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A effective DNA vaccine against diverse genotype J infectious hematopoietic necrosis virus strains prevalent in China

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ABSTRACT

Infectious hematopoietic necrosis virus (IHNV) is the most important pathogen threatening the aquaculture of salmonid fish in China. In this study, a DNA vaccine, designated pIHNch-G, was constructed with the glycoprotein (G) gene of a Chinese IHNV isolate SD-12 (also called Sn1203) of genotype J. The minimal dose of vaccine required, the expression of the Mx-1 gene in the muscle (vaccine delivery site) and anterior kidney, and the titers of the neutralizing antibodies produced were used to evaluate the vaccine efficacy. To assess the potential utility of the vaccine in controlling IHNV throughout China, the cross protective efficacy of the vaccine was determined by challenging fish with a broad range of IHNV strains from different geographic locations in China. A single 100 ng dose of the vaccine conferred almost full protection to rainbow trout fry (3 g) against waterborne or intraperitoneal injection challenge with IHNV strain SD-12 as early as 4 days post-vaccination (d.p.v.), and significant protection was still observed at 180 d.p.v. Intragenogroup challenges showed that the DNA vaccine provided similar protection to the fish against all the Chinese IHNV isolates tested, suggesting that the vaccine can be widely used in China. Mx-1 gene expression was significantly upregulated in the muscle tissue (vaccine delivery site) and anterior kidney in the vaccinated rainbow trout at both 4 and 7 d.p.v. Similar levels of neutralizing antibodies were determined with each of the Chinese IHNV strains at 60 and 180 d.p.v. This DNA vaccine should play an important role in the control of IHN in China.

1. Introduction

Infectious hematopoietic necrosis virus (IHNV) is a typical virus of the genus *Novirhabdovirus* and a major pathogen of salmon and trout [1]. Depending upon the differences on host species, virus strains, and fish farming environment, Epidemics of infectious hematopoietic necrosis (IHN) can cause mortality at rates exceeding 90% in some cases, depending upon the host species, viral strain, and fish-farming environment [2]. To reduce the economic losses caused by this pathogen, various candidate IHNV vaccines have been developed [3,4], including attenuated vaccines [5–10], killed virus [11], and vaccines based on recombinant DNA technology. However, traditional vaccines have not provided the desired protection to rainbow trout [12,13]. The first DNA vaccine developed against IHNV stimulated a strong immune response in rainbow trout fry with a single dose of 10 μg [14]. Another IHNV DNA vaccine based on the G gene of IHNV strain WRAC conferred almost full protection at a lower dose [2,12,15–17], and the vaccine, designated pIHNw-G, was very stable and effective under various conditions, including in various fish host species and life stages, during challenges with diverse strains, and when delivered with various strategies [12,15,17–19].

According to previous phylogenetic studies, the worldwide IHNV strains can be divided into five genogroups U, M, L [20–25], J [26,27] and E [28,29]. All Chinese IHNV isolates cluster in the J Nagano sub genogroup within genotype J, together with some Japanese IHNV isolates [30]. Although they all belong to genogroup J, the IHNV isolates that have evolved in Chinese salmon environments formed a separate branch [30]. Previous IHNV DNA vaccines were constructed with the G genes of the IHNV genogroup M and U

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strains [14,16], and Corbeil et al. [12] demonstrated that the pIHNw-G vaccine protects fish against a broad range of viral strains from different geographic locations, including strains from France and Japan. However, Chinese IHNV strains have not yet been tested. In this study, we constructed an IHNV DNA vaccine based on the G gene of the J genotype IHNV- SD12 strain (also called Sn1203) isolated from rainbow trout [31]. The protection efficacy of the DNA vaccine was determined with intragenogroup challenges using nine field IHNV isolates from different Chinese provinces, which together include almost all the salmon and trout aquaculture districts in China. To our knowledge this is the first study to construct an IHNV DNA vaccine using the G gene from a genogroup J IHNV strain. The protection efficacy of the DNA vaccine was assessed by intragengroup challenges with IHNV field strains recently prevalent in China. This DNA vaccine should play an important role in the control of IHN in China.

2. Material and methods

2.1. Ethics statement

In this study, all animal experiments were performed under the Guidelines of European Union Council Directive 2010/63/EU (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm) for the protection of animals used for scientific purposes.

2.2. Materials

Nine Chinese IHNV strains isolated from different geographic locations in China in 2012–2015 were from laboratory stocks: SD-12 (also called Sn1203), HLJ-15, LN-15, BJ-15, GS-15, XJ-14, QH-15, SC-15, and YN-15. The locations and detailed information on these IHNV strains were given in Fig. 1 and Table 1 in the Supplementary Materials. *Epithelioma papulosum cyprini* (EPC) cells were kindly provided by Professor Lingbing Zeng of the Changjiang River Fisheries Research Institute, Chinese Academy of Fishery Sciences, China.

2.3. DNA vaccine

The DNA vaccine, designated pIHNch-G, was constructed by cloning the G gene of SD-12 into plasmid pcDNA3.1. The vaccine pIHNch-G and plasmid pcDNA3.1 were propagated in *Escherichia coli* strain DH5a and prepared with an EndoFree plasmid extract kit (Tiangen, Shanghai, China). The expression of the G gene from the DNA vaccine in fish cells was confirmed by transfecting the fish EPC cell line with pIHNch-G, and an immunofluorescence antibody test (IFAT) as described in a previous study [32].

2.4. DNA vaccination

Specific-pathogen-free rainbow trout (mean weight, 3 g; Heilongjiang River Fishery Research Institute, Harbin, China) were anaesthetized by immersion in tricaine methane sulfonate (MS-222; Sigma, USA) and the DNA vaccine was delivered by intramuscular (i.m.) injection at the base of the dorsal fin. To determine the optimal dose of the DNA vaccine, 0.1, 1.0, 5.0 and 10.0 μg was injected in 50 μl of phosphate-buffered saline (PBS). The control fish were injected with the vector alone, PBS alone, or left unhandled, as specified in the text. Each treatment group was placed in a 50 L circulating water tank (15 °C) and fed a dry pelleted diet *ad libitum*.

2.5. Challenges of rainbow trout with SD-12

The rainbow trout was challenged by immersion or by intraperitoneal (i.p.) injection, depending on the size of the fish. In the immersion challenge experiment, duplicate groups of 50 fish each (mean weight $\leq$ 5 g) were exposed to waterborne SD-12 for 60 min with aeration. The concentration of SD-12 was 10^5 plaque-forming units (PFU)/ml, and the volume of water was 10× the total weight of the fish (g). In the i.p. injection challenge, each fish from duplicate groups of 50 fish (mean weight over 5 g) was anesthetized and injected i.p. with 10^2 PFU SD-12 in 50 μl of PBS. Mock infections were performed by replacing the viral suspension with PBS. The experimental groups were maintained separately in 50 L circulating tanks (15 °C) and fed a dry pelleted diet *ad libitum*. The cumulative percentage mortality (CPM) was monitored for 21 days. The relative percentage survival (RPS) was then calculated with the formula RPS = [1 − (% mortality of fish given vaccine/% mortality of fish given pcDNA3.1)] × 100 [33]. In the DNA vaccine dose experiments, the fish were challenged by immersion at 21 d.p.v. To determine the early protection and specific protection conferred by the DNA vaccine, rainbow trout were immersion challenged with SD-12 at 1, 2, 4, or 7 d.p.v. or with i.p. injection at 28, 120, or 180 d.p.v.

2.6. Intragenogroup challenge experiments

To determine the cross protection afforded by the DNA vaccine against diverse Chinese IHNV strains, duplicate groups of 50 fish were immersion challenged at 4 d.p.v. or challenged with i. p. injection at 60 or 180 d.p.v. Each IHNV strain (Table 1 in the Supplementary Materials) was used as a challenge strain, and the challenge dose was the same as that used for of SD-12 (Section 2.5).

2.7. Mx-1 gene expression assay

RNA from the muscle tissues (at the injection site) and anterior kidneys of the immunized fish (n = 5) was prepared with routine procedures. Quantitative reverse transcription-PCR (qRT-PCR) was performed with the One Step SYBR PrimeScript Plus RT-PCR Kit (Perfect Real Time) (Takara, Dalian, China, cat. no. RR096A). The housekeeping gene, acidic ribosomal phosphoprotein P0 (ARP) [34] was used as the reference gene to normalize the expression level of the Mx-1 gene. The fold-change in Mx-1 gene expression was calculated relative to that in pcDNA3.1 in the PBS-mock-vaccinated control group.

2.8. Neutralizing antibody titers

At 60 and 180 d.p.v., blood samples (n = 10) from the trout vaccinated with pIHNch-G or empty pcDNA3.1 (without viral challenge) were collected by caudal transection, and sera were prepared with a routine procedure. The IHNV-neutralizing antibody (NAb) titer of each serum was determined with a complement-dependent neutralization assay. Titers of $\geq$20 were considered positive, and a titer of <20 was negative [35].

2.9. Statistical analysis

Analysis of variance (ANOVA) was used to determine the differences in gene expression level replicates. Student's t test was also used to compare some paired samples. Values of $P < 0.05$ were deemed statistically significant.

3. Results

3.1. Expression of G gene from DNA vaccine in a fish cell line

The EPC cells were transfected with the DNA vaccine using a routine procedure. The transfected cells were permeabilized at 12 h post-transfection, and then incubated with a rabbit anti-glycoprotein polyclonal antibody [36] and fluorescein-isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody (eBioscience, shanghai, China). Specific green fluorescence was observed in the cells transfected with pIHNch-G, whereas no specific fluorescent signal was observed in the cells transfected with the pcDNA3.1 vector (Fig. 1). These results indicate that the G gene is efficiently expressed from the pIHNch-G vector in fish cells.

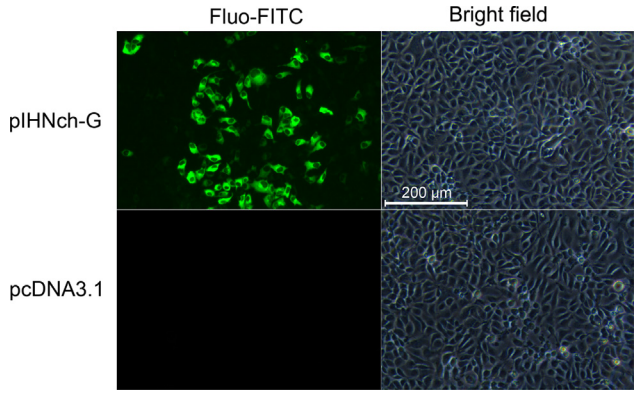

Fig. 1. Immunofluorescent micrographs of EPC cell cultures expressing the IHNV glycoprotein after transfection with the pIHNch-G DNA vaccine. Cells were stained with an IHNV-glycoprotein-specific rabbit polyclonal antibody and a FITC-conjugated goat anti-rabbit IgG antibody. Scale bar corresponds to 200 μm. EPC cells transfected with the pcDNA3.1 vector and treated identically, were used as the negative control. Fluo: fluorescence; FITC: fluorescein-isothiocyanate.

3.2. Determination of the minimal vaccine dose

To exclude any influence of the challenge method, the immunized rainbow trout were challenged with both immersion and i.p. injection. All groups vaccinated with the DNA vaccine had very low CPMs (4%–12%), whereas the mock-vaccinated rainbow trout had very high CPMs (>90%) (Fig. 2A). When fish were vaccinated with the same dose of DNA vaccine, there were no significant differences between the final CPMs of the rainbow trout challenged by immersion and those challenged by i.p. injection ($P < 0.05$) (Fig. 2A). Interestingly, rainbow trout vaccinated with 10.0 and 5.0 μg of the DNA vaccine were significantly higher than those of fry vaccinated with 1.0 or 0.1 μg of the DNA vaccine (4%–6%; $P < 0.05$), when challenged by either immersion or i.p. injection (Fig. 2B). Based on the results of the dose experiments, we used a dose of 0.1 μg of DNA vaccine per fish (mean weight, 3 g) as the standard dose in subsequent experiments. No mortality was observed in the sham-infected control groups, and no significant differences in mortality were observed between any replicates of any of the treatment groups in either study (data not shown).

3.3. Protection conferred by the DNA vaccine

To determine the early nonspecific protection stimulated by the vaccine, duplicate groups of 50 fish were challenged with SD-12 at 1, 2, 4, and 7 d.p.v. High mortality rates (92%–96%) were observed in all the treatment groups challenged at 1 and 2 d.p.v. (Fig. 3A). By 4 and 7 d.p.v., the DNA vaccine treatment groups were significantly protected ($P < 0.05$), with only 6%–8% mortality, compared with 92%–94% in the pcDNA3.1-vaccinated control groups (Fig. 3A).

To determine the long-term specific immunoreaction, the protection efficacy was determined at later time points (Fig. 3B). By 28 d.p.v., the pIHNch-G-treated groups was still significantly protected ($P < 0.05$), with 8% mortality, compared with 94% in the pcDNA3.1-treated control groups (Fig. 3B). By 120 and 180 d.p.v., the pIHNch-G treatment groups were significantly more strongly

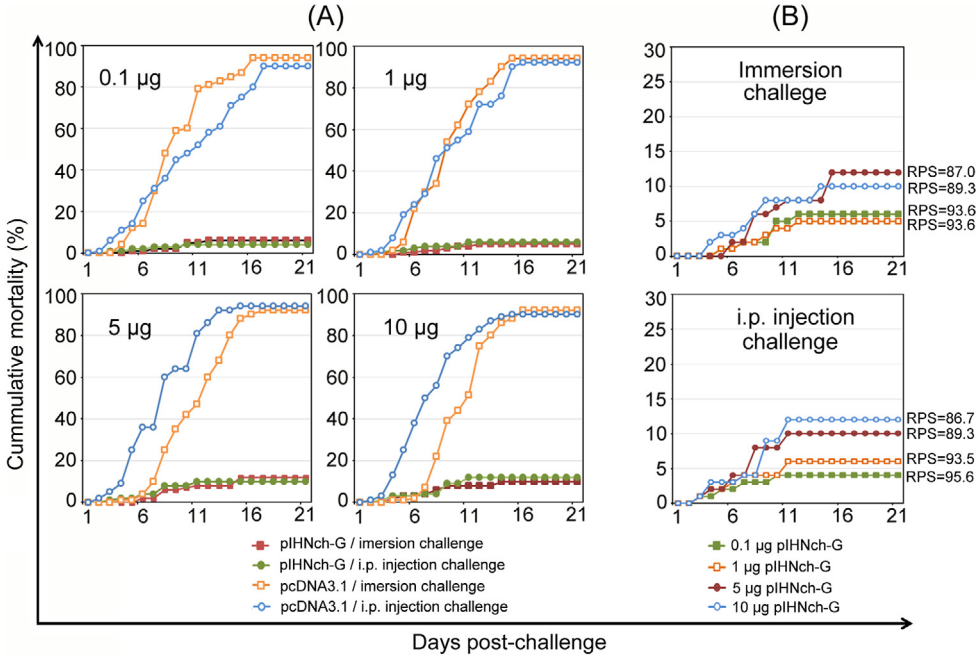

Fig. 2. Cumulative percentage mortality curves for rainbow trout challenged at 28 days post vaccination with 0.1, 1.0, 5.0, or 10.0 μg of DNA vaccine pIHNch-G. Rainbow trout injected with plasmid pcDNA3.1 (vector) were used as the negative controls. Duplicate groups of 50 fish were challenged by waterborne exposure to 10^5 IHNV SD-12 PFU/ml or intraperitoneal injection with 10^2 PFU IHNV SD-12 per fish. No significant differences were observed in mortality between any replicates within any treatment group. i.p. injection: intraperitoneal injection; RPS: relative percentage survival. Differences were analyzed and different letters are used to indicate significant differences ($P < 0.05$).

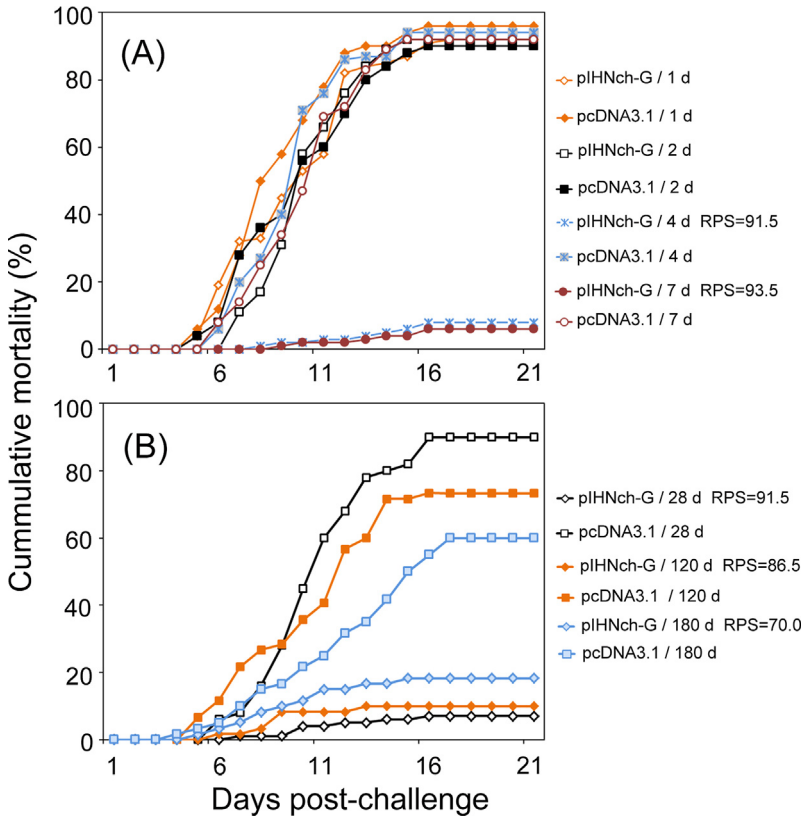

Fig. 3. Cumulative percentage mortality curves for vaccinated rainbow trout challenged at different time points post vaccination. Rainbow trout injected with plasmid pcDNA3.1 (vector) were used as the negative controls. Duplicate groups of 50 fish challenged at 1, 2, 4, and 7 days post vaccination (d.p.v.) were used to determine the nonspecific protection induced by the DNA vaccine in fish (A), and fish challenged at 28, 120, and 180 d.p.v. were used to determine the specific protection afforded by the vaccine (B). No significant differences were observed in mortality between any replicates within any treatment group. RPS: relative percentage survival. Differences were analyzed and different letters are used to indicate significant differences ($P < 0.05$).

protected than the pcDNA3.1-treated controls ($P < 0.05$). By 120 d.p.v., the average mortality in the pIHNch-G-vaccinated groups was only 10%, and a dramatic reduction in CPM (74%) was observed in the pcDNA3.1-treated control groups. By 180 d.p.v., the final CPM increased to 18% in the pIHNch-G-vaccinated fish, whereas the final CPM in the pcDNA3.1-treated control groups had decreased to 60% (Fig. 3B). No mortality was observed in the sham-infected control groups, and no significant differences in mortality were observed between any replicates of any of the treatment groups in either study (data not shown).

3.4. Cross-protection afforded by the DNA vaccine

In the intragenogroup challenge at 4 d.p.v., the pIHNch-G-treated groups were significantly protected ($P < 0.05$), with 4%–8% mortality, compared with 92%–96% mortality in the various control groups. There were no significant differences in the final CPMs of the pIHNch-G groups challenged with different IHNV strains ($P < 0.05$). Significant protection, similar to that observed in the pIHNch-G-vaccinated groups, was also observed in the fish challenged with different IHNV strains at 60 d.p.v. In the fish challenged at 180 d.p.v., there was no significant difference between the final CPM in each of the pIHNch-G-treated groups. Although the protection efficacy at 180 d.p.v. was still significantly higher than that in the various control groups ($P < 0.05$), the CPMs of the pIHNch-G groups increased to 18% on average, compared with 48%–62% mortality in the various control groups (Fig. 4).

3.5. Changes in Mx-1 gene expression

The Mx-1gene expression levels in the vaccinated fish were determined at 4 and 7 d.p.v. At 4 d.p.v., the changes in Mx-1 gene expression in the muscle tissue and anterior kidney in the pIHNch-G-vaccinated group were 86-fold and 9-fold, respectively, and at 7 d.p.v., they were 128-fold and 39-fold, respectively (Fig. 5). These results indicate that the expression of Mx-1 gene was significantly upregulated in the muscle and anterior kidney at 4 and 7 d.p.v. ($P < 0.05$).

3.6. NAb titration

At 60 and 180 d.p.v., sera from 10 fish vaccinated with pIHNch-G or empty pcDNA3.1 (without viral challenge) were tested for seroconversion. The NAb titers were determined for each of the IHNV strains in separate neutralization assays and are shown in Table 1. By 60 d.p.v., more than half the fish had a titer ≥ 160. However, at 180 d.p.v., fewer than half the fish had positive NAb titers, and most of the positive titers were 20–40 (except for two fish with titers of 80), much lower than that of the positive control sera (>160). In all the experiments, no NAb was detected in the sera

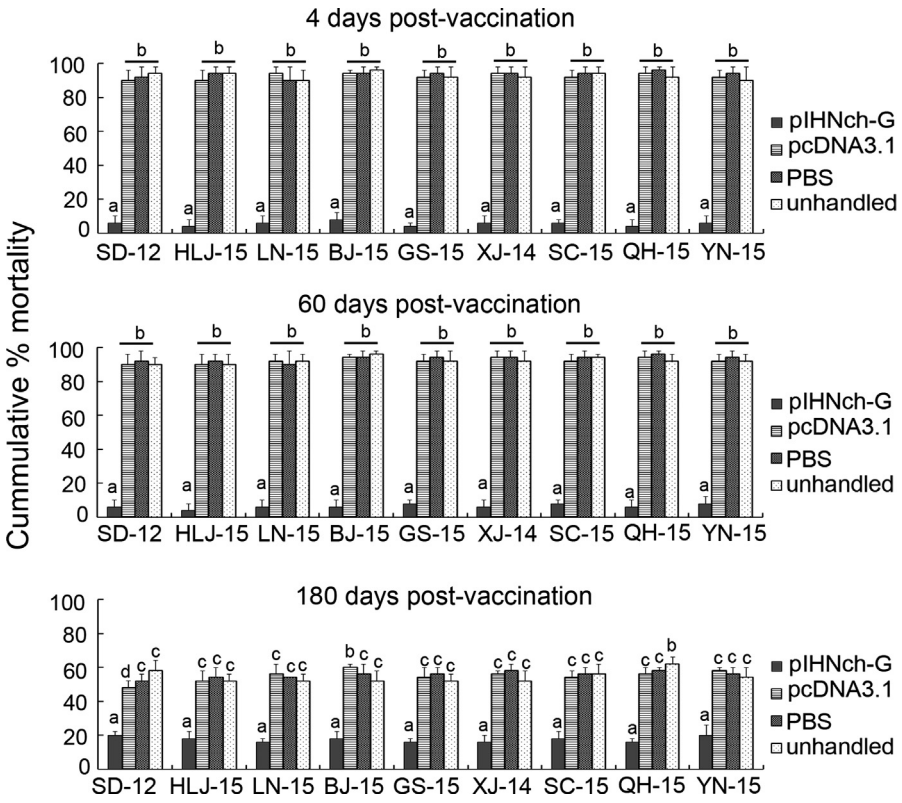

Fig. 4. Final cumulative percentage mortality at different time points post vaccination in rainbow trout challenged with diverse Chinese IHNV strains. Rainbow trout injected with plasmid pcDNA3.1 (vector), PBS, or untreated were used as the negative controls. Rainbow trout challenged at 4 d.p.v. were used to determine the nonspecific protection afforded by the DNA vaccine in the fish, and those challenged at 60 and 180 d.p.v. were used to determine the cross-specific protection afforded by the vaccine. Differences were analyzed and different letters are used to indicate significant differences ($P < 0.05$).

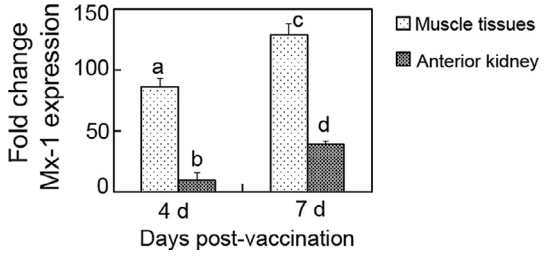

Fig. 5. Host Mx-1 gene expression was analyzed after an intramuscular injection of the DNA vaccine. The fold changes in Mx-1 gene transcription in individual muscle and anterior kidney samples from rainbow trout at 4 and 7 d.p.v., normalized to it expression in the pcDNA3.1-vaccinated group, are shown. Differences were analyzed and different letters are used to indicate significant differences ($P < 0.05$).

from the pcDNA3.1-vaccinated controls against any one of the viral strains (results not shown).

4. Discussion

The first outbreak of IHN occurred in Benxi, northeast China, in 1985, and 600,000 rainbow trout fry died in 3–15 days on a rainbow trout farm in 1986 [30]. IHNV was mainly isolated from salmon and trout fish farms in the northern part of China before 2006 [37–39], but since then, it has spread to almost every salmon and trout farm in China. IHN has become the most important pathogen threatening the development of the salmonid fish farming industry in China, and all Chinese IHNV isolates belong to genogroup J [26]. A previous study showed that the protection efficacy of a DNA vaccine can differ, depending on the infecting IHNV strain

Table 1
Neutralizing antibody (NAb) response induced by pIHNch-G vaccination at different time points.

Test time (d.p.v.)	Challenge IHNV strains	Number of pIHNch-G fish seroconverted/number tested	Nab titers[a] (number of individual fish at each titer)[b]
60	SD-12	10/10	80 (3), ≥160 (7),
	HLJ-15	10/10	40 (1), 80 (1), ≥160 (8)
	LN-15	10/10	40 (1), 80 (2), ≥160 (7)
	BJ-15	10/10	80 (2), ≥160 (8)
	GS-15	10/10	80 (3), ≥160 (7)
	XJ-14	10/10	40 (2), ≥160 (8)
	QH-15	10/10	40 (1), 80 (2), ≥160 (7)
	SC-15	10/10	80 (2), ≥160 (8)
	YN-15	10/10	40 (1), 80 (1), ≥160 (8)
180	SD-12	4/10	<20 (7), 20 (1), 40 (3)
	HLJ-15	4/10	<20 (6), 20 (2), 40 (2)
	LN-15	3/10	<20 (8), 20 (2) 40 (1)
	BJ-15	3/10	<20 (7), 20 (3)
	GS-15	4/10	<20 (6), 20 (2), 80 (2)
	XJ-14	4/10	<20 (6), 20 (2), 40 (2)
	QH-15	3/10	<20 (7), 20 (3)
	SC-15	3/10	<20 (7), 20 (1), 40 (2)
	YN-15	2/10	<20 (8), 20 (1), 40 (1)

[a] Positive control serum used in this study had a titer >160 in all assays.
[b] Sera from ten fish. Titers <20 were defined as negative. No detectable NAb was found in fish injected with the pcDNA3.1 vector.

[1]. Although there have been many studies of DNA vaccines against IHNV strains of different genogroups, to our knowledge, this is the first study to construct a DNA vaccine against IHNV genogroup J isolates. The intragenogroup protective efficacy of the DNA vaccine was determined with diverse IHNV strains from different geographic locations in China.

Great progress has been made in the development of IHNV DNA vaccines against the IHN viruses of genogroups U and M. In previous studies, nanogram quantities of the DNA vaccine pIHNw-G conferred almost full protection on rainbow trout fry against IHNV at 4 d.p.v. In this study, 100 ng of pIHNch-G also conferred approximately complete protection as early as 4 d.p.v. Surprisingly, a high dose of 5 μg and 10 μg did not provide better protection than lower doses, but in fact, provided significantly weaker protection than low doses ($P < 0.05$; Fig. 2B). This may be attributable to a lack of purity in the prepared DNA vaccine. In most IHNV challenge models, rainbow trout fry are usually challenged by immersion rather than by i.p. injection [2]. To exclude any possible effect of the challenge method on the protective efficacy of the vaccine, we used both immersion and i.p. injection to challenge the rainbow trout in the minimal dose experiment. The challenge method did not significantly affect the protection afforded by the DNA vaccine, and rainbow trout vaccinated with the same dose of DNA vaccine but challenged in a different way showed no significant difference in RPS ($P > 0.05$; Fig. 2A).

To analyze the onset of protection, rainbow trout were challenged by immersion at 1, 2, 4, and 7 d.p.v. As expected, the delivery of pIHNch-G by i.m. injection provided significant protection as early as 4 d.p.v. at 15 °C, as does the previously constructed DNA vaccine pIHNw-G [12]. It has recently been demonstrated that nasal vaccination with a live attenuated IHNV vaccine elicited significant protection at 7 d.p.v. (the earliest time point tested) [7,10]. These time points (4 d.p.v. and 7 d.p.v.) may reflect the kinetics of the innate immune response in an ectothermic vertebrate, such as the rainbow trout [10]. The interferon-stimulated gene (ISG) Mx-1 gene is recognized as a marker of the nonspecific immunity induced by a DNA vaccine in fish [1]. qRT–PCR showed that the expression of Mx-1 gene in the muscle tissues (vaccine delivery site) and anterior kidney tissues of vaccinated fish was strongly upregulated at 4 and 7 d.p.v., which may reflect the significant protection afforded at 4 d.p.v.

To detect the long-term protection conferred by the DNA vaccine, vaccinated rainbow trout were challenged at 28, 120, and 180 d.p.v., and the fish at each time point showed significantly lower CPMs than those in the corresponding control groups. This indicates that the DNA vaccine still provided specific protection at 180 d.p.v. However, with increasing time, this protection decreased to below the level observed at 28 d.p.v. In contrast, the CPMs of the control groups become progressively lower. This can be explained by the dynamics of the NAb titers in fish. The NAb titer in rainbow trout sera was significantly lower at 180 d.p.v. than at earlier time points. However, NAbs have been shown to be protective at low titers (plaque neutralization titers of 20–40) [40]. Therefore, significant protection still could be observed at 180 d.p.v. Rainbow trout fry, the most common IHNV host, are more sensitive to IHNV than the adult fish [41]. In this study, the rainbow trout were large and less sensitive to IHNV at 120 and 180 d.p.v. Therefore, it is reasonable that CPM declined at later time point, which was also observed in the intragenogroup challenges. Although we did not determine the protection efficacy at time points later than 180 d.p.v., it has been reported that an IHNV DNA vaccine provided specific protection in fish at 2 years post vaccination [41]. Because DNA vaccine pIHNch-G conferred almost the same protection as DNA vaccine pIHNw-G soon after vaccination, it is possible that the specific protection induced by pIHNch-G in fish would last for 2 years, like that induced by pIHNw-G.

The importance of developing vaccines that are effective against heterologous strains of a given pathogen has been emphasized in studies of IHNV. Despite the diversity of IHNV strains, only one serotype of IHNV has been identified in previous studies [12]. Furthermore, many IHNV isolates from different districts in China have recently been analyzed, and the similarity of their G genes was > 98% [30]. Despite this high similarity and the single serotype of IHNV, a previous study showed that single-site mutations in the G gene of IHNV can cause low virulence or lead to neutralization-resistant variant IHNV strains [42]. As we expected, the DNA vaccine pIHNch-G constructed in this study provided significant uniform protection to vaccinated rainbow trout at each time point in intragenogroup challenges with diverse IHNV strains. This might be attributable to the high homology among the Chinese IHNV strains. In other words, the evolution of the Chinese IHNV strains has not yet produced any neutralization-resistant variant IHNV strain.

5. Conclusion

A DNA vaccine was constructed by cloning the G gene of Chinese IHNV isolate SD-12 (genogroup J) into the pcDNA3.1 vector. A dose of 0.1 μg of the vaccine per rainbow trout (mean weight, 3 g) not only provided significant protection against challenge with the parental IHNV strain SD-12, but provided almost the same protection against intragenogroup challenge with other Chinese IHNV field strains (genogroup J). Significant protection was induced as early as 4 d.p.v. and persisted up to 180 d.p.v. This DNA vaccine should play an important role in the comprehensive control of IHN in China.

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Conflict of interest

The authors declare that they have no conflict of interest related to this work.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2017.03.047.

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