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

3

4 Dagoberto Sepúlveda^{a, e}, Ellen Lorenzen^a, Jesper Skou Rasmussen^a, Katja Einer-Jensen^b,
5 Bertrand Collet^{c, #}, C.J. Secombes^d, Niels Lorenzen^{a, e}

6 ^a *Department of Animal Science, Aarhus University, Denmark*

7 ^b *Qiagen, Aarhus, Denmark*

8 ^c *Marine Scotland, Aberdeen, United Kingdom*

9 ^d *School of Biological Sciences, University of Aberdeen, Aberdeen, United Kingdom*

10 ^e *Technical University of Denmark*

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19 [#] Present address: INRA, Virologie et Immunologie Moléculaires, Université Paris-Saclay, Jouy-
20 en-Josas, France.

21 **ABSTRACT**

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

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

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

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

45 In conclusion, the results revealed a more fish host dependent activity of the trout Mx
46 promoter compared to the traditionally used cross species-active CMV promoter, but

47 improvements will be needed for its application in DNA vaccines to ensure long term
48 protection.

49 **INTRODUCTION**

50 Viral haemorrhagic septicaemia virus (VHSV), a member of the *Rhabdoviridae* family [1],
51 causes a severe disease (VHS) in wild and farmed fish worldwide [2]. Several vaccination
52 strategies have been tested against VHS, including inactivated virus vaccines, live-attenuated
53 virus vaccines, and recombinant vaccines. However, due to low efficacy, high cost per dose, or
54 safety limitations, there is currently no commercially available vaccine against VHS [3-7]. In
55 1998, the evaluation of a DNA vaccine as an alternative immunization strategy against VHS
56 revealed the establishment of highly protective immunity [8]. Since then, several studies have
57 examined the protection induced by DNA vaccination against VHS and the related disease IHN
58 (infectious haematopoietic necrosis) under different experimental conditions, with promising
59 results [9, 10]. The DNA vaccines typically include an eukaryotic expression plasmid vector
60 encoding the viral surface glycoprotein (G), under the control of the human cytomegalovirus
61 promoter (CMV). The immunological protection follows a sequential three-phase scenario [10],
62 which involves:

- 63 (i) *The early antiviral response (EAVR)*, which comprise cross-reactive protection
64 associated with innate antiviral immune mechanisms. This protective phase starts
65 shortly (within days) after the intramuscular injection of the DNA vaccine but only
66 lasts for a few weeks. It is characterized by up-regulating the expression of
67 interferon type I (IFN I), and consequently also increased transcription of multiple
68 interferon-stimulated genes (ISG) [11-15].
- 69 (ii) *The specific antiviral response (SAVR)*, which starts a few weeks after vaccination,
70 when cross-protection against heterologous virus fades away and adaptive
71 immunity characterized by neutralizing antibodies and cytotoxic T lymphocytes
72 (CTL) appear [16-18].

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

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

84 Example of fish-derived promoters tested in DNA vaccines are the Interferon regulatory factor
85 1A (IRF1A) promoter, the Mx1 promoter and the carp β -actin (AE6) promoter [23, 24, 26-28].
86 Some of the examined DNA vaccines with alternative promoters have shown potential in terms
87 of inducing protective immunity [23, 24]. However, the reports published so far have not taken
88 the time-course scenario of the protective mechanisms into account considering both
89 specificity and duration of protection. The time-course study is important from a practical point
90 of view, where the ability of the vaccine to induce a fast, efficient, and long-lasting protection,
91 is essential.

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

97 This work aimed to analyze the capacity of a VHSV glycoprotein DNA vaccine with an IFN-
98 inducible trout-derived Mx 1 promoter to induce EAVR, SAVR, and LAVR in rainbow trout

99 fingerlings. To address the host range aspect of promoter activity, we also compared
100 expression of the recombinant protein in transfected cell lines derived from fish and humans.

101

102 **MATERIALS AND METHODS**

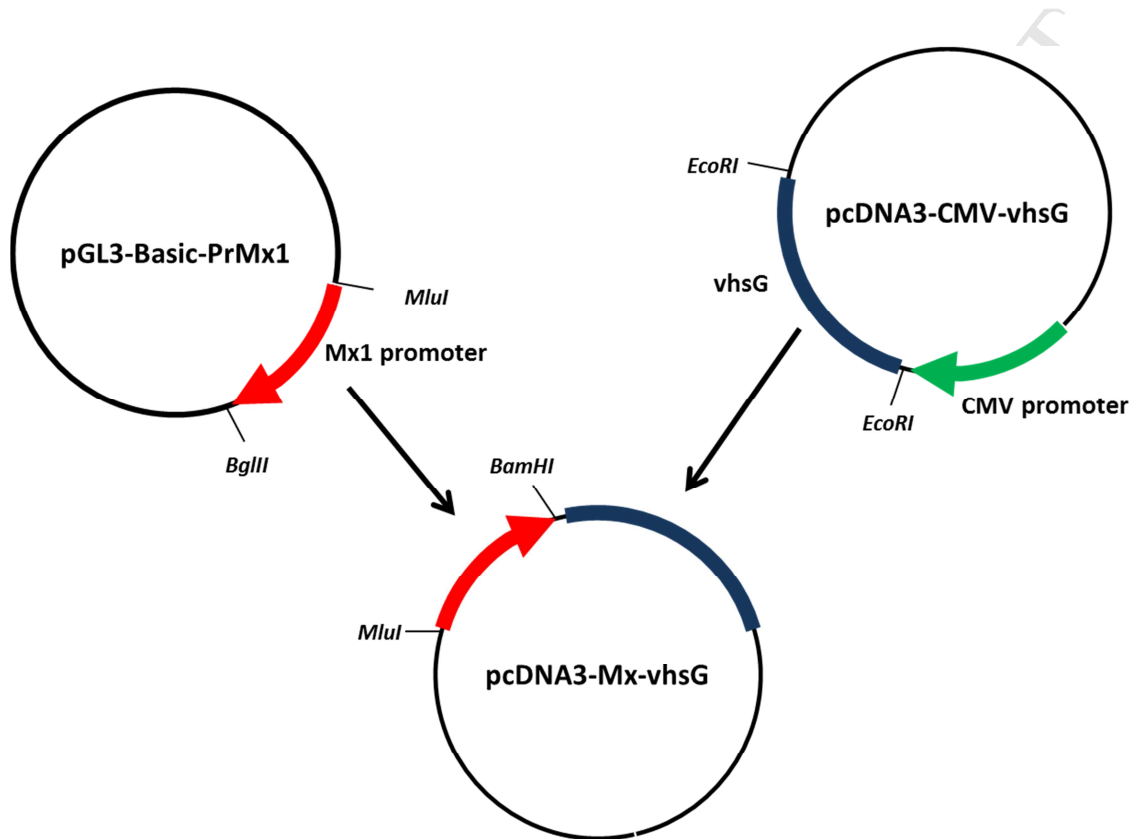
103

104 **Cell lines.** EPC cells (epithelioma papulosum cyprinid) [30], BF2 cells (bluegill fry fibroblast)
105 [31], HeLa cells (ATCC[®]CCL-2), and FHS74 (ATCC[®]CCL241) [32] were used in this work. BF2
106 and HeLa cells were maintained in minimum essential media (MEM), EPC cells in RPMI 1640
107 medium and FHS74 cells in Hybri-Care Medium (ATCC[®] 46-X[™]) supplemented with 30 ng/ml
108 epidermal growth factor (EGF). All media were supplemented with 10% fetal bovine serum
109 (FBS), 100 U/ml of Penicillin and 100 µg/ml of Streptomycin. EPC and BF2 were maintained at
110 15°C, while HeLa and FHS74 were maintained at 37°C.

111 **Virus.** A low passaged VHSV isolate (DK3592b) was propagated by inoculating freshly
112 passaged BF2 cells with a low MOI (multiplicity of infection). The infected cell cultures were
113 maintained at 15°C. When a complete cytopathic effect was observed, the medium was
114 centrifuged at 5,000 x g for 15 min at 4°C. The supernatant was collected and the titer of the
115 virus was determined using the method of 50% tissue culture infective doses (TCID₅₀) per ml
116 in BF2 cells [33]. Virus batches were stored in aliquots at -80°C.

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

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



129

130

131 **Figure 1. Schematic outline of the construction of the DNA vaccines with the IFN-**
 132 **inducible Mx promoter.** The Mx1 promoter was excised from pGL3-Basic-PrMx1 as a 600 bp
 133 *MluI*-*BglII* fragment and inserted into pcDNA3-CMV digested with *MluI* and *BamHI*. The IFN-
 134 regulated expression vector encoding the VHSV glycoprotein (pcDNA3-Mx-vhsG) was obtained
 135 by excising the VHSV glycoprotein gene (vhsG) from pcDNA3-vhsG [35] as a 1576 bp *EcoRI*-
 136 *EcoRI* fragment and ligating it into pcDNA3-Mx digested with *EcoRI*. The plasmid pcDNA3-vhsG
 137 was denominated pcDNA3-CMV-vhsG in this work. The glycoprotein gene was derived from
 138 VHSV isolate DK3592b. For the *in vitro* evaluation, the glycoprotein coding sequence from both
 139 pcDNA3-CMV-vhsG and pcDNA3-Mx-vhsG were excised with *EcoRI* and *NotI* and replaced with
 140 the EGFP gene, which was excised from pEGFP-N1 (Clontech) using the same endonucleases.
 141 The empty vectors with CMV and Mx promoters (pcDNA3-CMV and pcDNA3-Mx) were used as
 142 negative controls.

143

144 **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

145

146 **Evaluation of promoter activity in cell culture**

147 EPC, FHs74 and HeLa cells were seeded in 24-well plates (2×10^5 cells/well) one day before
 148 transfection. The transfection was performed with the plasmids: pcDNA3-CMV, pcDNA3-CMV-
 149 EGFP, pcDNA3-Mx, and pcDNA3-Mx-EGFP. Briefly, 0.75 μg of either construct was mixed with
 150 2 μg of PEI (Polyethylenimine) in 300 μl of medium without serum, and incubated for 20 min
 151 at room temperature. The medium from each well was removed and replaced by the
 152 transfection mix. At 6 h post-transfection for the EPC cells and 4 h for the HeLa and the FHs74
 153 cells, the cell culture medium was replaced by fresh medium with or without Poly(I:C)
 154 (P0913, Sigma) (a potent inducer of IFN) to a final concentration of 10 $\mu\text{g}/\text{ml}$.

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

159

160 **Evaluation of EAVR and SAVR**

161 Outbreed, all female rainbow trout with average weight of 3.5 g (EAVR) or 1.8 g (SAVR) were
 162 used for the vaccination trials. The fish were anesthetized in 0.01% benzocaine (Sigma) and
 163 then injected intramuscularly (I.M.) with 1 μg of either of the plasmids encoding VHSV G or

164 with the plasmids with no transgene (Table 1) in 25 µl of saline solution (0.9% NaCl). At one
165 week post-vaccination, the fish vaccinated with either pcDNA3-Mx or with pcDNA3-Mx-vhsG
166 were split into two subgroups. One subgroup was injected intraperitoneally (I.P.) with 1 µg of
167 Poly(I:C)/g fish in 50 µl of saline solution, and the second subgroup was injected with 50 µl of
168 saline solution (Fig. 2). After vaccination, the fish were maintained in 120 L aerated aquaria
169 supplied with recirculated water at 8-10°C in a pathogen-free laboratory facility until
170 challenge.

171 For evaluation of EAVR and SAVR, challenge with the virulent VHSV isolate DK3592b was
172 performed at 2 and 8 weeks post-vaccination, respectively (Fig. 2). The fish were kept in
173 aerated running fresh water in 8 L aquaria with 2-3 replicates of 25-33 fish per treatment. The
174 challenge was carried out by immersion in static freshwater with an infectious dose of $2-3 \times 10^5$
175 TCID₅₀ /ml of water. After 2 h, the water flow was re-established. During the following 3
176 weeks, temperature and mortality/survival were registered on a daily basis. Fish with evident
177 clinical signs were euthanized with an overdose of benzocaine. Representative samples of
178 dead/terminated fish were examined virologically from all aquaria as described earlier [16,
179 36].

180

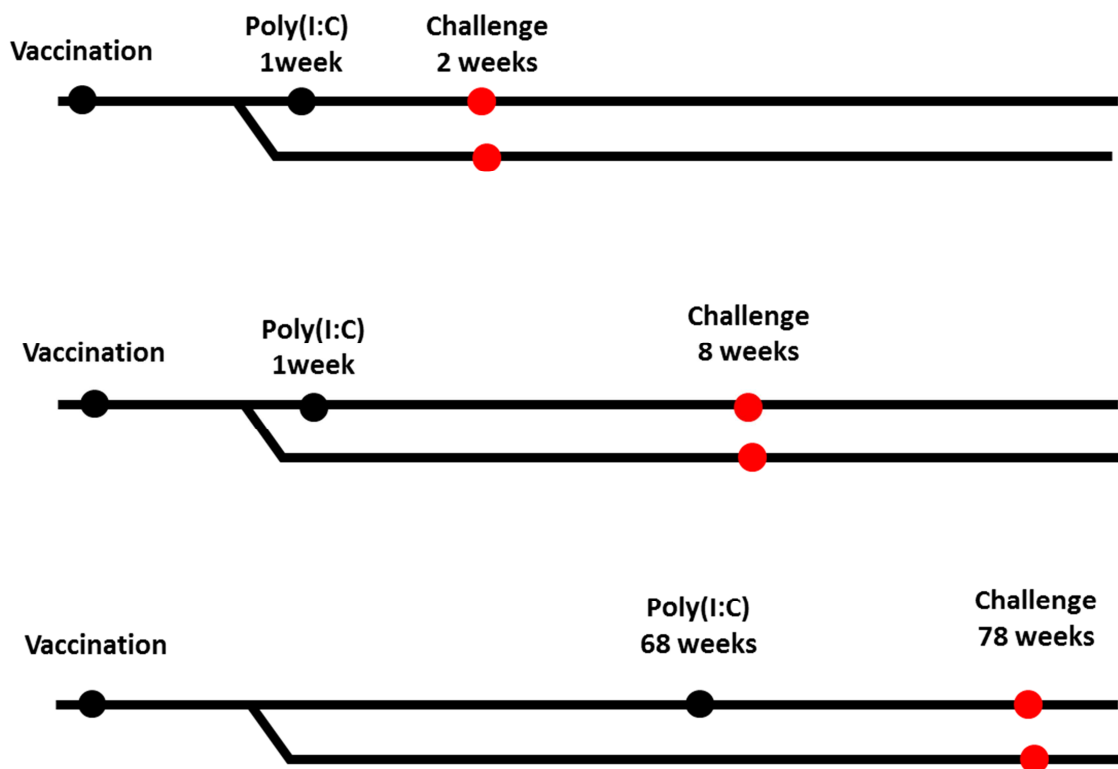
181 **Evaluation of the LAVR**

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

190 2). The fish injected with pcDNA3-CMV, and pcDNA3-CMV-vhsG were injected only with the
 191 saline solution. At this time, the weight of the fish was 40-70 g.

192 The challenge was performed at 78 weeks post-vaccination (10 weeks post-induction with
 193 Poly(I:C). The virus challenge was carried out as described above, with a virus titer of 3×10^4
 194 $TCID_{50}$ /ml in 3 replicate 8 L aquaria of 20 fish per treatment.

195 Temperature and mortality/survival were registered daily for 30 days. The fish with evident
 196 clinical signs were euthanized with an overdose of benzocaine. The relative percentage survival
 197 (RPS) was calculated: $RPS = [1 - (\% \text{ mortality of immunized fish} / \% \text{ mortality of control}$
 198 $\text{fish})] \times 100$.



199

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

201

202

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

206 **Statistical analysis**

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

210

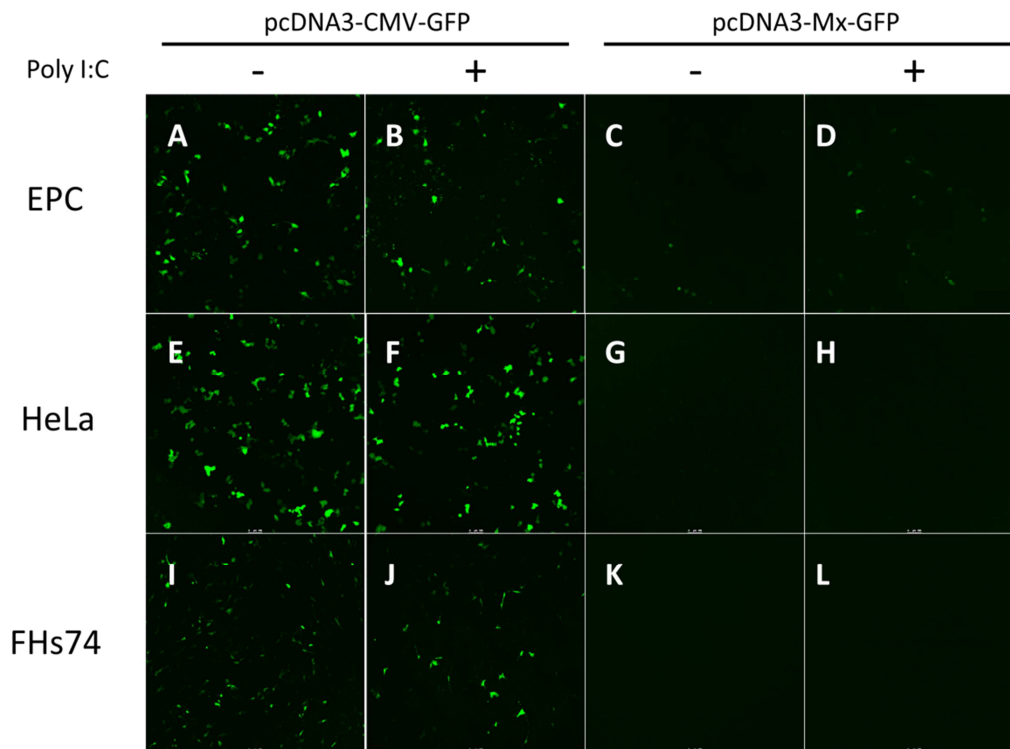
211 **RESULTS**

212 **Evaluation of promoter activity in cell culture**

213 EPC, HeLa, and FHS74 cell lines transfected with pcDNA3-CMV-EGFP expressed EGFP whether
214 treated with Poly(I:C) or not. The intensity of fluorescence and the number of EGFP-positive
215 EPC cells was higher than that of EPC cell cultures transfected with pcDNA3-Mx-EGFP (Fig. 3A,
216 B, E, F, I, J).

217 EPC cell cultures transfected with pcDNA3-Mx-EGFP but not treated with Poly(I:C) showed only
218 a few cells expressing EGFP with low intensity of fluorescence (Fig. 3C). When these cell
219 cultures were treated with Poly(I:C) more positive cells with higher intensity of fluorescence
220 were seen (Fig. 3D), indicating induction of the Mx promoter. The HeLa and FHS74 cell cultures
221 transfected with pcDNA3-Mx-EGFP showed no expression of EGFP even when stimulated with
222 Poly(I:C) (Fig. 3 G, H, K, L).

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



225

226 **Figure 3. Expression of EGFP in transfected cell cultures.** **A:** EPC cells transfected with
 227 pcDNA3-CMV-EGFP untreated with Poly(I:C). **B:** EPC cells transfected with pcDNA3-CMV-EGFP
 228 treated with Poly(I:C). **C:** EPC cells transfected with pcDNA3-Mx-EGFP untreated with
 229 Poly(I:C). **D:** EPC cells transfected with pcDNA3-Mx-EGFP treated with Poly(I:C). **E:** HeLa cells
 230 transfected with pcDNA3-CMV-EGFP untreated with Poly(I:C). **F:** HeLa cells transfected with
 231 pcDNA3-CMV-EGFP treated with Poly(I:C). **G:** HeLa cells transfected with pcDNA3-Mx-EGFP
 232 untreated with Poly(I:C). **H:** HeLa cells transfected with pcDNA3-Mx-EGFP treated with
 233 Poly(I:C). **I:** FHs74 cells transfected with pcDNA3-CMV-EGFP untreated with Poly(I:C). **J:**
 234 FHs74 cells transfected with pcDNA3-CMV-EGFP treated with Poly(I:C). **K:** FHs74 cells
 235 transfected with pcDNA3-Mx-EGFP untreated with Poly(I:C). **L:** FHs74 cells transfected with
 236 pcDNA3-Mx-EGFP treated with Poly(I:C). The images were obtained by Leica TCS SP2 laser
 237 scanning confocal microscope. Images were processed by the Leica confocal software.

238

239 Evaluation of the EAVR and the SAVR

240 The challenge performed at 2 wpv showed that the DNA vaccine with the IFN-inducible Mx
 241 promoter (pcDNA3-Mx-vhsG) was able to induce protection consistently (RPS=67.8) in all
 242 replicates when the fish were treated with Poly(I:C). Without Poly(I:C), protection was
 243 marginal, and although the fish vaccinated with the control plasmid (pcDNA3-Mx) treated with
 244 Poly(I:C) also showed slightly lower mortality than fish untreated with Poly(I:C), the combine

245 protective effect was still lower than that provided by pcDNA3-Mx-vhsG + Poly(I:C) (Table 2,
 246 Fig. 4A). When immunization with pcDNA3-Mx-vhsG was evaluated in a challenge at 8 wpv,
 247 protection was independent of Poly(I:C) stimulation (Table 3, Fig. 4B). The reference DNA
 248 vaccine with CMV promoter (pcDNA3-CMV-vhsG) induced high protection at both 2 and 8 wpv,
 249 while the CMV-plasmids without the G gene did not induce any protection against VHS (Fig. 4).
 250 The vaccine encoding the IHNV G protein only was able to induce protection against VHS
 251 challenge at 2 wpv, indication that cross-protective mechanisms of innate immunity were
 252 involved at this time point, while adaptive/specific mechanisms increasingly provided
 253 protection at 8wpv where only the homologous vaccine encoding the VHSV G protein protected
 254 the fish, as reported earlier [34].

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

Groups	Induction 1wpv	Cumulative Mortality(%)	Mortality in replicate aquaria** (%)	RPS*
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

256 *RPS: Relative percentage survival.

257 ** Replicate aquaria with 24-27 fish in each.

258

259

260

261

262

263

264

265 **Table 3:** Protection induced by the DNA vaccines against VHSV challenge at 8 weeks post-
 266 vaccination

Groups	Induction 1wpv	Cumulative Mortality(%)	Mortality in replicate aquaria** (%)	RPS*
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

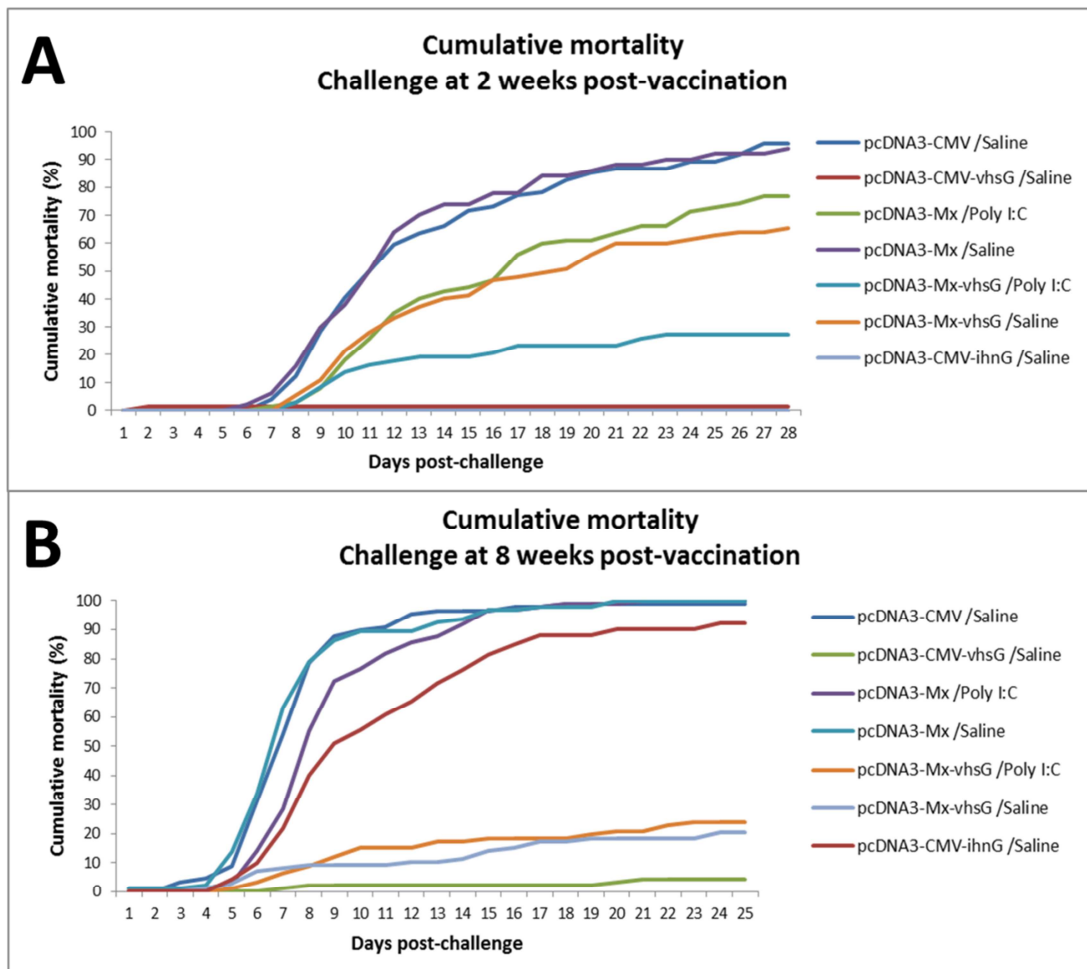
267 *RPS: Relative percentage survival.

268 **Replicate aquaria with 28-34 fish in each.

269

270

271



272

273

274 **Figure 4.** Development of mortality in groups of DNA vaccinated rainbow trout following
 275 immersion challenge with VHSV at 2 and 8 weeks post-vaccination. **A:** challenge performed at
 276 2 weeks post-vaccination. **B:** challenge performed at 8 weeks post-vaccination. The legend
 277 indicates the fish groups given I.M. injections of DNA plasmid followed by I.P. injections of
 278 saline or Poly(I:C) one week later.

279

280 Evaluation of the LAVR

281 The challenge performed at 78 wpv showed that immunization with the reference DNA vaccine
 282 pcDNA3-CMV-vhsG induced long-term protection against VHS, while the DNA vaccine with the
 283 Mx promoter did not elicit any protection at this time, independent of exposure to Poly(I:C)
 284 (Table 4). On average, the fish immunized with pcDNA3-Mx-vhsG showed a lower cumulative

285 mortality than the group given plasmid without insert, but this difference was not significant. A
 286 relatively high variability in mortality was evident between replicate aquaria in some of the
 287 groups, possibly reflecting that immersion challenge of relatively larger fish (50-70g) is more
 288 sensitive to small tank variations compared to challenge trials with small (3-10g) fish. (Fig. 5).

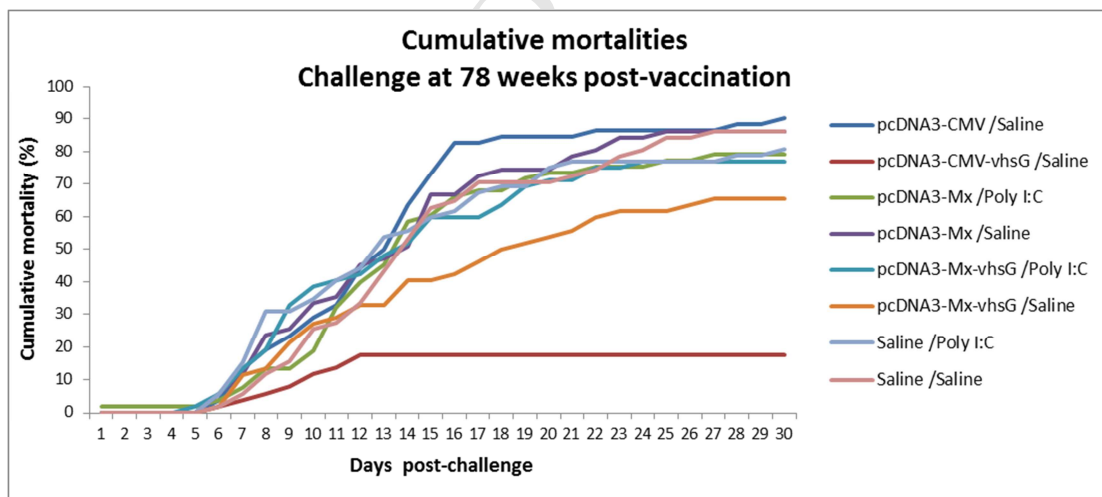
289 **Table 4.** Protection induced by the DNA vaccines against VHSV challenge at 78 weeks post-
 290 vaccination

Groups	Inductio n 68 wpv	Cumulative Mortality (%)	Mortality in replicate aquaria** (%)	RPS*
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	80.48
pcDNA3-Mx***	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***	Poly(I:C)	76.6	94.4; 88.2; 47.0	15.3
pcDNA3-Mx-vhsG***	Saline	65.2	72.2; 47.0; 76.5	27.8

291 *RPS: Relative percentage survival

292 **Replicate aquaria with 17-18 fish in each.

293 *** High variability among replicates



294

295 **Figure 5.** Development of mortality in groups of DNA vaccinated rainbow trout following
 296 immersion challenge with VHSV at 78 weeks post-vaccination. The legend indicates the fish
 297 groups given I.M. injections of DNA plasmid followed by I.P. injections of saline or Poly(I:C) 68
 298 weeks later.

299

300 **DISCUSSION**

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

306 The *in vitro* results showed that the DNA vaccine plasmid with the IFN-inducible Mx promoter
307 from rainbow trout was able to drive the expression of EGFP in transfected cells of the EPC fish
308 cell line, but not in the tested human cell lines, while the vaccine construct with the CMV
309 promoter drove strong expression of EGFP in both fish and human cell lines. The expression
310 activity of the Mx promoter in the EPC cells was partly IFN-dependent as it was increased by
311 Poly(I:C) treatment. Similar results were obtained using DNA vaccines encoding for the VHSV
312 glycoprotein (data not shown). The transcription factors involved in the intracellular induction
313 pathway for IFN-stimulated genes (ISGs), as well as Interferon-stimulated response elements
314 (ISREs), are well conserved between teleosts and higher vertebrates [27, 40]. Therefore, it
315 seems unlikely that lack of functionality of the trout Mx promoter in the HeLa and FHS74 cell
316 lines was due to a lack of cross-reactivity at the level of the transcription factors or ISREs.
317 However, considering that the IFN promoter sequences in human and trout are different [27,
318 41-43], and since EPC cells were grown at 15°C, while HeLa and FHS74 cells were grown at
319 37°C, one possibility could be that temperature-dependent conformation of the Mx promoter
320 region interfered with activation of transcription in human cell lines... Although there are
321 currently no concerns about consumers eating DNA-vaccinated fish each vaccine construct will
322 be evaluated on an individual basis [44] and the lack of activity of the trout Mx promoter in
323 human cells could make it attractive for some fish DNA vaccines.

324 Since the initial reports of the high efficacy of the CMV promoter-based DNA vaccines against
325 rhabdoviral fish diseases [35, 45], considerable efforts have been made to identify alternative

326 promoters that may be more appropriate for driving expression in aquacultured fish [23, 24,
327 28]. However, these studies have either been based on quantitative expression analysis *in*
328 *vitro* in transfected cell cultures and/ or a single challenge test *in vivo*, typically 4-6 weeks
329 post-vaccination. Our results demonstrate that to evaluate the applied potential of a DNA
330 vaccine, it is necessary to examine protection against disease *in vivo* in all three phases of the
331 immune response to the vaccine.

332 Back in 2003, Alonso et al. reported rather low protection (RPS=16) against IHN in 0.4 g
333 rainbow trout fry following DNA vaccination with a trout Mx promoter IHNV-G gene construct,
334 resembling that used for VHSV glycoprotein in the current study. However, since the challenge
335 was performed at one-month post-vaccination with fish kept at 13°C, the authors could not
336 exclude that the observed protection with the CMV promoter reference construct might be due
337 to innate mechanisms, i.e. the EARV (24). Indeed, in the present study we observed low
338 protection at the presumed time of EARV (RPS=31.8), in pcDNA3-Mx-vhsG vaccinated fish.
339 Injection of Poly(I:C) one week before challenge highly improved the protection obtained
340 (RPS=67.8), likely due to Poly(I:C) induced IFN upregulating the expression of VHSV G as well
341 as directly contributing to the protection seen [24, 26, 28]. The high protection (RPS=75.8)
342 against VHSV challenge at 8 wpv in fish given the pcDNA3-Mx-vhsG, even without Poly(I:C)
343 stimulation, suggested that the baseline activity of the Mx promoter (as visualized in the *in*
344 *vitro* evaluation) was sufficient to trigger a protective SAVR. However, since no heterologous
345 pcDNA3-Mx-ihnG was included, we cannot fully exclude that EARV mechanisms might have
346 contributed to the protection. [13, 14]. In mammals, IFN and related innate antiviral immune
347 mechanisms are important not only for protection at the early stage of viral infection but also
348 for paving the way for an effective adaptive response [46, 47]. Assuming that this also occurs
349 in salmonid fish, as indicated by an adjuvant effect of type I IFNs [46, 48], the combination of
350 poor early protection with high (assumed) - specific protection in fish vaccinated with pcDNA3-
351 Mx-vhsG suggests that there may be qualitative and/or quantitative differences between the
352 IFN response elements activating the EARV mechanisms involved in protection and the ones

353 promoting an efficient induction of SAVR /LAVR. Also, there may be distinct requirements to
354 reach a protective LAVR on top of, or separate to, those needed for a protective SARV. Further
355 experiments including examination of the specificity of the protection induced by the pcDNA3-
356 Mx-vhsG vaccine at 8 wpv along with immune gene expression analysis are needed to resolve
357 this aspect. Interestingly, Chang et. al. (2015) showed that while IFNa, IFNb, or IFNc plasmid
358 constructs all had an adjuvant effect, promoting generation of a protective adaptive immune
359 response to a co-injected DNA vaccine against Infectious salmon anaemia (ISA) in Atlantic
360 salmon, only IFNc was able to provide protection against this disease without needing the
361 expression of the viral antigen [46].

362 The protective effect at 78 wpv reported here for the pcDNA3-CMV-vhsG vaccinated fish along
363 with earlier observations by Kurath et al demonstrating immunity to IHN 2 years post-
364 vaccination [18] suggests that the salmonid rhabdovirus G-gene DNA vaccines with CMV-
365 promoter driven expression induce long lasting immunity covering the typical 2-3-year lifespan
366 for cultured rainbow trout. However, such long term studies have not been conducted for other
367 fish DNA vaccines, and a similar duration of immunity may not be expected. For the Mx
368 promoter DNA vaccine construct tested here, our initial hypothesis was that without Poly(I:C)
369 or IFN stimulation, no or low levels of vaccine antigen expression would occur in fish
370 vaccinated with pcDNA3-Mx-vhsG. In that case, elimination of cells harboring the plasmid by
371 the local inflammatory response, described in fish vaccinated with pcDNA3-CMV-vhsG [29],
372 would be less likely to occur or at least delayed. This could extend the lifetime of the vaccine in
373 the fish and possibly allow post-vaccination management of the fish immune status by internal
374 or external IFN stimulation. However, even with Poly(I:C) stimulation 10 weeks earlier,
375 vaccination with pcDNA3-Mx-vhsG failed to protect the fish against VHS. It remains to be
376 determined whether this was due to elimination of transfected cells before the Poly(I:C)
377 stimulation, or because the baseline expression level of the antigen by the Mx promoter was
378 insufficient to induce long-term memory. In applied terms, future research should address
379 whether the fish specificity of the trout Mx promoter could be combined with the high

380 expression capacity of other promoters by designing hybrid promoters as previously attempted
381 by Martinez-Lopez et al. (2013) [28]. Also, it must be analyzed whether the trout Mx promoter
382 might increase the chance of integration by homologous recombination in vaccinated rainbow
383 trout. Since the trout Mx promoter also worked in cyprinid cells, this issue could be addressed
384 by the use of heterologous fish promoters when designing DNA vaccines for a particular fish
385 species.

386

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388

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Highlights

- DNA vaccine with Poly(I:C) inducible Mx promoter.
- Selective expression in fish cells.
- Immunity to VHS induced by DNA vaccine with CMV promoter last more than 17 months.
- No direct link between early and specific protection following DNA vaccination.