Orginial Articles

MARINE BIOTECHNOLOGY

Gene Expression in Tilapia Following Oral Delivery of Chitosan-Encapsulated Plasmid DNA Incorporated into Fish Feeds

Erwin A. Ramos, Jenne Liza V. Relucio, Celia Aurora T. Torres-Villanueva

National Institute of Molecular Biology and Biotechnology, College of Science, University of the Philippines, Diliman, Quezon City, 1101, Philippines

Received: 10 March 2003 / Accepted: 3 December 2004 / Online publication: 11 March 2005

Abstract

DNA delivery into fish is important for transient gene expression, (e.g., DNA vaccination). Previous studies have generally focused on intramuscular injection of DNA vaccines into fish. However, this method is obviously impractical and laborious for injecting large numbers of fishes. This study reports oral delivery of a construct expressing the β-galactosidase reporter gene into fish by encapsulating the DNA in chitosan and incorporating it into fish feeds. We found that β-galactosidase expression could be observed in the stomachs, spleens, and gills of fishes fed with flakes containing the chitosan-DNA complex. These results suggest that DNA vaccines and other constructs can be easily and cheaply delivered into fishes orally by use of carriers and incorporation into fish feeds.

Key words: oral gene delivery — transient gene expression — plasmid DNA

Introduction

Gene transfer for transient gene expression in fish is important for the application of DNA vaccines to prevent economically important diseases in commercial species (Anderson et al., 1996). This method of gene transfer, also known as genetic or DNA immunization, is also useful for the delivery of other biologically important molecules that may be expressed from foreign recombinant DNA (Robinson and Torres, 1997). Transient transfer and expression of such foreign genes in animals that will be consumed by the public is relevant in the context of public concerns about genetically modified (GM) foods (Sieu, 2000). Unlike the case of GM organisms in which the foreign DNA has been integrated into the animal genome, the foreign gene following DNA immunization is not integrated into the animal's genome, but rather is lost after some time (Winegar et al., 1996).

Successful DNA immunization for transient gene expression in fish has been described previously (Hansen et al., 1991; Anderson et al., 1996; Sulaiman et al., 2000). However, most of these studies have relied on intramuscular injection into fish. This method is obviously impractical and not feasible for commercial fisheries, which would require injection of hundreds to thousands of fishes. DNA vaccines have also been administered into fishes by intrabuccal administration or by immersion (Corbeil et al., 2000; Fernandez-Alonso et al., 2001). In one study neither intrabuccal nor immersion delivery resulted in protection against challenge (Corbeil et al., 2000), while in another study (Fernandez-Alonso et al., 2001) delivery of DNA vaccines by immersion elicited protective humoral responses. In mice, DNA vaccines have been delivered orally using a variety of carriers (Eldridge et al., 1990; Chen et al., 1997; Leong et al., 1997). Gene expression following such oral DNA immunization was reported in these previous studies.

In this study we examined the feasibility of gene transfer into fish by encapsulation into chitosan, a natural polysaccharide derived from crustacean shells. (Leong et al., 1997). The chitosan-DNA complexes either were delivered intrabuccally directly into fishes, or were incorporated into fish feeds, which were then fed into *Oreochromis niloticus* (commonly known as tilapia) fishes.

Correspondence to: Celia Aurora T. Torres-Villanueva; E-mail: celia.torres_villanueva@up.edu.ph

Materials and Methods

Plasmid. The plasmid DNA pCMV · SPORT-βgal was purchased from Gibco BRL (Life Technologies TECH-LINE). Milligram amounts of plasmids were prepared from DH5α *Escherichia coli* transformants using either alkaline lysis protocols as described (Sambrook et al., 1989) or methods utilizing Nucleobond Plasmid Purification Kits (Clontech Laboratories).

Fishes. *Oreochromis niloticus*, or the regular fishpond black Tilapia (generously provided by Mr. Remus Villanueva, Philippines), was used in this study. The fishes used in the transfection experiments had lengths ranging from 5 to 10 cm and average weights of 33 to 40 g for the DNA-chitosan treatments. Fishes were held in a common tank and later placed in separate fish tanks (2 fishes per tank) containing purified water (Barnsted, ULTRApure Reverse Osmosis System Series 682, Barnstead/Thermolyne Corp.). Fishes were handled upon instructions of a licensed veterinarian and following prescribed national guidelines. With the exception of the fishes fed with the baked fish feed flakes, all were fed with commercial fish pellets.

Synthesis of DNA-Chitosan Nanospheres. The plasmid DNA was encapsulated in chitosan via complex-coacervation as described (Leong et al., 1997; Roy et al., 1999). Briefly, 100 μl of 50 mmol/L Na_2SO_4 (containing 10 μg of DNA) and 100 μl 0.02% w/v powdered chitosan (gift from Vanson Chemicals) in solution (25 mmol/L NaOAc-HOAc buffer, pH 5.5) were combined while vortexing at high speed for 20 seconds immediately after heating both solutions at 55°C for 1 minute. This process was repeated until several preparations, each containing the required amount of complexed DNA per fish (30 μg for intramuscular injection and 50 μg for intrabuccal delivery), were obtained. These preparations of DNA-chitosan complex solutions were lyophilized (Labconco, Freezone 6 Plus, Labconco Corp.) at −79°C to −82°C with a vacuum pressure of 6.6 Pa for at least 8 hours or until the samples were completely dried.

Confirmation of DNA Encapsulation in Chitosan. DNA encapsulation was verified by release from the complex using chitosanase (Life Technologies TECH-LINE). DNA-chitosan complexes before and after enzyme digestion were electrophoresed in 1% agarose gel after centrifugation at 12000 *g* at 4°C for 5 minutes (Figure 1).

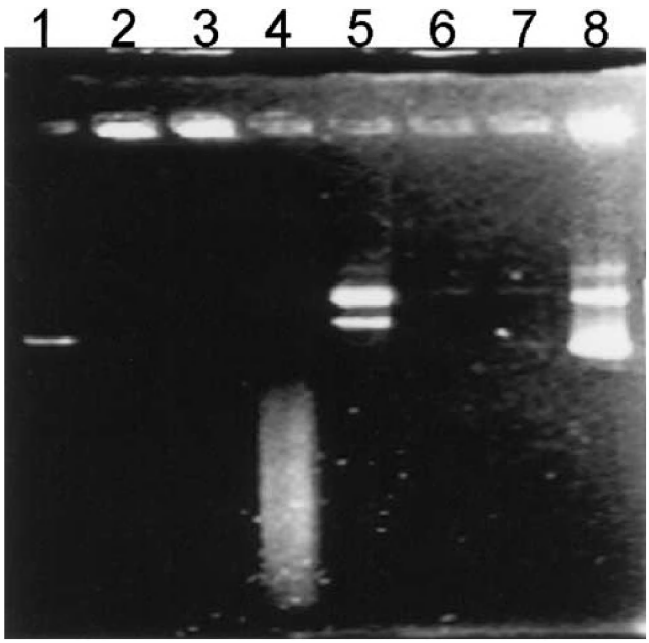

Fig. 1. Electrophoretic migration of DNA before and after encapsulation with chitosan: (**1**) untreated plasmid DNA; (**2**) pre-enzyme treatment coacervate (7000 *g*); (**3**) pre-enzyme treatment coacervate (4000 *g*); (**4**) postenzyme digestion spin (7000 *g*); (5) postenzyme digestion spin (4000 *g*); (**6**) and (7) fresh complex coacervate samples; (**8**) plasmid DNA in sulfate solution before complex coacervation.

Preparation of Fish Feeds. The fish feeds were made from a 10:1:5 mixture of sifted flour, mashed fry (commercially available fish meal) and distilled water (1 ml per flake meal). These ingredients were mixed until a very soft biscuit-textured dough was achieved. Each of the lyophilized DNA-chitosan complex preparations was added to a 1 g (dry weight) fish flake dough and was spread thinly (approx. 1.5 mm) on a cupcake-sized mold of a no-stick baking pan. The dough was heated at 35°C for no more than 45 minutes in a drying oven and flaked by crumbling.

Gene Delivery. The experiment design is summarized in Table 1. There were 2 fishes per treatment per time point. Treatments 1 to 4 employ oral gene delivery methods using chitosan as carrier.

Gene Delivery Through Feeding. The fishes fed with fish flakes were starved for 1 day immediately prior to gene delivery. Baked fish flakes containing 50 μg plasmid complexed with chitosan per fish were put in the tank water.

Intrabuccal Delivery. Fishes received 50 μg of plasmid by delivery through the mouth into the throat of the fish using a needleless 1-ml tuberculin

Table 1. Experiment Design

	2 Days	*5 Days*	*7 Days*
1) feeding with fish flakes containing DNA-chitosan complex (50 μg plasmid DNA)	4 fishes	6 fishes	4 fishes
2) intrabuccal delivery of DNA-chitosan complex in solution (50 μg plasmid DNA)	4 fishes	6 fishes	4 fishes
3) intrabuccal delivery of plasmid DNA (50 μg plasmid DNA)	4 fishes	6 fishes	4 fishes
4) Intrabuccal delivery of PBS only	4 fishes	6 fishes	4 fishes
5) intramuscular injection of DNA-chitosan complex (30 μg plasmid DNA)	4 fishes	6 fishes	4 fishes
6) intramuscular injection of plasmid DNA only (30 μg plasmid DNA)	4 fishes	6 fishes	4 fishes
7) intramuscular injection of PBS only	4 fishes	6 fishes	4 fishes

syringe with a 200-μl micropipette tip (adjusted by cutting off 0.5 cm from the attachment end) attached to the syringe tip. The DNA-chitosan complexes were dissolved in 100 μl phosphate-buffered saline (PBS). Control treatments of intrabuccal administration with those administered with 50 μg of naked plasmid pCMV · SPORT-βgal in 100 μl 1× PBS and those with 100 μl 1× PBS.

Intramuscular Injection. Fishes were injected with DNA chitosan complex containing 30 μg of the plasmid pCMV · SPORT-βgal using a 1-ml tuberculin syringe with a 26-G needle. The DNA-chitosan solution was injected at the muscle in the trunk region above the lateral line directly in line with the first or second dorsal fins. The needle was maintained at a depth of at least 2 to 3 mm for 1 to 2 seconds to minimize loss of the injected solution. Fishes injected with 30 μg of the plasmid in 100 μl 1× PBS and those with 100 μl of 1× PBS were set as controls.

Tissue Staining with X-gal. The fishes were sacrificed at 2, 5, and 7 days after DNA delivery. Fish gills, stomachs, small intestines, and spleens (for those treated by feeding of fish flakes and intrabuccal delivery) and muscles (i.m. injected) were recovered and stained with X-gal (Gibco BRL) working solution as described (Heppell and Davis, 2000). The working solution (1 mg/ml) was prepared immediately prior to staining by diluting the stock (20 mg/ml X-gal in dimethylformamide) with the X-gal diluent (10 mmol/L Na_3PO_4, 150 mmol/L NaCl, 3.3 mmol/L $K_4Fe(CN)_6$, 3.3 mmol/L $K_3Fe(CN)_6$). Stained tissues were photographed (Olympus, DP10, Olympus Optical Co.) under a stereomicroscope (Olympus, SZX 12, Olympus Optical Co.).

Microsectioning. Tissue samples were embedded in paraffin wax following a modification of a protocol by Zeller (1997). The paraffin blocks containing the samples were then cut into approximately 4-μm-thick tissue sections using a microtome. The sections were transferred onto glass slides and serially treated with xylene for deparaffinization and ethanol for rehydration. Tissue sections were then mounted with Canada balsam (China National Chemicals Import and Export Corporation).

Quantitation of Intensity of Staining. Intensities (optical densities, or OD) of X-gal staining as a measure of β-galactosidase expression were quantified using Scion Image™ (Scion Corporation, Frederick, MD, USA) from digital photographs of the stained tissues. Only good images with comparable qualities (such as brightness, contrast, background color, focus, etc.) were used for quantitation. The Mann-Whitney test (Daniel, 1991) was used for statistical analysis of differences between optical densities of tissues from treated and untreated fishes.

Results and Discussion

Oral delivery of plasmid DNA encapsulated in chitosan, either through feeding with fish feeds incorporated with DNA-chitosan, or by intrabuccal delivery, resulted in expression of the reporter gene β-galactosidase (β-gal) in the spleen, stomach, and gills of these fishes (Figures 2, 3, 4) and not in controls or in fishes that received plasmid DNA through intramuscular injection (data not shown). Gene expression in stomachs (Figure 2), spleens (Figure 3), and gills (Figure 4) was observed in fishes administered with DNA in chitosan but not in the chitosan-fed and buffer-fed controls.

Chitosan enhances paracellular and transcellular transport across epithelial cells (Roy et al., 1999). Gene expression in these tissues was consistent with this property of chitosan because most of the

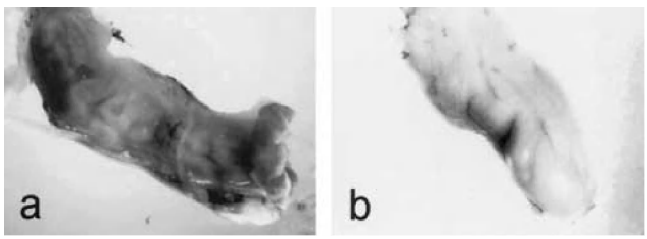

Fig. 2. Stomach tissues at 2 days; (**a**) from chitosan-DNA treated fishes; (**b**) negative control.

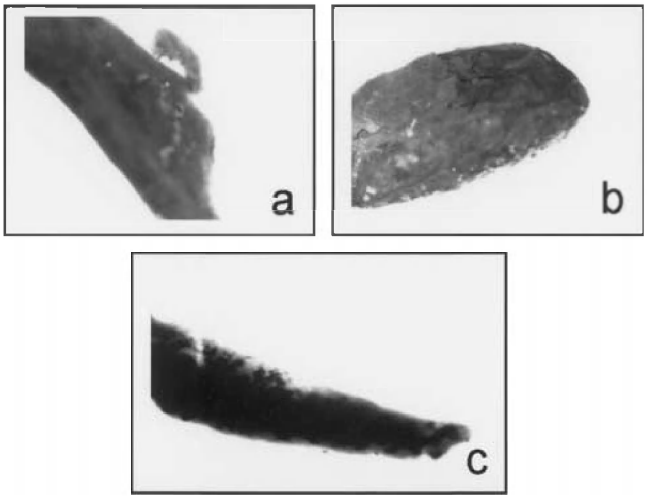

Fig. 3. Spleen tissues at 2 days: (**a** and **b**) from chitosan-DNA treated fishes; (**c**) negative control.

organs sampled were composed of epithelial tissues (Herrera, 1996). No staining was studied in muscles sampled from orally treated fishes.

The muscles of the fishes that received naked DNA by intramuscular injection were positive for β-gal expression for all time points, while those of fishes that were injected either with DNA in chitosan coacervates or with buffer alone were negative (Figure 5).

Intracellular localization of β-gal expression was observed in the stomachs, spleens, and intestines of fishes treated with chitosan-DNA complexes but not in tissues from negative controls (Figure 6). These results further demonstrate that gene delivery by

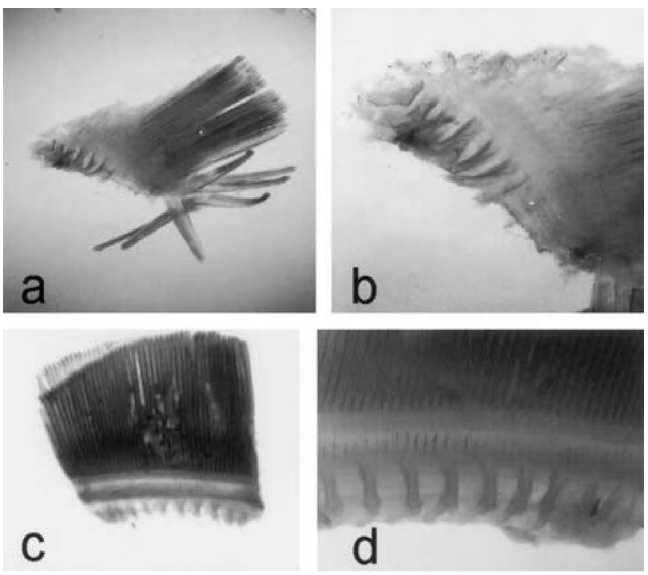

Fig. 4. Gills at 2 days: (**a** and **b**) from chitosan-DNA treated fishes; (**c** and **d**) negative controls.

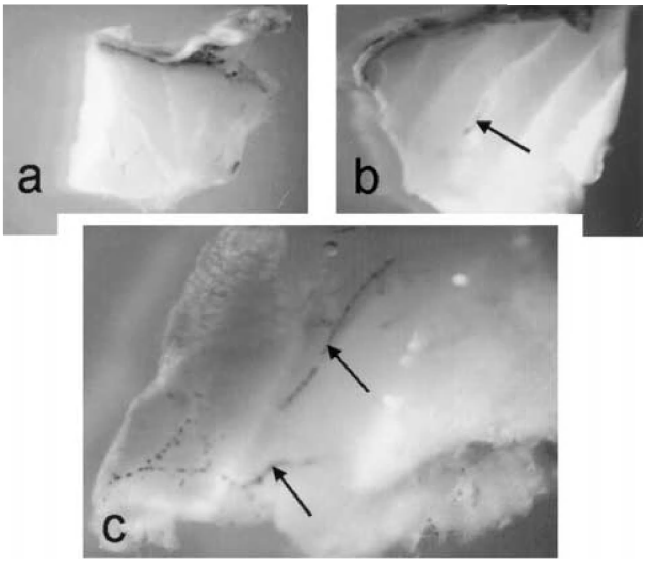

Fig. 5. Muscle tissues at 2 days: (**a**) negative control; (**b** and **c**) from plasmid DNA-injected fishes.

oral delivery of chitosan-DNA into fish results in functional gene expression.

Quantitation of intensity of staining through computer-aided image analysis was performed in order to obtain an approximation of relative levels of genes expression. Our measurements show that despite a wide variability in the values of optical density between different samples, tissues from treated fishes were more intensely stained than tissues from untreated (negative control) fishes (Figure 7). Using the Mann-Whitney test (Daniel, 1991), we found that the optical density of the stomachs, intestines, and gills from fishes that were orally administered chitosan-DNA complexes was significantly greater than the intensity of staining of the tissues from the negative controls.

Background staining in control fishes was observed in the intestines (data not shown). Background staining in tissues of the gastrointestinal tract may be due to the endogenous β-gal gene expression in these tissues due to the normal bacterial flora residing there, and have been observed by others (Roy et al., 1999).

The use of chitosan for oral delivery of plasmid DNA has been demonstrated in mice (Roy et al., 1999). Other types of carriers have been used for both intrabuccal DNA delivery and delivery by immersion of fishes (Chen et al., 1997; Fernandez-Alonso et al., 2001). The use of such carriers presumably aids in protecting the plasmid DNA from degradation by nucleases and acid in the esophageal and gastrointestinal tract. Such use of carriers is unnecessary for intramuscular injection (Robinson and Torres, 1997),

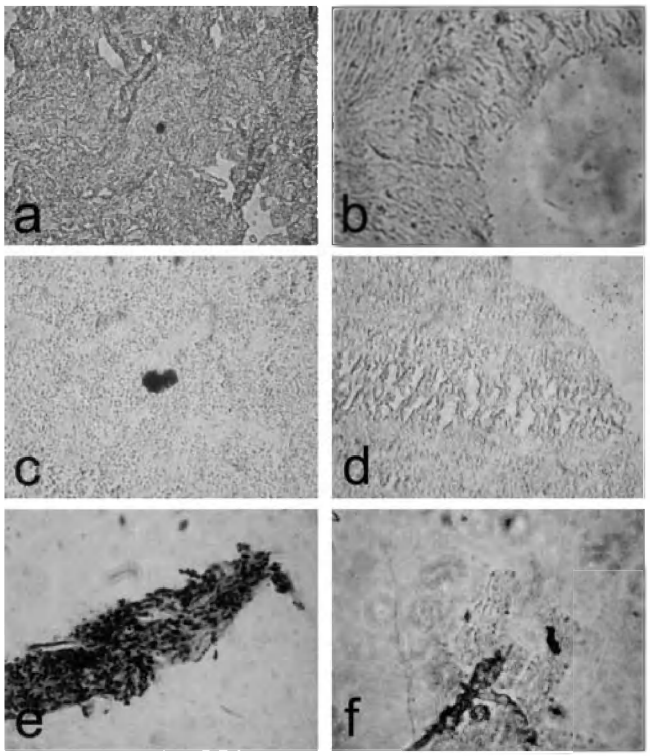

Fig. 6. Microsections of stomach (**a** and **b**), spleen (**c** and **d**), and intestines (**e** and **f**) from chitosan-DNA treated fishes (**a, c, e**) and from negative controls (**b, d, f**).

and it may hinder transfection following intramuscular delivery, as suggested by the results of this study.

It is speculated that gene expression in gills was made possible through the release of DNA from chitosan trapped over gill filaments via the flow of water as gills are mostly composed of epithelial tissues (Herrera, 1996). The blood route is another possibility. Gene expression in spleen gives evidence of some DNA circulation in blood through the gastrointestinal tract. Gills, being heavily innervated with blood vessels, may possibly express foreign genes.

The use of chitosan as plasmid DNA carrier is economical, since chitosan is cheap and chitosan encapsulation is also a simple method and does not require complex synthesis. In addition, chitosan is widely used in controlled drug delivery and in commercial diet supplements (Roy et al., 1999), and hence deemed safe even for human consumption.

The incorporation of chitosan-encapsulated plasmid DNA into feeds, instead of delivering the plasmid by intrabuccal or intramuscular administration, is more economical and practical for immunization or inoculation of large numbers of fishes in commercial fisheries.

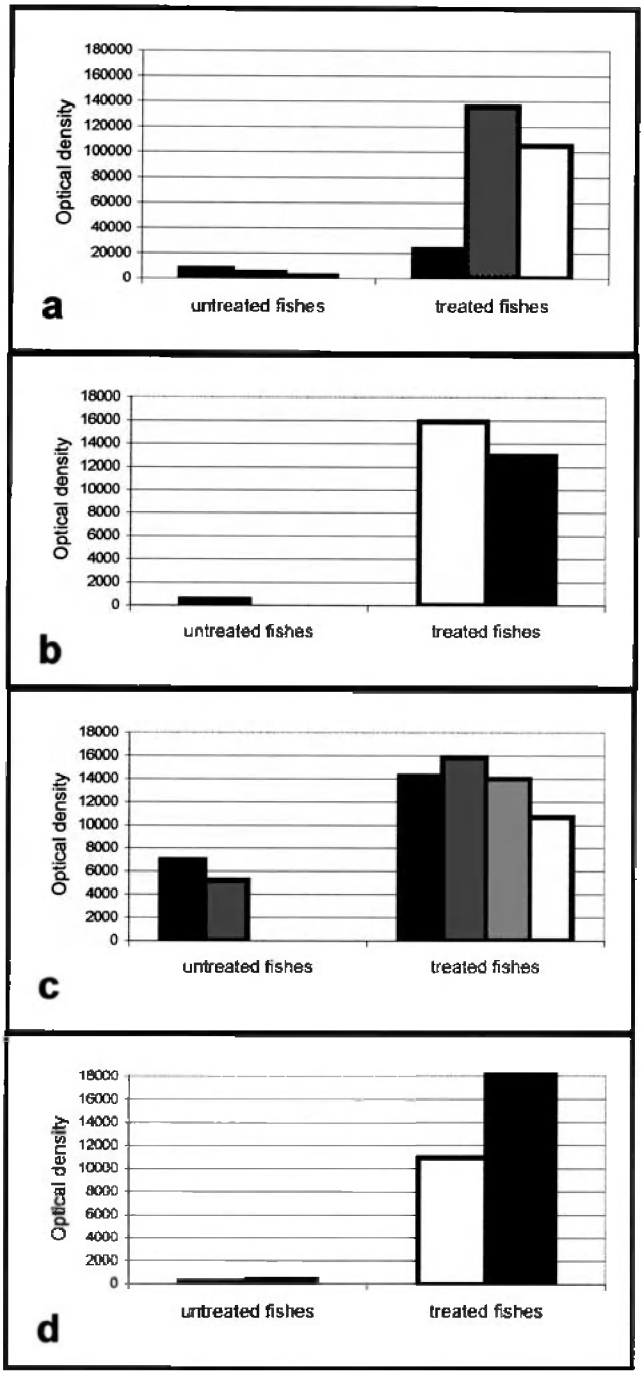

Fig. 7. Relative staining intensities optical density (OD) of (**a**) stomach, (**b**) spleen, (**c**) intestines, and (**d**) gills from negative controls (lefr bars) and chitosan-DNA treated fishes (right bars). Images were analyzed using Scion Imaging ™ software (Scion Corporation, Frederick, MD, USA).

This study indicates that gene expression following oral administration of DNA in gene delivery vehicles is possible in fishes. Further investigations on the optimal or minimal plasmid DNA dosage required for gene expression, quantitation of gene

expression with time, and other important aspects of the method are ongoing. Nevertheless, the preliminary results reported here are promising because, to date, no other investigator has reported success with oral delivery of either DNA incorporated into feeds (Heppell and Davis, 2000). The applications of this simple, practical, and effective method of gene delivery into fish are numerous and broad, ranging from the delivery of vaccines to delivery of growth-enhancing genes, and other genes with commercial applications.

Acknowledgments

We thank Mr. Remus C. Villanueva for his invaluable support and indispensable role in making this project possible; Dr. Cynthia Nalo-Ochona, Ms. Hazel B. Alfon, Ms. Eloise Prieto, and Mr. Adrian Constantino for their technical assistance and support in the handling and staining of specimens; Dr. Gisela P. Concepcion for the use of her orbital shaking incubator and microtome; and Dr. Cynthia P. Saloma for the use of her microscope and camera.

References

1. Anderson ED, Mourich DV, Fahrenkrug SC, La Patra S, Leong JC, Shepherd J (1996) Genetic immunization of rainbow trout (*Oncorhynchus mykiss*) against infectious necrosis hematopoietic virus. Mol Mar Biol Biotechnol 5, 114–122
2. Anderson ED, Leong JC (2000) Development of DNA vaccines for salmonid fish. In: *DNA vaccines: Methods and Protocols Totowa*, Lowrie DB, Whalen RG, eds. (Totowa, NJ: Humana Press) pp 105–122
3. Chen SC, Jones DH, Fynan EF, Farrar GH, Clegg JCS, Greenberg HB, Herrmann JE (1997) Protective immunity induced by oral immunization with a rotavirus DNA vaccine encapsulated in microparticles. J Virol 72, 5757–5761
4. Corbeil S, Kurath G, LaPatra SE (2000) Fish DNA vaccine against infectious hematopoietic necrosis virus: efficacy of various routes of immunization. Fish Shellfish Immunol 10, 711–723
5. Daniel WW (1991) Biostatistics: A Foundation for Analysis in the Health Sciences. (Hopoken, NJ: John Wiley and Sons)
6. Eldridge JH, Hammond CJ, Meulbroek JA, Staas JK, Gilley RM, Tice TR (1990) Controlled vaccine release in the gut associated lymphoid tissues: orally administered biodegradable microspheres target the Peyer's patches. J Controlled Release 11, 205–214
7. Fernandez-Alonso M, Rocha A, Coll JM (2001). DNA vaccination by immersion and ultrasound to trout viral haemmorrhagic septicaemia virus. Vaccine 19, 3067–3075
8. Hansen E, Fernandez K, Goldspink G, Butterworth P, Umeda PK, Chang K-C (1991) Strong expression of foreign genes following direct injection into fish muscle. Fed Eur Biochem Soc 290, 73–76
9. Heppell J, Davis HL (2000) Intramuscular injection of DNA vaccines in fish. In: *DNA Vaccines: Methods and Protocols*, Lowrie DB, Whalen RG, eds. (Totowa, NJ: Humana Press) pp 99–104
10. Herrera, AA (1996) *Histology of Tilapia*: Oreochromis niloticus Quezon City, Philippines: Bureau of Fisheries and Aquatic Resources, Department of Agriculture, pp 1–35
11. Leong KW, Mao HQ, Truong-Le VL, Roy K, Walsh SM, August JT (1997) DNA-polycation nanospheres as nonviral gene delivery vehicles. J Controlled Release 53, 183–193
12. Robinson HL, Torres CAT (1997) DNA vaccines. Semin Immunol 9, 271–283
13. Roy K, Mao H-Q, Huang S-K, Leong KW (1999) Oral gene delivery with chitosan-DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. Nat Med 5, 387–391
14. Sambrook J, Maniatis T, Fritsch EF (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press)
15. Savas H, Güven O (2001) Investigatzion of active substance release from poly (ethylene oxide) hydrogels. Int J Pharm 224, 151–158
16. Sieu CT (2000) Seeds of division. Asiaweek 26, 26–31
17. Sulaiman, ZH, Chan, RHM, Simanjuntak, PM, and Jami, YHA (2000) Gene transfer in food fish and prawn Presented at the 7th ASEAN Food Conference, Manila, Philippines, November 19–22, 2000
18. Winegar RA, Montefiore JA, Suing KD, O'Loughlin KG, Rudd CJ, MacGregor JT (1996) Determination of tissue distribution of an intramuscular plasmid vaccine using PCR and in situ DNA hybridization. Hum Gene Ther 7, 2185–2194
19. Zeller R (1997) Fixation, embedding, and sectioning of tissues, embryos, and single cells. In: *Short Protocols in Molecular Biology*, Ausubel, Brent, Kingston, Moore, Seidman, Smith, Struhl, eds. (Mississauga, Ont.: John Wiley & Sons)