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Short Communication

A single dose of a suicidal DNA vaccine induces a specific immune response in salmonids

M L Ogas Castells, J L La Torre, P R Grigera and T V Poggio

Centro de Virología Animal (CEVAN), Instituto de Ciencia y Tecnología "Dr. Cesar Milstein", Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Ciudad de Buenos Aires, Buenos Aires, Argentina

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DNA encoding antigenic proteins (Donnelly *et al.* 1997) constitute a promising alternative to traditional pathogen-inactivated vaccines in the control and prevention of viral diseases in the aquaculture industry (Heppell & Davis 2000; Gillund *et al.* 2008). Genetic immunization using classical DNA vaccines against two main viral pathogens of salmonids, the infectious haemopoietic necrosis virus (IHNV) and the viral haemorrhagic septicaemia (VHS), induced specific antibodies and protection in rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) (Anderson *et al.* 1996; Lorenzen *et al.* 1998; Lorenzen & LaPatra 2005). In 2005, the IHNV DNA vaccine became the first genetic vaccine to gain approval by the Canadian Food Inspection Agency (Lorenzen & LaPatra 1999). A new alternative to improve DNA vaccine performance is to express the target antigen under the control of Semliki Forest virus (SFV) replicase with the premise of using the ability of alphavirus to produce large amounts of viral mRNA (Zhou *et al.* 1994). The major characteristics of alphavirus-based replicon are self-amplification, high-level expression of heterologous protein, induction of apoptosis in infected cells and induction of strong type I IFN response, which can enhance the efficacy of the vaccine and activate a robust immune response *in vivo* (Kohno *et al.* 1998; Wolf *et al.* 2014). Vaccination with alphavirus-derived DNA replicons containing heterologous antigens has shown therapeutic efficacy at doses several logs lower than those required by non-replicative pcDNA plasmids in conventional genetic vaccines (Hariharan *et al.*1998; Leitner *et al.* 2000).

The early research in fish DNA vaccination has been directed to study the long-term expression, tissue distribution and immune response induced by several reporter genes (Hansen *et al.* 1991; Gomez-Chiarri *et al.* 1996; Heppell *et al.* 1998; Russell *et al.* 2000). Russell *et al.* (1998), for instance, have shown both β-Gal activity at the site of injection and induction of specific antibodies in goldfish serum after conventional genetic vaccination with the LacZ gene cloned under the early cytomegalovirus (CMV) promoter in non-replicative pcDNA.

In vitro, it has been demonstrated that SFV vectors could successfully be used to express heterologous proteins in salmon cell lines at temperatures supportive of fish virus growth (Phenix *et al.* 2000); however, no studies have been carried out to evaluate the ability of SFV-based constructs to deliver antigen *in vivo* and induce gene expression in salmonids kept in regular farming systems. Here, we describe a functional alphavirus replicon encoding β-Gal protein and report its ability to induce a specific immune response in salmonids under commercial aquaculture conditions.

Plasmids pSFV-LacZ encoding *Escherichia coli* β-Gal protein have been described elsewhere

Correspondence *T V Poggio, Centro de Virología Animal (CEVAN), Instituto de Ciencia y Tecnología "Dr. Cesar Milstein", Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Saladillo 2468 C1440FFX, Ciudad de Buenos Aires, Buenos Aires, Argentina (e-mail: vpoggiocevan@centromilstein.org.ar)*

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(DiCiommo & Bremner 1998). The recombinant plasmid was confirmed by sequence analysis.

In Situ X-Gal Staining: The DNA plasmid pSFV-LacZ (10 μg) was transfected into BHK-21 and HEK-293 cells using Lipofectamine 2000 reagent (11668-027 Invitrogen) and incubated at 37 °C according to the method described elsewhere (Berglund *et al.* 1998). Mock-transfected cells and conventional plasmid vector pCDNA 3.1 coding Lacz were included as controls. The resulting β-Gal expression was visualized by *in situ* X-Gal staining (5-bromo-4-chloro-3-indolyl-b-D-galactoside, V3941 Promega) 48 h after transfection. Briefly, BHK-21 cell monolayers were fixed in 0.5% glutaraldehyde in 100 mM PBS containing 1 mM MgCl2 at 4 °C for 10 min. After one wash with detergent solution (100 mM PBS, 1 mM MgCl2, 0.02% Nonidet™ P-40), the cells were incubated at 37 °C in X-Gal staining solution (10 mM PBS, 1 mM MgCl2, 150 mM NaCl, 33 mM K4Fe(CN) 6, 33 mM K3Fe(CN)6, 0.1% X-Gal) until colour development. Slides were examined by light microscopy.

β-Gal Activity Detection: The β-Gal activity was measured in cell extracts with o-nitrophenyl-β-d-galactopyrannoside (ONPG) as a substrate 48 h after transfection. HEK-293 cells monolayers were washed once with phosphate-buffered saline (PBS). Then, cells were lysed with 10 mM Tris, pH 7.5, 150 mM NaCl and 1% Triton® X-100 with protease inhibitor cocktail (05892970001-Roche) (Buffer lysis) for 30 min on ice, and β-Gal activity was assayed (Sambrook, Fritsch & Maniatis 1989).

β-Gal Control Enzyme (G4538-Sigma) was used to convert optical density 415 nm into nanograms of protein.

BHK-21-transfected cells were washed twice with PBS (pH 7.4) and lysed in buffer lysis solution 48 h after transfection. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970). Separated proteins were transferred to a nitrocellulose membrane (cat RPN137E Hybond-C Extra, GE Healthcare Sciences), and immunoblotting was performed by standard procedures (Burnette 1981). To eliminate possible non-specific binding, the membrane was blocked with 10% skimmed milk in PBST (0.5% Tween-20 in PBS) overnight and incubated with monoclonal anti-β-Gal antibody (G6282 SIGMA) (1:1000) for 1 h at room temperature. The horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (cat W4021 Promega) was used as the secondary antibody. The protein bands were visualized by 3,30-diaminobenzidine (DAB) tetrahydrochloride hydrate (cat D5637 Sigma-Aldrich) staining.

The study including fish rearing and experimentation was performed following ethical guidelines approved by the Institutional Committee for Animal Experimentation. All fish were reared under pathogen-free conditions and transferred to aerated fresh water tanks with internal biofilters for 4 days before vaccination at the Aquaculture Unit from the Industrial Park Mercedes (Mercedes, Prov. Buenos Aires- Argentina). They were fed approximately 1% biomass of dry feed pellet (ECOFEED S.A ICTUS 3800). Rainbow trout specimens (n = 25/group) in the size range of 25–30 g were placed in 250-L tanks containing freshwater. The water was led into each tank at 10 L min^{-1}. During the experiment, water temperature was recorded daily (14 ± 1 °C). Fish were anesthetized by immersion in a solution of benzoacaine (10% in ethanol, 1 mL L^{-1}) previous to vaccination.

The DNA vaccine was prepared by diluting the purified DNA plasmid pSFV-LacZ preparation to 1 mg mL^{-1} in sterile PBS. A group of 25 rainbow trout specimens were inoculated once with 100 ug pSFV-LacZ. Another group received sterile PBS which served as the negative control (PBS control). The diluted DNA was injected intramuscularly (im) in the epaxial muscle ventrally of the dorsal fin using a 26-gauge needle.

Blood collected from the caudal vein of each fish was centrifuged (3500× ***g*** for 15 min), and serum was kept at −85 °C. Total anti- β-Gal antibodies were tested by indirect ELISA as described in the Methods.

Serum samples from all of the fish were analysed by an indirect ELISA test using commercial β-Gal (10-1101-25 Medicago) as the antigen. The ELISA test was routinely performed in 96-well flat-bottomed plates (cat 44-2404-21 Nunc Maxisorp) coated with 1 ug mL^{-1} β-Gal in 0.1 M carbonate/bicarbonate buffer (pH9.6) and incubated overnight at 4 °C. All the incubations were performed at 37 °C for 1 h. After blocking with SD-A (10% adult horse serum, 0002% Thimerosal in PBS), plates were incubated with duplicate twofold serial dilutions of test sera. Twofold serial dilutions of sera starting from 1:2 were added to the wells. Anti-IgM of coho salmon *Oncorhincus kisutch* IgG fraction monoclonal antibody (3H7/E1 Grupo Bios –

Chile) at a 1:100 dilution was then added, followed by the addition of horseradish peroxidase-conjugated rabbit anti-mouse IgG. The substrate 2,2 azino –bis (3 ethylbenzthiazoline 6-sulfonic acid) (ABTS) was used, and the optical density (O.D.) determined at 405 nm with a Bio-Rad microplate reader.

Representative muscle biopsy samples (blocks of 3 cm long, 3 cm wide and 2 cm deep (18 cm^3), centred on the injection point and surrounding area, were placed directly in formaldehyde solution 4% buffered, embedded in paraffin and sectioned 5 μm thick before staining with periodic acid-Schiff (PAS), Masson-trichrome and haematoxylin and eosin (H&E) staining for examination with an Olympus IX-51 inverted microscope (Olympus America). Biopsy samples were taken 48 h after inoculation in five vaccinated and control fish.

Evaluation of immune responses among the different groups was assessed by analysis with Student's *t*-test. *P*-Values of <0.05 were considered statistically significant.

The reporter LacZ gene that codes for the β-Gal is widely used to evaluate both transfection efficiency and transcriptional activity. β-Gal protein expression levels can be assessed by measuring β-Gal protein enzymatic activity in cell lysates, by histochemical staining on fixed cells or by immunological methods (Alam & Cook 1990; Ramos *et al.* 2005).

To test the ability of SFV alphavirus replicon to express foreign antigens, the lacZ gene was cloned in plasmid pSFV carrying the Semliki Forest alphavirus replicon under control of the CMV promoter as described in the Methods. The resulting plasmid (pSFV-LacZ) contains the foreign LacZ replacing the SFV capsid protein gene downstream of the non-structural SFV replicase gene within the replicon.

Plasmid pSFV-LacZ was transfected into BHK-21 cells, and expression of the reporter gene was evaluated. Non-transfected cell controls were included as background references.

Figure 1 A shows representative microscope fields of BHK21 cells transfected with 10 μg pSFV-LacZ that stained positive after fixation and incubation with X-Gal substrate demonstrating expression and accumulation of reporter β-Gal protein.

The replicon vector was able to cause obvious morphological and functional damage to the BHK-21 cells. The initial transfection of BHK-21 cells by the pSFV-LacZ replicon resulted in a clear and efficient cytopathic effect after 48 h (Fig. 1c). Replicon vector expression in cells leads to inhibition of host macromolecular synthesis, culminating in eventual cell death by an apoptotic mechanism (Perri *et al.* 2000).

On the other hand, mock and pCDNA 3.1-LacZ-transfected BHK-21 cells do not shown changes (Fig. 1 b–d) after transfection.

The functionality of the reporter β-Gal protein expressed by pSFV-LacZ was confirmed by ONPG hydrolysis. The results showed that β-Gal expression increased with increasing numbers of HEK 293-transfected cells (Fig. 2a).

These results illustrate that our SFV expression system was able to be used in mammalian cell lines from different species. The β-Gal protein identity was confirmed by immunoblotting analysis with a specific antibody (Fig. 2b).

The results demonstrated that the pSFV-LacZ plasmid was able to direct the expression of the

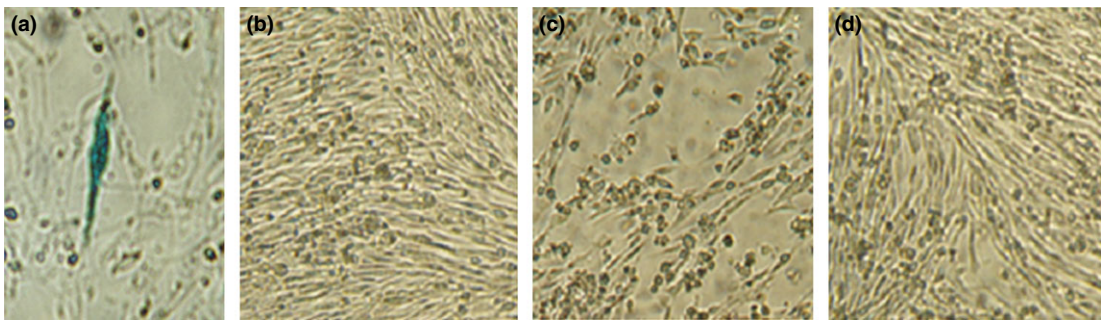

Figure 1 *In situ* β-Gal staining. (a) BHK-21 cells transfected with pSFV-LacZ (40×) and expressing β-Gal appear blue following fixation and incubation with X-Gal substrate. Cytopathic effect induced by the viral replicon after 48 h. (b) Mock-transfected BHK-21 cells, (c) BHK-21 cells transfected with pSFV-LacZ, (d) BHK-21 cells transfected with pcDNA 3.1-LacZ.

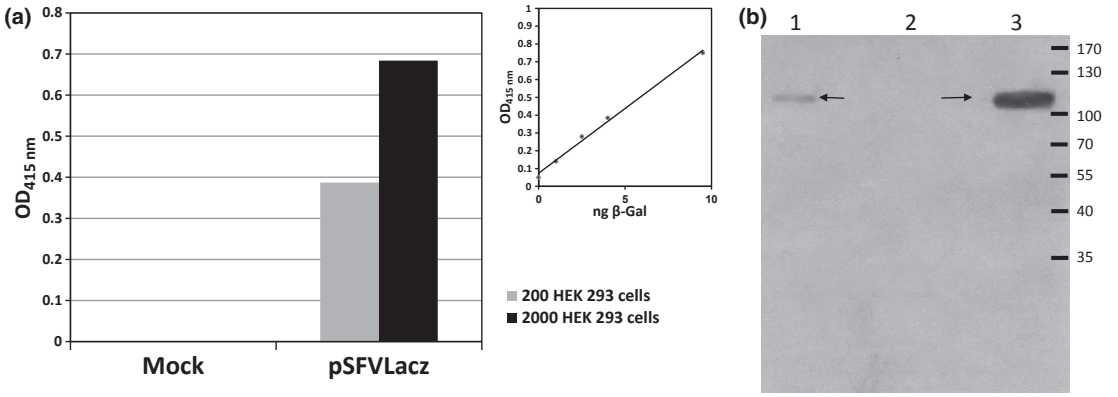

Figure 2 (a) β-Gal gene expression. O-nitrophenyl-β-d-galactopyrannoside (ONPG) artificial chromogenic substrate was used to provide a quick and sensitive method for quantifying β-Gal expression in 200 and 2000 HEK-293 cells at 48 h post-transfection. A curve generated with commercial β-Gal was used to convert optical density 415 nm into nanograms of protein. (b) Immunoblot analysis of β-Gal expression in transfected BHK-21 cells lysates. A SDS-PAGE gel was transferred into nitrocellulose membrane, and β Gal was visualized as described in the methods. Arrows indicate position of β-Gal. Lane 1. BHK-21 cells transfected with pSFV- LacZ. Lane 2. (negative control) BHK-21 cells transfected with pSFV vector coding no antigen. Lane 3. (positive control) Commercial recombinant β-Gal protein.

reporter gene in transfected BHK-21 and HEK 293 cells. We next proceeded to investigate whether the pSFV-LacZ replicon was able to express the heterologous protein *in vivo* and induce a specific immune response in aquacultured fish. With that aim, 25 rainbow trout specimens received one intramuscular immunization with 100 ug of purified plasmid pSFV-LacZ. At the same time, a control group was injected with PBS buffer; both groups were maintained under aquaculture conditions described in the Methods. Figure 3 shows the optical density (OD) profile obtained as a function of serial dilutions of sera from pSFV-LacZ injected and control rainbow trout specimens. The curve shows a linear correlation indicative of specific binding to the β-Gal substrate and demonstrates that rainbow trout specimens vaccinated with β-Gal coding replicon induced a significant anti-β-Gal response of the IgM type after 45 days of the initial inoculation. Genetic vaccines based on alphavirus replicons have been shown to elicit both humoral and cellular responses in a variety of animal challenge models, including mice, ferrets, chickens and non-human primates (Berglund *et al.* 1997, 1999; Phenix *et al.* 2001; Sun *et al.* 2010; Yager *et al.* 2013). No studies have previously investigated the capacity for pSFV-based replicon DNA vaccine to induce adaptative immune responses in fish. The data presented here confirm that pSFV alphavirus

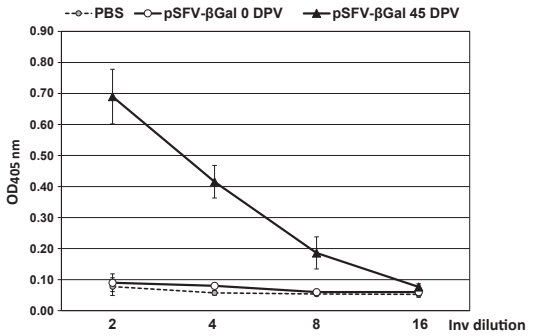

Figure 3 Vaccination of salmonids with the SFV replicon coding for the β-Gal protein. Vaccinated group of fish received 100 μg pSFV-LacZ per fish on day 0. A control group of fish was included as a negative control and received PBS. Antibody responses were assessed by ELISA with recombinant β-Gal as the capture antigen. Diluted samples were individually tested in triplicate. Each bar represents the means of $OD_{405} \pm SD$. The statistical significance was calculated by analysis with Student's *t*-test. Differences were considered to be significant with $P < 0.05$.

replicons are also functional in fish and able to induce antigen-specific antibody response in aquacultured conditions. There was evidence that SFV expression systems functioned inefficiently at 15 °C in salmon cell lines such as CHSE-214, Atlantic salmon AS and F95/9, (Phenix *et al.* 2000). It is worth mentioning that fish were kept at 14–15 °C throughout our experiment, a

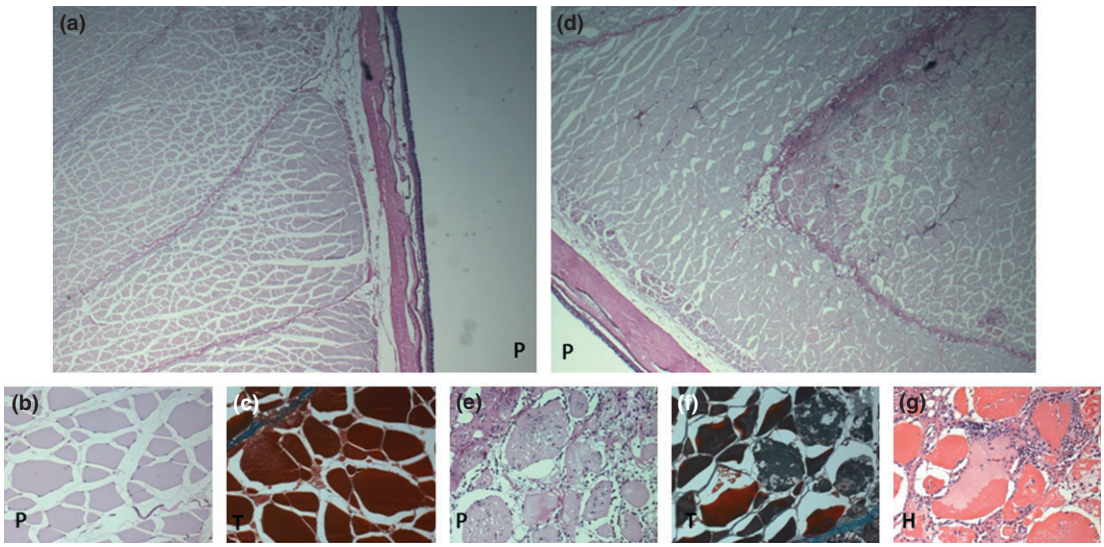

Figure 4 Histopathological analysis of fish muscle samples. Biopsy samples were taken 48 h after inoculation. Representative section of muscle stained with periodic acid-Schiff (PAS) (P), Masson-trichrome (T) and H&E (H) from PBS control (a–c) and pSFV-LacZ vaccinated fish (d–g). Picture a and d were taken at 40× magnification. Picture b–c–e–f and g were taken at 400× magnification.

suboptimal temperature for *in vitro* antigen expression, and they still were able to develop a specific immune response.

In vitro studies carried out by Olsen *et al.* (2013) using a replicon based on salmonid alphaviruses have shown that this replicon was functional in mammalian, insect and fish cell lines within a wide range of temperatures. They also probed that the vector was functional *in vivo* in arthropods *Litopenaus vanamei*; however, no studies have been carried out in fish. In our study, we have used a replicon based on a mammalian alphavirus and have shown that it is functional *in vivo*, and induce humoral response in rainbow trout specimens.

Wolf *et al.* (2014) also constructed a replicon based on salmonid alphaviruses encoding the antigen salmon anaemia virus (ISAV) haemagglutinin-esterase. They used it as a vaccine, but were not able to detect an antibody response. However, using a replicon based on Semliki virus, we have detected a specific humoral response to the β-Galactosidase antigen.

Histopathological examination with PAS and Masson-trichrome staining of muscle sections from fish injected with PBS buffer solution ($n = 5$) revealed healthy tissue (Fig. 4b–a–c), while muscle injected with pSFV-LacZ ($n = 5$) and sampled at 48 h after treatment were characterized by focal inflammatory sites and prominent neutrophilic infiltrates and morphological evidence of occasional small foci of necrosis at the site of injection compared with the control samples. Focal increases in the number of epithelial layers, and in the number and diameter of mucus-secreting cells have been observed in tissue treated once with pSFV-LacZ (Fig. 4e–f).

On day 45, no findings of unusual pigmentation (melanosis) or intra-abdominal adhesions have been described at the intramuscular injection site after vaccination.

The induction of cytopathogenicity by SFV replicons, probably due to the accumulation of double-strand RNA intermediates, is also a contributing factor to the immunogenicity of these constructions and gives them an edge over traditional pcDNA3.1-based genetic vaccines. Previous studies in salmon cell lines have shown that the cytophatic effect was not detectable at temperatures below 20 °C (Phenix *et al.* 2000). This feature also improves biosafety as all the cell lines that have been transfected with the replicon undergo apoptosis. Future work will be needed to determine the persistence of the vector in fish muscle.

In summary, this study revealed that the intramuscular administration of SFV-based construct proved to be functional and capable of delivering antigen to the immune system of salmonids under cold water conditions. These vectors could be used as an alternative in prophylactic vaccination strategies for farm-raised salmonid.

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