immersion and oral routes to *Salmo gairdneri*. Fish Pathol 19:263–268

Piganelli JD, Zhang JA, Christensen JM, Kattari SL (1994) Enteric coated microspheres as an oral method for antigen delivery to salmonids. Fish Shellfish Immunol 4:179–188

Polk AE, Amsden B, Scarrat DJ, Gonzal A, Okahmafe AO, Goosen MFA (1994) Oral delivery in aquaculture: controlled release of proteins from chitosan-alginate microcapsules. Aquacult Eng 13:311–323

Post G (1966) Response of rainbow trout (*Salmo gairdneri*) to antigens of Aeromonas hydrophila. J Fish Res Board Can 23:1487–1494

Press CMcL, Evensen O, Reitan LJ, Landsverk T (1996) Retention of furunculosis vaccine components in Atlantic salmon, *Salmo salar* L., following different routes of vaccine administration. J Fish Dis 19:215–224

Quentel C, Vegneulle M (1997) Antigen uptake and immune responses after oral vaccination. In: Gudding R, Lillehaug A, Midtlying PJ, Brown F (eds) Fish vaccinology. Dev Biol Stand. Basel, Karger, 90, p 69–78

Rombout JHWM, Van Den Berg AA (1989) Immunological response of the second gut segment of carp. I. Uptake and processing of antigens by epithelial cells and macrophages. J Fish Biol 35:13–22

Rombout JWHM, Lamers CHJ, Helfrich MH, Dekker A, Taverne-Thiele AJ (1985) Uptake and transport of intact macromolecules in the intestinal epithelium of carp (*Cyprinus carpio* L.) and the possible immunological implications. Cell Tissue Res 239:519–530

Rombout JHWM, Blok LJ, Lamers CHJ, Egberts E (1986) Immunization of carp with *Vibrio anguillarum* bacterin: indications for a common mucosal immune system. Dev Comp Immunol 10:341–351

Rombout JHWM, Van Den Berg AA, Van Den Berg CTGA, White P, Egberts E (1989) Immunological response of the second gut segment of carp. III. Systemic and/or mucosal responses after immunization with soluble or particulate antigen. J Fish Biol 35:179–186

Rombout JHWM, Taverne P, Thiele AJ, Valenna MJ (1993) The gut associated lymphoid tissue (GALT) of carp (*Cyprinus carpio* L.): an immunocyto-chemical analysis. Dev Comp Immunol 17:55–66

Schachte JH (1978) Immunization of channel catfish, *Ictalurus punctatus* against two bacterial diseases. Mar Fish Rev 40:18–24

Shankar KM, Yamamoto T (1994) Epitope analysis of infectious pancreatic necrosis virus (IPNV) isolated from lake trout, *Salvelinus namayacush* (Walbaum) by monoclonal antibodies. J Fish Dis 17:471–482

Tatner MF, Johnson CM, Horne MT (1984) The tissue localisation of *Aeromonas salmonicida* in rainbow trout, *Salmo gairdneri* Richardson, following three methods of administration. J Fish Biol 25:95–108

Vigneulle M, Baudin Lauracin F (1991) Uptake of *Vibrio anguillarum* bacterin in the posterior intestine of rainbow trout *Oncorhyncus mykiss*, sea bass *Dicentrarchus labrax* and turbot *Scoptholmus maximus* after oral administration or anal intubation. Dis Aquat Org 11:85–92

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