Full length article

Time-course study of the protection induced by an interferon-inducible DNA vaccine against viral haemorrhagic septicaemia in rainbow trout

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ABSTRACT

The highly effective DNA vaccines against diseases caused by fish rhabdoviruses in farmed fish consist of a DNA plasmid vector encoding the viral glycoprotein under the control of a constitutive cytomegalovirus promoter (CMV). Among others, attempts to improve efficacy and safety of these DNA vaccines have focused on regulatory elements of plasmid vectors, which play a major role in controlling expression levels of vaccine antigens. Depending on the context, use of a fish-derived promoter with minimal activity in mammalian cells could be preferable. Another aspect related to the CMV promoter is that constitutive expression of the vaccine antigen may lead to rapid elimination of antigen expressing cells in the fish and thereby potentially reduce the long-term effects of the vaccine.

In this study, we compared DNA vaccines with the interferon-inducible Mx promoter from rainbow trout and the CMV promoter, respectively. Plasmid constructs encoding the enhanced green fluorescent protein (EGFP) were used for the *in vitro* analysis, whereas DNA vaccines encoding the glycoprotein (G) of the viral haemorrhagic septicaemia virus (VHSV) were applied for the *in vivo* examination.

The *in vitro* analysis showed that while the DNA vaccine with the CMV promoter constitutively drove the expression of EGFP in both fish and human cell lines, the DNA vaccine with the Mx promoter inducibly enhanced the expression of EGFP in the fish cell line.

To address the impact on protection, a time-course model was followed as suggested by Kurath et al. (2006), where vaccinated fish were challenged with VHSV at 2, 8 and 78 weeks post-vaccination (wpv). The DNA vaccine with the CMV promoter protected at all times, while vaccination with the DNA vaccine containing the Mx promoter only protected the fish at 8 wpv. However, following induction with Poly (I:C) one week before the challenge, high protection was also evident at 2 wpv.

In conclusion, the results revealed a more fish host dependent activity of the trout Mx promoter compared to the traditionally used cross species-active CMV promoter, but improvements will be needed for its application in DNA vaccines to ensure long term protection.

1. Introduction

Viral haemorrhagic septicaemia virus (VHSV), a member of the *Rhabdoviridae* family [1], causes a severe disease (VHS) in wild and farmed fish worldwide [2]. Several vaccination strategies have been tested against VHS, including inactivated virus vaccines, live-attenuated virus vaccines, and recombinant vaccines. However, due to low efficacy, high cost per dose, or safety limitations, there is currently no commercially available vaccine against VHS [3–7]. In 1998, the evaluation of a DNA vaccine as an alternative immunization strategy against VHS revealed the establishment of highly protective immunity [8]. Since then, several studies have examined the protection induced by DNA vaccination against VHS and the related disease IHN (infectious haematopoietic necrosis) under different experimental conditions, with promising results [9,10]. The DNA vaccines typically include an eukaryotic expression plasmid vector encoding the viral surface

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glycoprotein (G), under the control of the human cytomegalovirus promoter (CMV). The immunological protection follows a sequential three-phase scenario [10], which involves:

(i) *The early antiviral response* (EAVR), which comprise cross-reactive protection associated with innate antiviral immune mechanisms. This protective phase starts shortly (within days) after the intramuscular injection of the DNA vaccine but only lasts for a few weeks. It is characterized by up-regulating the expression of interferon type I (IFN I), and consequently also increased transcription of multiple interferon-stimulated genes (ISG) [11–15].

(ii) *The specific antiviral response* (SAVR), which starts a few weeks after vaccination, when cross-protection against heterologous virus fades away and adaptive immunity characterized by neutralizing antibodies and cytotoxic T lymphocytes (CTL) appear [16–18].

(iii) *The long-term antiviral response* (LAVR), which follows the SAVR, is characterized by slightly lower protection than in SAVR and minimal levels or absence of neutralizing antibodies in fish which have not been exposed to virus. This phase like the SAVR, is thought to be associated with specific adaptive immune mechanisms, although this remains to be demonstrated [10,18].

Considering that the persistence of a plasmid in fish tissue could last up to 535 days after intramuscular injection [19,20], DNA vaccine residues might be present at the time of slaughter. Although there are no indications of consumer safety issues related to the CMV promoter [21,22], this aspect has earlier driven the search for alternative, fish-derived, promoters for DNA vaccines to be used in aquacultured fish [23,24], and was also discussed more recently by Alonso and Leong [25].

Example of fish-derived promoters tested in DNA vaccines are the Interferon regulatory factor 1A (IRF1A) promoter, the Mx1 promoter and the carp β-actin (AE6) promoter [23,24,26–28]. Some of the examined DNA vaccines with alternative promoters have shown potential in terms of inducing protective immunity [23,24]. However, the reports published so far have not taken the time-course scenario of the protective mechanisms into account considering both specificity and duration of protection. The time-course study is important from a practical point of view, where the ability of the vaccine to induce a fast, efficient, and long-lasting protection, is essential.

Another aspect of the strong constitutive antigen expression mediated by the CMV promoter in vaccinated animals relates to the rather efficient elimination of transfected cells by infiltrating leucocytes [29]. Reduced persistence of antigen may imply shorter duration of immunity, and it may be speculated that more long lasting and possibly controlled onset of immunity could be obtained by a using an inducible promoter.

This work aimed to analyze the capacity of a VHSV glycoprotein DNA vaccine with an IFN-inducible trout-derived Mx 1 promoter to induce EAVR, SAVR, and LAVR in rainbow trout fingerlings. To address the host range aspect of promoter activity, we also compared expression of the recombinant protein in transfected cell lines derived from fish and humans.

2. Materials and methods

2.1. Cell lines

EPC cells (epithelioma papulosum cyprinid) [30], BF2 cells (bluegill fry fibroblast) [31], HeLa cells (ATCC®CCL-2), and FHs74 (ATCC®CCL241) [32] were used in this work. BF2 and HeLa cells were maintained in minimum essential media (MEM), EPC cells in RPMI 1640 medium and FHs74 cells in Hybri-Care Medium (ATCC® 46-X™) supplemented with 30 ng/ml epidermal growth factor (EGF). All media were supplemented with 10% fetal bovine serum (FBS), 100 U/ml of
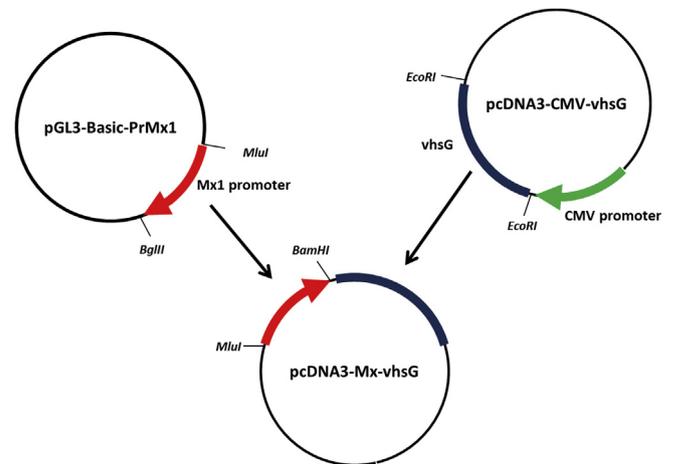

Fig. 1. Schematic outline of the construction of the DNA vaccines with the IFN-inducible Mx promoter. The Mx1 promoter was excised from pGL3-Basic-PrMx1 as a 600 bp *Mlu*I-*Bgl*III fragment and inserted into pcDNA3-CMV digested with *Mlu*I and *Bam*HI. The IFN-regulated expression vector encoding the VHSV glycoprotein (pcDNA3-Mx-vhsG) was obtained by excising the VHSV glycoprotein gene (vhsG) from pcDNA3-vhsG [35] as a 1576 bp *Eco*RI-*Eco*RI fragment and ligating it into pcDNA3-Mx digested with *Eco*RI. The plasmid pcDNA3-vhsG was denominated pcDNA3-CMV-vhsG in this work. The glycoprotein gene was derived from VHSV isolate DK3592b. For the *in vitro* evaluation, the glycoprotein coding sequence from both pcDNA3-CMV-vhsG and pcDNA3-Mx-vhsG were excised with *Eco*RI and *Not*I and replaced with the EGFP gene, which was excised from pEGFP-N1 (Clontech) using the same endonucleases. The empty vectors with CMV and Mx promoters (pcDNA3-CMV and pcDNA3-Mx) were used as negative controls.

Penicillin and 100 μg/ml of Streptomycin. EPC and BF2 were maintained at 15 °C, while HeLa and FHs74 were maintained at 37 °C.

2.2. Virus

A low passaged VHSV isolate (DK3592b) was propagated by inoculating freshly passaged BF2 cells with a low MOI (multiplicity of infection). The infected cell cultures were maintained at 15 °C. When a complete cytopathic effect was observed, the medium was centrifuged at 5000 × g for 15 min at 4 °C. The supernatant was collected and the titer of the virus was determined using the method of 50% tissue culture infective doses (TCID$_{50}$) per ml in BF2 cells [33]. Virus batches were stored in aliquots at −80 °C.

2.3. Plasmid constructs

An expression vector with Mx promoter (pcDNA3-Mx) was constructed by replacing the CMV promoter of pcDNA3 (Invitrogen), with the Mx1 promoter from pGL3-Basic-PrMx1 [27]. The pcDNA3 vector was named pcDNA3-CMV in this work. Further details in Fig. 1. To evaluate the specificity of the immune protection induced by the VHS-DNA vaccine in the challenges performed at 2 and 8 wpv, a group of trout was vaccinated with the plasmid pcDNA3.1-CMV-ihnG, encoding the glycoprotein of the IHNV (isolate US-WRAG) [34]. This IHNV-DNA vaccine should be able to induce EAVR (innate immune mechanisms) but not SAVR (Adaptive immune mechanisms) against VHS in vaccinated rainbow trout.

Escherichia coli transformed with each plasmid were propagated overnight in 4 L of LB broth containing ampicillin (100 μg/ml), at 37 °C. An endofree plasmid purification kit (Gigaprep kit Qiagen) was used for the further purification of the DNA constructs. The DNA constructs for the vaccination trials and the *in vitro* evaluations are listed in Table 1.

Table 1

Plasmid constructs.

Plasmids	Promoter	Encoded protein
pcDNA3-CMV	CMV	–
pcDNA3-CMV-vhsG	CMV	VHSV glycoprotein
pcDNA3-CMV-EGFP	CMV	EGFP
pcDNA3-Mx	IFN-inducible Mx promoter	–
pcDNA3-Mx-vhsG	IFN-inducible Mx promoter	VHSV glycoprotein
pcDNA3-Mx-EGFP	IFN-inducible Mx promoter	EGFP
pcDNA3.1-CMV-ihnG	CMV	IHNV glycoprotein

2.4. Evaluation of promoter activity in cell culture

EPC, FHs74 and HeLa cells were seeded in 24-well plates (2×10^5 cells/well) one day before transfection. The transfection was performed with the plasmids: pcDNA3-CMV, pcDNA3-CMV-EGFP, pcDNA3-Mx, and pcDNA3-Mx-EGFP. Briefly, 0.75 μg of either construct was mixed with 2 μg of PEI (Polyethylenimine) in 300 μl of medium without serum, and incubated for 20 min at room temperature. The medium from each well was removed and replaced by the transfection mix. At 6 h post-transfection for the EPC cells and 4 h for the Hela and the FHs74 cells, the cell culture medium was replaced by fresh medium with or without Poly (I:C) (P0913,Sigma) (a potent inducer of IFN) to a final concentration of 10 μg/ml.

The EPC cell culture was evaluated at 7 days post-transfection, while the HeLa and FHs74 cell cultures were evaluated by fluorescence microscopy at 2 days after transfection. The images were obtained with a Leica TCS SP2 laser scanning confocal microscope (Leica, Germany), and processed by the Leica confocal software.

2.5. Evaluation of EAVR and SAVR

Outbreed, all female rainbow trout with average weight of 3.5 g (EAVR) or 1.8 g (SAVR) were used for the vaccination trials. The fish were anesthetized in 0.01% benzocaine (Sigma) and then injected intramuscularly (I.M.) with 1 μg of either of the plasmids encoding VHSV G or with the plasmids with no transgene (Table 1) in 25 μl of saline solution (0.9% NaCl). At one week post-vaccination, the fish vaccinated with either pcDNA3-Mx or with pcDNA3-Mx-vhsG were split into two subgroups. One subgroup was injected intraperitoneally (I.P.) with 1 μg of Poly (I:C)/g fish in 50 μl of saline solution, and the second subgroup was injected with 50 μl of saline solution (Fig. 2). After vaccination, the fish were maintained in 120 L aerated aquaria supplied with recirculated water at 8–10 °C in a pathogen-free laboratory facility until challenge.
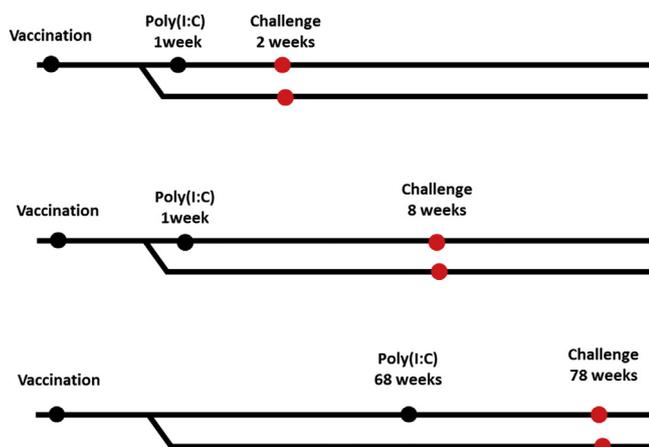

Fig. 2. Timelines of vaccination/challenge trials. (A) EAVR, (B) SAVR, (C) LAVR.

For evaluation of EAVR and SAVR, challenge with the virulent VHSV isolate DK3592b was performed at 2 and 8 weeks post-vaccination, respectively (Fig. 2). The fish were kept in aerated running fresh water in 8 L aquaria with 2–3 replicates of 25–33 fish per treatment. The challenge was carried out by immersion in static freshwater with an infectious dose of 2–3x10^5 TCID$_{50}$/ml of water. After 2 h, the water flow was re-established. During the following 3 weeks, temperature and mortality/survival were registered on a daily basis. Fish with evident clinical signs were euthanized with an overdose of benzocaine. Representative samples of dead/terminated fish were examined virologically from all aquaria as described earlier [16,36].

2.6. Evaluation of the LAVR

Outbreed all female rainbow trout (2–5 g) were divided into 5 groups, 4 of them injected I.M. with 1 μg of either of the plasmids applied in the first vaccination trial in 25 μl of saline solution, and one group injected with 25 μl of a saline solution. Each group contained 120 fish, which were maintained in 120 L aerated aquaria supplied with recirculated water at 8–10 °C in a pathogen-free laboratory facility. At 68 weeks post-vaccination, the groups of fish previously injected with saline solution, pcDNA3-Mx, or pcDNA3-Mx-vhsG were split into 2 subgroups. The fish of one subgroup were injected I.P. with 100 μl of saline solution, while the fish of the second subgroup were injected I.P. with 100 μg of Poly (I:C) in 100 μl of saline solution (Fig. 2). The fish injected with pcDNA3-CMV, and pcDNA3-CMV-vhsG were injected only with the saline solution. At this time, the weight of the fish was 40–70 g.

The challenge was performed at 78 weeks post-vaccination 10 weeks post-induction with Poly (I:C). The virus challenge was carried out as described above, with a virus titer of 3×10^4 TCID$_{50}$/ml in 3 replicate 8 L aquaria of 20 fish per treatment.

Temperature and mortality/survival were registered daily for 30 days. The fish with evident clinical signs were euthanized with an overdose of benzocaine. The relative percentage survival (RPS) was calculated: RPS = [1-(% mortality of immunized fish/% mortality of control fish)] × 100.

2.7. Animal experiments

All animal experiments were performed according to European and Danish rules for the use of experimental animals. The experiments were approved by Danish Animal Experiments Inspectorate under license No. 2014-15-0201-00379.

2.8. Statistical analysis

Statistically significant differences of the cumulative mortalities were performed using RStudio v. 1.0.143 [37,38] by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test [39], with $p < 0.05$ considered statistically significant.

3. Results

3.1. Evaluation of promoter activity in cell culture

EPC, HeLa, and FHs74 cell lines transfected with pcDNA3-CMV-EGFP expressed EGFP whether treated with Poly (I:C) or not. The intensity of fluorescence and the number of EGFP-positive EPC cells was higher than that of EPC cell cultures transfected with pcDNA3-Mx-EGFP (Fig. 3A, B, E, F, I, J).

EPC cell cultures transfected with pcDNA3-Mx-EGFP but not treated with Poly (I:C) showed only a few cells expressing EGFP with low intensity of fluorescence (Fig. 3C). When these cell cultures were treated with Poly (I:C) more positive cells with higher intensity of fluorescence were seen (Fig. 3D), indicating induction of the Mx promoter. The HeLa
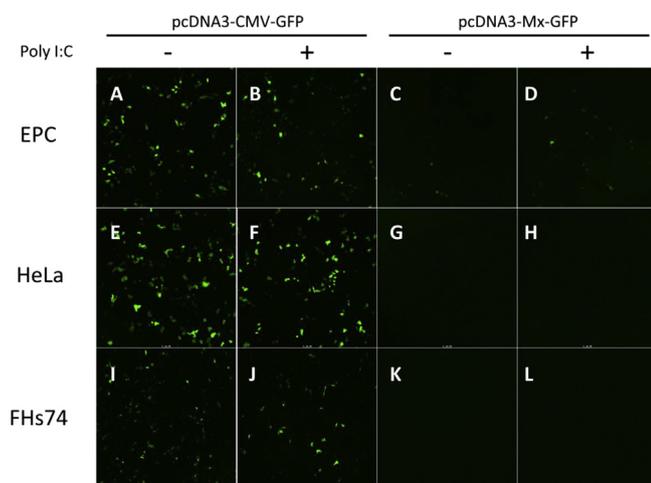
Fig. 3. **Expression of EGFP in transfected cell cultures. A**: EPC cells transfected with pcDNA3-CMV-EGFP untreated with Poly (I:C). **B**: EPC cells transfected with pcDNA3-CMV-EGFP treated with Poly (I:C). **C**: EPC cells transfected with pcDNA3-Mx-EGFP untreated with Poly (I:C). **D**: EPC cells transfected with pcDNA3-Mx-EGFP treated with Poly (I:C). **E**: HeLa cells transfected with pcDNA3-CMV-EGFP untreated with Poly (I:C). **F**: HeLa cells transfected with pcDNA3-CMV-EGFP treated with Poly (I:C). **G**: HeLa cells transfected with pcDNA3-Mx-EGFP untreated with Poly (I:C). **H**: HeLa cells transfected with pcDNA3-Mx-EGFP treated with Poly (I:C). **I**: FHs74 cells transfected with pcDNA3-CMV-EGFP untreated with Poly (I:C). **J**: FHs74 cells transfected with pcDNA3-CMV-EGFP treated with Poly (I:C). **K**: FHs74 cells transfected with pcDNA3-Mx-EGFP untreated with Poly (I:C). **L**: FHs74 cells transfected with pcDNA3-Mx-EGFP treated with Poly (I:C). The images were obtained by Leica TCS SP2 laser scanning confocal microscope. Images were processed by the Leica confocal software.

and FHs74 cell cultures transfected with pcDNA3-Mx-EGFP showed no expression of EGFP even when stimulated with Poly (I:C) (Fig. 3 G, H, K, L).

No fluorescence signal was observed in all cell lines transfected with pcDNA3-CMV and pcDNA3-EGFP (data not shown).

3.2. Evaluation of the EAVR and the SAVR

The challenge performed at 2 wpv showed that the DNA vaccine with the IFN-inducible Mx promoter (pcDNA3-Mx-vhsG) was able to induce protection consistently (RPS = 67.8) in all replicates when the fish were treated with Poly (I:C). Without Poly (I:C), protection was marginal, and although the fish vaccinated with the control plasmid (pcDNA3-Mx) treated with Poly (I:C) also showed slightly lower mortality than fish untreated with Poly (I:C), the combine protective effect was still lower than that provided by pcDNA3-Mx-vhsG + Poly (I:C) (Table 2, Fig. 4A). When immunization with pcDNA3-Mx-vhsG was evaluated in a challenge at 8 wpv, protection was independent of Poly (I:C) stimulation (Table 3, Fig. 4B). The reference DNA vaccine with CMV promoter (pcDNA3-CMV-vhsG) induced high protection at both 2 and 8 wpv, while the CMV-plasmids without the G gene did not induce any protection against VHS (Fig. 4). The vaccine encoding the IHNV G protein only was able to induce protection against VHS challenge at 2 wpv, indication that cross-protective mechanisms of innate immunity were involved at this time point, while adaptive/specific mechanisms increasingly provided protection at 8wpv where only the homologous vaccine encoding the VHSV G protein protected the fish, as reported earlier [34].

3.3. Evaluation of the LAVR

The challenge performed at 78 wpv showed that immunization with the reference DNA vaccine pcDNA3-CMV-vhsG induced long-term

Table 2
Protection by the DNA vaccines against VHSV challenge at 2 weeks post-vaccination.

Groups	Induction 1wpv	Cumulative Mortality (%)	Mortality in replicate aquaria[b] (%)	RPS[a]
pcDNA3-CMV	Saline	97.3	100; 96; 96	0.0
pcDNA3-CMV-vhsG	Saline	0.0	0.0; 0.0; 0.0	100
pcDNA3-Mx	Poly (I:C)	76.3	80; 68; 81	21.5
pcDNA3-Mx	Saline	96	96; 96	1.3
pcDNA3-Mx-vhsG	Poly (I:C)	28.6	28; 38; 20	67.8
pcDNA3-Mx-vhsG	Saline	66.3	69; 58; 72	31.8
pcDNA3.1-CMV-ihnG	Saline	0.0	0.0; 0.0; 0.0	100

[a] RPS: Relative percentage survival.
[b] Replicate aquaria with 24–27 fish in each.

protection against VHS, while the DNA vaccine with the Mx promoter did not elicit any protection at this time, independent of exposure to Poly (I:C) (Table 4). On average, the fish immunized with pcDNA3-Mx-vhsG showed a lower cumulative mortality than the group given plasmid without insert, but this difference was not significant. A relatively high variability in mortality was evident between replicate aquaria in some of the groups, possibly reflecting that immersion challenge of relatively larger fish (50–70 g) is more sensitive to small tank variations compared to challenge trials with small (3–10 g) fish. (Fig. 5).

4. Discussion

In this study, we performed a functional characterization of a DNA vaccine with an IFN-inducible Mx promoter derived from rainbow trout. This involved an analysis of the expression activity in both human and fish cell lines, and an evaluation of the protection during the three antiviral immune response phases. These phases, EAVR, SAVR, and LAVR were classified according to the timing and nature of the presumed protective mechanisms [10,18].

The *in vitro* results showed that the DNA vaccine plasmid with the IFN-inducible Mx promoter from rainbow trout was able to drive the expression of EGFP in transfected cells of the EPC fish cell line, but not in the tested human cell lines, while the vaccine construct with the CMV promoter drove strong expression of EGFP in both fish and human cell lines. The expression activity of the Mx promoter in the EPC cells was partly IFN-dependent as it was increased by Poly (I:C) treatment. Similar results were obtained using DNA vaccines encoding for the VHSV glycoprotein (data not shown). The transcription factors involved in the intracellular induction pathway for IFN-stimulated genes (ISGs), as well as Interferon-stimulated response elements (ISREs), are well conserved between teleosts and higher vertebrates [27,40]. Therefore, it seems unlikely that lack of functionality of the trout Mx promoter in the HeLa and FHs74 cell lines was due to a lack of cross-reactivity at the level of the transcription factors or ISREs. However, considering that the IFN promoter sequences in human and trout are different [27,41–43], and since EPC cells were grown at 15 °C, while HeLa and FHs74 cells were grown at 37 °C, one possibility could be that temperature-dependent conformation of the Mx promoter region interfered with activation of transcription in human cell lines Although there are currently no concerns about consumers eating DNA-vaccinated fish each vaccine construct will be evaluated on an individual basis [44] and the lack of activity of the trout Mx promoter in human cells could make it attractive for some fish DNA vaccines.

Since the initial reports of the high efficacy of the CMV promoter-based DNA vaccines against rhabdoviral fish diseases [35,45], considerable efforts have been made to identify alternative promoters that may be more appropriate for driving expression in aquacultured fish [23,24,28]. However, these studies have either been based on quantitative expression analysis *in vitro* in transfected cell cultures and/or a single challenge test *in vivo*, typically 4–6 weeks post-vaccination. Our
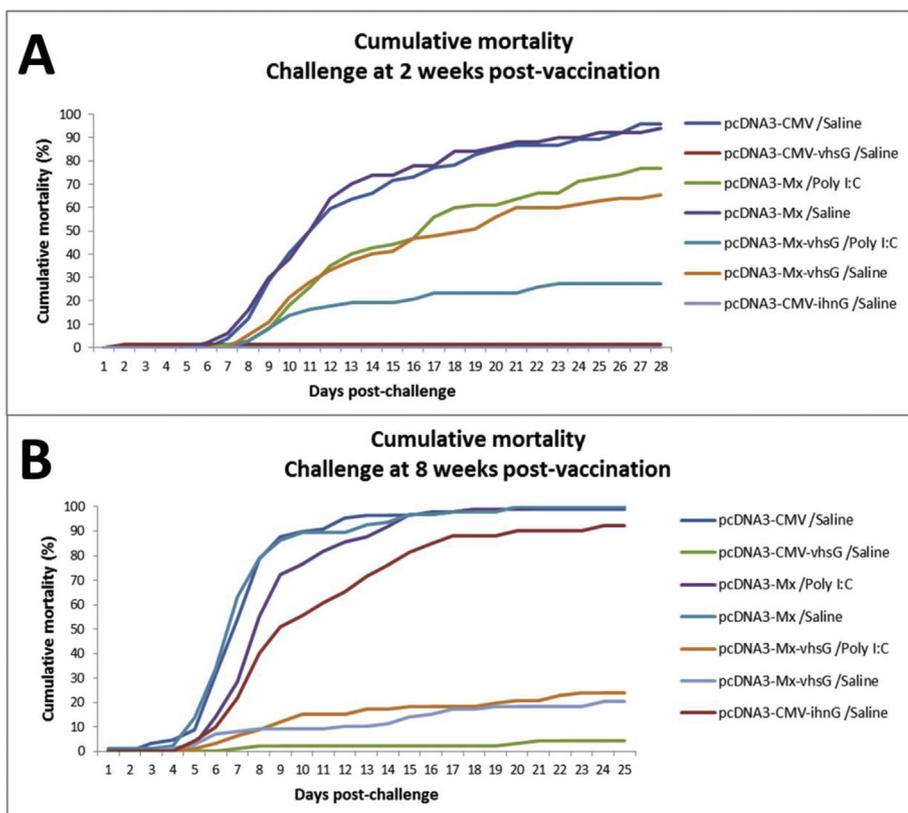
Fig. 4. Development of mortality in groups of DNA vaccinated rainbow trout following immersion challenge with VHSV at 2 and 8 weeks post-vaccination. A: challenge performed at 2 weeks post-vaccination. B: challenge performed at 8 weeks post-vaccination. The legend indicates the fish groups given I.M. injections of DNA plasmid followed by I.P. injections of saline or Poly (I:C) one week later.

Table 3
Protection induced by the DNA vaccines against VHSV challenge at 8 weeks post-vaccination.

Groups	Induction 1wpv	Cumulative Mortality (%)	Mortality in replicate aquaria[b] (%)	RPS[a]
pcDNA3-CMV	Saline	98.9	100; 100; 96.8	0.0
pcDNA3-CMV-vhsG	Saline	4.2	3.3; 0.0; 9.4	95.7
pcDNA3-Mx	Poly (I:C)	100	100; 100; 100	−1.1
pcDNA3-Mx	Saline	100	100; 100; 100	−1.1
pcDNA3-Mx-vhsG	Poly (I:C)	23.9	25.8; 23.3; 22.6	75.8
pcDNA3-Mx-vhsG	Saline	20.4	23.5; 19.3; 18.2	79.4
pcDNA3.1-CMV-ihnG	Saline	92.4	89.7; 93.5; 93.8	6.7

[a] RPS: Relative percentage survival.
[b] Replicate aquaria with 28–34 fish in each.

Table 4
Protection induced by the DNA vaccines against VHSV challenge at 78 weeks post-vaccination.

Groups	Induction 68 wpv	Cumulative Mortality (%)	Mortality in replicate aquaria[b] (%)	RPS[a]
pcDNA3-CMV	Saline	90.41	88.9; 88.2; 94.1	0.0
pcDNA3-CMV-vhsG	Saline	17.6	11.8; 35.3; 5.9	80.5
pcDNA3-Mx[c]	Poly (I:C)	79.4	50.0; 100.0; 88.2	12.2
pcDNA3-Mx	Saline	86.3	70.6; 94.1; 94.1	4.6
pcDNA3-Mx-vhsG[c]	Poly (I:C)	76.6	94.4; 88.2; 47.0	15.3
pcDNA3-Mx-vhsG[c]	Saline	65.2	72.2; 47.0; 76.5	27.8

[a] RPS: Relative percentage survival.
[b] Replicate aquaria with 17–18 fish in each.
[c] High variability among replicates.

results demonstrate that to evaluate the applied potential of a DNA vaccine, it is necessary to examine protection against disease *in vivo* in all three phases of the immune response to the vaccine.

Back in 2003, Alonso et al. reported rather low protection (RPS = 16) against IHN in 0.4 g rainbow trout fry following DNA vaccination with a trout Mx promoter IHNV-G gene construct, resembling that used for VHSV glycoprotein in the current study. However, since the challenge was performed at one-month post-vaccination with fish kept at 13 °C, the authors could not exclude that the observed protection with the CMV promoter reference construct might be due to innate mechanisms, i.e. the EARV (24). Indeed, in the present study we observed low protection at the presumed time of EARV (RPS = 31.8), in pcDNA3-Mx-vhsG vaccinated fish. Injection of Poly (I:C) one week before challenge highly improved the protection obtained (RPS = 67.8), likely due to Poly (I:C) induced IFN upregulating the expression of VHSV G as well as directly contributing to the protection seen [24,26,28]. The high protection (RPS = 75.8) against VHSV challenge at 8 wpv in fish given the pcDNA3-Mx-vhsG, even without Poly (I:C) stimulation, suggested that the baseline activity of the Mx promoter (as visualized in the *in vitro* evaluation) was sufficient to trigger a protective SAVR. However, since no heterologous pcDNA3-Mx-ihnG was included, we cannot fully exclude that EARV mechanisms might have contributed to the protection [13,14]. In mammals, IFN and related innate antiviral immune mechanisms are important not only for protection at the early stage of viral infection but also for paving the way for an effective adaptive response [46,47]. Assuming that this also occurs in salmonid fish, as indicated by an adjuvant effect of type I IFNs [46,48], the combination of poor early protection with high (assumed) -specific protection in fish vaccinated with pcDNA3-Mx-vhsG suggests that there may be qualitative and/or quantitative differences between the IFN response elements activating the EAVR mechanisms involved in protection and the ones promoting an efficient induction of SAVR/LAVR. Also, there may be distinct requirements to reach a protective LAVR on top of, or separate to, those needed for a protective SARV. Further experiments including examination of the specificity of the protection induced by the pcDNA3-Mx-vhsG vaccine at 8 wpv along with immune gene expression analysis are needed to resolve this aspect. Interestingly, Chang et al. (2015) showed that while IFNa, IFNb, or
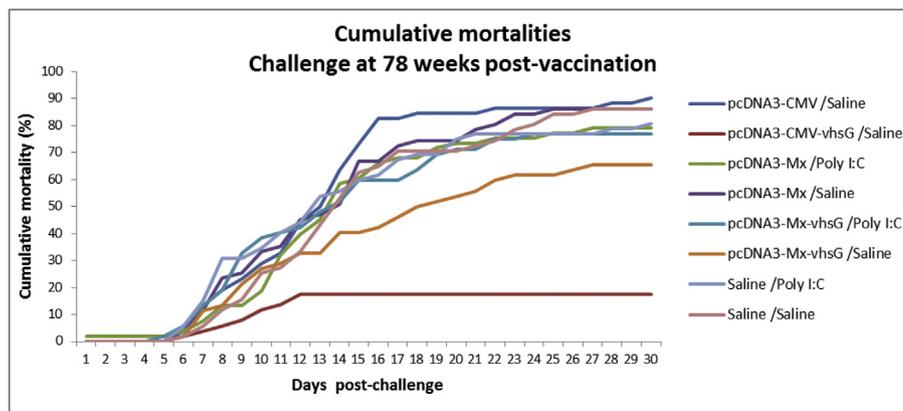
Fig. 5. Development of mortality in groups of DNA vaccinated rainbow trout following immersion challenge with VHSV at 78 weeks post-vaccination. The legend indicates the fish groups given I.M. injections of DNA plasmid followed by I.P. injections of saline or Poly (I:C) 68 weeks later.

IFNc plasmid constructs all had an adjuvant effect, promoting generation of a protective adaptive immune response to a co-injected DNA vaccine against Infectious salmon anaemia (ISA) in Atlantic salmon, only IFNc was able to provide protection against this disease without needing the expression of the viral antigen [46].

The protective effect at 78 wpv reported here for the pcDNA3-CMV-vhsG vaccinated fish along with earlier observations by Kurath et al. demonstrating immunity to IHN 2 years post-vaccination [18] suggests that the salmonid rhabdovirus G-gene DNA vaccines with CMV-promoter driven expression induce long lasting immunity covering the typical 2–3-year lifespan for cultured rainbow trout. However, such long term studies have not been conducted for other fish DNA vaccines, and a similar duration of immunity may not be expected. For the Mx promoter DNA vaccine construct tested here, our initial hypothesis was that without Poly (I:C) or IFN stimulation, no or low levels of vaccine antigen expression would occur in fish vaccinated with pcDNA3-Mx-vhsG. In that case, elimination of cells harboring the plasmid by the local inflammatory response, described in fish vaccinated with pcDNA3-CMV-vhsG [29], would be less likely to occur or at least delayed. This could extend the lifetime of the vaccine in the fish and possibly allow post-vaccination management of the fish immune status by internal or external IFN stimulation. However, even with Poly (I:C) stimulation 10 weeks earlier, vaccination with pcDNA3-Mx-vhsG failed to protect the fish against VHS. It remains to be determined whether this was due to elimination of transfected cells before the Poly (I:C) stimulation, or because the baseline expression level of the antigen by the Mx promoter was insufficient to induce long-term memory. In applied terms, future research should address whether the fish specificity of the trout Mx promoter could be combined with the high expression capacity of other promoters by designing hybrid promoters as previously attempted by Martinez-Lopez et al. (2013) [28]. Also, it must be analyzed whether the trout Mx promoter might increase the chance of integration by homologous recombination in vaccinated rainbow trout. Since the trout Mx promoter also worked in cyprinid cells, this issue could be addressed by the use of heterologous fish promoters when designing DNA vaccines for a particular fish species.

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