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DNA vaccination by immersion and ultrasound to trout viral haemorrhagic septicaemia virus

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Abstract

This work reports preliminary data on the application of a novel method, ultrasound, for the DNA vaccination of rainbow trout. First, the best formulations were selected that increased the transfer by immersion of a plasmid coding for the green fluorescent protein (GFP) gene into trout fry. Quantification of GFP expression by fluorescence in the fin cells was used to study time course, DNA concentration dependence and comparison of different formulations. The best GFP expression results were obtained with short pulses of ultrasound, DOTAP liposomes and recombinant bacteria or bactofection. Other liposomes or microencapsulation formulations resulted in a GFP fluorescence similar to background values. Second, DNA immersion-vaccination of immunocompetent fingerling trout with the selected formulations was performed by using a plasmid coding for the glycoprotein G gene of the viral haemorrhagic septicaemia virus (VHSV). The immunization of fingerling trout was estimated by measuring humoral antibody, lymphoproliferation and VHSV challenge responses. Short pulses of low intensity ultrasound were the only method by which both humoral antibody responses and survival after VHSV challenge were obtained. Immersion DNA-vaccination using short pulses of ultrasound could eventually lead to a practical way to vaccinate small fish.

Keywords: DNA immersion-vaccination; Ultrasound; Trout; Viral haemorrhagic septicaemia

1. Introduction

Expression of the G protein from infectious haematopoietic necrosis virus (IHNV) [1] and viral haemorrhagic septicaemia virus (VHSV) [2], production of trout antibodies against G [3] and trout protection against viral challenges have been demonstrated following intramuscular injection of plasmids coding for the corresponding rhabdoviral genes [4–6]. However, practical injection-vaccination methods are restricted to medium sized fish. To enable DNA-vaccination of smaller fish, immersion-vaccination methods need to be developed [7,8].

The protein G of VHSV induces trout neutralizing antibodies [9] and T-cell immunoproliferation [10,11]. However, recombinant protein G conferred low protection to VHSV challenges when expressed in *Escherichia coli* [12], *Aeromonas salmonicida* [13] or *Yersinia ruckeri* [10]. Furthermore, only moderate protection was obtained when expressed in insect [14] or in yeast [10] cells. Successful DNA-vaccination against the VHSV disease, which causes important economic losses throughout the world, is therefore one of the expectations of this new technology.

A model to study DNA immersion-vaccination was recently described [15] using trout fry and plasmid coding for the green fluorescent protein (GFP) [16,17]. This work quantifies expression of GFP in the fins of trout fry, which depends on time course, DNA concentration and formulation. With the best formulations, DNA immersion-vaccination of fingerling trout with the protein G gene of VHSV showed that short pulses of low intensity ultrasound obtained a significant humoral antibody response and 50% protection against VHSV challenge.

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2. Material and methods

2.1. Plasmid constructs and large scale preparation of plasmids

The plasmid coding for the GFP, cloned downstream of the cytomegalovirus (CMV) promoter (pQBI$_{25}$, Quantum Biotech. Inc., Montrevil-sous-bois, France) was used for the experiments in trout fry species. The plasmid G3-pcDNAI/Amp coding for the glycoprotein G gene of the VHSV (French isolate 07.71) [22] described before [15] was used for the experiments in fingerling trout. The plasmid constructs were transferred and multiplied in the *E.coli* strains XL2 or Top10F, respectively. Large amounts of plasmids were prepared from recombinant *E.coli* pellets obtained by centrifugation at $10000 \times g$ during 20 min of bacteria cultures from fermentation of 12 l using the Wizard plus Megaprep DNA purification system (Promega, Madison, USA). Plasmid solutions were adjusted to 1 mg/ml of total DNA (absorbance at 260 nm). Plasmid DNA contained 70–90% of GFP-pQBI$_{25}$ or G3-pcDNAI/Amp depending on the preparation as shown by agarose gel electrophoresis, the rest being other contaminant bacterial DNA. Epithelial papulosum cyprini (EPC) cells transfected with the GFP-pQBI$_{25}$ plasmid showed green fluorescence and transfected with the G3-pcDNAI/Amp plasmid showed G expression as shown by cytofluorometry by using the anti-G VHSV monoclonal antibody 3F1A12 [15].

2.2. Plasmid formulations for the immersion method

Formulations tested were short pulses of ultrasound, DOTAP liposomes, exposure to recombinant bacteria or bactofection, microencapsulation and fugene liposomes. For the ultrasound formulations, a 250 ml cylindrical bath sonicator of 8×5 cm (Selecta, Barcelona, Spain) at 40 W and 40 kHz was used. For the trout fry, we used two short pulses of 1 s, followed by addition of the plasmid and two additional short pulses of 1 s. For the fingerling trout, we used two short pulses of 10 s, followed by addition of the plasmid and two additional short pulses of 2 s. The trout were allowed to rest 1 min between pulses. For the DOTAP (1,2, dioleoyl-3-4 trimethyl ammonium propane) (Avanti Polar Lipids, Alabaster, AL, USA) formulations, we used 20 mM DOTAP, 20 mM cholesterol and 20 mM phosphatidylcholine. The lipid-dried mixtures were, sonicated and lyophilized with 20–200 μg of DNA. They were reconstituted with water-glucose just prior to use according to the earlier reported preparation methods [18]. For the bactofection formulations, the *E.coli* XL2 containing the GFP-pQBI$_{25}$ or *E.coli* Top10F containing the G3-pcDNAI/Amp plasmids were added to the trout at several (see Figures) concentrations (5 g recombinant *E.coli* pellets were equivalent to 5 μg of plasmid). For the microencapsulation formulations, copolymers of vinylpyrrolidone and dimethylacrylamide were used at 1 mg/ml [19]. For the fugene formulations, fugene-6, a commercial liposome (Roche, Barcelona, Spain) was used at 8 μl/ml. Transfection of EPC cells was obtained with fugene-6 with greater efficiency [20]. As a positive control, trout fry were intramuscularly injected with 1 μg (1 μl per trout) of GFP-pQBI$_{25}$ DNA using a 10 μl Hamilton Syringe [2].

2.3. Trout fry immersion assays

Five trout fry (*Oncorhynchus mykiss*, Walbaum) of 0.2–0.5 g body weight (Las Zayas, León, Spain) was maintained in a beaker with 20 ml of aquarium water at 10°C with the GFP-pQBI$_{25}$+ formulations. For the ultrasound treatment, the beaker was maintained inside a cylindrical bath sonicator (Electra, Barcelona, Spain) filled with 150 ml of water. After an exposure of 4 s for sonication or 30 min for the rest of formulations, trout fry were then placed in 500 ml beakers filled with aquarium water and maintained at 4–10°C for 1–3 weeks. Three trout fry per experimental point were selected and analyzed for GFP expression. The plasmid DNA remaining in the water after trout fry immersion showed no variations in the amount or band patterns as shown by agar electrophoresis (not shown).

2.4. Fluorescence assays for GFP

Two days after immersion in the plasmid DNA, the fish were anaesthetized with 20 mg/l of MSS-222 (3-aminobenzoic ac.ethyl ester, Sigma Chem. Co., St. Louis, MO, USA), placed on a microscope slide and their caudal fins covered with a coverslip. Caudal fins were then examined and photographed at 40× (CCDOPS program VSSS2.1) using a Nikon Diaphot inverted microscope fitted with a mercury lamp using a FITC filter set (DM510 B-2A) and a SBIG ST-7 camera (Santa Bárbara Instrument Group, St. Barbara, CA, USA). Identical fluorescent background and range values were chosen for comparison between the different photographs. Three trout per experimental point were examined. Three photographs of the caudal fins were made from each trout. Absolute fluorescence values were measured in each of the individual cells (50–120 cells per photograph) by defining a square of 3×3 (CCDOPS program St Barbara Instrument Group, Santa Bárbara, CA, USA.) corresponding to about 9 μm^2. Background fluorescence was calculated as the average of the absolute fluorescence values obtained from five melanomacrophages in the same photograph (melanomacrophages were never fluorescent). Results were expressed as relative fluorescence by the formula, fluorescence in cell − background fluorescence/back-

ground fluorescence × 100. Significant fluorescent was defined in those cells with a relative fluorescence above that of the cells from trout not exposed to plasmid (average relative fluorescence + 2 × standard deviation (S.D.)). The fluorescent cells with a significant fluorescence value were counted and expressed as the percentage of the total fluorescent cells (150–350 cells per experiment) (Fig. 1). Curve smoothing of some data was performed for clarity purposes according to the least squares averaging method by using the FigureP program (Biosoft, Cambridge, UK).

2.5. Cells and VHSV virus for the challenges

EPC cells [21] were grown at 28°C with RPMI Dutch modified cell culture medium buffered with 20 mM HEPES and supplemented with 10% fetal calf serum (FCS). The VHSV-07.71 isolated in France from rainbow trout was grown, an assayed for infectivity in EPC cells [22].

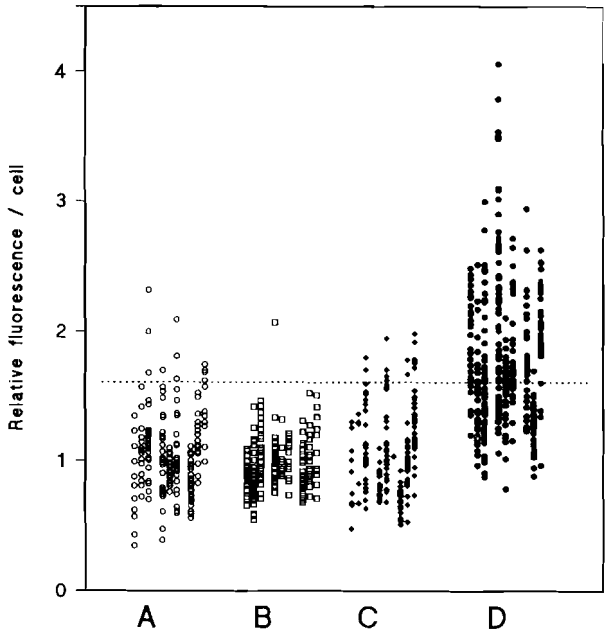

Fig. 1. Quantification of fluorescence in the caudal fin cells of trout fry exposed to GFP-pQBI$_{25}$ + ultrasound. Trout fry were immersed in a GFP-pQBI$_{25}$ + ultrasound (as detailed in Section 2). Three fields from the fins of each of the trout fry from three trout per experiment were photographed at 40 ×. The fluorescence from each of 60–120 cells per trout was estimated 2 days after immersion by using the CCDOPS program. Each point in the figure represents one cell. Cells from each trout are separated by an empty vertical space. Results were expressed by the following formula: fluorescence of each cell/average fluorescence of the cells in trout fry not exposed to plasmid × 100. The horizontal dashed line represents the average relative fluorescence of group A + 2 S.D. ○, A, ultrasound (n = 212). □, B, 10 μg/ml of G3-pcDNAI/Amp + ultrasound (n = 210). ◆, C, 1 μg/ml of GFP-pQBI$_{25}$ + ultrasound (n = 184). ●, D, 10 μg/ml of GFP-pQBI$_{25}$ + ultrasound cells (n = 366). At the 0.01 level, the means of A, B or C were not significatively different but different from D, assuming a normal distribution as calculated by the 2-population t-test (OriginLab Corporation, Northampton, MA, USA).

2.6. Immersion vaccination and challenge of fingerling trout

About 20–30 fingerling trout of 5–7 g body weight (El Molino, Madrid, Spain) were placed with 100 ml of aquarium water with strong aeration (total volume ~ 250 ml) during 24 s for ultrasound or 30 min for the rest of formulations. Trout were then released into 30 l closed-system aquaria maintained at 10°C. One month later, VHSV was injected to each trout (100 μl containing 10^6–10^7 plaque forming units of VHSV per trout) and mortalities recorded during the next 30 days. Control aquaria containing non-vaccinated trout were included in each of the experiments. Relative percent survival (RPS) was calculated by the following formula, 1 − mortality of trout treated with plasmid formulations/mortality in non-vaccinated trout × 100.

2.7. ELISA

To assay for trout anti-viral G antibodies, polystyrene plates of 96-wells (Dynatech, Plochingen, W. Germany) were coated with 1 μg of purified G4 or 2 μg of purified frg # 11 per well in 100 μl distilled water overnight at 37°C. G4 is a recombinant protein from the protein G of VHSV from aa 9 to 443 made in yeast as described before [10]. Frg # 11 is a recombinant peptide from the protein G of VHSV from aa 56 to 113 made in *E.coli* as described before [23]. Serum was obtained from three to six trout 1 month after immersion-vaccination and 1 day before VHSV challenge. The trout antibodies were three-fold serially diluted from 20 to 2500 fold in dilution buffer (130 mM NaCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.24 mM merthiolate, 5 g/l bovine serum albumin (BSA), 0.3% rabbit serum, 0.5 g/l Tween 20, 50 mg/l phenol red, pH 6.8). The plates were incubated for 60 min at room temperature with 100 μl per well of diluted trout antibodies. After they were incubated during 30 min with anti trout immunoglobulin monoclonal antibody (MAb) 1G7 [24]. Other details as described before [25].

2.8. Neutralization assays

To assay for trout anti VHSV neutralizing antibodies, 10^3 $TCID_{50}$ per ml VHSV 07.71 were incubated overnight at 4°C with three-fold serial dilutions of the trout serum. Trout serum was obtained from three to six trout 1 month after immersion-vaccination and 1 day before VHSV challenge. Then the virus antibody mixtures were added to monolayers of EPC cells in 96-well plates, adsorbed during 2 h at 14°C and incubated overnight at 14°C. The VHSV infected EPC monolayers were then fixed during 10 min in cold methanol and air dried. To detect the N antigen of VHSV, the MAb 2C9 [25] was used. Other details as

described before [26]. The absorbances were measured at 492–620 nm at several serum dilutions, the 36-fold dilutions were chosen for further analysis. The results are expressed as relative absorbance = absorbance of each serum/average absorbance of control serum × 100. Control serum were obtained from phosphate buffered saline (PBS) injected trout for the injection control or no plasmid exposed trout for the immersion control (three trout per experiment).

2.9. Lymphoproliferation assays

To assay for trout cellular immune responses, kidney cells were extracted [11] from three to six trout 1 month after immersion-vaccination and 1 day before VHSV challenge. They were incubated during a week with 1 μg/ml of G4 at 20°C. Proliferation was estimated by using tritiated thymidine as described in detail earlier [11].

3. Results

3.1. Trout fry experiments

Two days after exposure to GFP-pQBI$_{25}$ + ultrasound, GFP-pQBI$_{25}$ + DOTAP liposomes, or GFP-pQBI$_{25}$ + recombinant *E.coli* (bactofection), trout fry fins showed abundant fluorescent spots of 10–20 μm of diameter brighter than background, confirming the results reported before by using GFP-pQBI$_{25}$ + DOTAP [15]. No cell fluorescences higher than background were obtained by using GFP-pQBI$_{25}$ and either microencapsulation in copolymers of vinylpyrrolidone or complexing with Fugene-6 (not shown). Fig. 1 shows a representative experiment obtained from trout exposed to GFP-pQBI$_{25}$ + ultrasound. We assigned a relative fluorescence of 1 ± 0.3 (number of cells, $n = 212$) to the average absolute fluorescence of the fin cells measured from ultrasound exposed trout not treated with GFP-pQBI$_{25}$ (control of relative fluorescence). Relative fluorescences of 0.96 ± 0.2 ($n = 210$) and 1.06 ± 0.3 ($n = 366$) were then calculated when trout were exposed to 10 μg/ml of G3-pcDNAI/Amp (a plasmid coding for a nonfluorescent protein) + ultrasound or to 1 μg/ml of GFP-pQBI$_{25}$ + ultrasound. In contrast, a relative fluorescence of 1.7 ± 0.5 ($n = 366$) was obtained by using 10 μg/ml of GFP-pQBI$_{25}$ + ultrasound. To best show the differences between both kinds of results we compared the percentage of fluorescent cells with relative fluorescences higher than the average fluorescence of control + 2 S.D. (90% of significance assuming a normal distribution). Thus, whereas ultrasound only control, 10 μg/ml of G3-pcDNAI/Amp + ultrasound exposed or 1 μg/ml of GFP-pQBI$_{25}$ + ultrasound exposed trout showed 5.1, 0.4 or 7.6% of the cells with a fluorescence value above the significant relative fluorescence, respectively, trout exposed to 10 μg/ml of GFP-pQBI$_{25}$ + ultrasound showed 50.2% of the cells with fluorescences equal to or higher than the threshold of significant relative fluorescence value (Fig. 1). Therefore, this parameter was used throughout the experiments to quantitate and to compare the influence of the variables when using different immersion concentrations, exposure times and/or formulations.

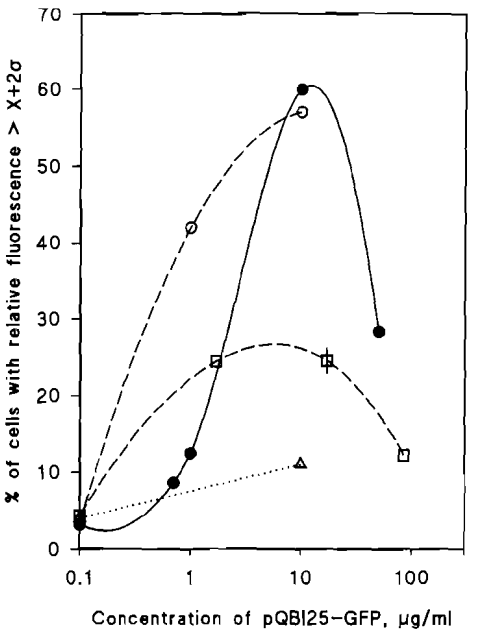

Fig. 2. GFP fluorescence in the caudal fin cells of trout fry and its dependence of immersion in GFP-pQBI$_{25}$ concentrations + formulations. GFP-pQBI$_{25}$ was used for immersion at different concentrations with different formulations. The caudal fins of the trout were examined after 2 days. The percentages of fluorescent cells with fluorescence values greater than 90% of the cells in the control are represented (average, $x + 2$ S.D., o). Some of the results were best fitted into a curve for clarity purposes, curve smoothing by the least squares averaging (FigP program Biosoft, Cambridge, UK). ●—●, GFP-pQBI$_{25}$ + ultrasound. ○- -○, GFP-pQBI$_{25}$ + DOTAP. □—□, GFP-pQBI$_{25}$ + bactofection. △—△, immersion in GFP-pQBI$_{25}$.

Fin cells with significant relative fluorescences were obtained after immersion in 10 μg/ml of GFP-pQBI$_{25}$ + ultrasound, 10 μg/ml of GFP-pQBI$_{25}$ + DOTAP or 10 μg/ml of GFP-pQBI$_{25}$ + bactofection. For each of the three immersion methods, higher or lower DNA concentrations, resulted in a lower percentage of cells with significant relative fluorescences (Fig. 2). Immersion in 10 μg/ml of GFP-pQBI$_{25}$ resulted in no significant relative fluorescences above background (< 10% of the cells).

The percentage of cells with significant relative fluorescence remained at about 35% from 2 to 20 days when trout were exposed to 10 μg/ml of GFP-pQBI$_{25}$ + ultrasound (Fig. 3). Immersion in GFP-pQBI$_{25}$ + DOTAP induced about 40% of significant relative fluorescent cells after 2 days but 7 days later

only background fluorescences were obtained. Immersion in GFP-pQBI$_{25}$ + bactofection also induced about 40% of fluorescent cells after 4 days but most of the trout died thereafter. Only 3–12% of the fluorescent cells showed significant relative fluorescences from 0 to 20 days when trout were immersed in GFP-pQBI$_{25}$.

3.2. Fingerling trout experiments

Immersion in G3-pcDNAI/Amp with the best immersion formulations selected above (ultrasound, DOTAP or bactofection) was then performed with larger immunocompetent fingerling trout. The experiments included aquaria with trout injected with G3-pcDNAI/Amp, thus providing a positive control to interpret the results, since this method has been shown to immunize and protect fingerling trout [2,5,6]. Aquarium containing trout not exposed to any plasmid but treated with the formulations or immersed in G3-pcDNAI/Amp was also included.

One month after immersion-vaccination and 1 day before the VHSV challenge, serum and kidney cells from three to six fingerling trout per aquarium per experimental point were removed and assayed for anti-G VHSV trout serum antibodies by ELISA, VHSV neutralizing trout serum antibodies and trout kidney

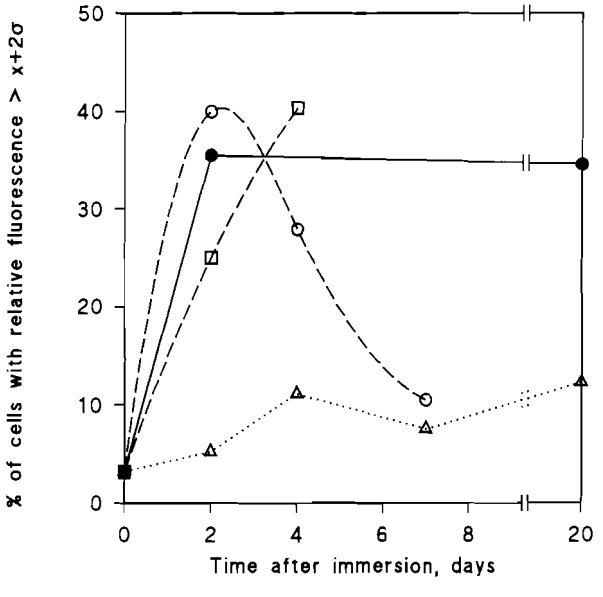

Fig. 3. Time course of GFP fluorescence in the caudal fin cells of trout fry after immersion in GFP-pQBI$_{25}$ formulations. GFP-pQBI$_{25}$ was used at 10 μg/ml. Five trout fry were exposed to immersion formulations at each time point and three of them were examined for fluorescence. The percentages of fluorescent cells with fluorescence values greater than 90% of the cells in the control are represented (average, $x+2$ S.D., o). Some of the results were best fitted into a curve for clarity purposes, curve smoothing by the least squares averaging (FigP program Biosoft, Cambridge, UK). ●—●, GFP-pQBI$_{25}$ + ultrasound. ○—○, GFP-pQBI$_{25}$ + DOTAP. □—□, GFP-pQBI$_{25}$ + bactofection. △—△, GFP-pQBI$_{25}$.

Table 1
Anti-G4 and anti-Frg # 11 antibodies in serum from trout immunized by immersion in G3-pcDNAI/Amp +formulations detected by ELISA[a]

Plasmid + formulation	Relative absorbance	
	G4 plates	Frg # 11 plates
G3-pcDNAI/Amp + ultrasound	1.3 ± 0.5 (9) 2	**4.1 ± 1.9 (9) 2**
G3-pcDNAI/Amp + DOTAP	0.9 ± 0.1 (12) 3	1.7 ± 0.7 (9) 2
G3-pcDNAI/Amp + bactofection	0.8 ± 0.2 (12) 2	[b]
G3-pcDNAI/Amp	1.4 ± 0.6 (9) 2	1.6 ± 0.3 (9) 2
G3-pcDNAI/Amp + injection[c]	1.8 ± 0.7 (21) 3	**2.2 ± 0.4 (24) 3**
pcDNAI/Amp + injection[d]	1.2 ± 0.1 (18) 3	0.8 ± 0.2 (12) 2

[a] Trout were immersed in 10 μg/ml of G3-pcDNAI/Amp and serum was obtained 1 month after vaccination and 1 day before challenge. Plates were coated with 1 μg per well of G4 or Frg # 11. Results are expressed in relative absorbances to control serum obtained either from PBS-injected trout for injection or from no plasmid-exposed trout for immersion (three trout per control). Results are expressed as the average and standard deviation from a total of n trout (number of parenthesis) obtained from different experiments, three to six trout per experiment (number after the parenthesis). Results are in bold when the means were significatively different from the G3-pcDNAI/Amp controls ($P<0.01$), assuming a normal distribution as calculated by the 2-population t-test (OriginLab Corporation, Northampton, MA, USA).

[b] Not done

[c] Injection of 1 μg of G3-pcDNAI/Amp per trout.

[d] Injection of 1 μg of pcDNAI/Amp per trout.

cell immunoproliferation. Table 1 shows that the highest anti-VHSV frg # 11 G antibody titres were obtained with serum from trout immersed in G3-pcDNAI/Amp + ultrasound (4.1 ± 1.9) or injected with G3-pcDNAI/Amp (2.2 ± 0.4). No VHSV G4 significant ELISA antibody titres were obtained with any of the sera examined ($n = 81$) from either the immersed or the injected trout (Table 1). No VHSV neutralizing trout antibodies could be detected in any of the serum examined ($n = 154$) from either the immersed or the injected trout (results not shown). In contrast, to the above referred results pooled serum from two adult trout injected with purified VHSV [27] showed an ELISA titre of 8.8 ± 1.5 and 100% of neutralization at a dilution of 1/200 (data not shown) in parallel ELISA and neutralization assays, respectively.

The immunoproliferation index of trout lymphocytes immunized with G3-pcDNAI/Amp + ultrasound (1 ± 0.1, $n = 3$), G3-pcDNAI/Amp + bactofection (1.2 ± 0.1, $n = 2$), G3-pcDNAI/Amp + DOTAP (2.4 ± 1.4, $n = 3$), G3-pcDNAI/Amp (1.4 ± 0.2, $n = 3$), were not different from either trout immersed in PBS (0.7 ± 0.2, $n = 3$), or injected with PBS (2.2 ± 0.7, $n = 6$) or injected with G3-pcDNAI/Amp (2.3 ± 1.9, $n = 9$).

Fig. 4 shows the time course of average relative percent mortalities (RPM, expressed in %) after VHSV challenge in immersed-vaccinated trout in two different experiments. To rule out a possible non-specific response despite the time lag between formulation exposures and challenges, trout exposed to formulations alone or to formulations + pcDNAI/Amp were included in some of the experiments. The RPM were not different from plasmid exposed controls (not shown). The rate of mortality in non-vaccinated trout was highest 5–10 days after challenge as typical of VHSV infections. Trout injected with G3-pcDNAI/Amp or exposed to G3-pcDNAI/Amp + ultrasound gave the lower rates of mortalities. Trout not exposed to plasmid, not exposed to plasmid but only to formulations or injected with PBS (controls used to calculate RPM), exposed to G3-pcDNAI/Amp, exposed to G3-pcDNAI/Amp + DOTAP or exposed to G3-pcDNAI/Amp + bactofection gave the highest mortalities.

Table 2 shows the combined final relative percent survival (RPS) from three different experiments (the time course in one of them was not followed up). Trout injected with G3-pcDNAI/Amp (71.5%) or exposed to G3-pcDNAI/Amp + ultrasound (50.1 ± 3.2%) gave an statistically significant higher rate of survival than trout not exposed to plasmid (0%), injected with PBS (0%), exposed to G3-pcDNAI/Amp (8.8 ± 0.5%), exposed to G3-pcDNAI/Amp + DOTAP (12.3 ± 10.9%) or exposed to G3-pcDNAI/Amp + bactofection (20.7 ± 14.0%). The combination of G3-pcDNAI/Amp + ultrasound + DOTAP (0%) or G3-pcDNAI/Amp + ultrasound + bactofection (14.3%) produced RPS statistically similar to the non-vaccinated controls (not shown).

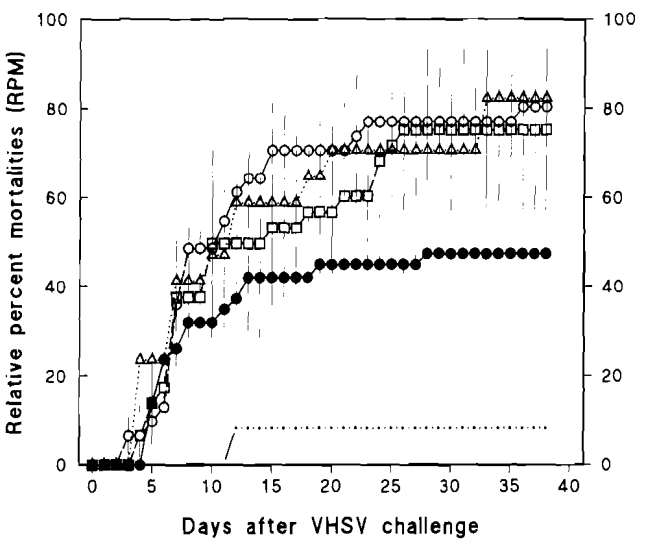

Fig. 4. Time course of the relative percent mortalities (RPM) of DNA immersion-vaccinated fingerling trout after challenge with VHSV. About 20 fingerling trout per aquarium were immersion-vaccinated with G3-pcDNAI/Amp + formulations. Thirty days later the trout were challenged with VHSV. Mortalities in control not exposed to plasmid fingerling trout were 100 and 87.5%, respectively. Relative percent mortalities (RPM) were calculated by the formula, mortality of fish treated with plasmid formulations/mortality in control not exposed to plasmid fingerling × 100. Averages and standard deviations (vertical lines in each point) from the two experiments were calculated. ● —●, G3-pcDNAI/Amp + ultrasound. ○— ○, G3-pcDNAI/Amp + DOTAP. □— □, G3-pcDNAI/Amp + bactofection. △ —△, G3-pcDNAI/Amp. ····, intramuscular injection of 1 μg of G3-pcDNAI/Amp per trout.

Table 2
Relative percent survival (RPS) of fingerling trout after VHSV challenge 1 month after immersion-vaccination in G3-pcDNAI/Amp+ formulations[a]

Plasmid + formulation	RPS (number of experiments)	P
G3-pcDNAI/Amp + ultrasound	**50.1 ± 3.2 (3)**	**<0.01**
G3-pcDNAI/Amp + DOTAP	12.3 ± 10.9 (3)	>0.01
G3-pcDNAI/Amp + bactofection	20.7 ± 14.0 (2)	>0.01
G3-pcDNAI/Amp	8.8 ± 0.5 (2)	>0.01
G3-pcDNAI/Amp + injection[b]	**71.5 (1)**	**<0.01**

[a] About 20 trout (5–10 g body weight per trout) per treatment point per aquaria were immersed into 10 μg/ml of G3-pcDNAI/Amp and several formulations. One or two non-vaccinated (either not exposed to plasmid or injected with PBS) control aquaria were included in each of the three experiments to calculate RPS. Mortality in non-vaccinated aquaria was 100, 87.5 and 63.6%, respectively, for each of the three experiments used for the calculations. The experiment with mortality 63.6% was not included in the time course Fig. 4 because only final mortality could be measured. Averages and standard deviations were calculated for the number of experiments shown under parenthesis. Results are in bold when the means were significatively different from the G3-pcDNAI/Amp control ($P<0.01$), assuming a normal distribution as calculated by the 2-population t-test (OriginLab Corporation, Northampton, MA, USA).

[b] Injection of 1 μg of G3-pcDNAI/AMP per trout.

4. Discussion

The results show that fingerling trout (minimal size trout can be vaccinated) were immunized and protected against VHSV by using DNA immersion-vaccination and ultrasound. With short pulses of low intensity ultrasound, the longest duration of GFP expression in the fins of trout fry correlated with the highest titre of VHSV immunization and with 50.1% protection to VHSV challenge in fingerling trout.

By comparing the three methods, the longer the duration of GFP expression in the trout fry the more reproducible the immunization, the best antibody response and the best protection against VHSV in fingerling trout. Therefore, the results with trout fry experiments with the GFP gene had a predictive value

for the results of fingerling trout with the G viral gene. Since the protection obtained by immersion using ultrasound was lower than the protection obtained by injection, as shown in this work (71.5% of survival) or in earlier reports [4], more work should be done to improve the protection results. Experiments with the GFP/trout fry model could then be used to further fine tune the variables involved in immersion-vaccination of fingerling trout before the costly challenge experiments are performed.

The fluorescence of the fin cells in trout exposed to the GFP gene is apparently due to induced transfection of the skin cells by the formulations used, for instance by ultrasound. The mechanisms by which ultrasound would induce transfection of the trout skin cells are not known. An ultrasound intensity of 1.7 W/cm^2 at 3000 kHz during 30–90 s produced intercellular space widening, as well as the disruption of some desmosomes connecting between the cells on the external epithelia of fish skin [28]. Perforation of the cell membranes required higher intensities of ultrasound (2.2 W/cm^2 at 1000 kHz during 30–90 s) [28]. The ultrasound pulses given to the trout in this work could not produce those effects nor hyperthermia [29] or cavitation damage [30] on the external epithelia of trout skin because of its lower intensity and short duration (0.4–0.6 W/cm^2 at 40 kHz during 4 s for trout fry or 24 s for fingerling trout). The total time of ultrasound exposure was about half of the time for it to have deleterious observable effects for either trout. Numerous other mechanisms [31] may be involved to explain the effects measured in this work but this needs further research.

Immersion-vaccination is an effective and practical method for mass vaccination of fish and most commercial bacterins are currently administered by this method. Most probably bactofection with bacteria harboring plasmids bearing eucaryotic promoters from either virus [32] or fish [33] could be used to DNA vaccinate against a variety of fish diseases. Of the immersion formulations, which induced expression of GFP in trout fry, bactofection could probably be the cheapest and the most practical. However, a suitable bacterial carrier would have to be chosen because *E.coli* did not induce any detectable antibody responses or protection in fingerling trout. Perhaps the use of an intracellular fish pathogen bacteria like *Renibacterium*, *Yersinia* or *Aeromonas* would be more efficacious than *E.coli*. Further work should explore those possibilities (attempts to transform *Yersinia ruckeri* with G3-pcDNAI/Amp were unsuccessful).

As mentioned above, the experiments reported here with GFP and trout fry indicate that this might be a model with a predictive value on protection, however, it required a labor intensive work to gather and to analyze the results. A more practical alternative would be an extract assay using β-galactosidase or luciferase as reporter genes. The first attempts to use a β-galactosidase expressing plasmid and detection of β-galactosidase expression by chemiluminescence could not measure any activity in trout fry extracts. It is not yet known if that failure was due to low sensitivity to detect the few cells that are transfected (according to the results obtained with GFP) or to the inhibition of the β-galactosidase activity by the trout extracts. Nevertheless if such an assay works, the use of such a model for optimization of the variables of immersion-vaccination might be easier.

Whereas the use of short pulses of ultrasound could be easily scaled up for a practical use in farms, the amount of plasmid DNA required seems too high for that purpose. Thus optimal concentrations of plasmid DNA for any of the successful formulation methods (ultrasound, DOTAP or bactofection) were all around 10 μg/ml for 30 trout compared with the ng amounts per trout needed for injection DNA vaccination [6]. Research to minimize that amount by manipulation of the plasmid or by introducing a new adjuvant in the formulations to make it more efficient should be performed. Introducing fish cytokines [34–36], CpG motifs [37] or stronger promoters [33] could be some of the practical solutions. The further development of the potential use of ultrasound as a practical immersion formulation for DNA vaccination of fish should still address issues such as the possibility of reuse of the plasmid (no alteration of the plasmid remaining in the water after the ultrasound treatment could be demonstrated), the length and distribution of the pulses, the intensity and frequency of the ultrasound, the geometry of the ultrasound vessel, the optimal size of the fish, the effects of stress, pH and salt concentrations, the water temperature, etc. [38].

Although the injection of G3-pcDNAI/Amp elicits lower levels of antibody reacting with Frg # 11 than an ultrasound bath with G3-pcDNAI/Amp, yet the former treatment leads to 71.5% RPS compared with 50.1% for the latter. However, this result is not so strange in fish vaccinated against VHSV, where there is a large variation on immunological responses, which not always correlate with protection. For instance, only 50% of fish survivors to the VHSV had neutralizing antibodies to VHSV [2,4,8]. Furthermore, the route on injection and the immersion might not have the same effects on inducing antibodies. The induction of G expression on the skin cells could be a better way to immunize trout against VHSV than by injection because the first targets of VHSV infection might be the external tissues. Thus, high titres of rhabdovirus have been found in the external mucus [39] and replication of VHSV in skin cells [40], in excised fin tissue [41] and in fin cell explants [42] of trout have been demonstrated to occur early in the infection course. If the induction of a local immune response is important to fight against VHSV,

immersion DNA vaccination could afford a good protection much more easily than injection DNA vaccination.

Since the data on protection of trout after ultrasound vaccination are preliminary, more work remains to be done to explore this and other possibilities, nevertheless immersion DNA-vaccination using ultrasound could eventually lead to a practical way to vaccinate small fish.

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References

[1] Anderson ED, Mourich DV, Fahrenkrug SC, LaPatra SC, Shepherd J, Leong JC. Genetic immunization of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. Mol Mar Biol Biotech 1996;5:114–22.

[2] Heppell J, Lorenzen N, Armstrong NK, Wu T, Lorenzen E, Einer-Jensen K, Ahrens P, Schorr J, Davis HL. Development of DNA vaccines for fish: vector design, intramuscular injection and antigen expression using viral haemorrhagic septicemia virus genes as model. Fish Shellfish Immunol 1998;8:271–87.

[3] Bourdinot P, Blanco M, DeKinkelin P, Benmansour A. Combined DNA immunization with the glycoprotein gene of viral hemorrhagic septicemia virus and infectious hematopoietic necrosis virus induces double-specific protective immunity and nonspecific response in rainbow trout. Virology 1998;249:297–306.

[4] Lorenzen N, Lorenzen E, Eoner-jensen K, Heppell J, Wu T, Davis H. Protective immunity to VHS in rainbow trout (*Oncorhynchus mykiss*, Walbaum) following DNA vaccination. Fish Shellfish Immunol 1998;8:261–70.

[5] Traxler GS, Anderson E, LaPatra SE, Richard J, Shewmaker B, Kurath G. Naked DNA vaccination of Atlantic salmon Salmo salar against IHNV. Dis Aquatic Organ 1999;38:183–90.

[6] Corbeil S, LaPatra SE, Anderson ED, Kurath G. Nanogram quantities of a DNA vaccine protect rainbow trout fry against heterologous strains of infectious hematopoietic necrosis virus. Vaccine 2000;18:2817–24.

[7] Fernandez-Alonso, M, F Alvarez, Estepa A, Coll JM. Vacunas DNA en Acuicultura.AquaTIC 1998; 4:http://aquatic.unizar.es/N1/art401/DNA_vac.htm.

[8] Gudding R, Lillehaugh A, Evensen O. Recent developments in fish vaccinology. Vet Immunol Immunopathol 1999;72:203–12.

[9] Lorenzen N, Olesen NJ, Vestergaard-Jorgensen PE. Neutralization of Egtved virus pathogenicity to cell cultures and fish by monoclonal antibodies to the viral G protein. J Gen Virol 1990;71:561–7.

[10] Estepa A, Thiry M, Coll JM. Recombinant protein fragments from haemorrhagic septicaemia rhabdovirus stimulate trout leucocyte anamnestic in vitro responses. J Gen Virol 1994;75:1329–38.

[11] Lorenzo GA, Estepa A, Chilmonczyk S, Coll JM. Different peptides from haemorrhagic septicemia rhabdoviral proteins stimulate leucocyte proliferation with individual fish variation. Virology 1995;212:348–55.

[12] Lorenzen N, Olesen NJ, Vestergaard-Jorgensen PE, Etzerodt M, Holtet TL, Thorgersen MC. Molecular cloning and expression in *Escherichia coli* of the glycoprotein gene of VHS virus and immunization of rainbow trout with the recombinant protein. J Gen Virol 1993;74:623–30.

[13] Noonan B, Enzmann PJ, Trust TJ. Recombinant infectious necrosis virus and viral hemorrhagic septicemia virus glycoprotein epitopes expressed in *Aeromonas salmonicida* induce protective immunity in rainbow trout (*Oncorynchus mykiss*). Appl Environ Microbiol 1995;61:3586–91.

[14] Lecocq-Xhonneux F, Thiry M, Dheur I, Rossius M, Vanderheijden N, Martial J, DeKinkelin P. A recombinant viral haemorrhagic septicaemia virus glycoprotein expressed in insect cells induces protective immunity in rainbow trout. J Gen Virol 1994;75:1579–87.

[15] Fernandez-Alonso M, Alvarez F, Estepa A, Blasco R, Coll JM. A model to study fish DNA immersion-vaccination by using the green fluorescent protein. J Fish Dis 1999;22:237–41.

[16] Cormack BP, Valdivia RH, Falkow S. FACS-optimized mutants of the green fluorescent protein (GFP). Gene 1996;173:33–8.

[17] Tsien RY. The green fluorescent protein. Ann Rev Biochem 1998;67:509–44.

[18] Gregoriadis G, Saffie R, BriandeSouza J. Liposome-mediated DNA vaccination. FEBS Lett 1997;402:107–10.

[19] Queiroz, AAA, Gallardo A, SanRoman J. Vinylpyrrolidone-*N'N*-dimethylacrylamide water soluble copolymers: synthesis, physical-chemical properties and proteic interactions, in press.

[20] Lopez, A, Fernandez-Alonso M, Rocha A, Estepa A, Coll JM. Epithelioma papulosum cyprini (EPC) carp cells transfection, Biotechnol Lett, in press.

[21] Fijan N, Sulimanovic D, Bearzotti M, Muzinic D, Zwillenberg LOZ, Chilmonczyk S, Vautherot JF, Kinkelin P. Some properties of the epithelioma papulosum cyprini (EPC) cell line from carp *Cyprinus carpio*. Annals Virol (Institute Pasteur) 1983;134:207–20.

[22] LeBerre M, De Kinkelin P, Metzger A. Identification sérologique des rhabdovirus des salmonidés. Bull Off Int Epiz 1977;87:391–3.

[23] Estepa A, Fernandez-Alonso M, Coll JM. Structure, binding and neutralization of VHSV with synthetic peptides. Virus Res 1999;63:27–34.

[24] Sanchez C, Coll JM, Dominguez J. One step purification of rainbow trout immunoglobulin. Vet Immunol Immunopathol 1991;27:383–92.

[25] Sanz FA, Coll JM. Detection of hemorrhagic virus of samonid fishes by use of an enzyme-linked immunosorbent assay containing high sodium chloride concentration and two non-competitive monoclonal antibodies against early viral nucleoproteins. Am J Vet Res 1992;53:897–903.

[26] Lorenzo G, Estepa A, Coll JM. Fast neutralization/immunoperoxidase assay for viral haemorrhagic septicemia with anti-nucleoprotein monoclonal antibody. J Virol Methods 1996;58:1–6.

[27] Fernandez-Alonso M, Alvartez F, Estepa A, Coll JM. G disulphide bond native conformation is required to elicit trout neutralizing antibodies against VHSV. J Fish Dis 1999;22:219–22.

[28] Frenkel V, Kimmel E. Ultrasound-induced intercellular space widening in fish epidermis. Ultrasound Med Biol 2000;26:473–80.

[29] Diederich CJ, Hynynen K. Ultrasound technology for hyperthermia. Ultrasound Med Biol 1999;25:871–87.
[30] Frenkel V, Kimmel E, Iger Y. Ultrasound-induced cavitation damage to external apithelia of fish skin. Ultrasound Med Biol 1999;25:1295–303.
[31] Martin CJ, Pratt BM, Watmough DJ. Observations of ultrasound-induced effects in the fish *Xiphophorus maculatus*. Ultrasound Med Biol 1983;9:177–83.
[32] Lewis PJ, Babiuk LA. In: Maramorosch K, Murphy FA, Shatkin AJ, editors. DNA vaccines: a review. Adv.Virus Res. 1999; 54: 129–188.
[33] Gomez-Chiarri M, Chiaverini LA. Evaluation of eukaryotic promoters for the construction of DNA vaccines for aquaculture. Gen Anal Biomol Eng 1999;15:121–4.
[34] Davis HL, Millan CL, Mancini M, McCluskie MJ, Hadchovel M, Comanita L, Tiollais P, Whallen RG, Micnel ML. DNA-based immunization against hepatitis B surface antigen (HBsAg) in normal and HBsAg transgenic mice. Vaccine 1997;15:849–52.
[35] Xiang ZQ, He Z, Wang Y, Ertl CJ. The effect of interferon-gamma on genetic immunization. Vaccine 1997;15:896–8.
[36] Kim JJ, Trivedi NN, Nothingham LK, Morrison L, Tsai A, Hu Y, Mahalinggam S, Dang K, Ahn L, Doyle NK, Wilson DM, Chatetergoon MA, Chalian AA, Boyer JD, Agadjanyan MG, Weiner DB. Modulation of amplitude and direction of in vivo immune responses by co-administration of cytokine gene expression cassettes with DNA immunogens. Eur J Immunol 1998;28:1089–103.
[37] Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, Koretzky GA, Klinman DM. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 1995;374:546–9.
[38] Nakanishi T, Ototake M. Antigen uptake and inmune responses after immersion vaccination. Dev Biol Stand 1997;90:59–68.
[39] LaPatra SE, Rohovec JS, Fryer JL. Detection of infectious hematopoietic necrosis virus in fish mucus. Fish Pathol 1989;24:197–202.
[40] Yamamoto T, Batts WN, Winton JR. In vitro infection of salmonid epidermal tissues by infectious hematopoietic necrosis virus and viral haemorrhagic septicemia virus. J Aquat An Health 1992;4:231–9.
[41] Dorson M, Torchy C. Viral haemorrhagic septicaemia virus replication in external tissue excised from rainbow trout, *Oncorhynchus mykiss* (Walbaum), and hybrids of different susceptibilities. J Fish Dis 1993;16:403–8.
[42] -Estepa A, Frías D, Coll JM. In vitro susceptibility of rainbow trout fin cell lines to viral haemorrhagic septicaemia virus. Dis Aquat Organ 1993;15:35–9.