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DNA vaccination for finfish aquaculture

Catherine Collins[a], Niels Lorenzen[b], Bertrand Collet[a,c,*]

[a] Marine Scotland, Aberdeen, United Kingdom
[b] Danish Technical University (DTU), Lyngby, Denmark
[c] Virologie et Immunologie Moléculaires, Institut National de la Recherche Agronomique (INRA), Université Paris-Saclay, Jouy-en-Josas, France

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ABSTRACT

In fish, DNA vaccines have been shown to give very high protection in experimental facilities against a number of viral diseases, particularly diseases caused by rhabdoviruses. However, their efficacy in generating protection against other families of fish viral pathogens is less clear. One DNA vaccine is currently in use commercially in fish farms in Canada and the commercialisation of another was authorised in Europe in 2017. The mechanism of action of DNA vaccines, including the role of the innate immune responses induced shortly after DNA vaccination in the activation of the adaptive immunity providing longer term specific protection, is still not fully understood. In Europe the procedure for the commercialisation of a veterinary DNA vaccine requires the resolution of certain concerns particularly about safety for the host vaccinated fish, the consumer and the environment. Relating to consumer acceptance and particularly environmental safety, a key question is whether a DNA vaccinated fish is considered a Genetically Modified Organism (GMO). In the present opinion paper these key aspects relating to the mechanisms of action, and to the development and the use of DNA vaccines in farmed fish are reviewed and discussed.

1. Introduction

The concept of DNA vaccination was first introduced in 1992 when a DNA plasmid encoding for the human Growth Hormone was injected intradermally in mice and found to generate specific antibodies [1]. Prior to that, the very first observation of expression of a foreign protein encoded by a DNA plasmid injected into the muscle tissue was in mice by Wolff et al. [2], followed by Hansen et al. in fish [3]. Five years were required for these results to be put into practice in fish vaccinology studies [4]. The developed plasmid constructs encoding the viral glycoprotein proved to be extremely efficient for protecting fish against rhabdoviruses [5–7] whereas in most larger mammals, and humans in particular, the efficacy of DNA vaccination did not reach the expected levels of protection [8–11]. In 2005, APEX-IHN (Novartis/Elanco) for protecting Atlantic salmon against Infectious Hematopoietic Necrosis Virus (IHNV) in British Colombia became the first DNA vaccine licensed for commercial use in aquaculture and was also the first DNA vaccine licensed for veterinary use in animal husbandry production worldwide [12]. Two other DNA vaccines have been authorised for veterinary use: “West Nile Innovator - DNA” (Fort Dodge Animal Health/Pfizer) for protection against West Nile virus in condors and horses, and “Oncept” (Merial) against dog melanoma [13]. In 2017, CLYNAV (Elanco), a polyprotein-encoding DNA vaccine against Salmon Pancreas Disease Virus (SPDV) infection in Atlantic salmon *Salmo salar* was given marketing authorisation by the European Commission through the European Medicines Agency (EMA) for use within the EU, based on a positive risk benefit assessment following examination of data. It was authorised for use in Norway by the Norwegian Medicines Agency

Abbreviations used: **ADCC**, Antibody-Dependent Cellular Cytotoxicity; **AHNV**, Atlantic Halibut NodaVirus; **APC**, Antigen Presenting Cells; **CCV**, Channel Catfish Virus; **CFIA**, Canadian Food Inspection Agency; **CVMP**, Committee for Veterinary Medicinal Products; **DOI**, Duration of Immunity; **EEA**, European Economic Area; **EFSA**, European Food Standards Agency; **EU**, European Union; **GM**, Genetically Modified; **GMO**, Genetically Modified Organisms; **IHNV**, Infectious Hematopoietic Necrosis Virus; **IHV**, Ictalurid Herpes Virus; **IPNV**, Infectious Pancreatic Necrosis Virus; **ISAV**, Infectious Salmon Anaemia Virus; **KHV**, Koi Herpes Virus; **MAA**, Marketing Authorisation Application; **MGNNV**, Malabaricus Grouper Nervous Necrosis Virus; **NNV**, nervous necrosis virus (=Betanodavirus); **NoMA**, Norwegian Medicines Agency; **OOI**, Onset of Immunity; **ORF**, Open Reading Frame; **PAMP**, Pathogen Associated Molecular Patterns; **PCPS**, Proteasome-Catalyzed Peptide Splicing; **QPCR**, Quantitative polymerase chain reaction; **RGNNV**, Redspotted Grouper Nervous Necrosis Virus; **RPS**, Relative Percentage of Survival; **RSIV**, Red Sea Bream Iridovirus; **SPDV**, Salmon Pancreatic Disease Virus; **SVCV**, Spring Viremia of Carp Virus; **VHSV**, Viral Haemorrhagic Septicaemia Virus

* Corresponding author. Infection et immunité des poissons, Unité de virologie et immunologie moléculaires, Centre de Recherche INRA de Jouy-en-Josas, Domaine de Vilvert, F-78352 Jouy-en-Josas Cedex, France.
E-mail addresses: ccollins@collpicto.com, Catherine.Collins@gov.scot (C. Collins), Lorenzennilo@aqua.dtu.dk (N. Lorenzen), bertrand.collet@inra.fr (B. Collet).

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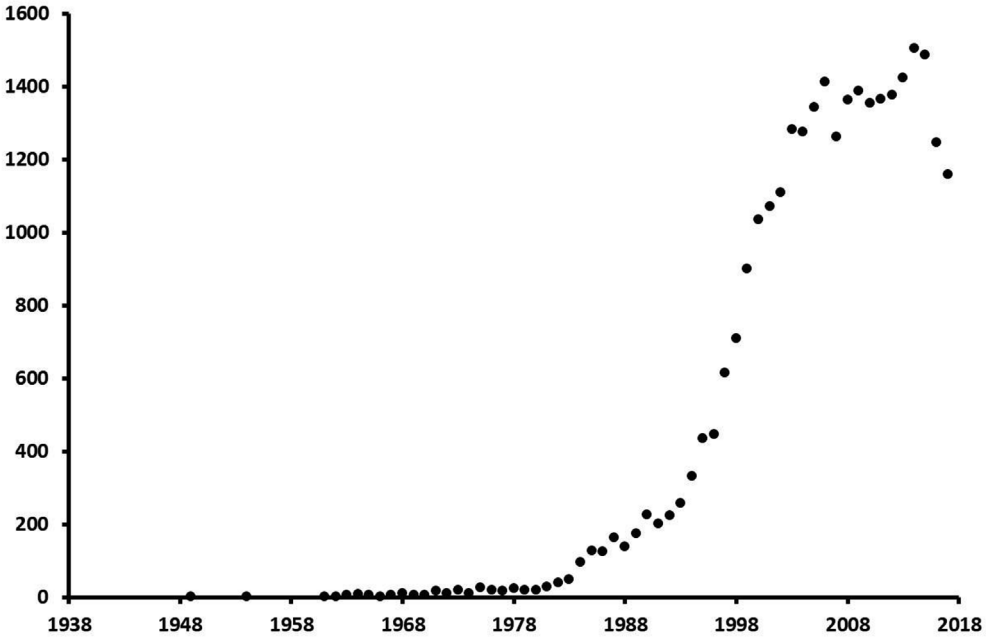

Fig. 1. Publication counts over the years. Number of publications returned with "DNA vaccine" keywords. Y-axis is the number of publications returned, X-axis is the year of publication. (NCBI https://www.ncbi.nlm.nih.gov/).

(NoMA).

Apart from their potential to generate very high protection against disease, DNA vaccines offer a great flexibility in incorporating additional features to the vaccine to direct or augment the immune response, as well as to modify the antigen [8,14]. Interest in DNA vaccination development is reflected in the continuing year on year increase in the number of publications relating to DNA vaccines and vaccination (Fig. 1).

In this article, we will give an update on the recent development of DNA vaccines for aquaculture, focusing on finfish and viral diseases. Experimental DNA vaccines have also been generated against a number of bacterial diseases in farmed fishes [15,16] and have also been attempted, with no or limited success, for the fish parasites *Ichthyophthirus multifiliis,* [17] and *Cryptobia salmositica* [18]. However, because bacteria usually can be cultured on simple broth media and efficient conventional vaccines often can be generated cost effectively from inactivated whole bacteria [19,20], there is a greater need to investigate DNA vaccines for viral fish diseases.

In the first section, we will provide an overview of the various attempts to date to induce protective immunity by DNA vaccination under experimental conditions for some important viral pathogens in aquaculture. In the second section, we will discuss the mechanisms of action of DNA vaccines and highlight some knowledge gaps in their understanding. In the final section, aspects of safety for the host, the end consumer and the environment will be discussed along with guidelines and regulations associated with the marketing authorisation process for DNA vaccines in the EU, and key points relating to the recent approval of the CLYNAV (Elanco) DNA vaccine.

2. DNA vaccines against fish viral diseases – variable protective efficacy

Despite the general opinion that DNA vaccines result in an improved immune response and protection because they stimulate both humoral and cellular adaptive immunity [21–23], along with some innate immune response mechanisms [8,13,24], most DNA vaccines for farmed fish remain for the moment experimental. Their performance in the field or clinical trials is still to be determined along with some concerns over potential for adverse immune responses in the presence of other vaccines or stressors or the development of tolerance or auto-immunity [25]. It is also apparent when reviewing scientific publications on experimental DNA vaccines for fish, that there is a lot of variation in the applied test conditions and in the evaluation of efficacy.

2.1. Rhabdoviruses

The DNA vaccines based on the gene encoding the G protein of the novirhabdoviruses Viral Haemorrhagic Septicaemia Virus (VHSV) and IHNV consistently generate very effective and long-lasting protection against the respective diseases in salmonids [6,7]. While VHSV and IHNV are morphologically similar viruses, both leading to lethal systemic infections in rainbow trout, they are genetically and antigenically distant. This makes them ideal for studies of specificity of vaccine induced protection. Following time-course studies of protection after DNA vaccination of rainbow trout against VHSV and IHNV respectively and reciprocal challenge experiments, a dual-phase kinetics of the immune response in the vaccinated fish was observed, including a rapidly activated transient nonspecific interferon (IFN) related antiviral protection followed by a long lasting specific immunity [26,27]. It was suggested that the vaccines' high efficacy might relate to their ability to induce an immune response mirroring that induced by a virus infection (Fig. 2; [28]). Understanding of the underlying mechanisms is important for future development of this type of vaccine for aquaculture, since the efficacy of DNA vaccines generated against many other fish viral pathogens has been less consistent [19]. However, despite the high efficacy of the DNA vaccines against novirhabdoviruses in salmonids, which is often being used as a "typical" example to illustrate the high potential of DNA vaccines for fish, the recent reports of limited protection of Pacific herring and muskellunge against VHSV following DNA vaccination stresses the importance of not making general conclusions based on a specific host-pathogen combination [29,30].

For another fish rhabdovirus, not belonging to the novirhabdoviridae, namely Spring Viremia of Carp Virus (SVCV) DNA vaccination has shown mixed results, with poor or moderate to high protection. Low to moderate Relative Percentage Survival (RPS) up to 48% and up to 40% were obtained in common carp by Kanellos et al. [31] and Zhang et al. [32], respectively, following DNA vaccination by injection and/or immersion with the SVCV G gene and SVCV challenge by i.m. or i.p., with

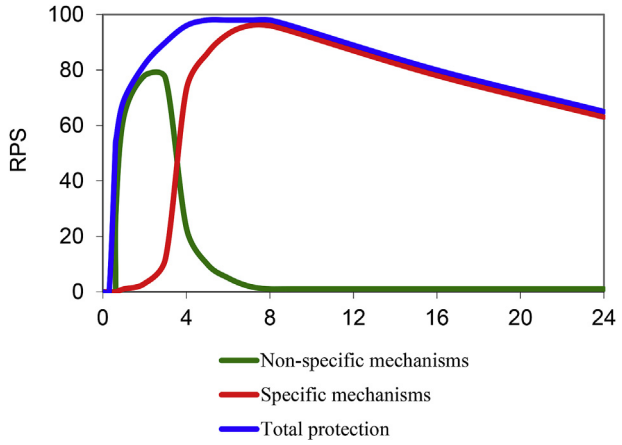

Fig. 2. Schematic illustration of the dual phase kinetics of the protective mechanisms following DNA vaccination of rainbow trout against VHS and IHN. An early temporary cross protective (green graph) immunity is substituted by a long lasting specific immunity (red graph). The X-axis units indicate weeks post vaccination. The underlying data were obtained with fish kept at 12–15°C, [28]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

increased protection dependent on the promoter used or on increasing plasmid dose. A RPS of up to 57.5% was seen by Zhang et al. [32] when the plasmid was delivered incorporated in single walled carbon nanotubes. Emmenegger and Kurath [33] obtained higher protection levels in Koi following SVCV G gene i.m. vaccination and challenge by i.p. resulting in an RPS reaching 88%. Embregts et al. [34] reported the best results to date for SVCV DNA vaccination, with an RPS of 83.3–100% in common carp of 1.5–2 g in weight following i.m. delivery and immersion challenge. These authors suggested the potential benefit of vaccinating at high temperatures (within range of host) in promoting specific T cell and antibody formation. Differences in the Embregts et al. [34] study compared to the other reports on the protective effect of DNA vaccination against SVC, were that in the former, challenge with virus was performed by immersion rather than injection, and with a longer period elapsing between vaccination and challenge.

2.2. Alphaviruses

As mentioned above, the second DNA vaccine to become licensed for use in aquaculture is the CLYNAV vaccine against Pancreas Disease (PD) in Atlantic salmon. Compared to the 2 years (or longer) duration of protection for the DNA vaccines against the novirhabdoviruses in salmonids [35], CLYNAV has only been assessed up to three months post vaccination for its ability to provide significant protection against SPDV subtype 3, following intramuscular vaccination (i.m.) (http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/veterinary/medicines/002390/vet_med_000334.jsp&mid=WC0b01ac058001fa1c). Depending on the number of degree days for the vaccine to induce protection, this seems to leave a narrow window for taking benefit of vaccination. However, though farmed salmon can become infected by SPDV at any stage of their production cycle there is some evidence that younger fish, post sea water transfer, are more susceptible [36]. Chang et al. [37]; reported significant suppression of viral replication and lesion development following i.p. challenge, in fish vaccinated i.p./i.m. with a DNA vaccine encoding the entire structural polyproteins of SPDV subtype 3. Interestingly, the reduction in virus load and disease pathology exceeded that obtained with an inactivated whole virus vaccine. However, a DNA vaccine containing the gene encoding the envelope glycoprotein E2 only did not differ from non-vaccinated controls in terms of protection. Xu et al. [38] also found little protection generated by i.m. vaccination of DNA plasmids containing the E1 or E2 SPDV genes only, in comparison with recombinant E1 or E2 proteins or whole inactivated virus delivered i.p, followed by i.m. challenge. It has been subsequently shown that the E1 and E2 structural proteins form a heterodimer surface protein and co-expression is required for cell surface expression [39]. Therefore, if responsible for the observed differences, an understanding of how viral proteins are transported and presented on the host cell surface may be required for some targets to ensure proper presentation for effective activation of immune pathways targeted through DNA vaccination.

2.3. Orthomyxoviruses

In addition to the location of the antigen expressed following DNA vaccination, attention should also be paid to the fact that some virus proteins are involved in suppressing the host response, and may hinder successful vaccine protection [40]. For example, Mikalsen et al. [41] and Chang et al. [37] obtained an RPS/survival of 40–60% and 14–60%, respectively, following i.p. challenge with Infectious Salmon Anaemia virus (ISAV) (< 5% survival in controls in the Chang et al. study) of Atlantic salmon i.m. vaccinated with a DNA plasmid expressing the viral Hemagglutinin Esterase (HE) protein. When DNA plasmids expressing any of the type I IFN subtypes, IFN a, b, c, were co-administered i.m. with the HE DNA plasmid, survival of 64–94% was obtained, significantly higher than for the pHE (14–60%) or pIFN a, b (10–20%) plasmids administered individually [42]. It has been found that the ISAV HE protein suppresses the IFN responses [40], and therefore this may have contributed to the low to moderate survival obtained with the HE-encoding DNA vaccine alone. The example illustrates the need for consideration of the vaccine product in terms of its activity in the host environment. It also illustrates that some DNA vaccines may need adjuvants to generate a strong protective response, and depending on what these are, they may generate additional safety and welfare issues. Chang et al. [43] further observed that i.m. DNA vaccination (pHE + pIFN adjuvant) against ISAV gave greater protection to an immersion/co-habitation challenge compared to challenge by i.p. injection. As observed for SVCV [34], using a challenge route that bypasses the natural barriers to infection may not give results which reflect the potency of the vaccine under natural conditions.

2.4. Birnaviruses

As reported for SPDV E1 and E2 antigen presentation [39], it has also been reported that interaction between VP2 and VP3 is important for the particle assembly in birnaviruses [44]. DNA i.m. vaccination expressing a polyprotein containing VP2, VP3 and VP4 resulted in a high RPS of 84% [45], or significant reduction in IPNV viral load (the i.p. challenge did not induce high mortality in controls) [46] in vaccinated fish. Cuesta et al. [46] reported induction of one of the type I IFN genes and the IFN- induced anti-viral gene *mx* following DNA vaccination with the IPNV polyprotein plasmid. However, *in vitro* transfection individually with plasmids expressing IPNV viral proteins VP2, VP3, VP4 and VP5, inhibited IFN promoter activity [47].

Protection obtained following DNA vaccination with the IPNV VP2 gene only has given varying results, which interestingly seems to partly depend on the route of vaccine delivery. Poor protection (RPS of 29%) was thus reported by Mikalsen et al. [45] after vaccination by i.m. injection and immersion challenge, while higher protection (in terms of reduced viral load, clinical signs and survival following challenge) through oral delivery of alginate, chitosan or liposome formulated with DNA has been reported by de las Heras et al. [48]; Ballesteros et al. [49] and Ahmadivand et al. [50]. The reported RPS values were above 80 with higher vaccine doses, and in the two latter experiments the vaccine was incorporated into the feed, demonstrating the application of oral vaccine delivery. The experiments were performed with small rainbow trout of 1–3 g each and high doses of DNA were used (10–25 μg/fish). Intraperitoneal injection or immersion challenge was performed 15 or 30 days post vaccination. Both innate and adaptive immune

Table 1
Overview of experimental DNA vaccination against non novirhabdoviruses in fish. Important parameters such as animal size, temperature, route of vaccination, route of infection and RPS are indicated.

Reference	Virus	Host (size)	Vaccine target	Adjuvant	Vaccine amount	No. Vaccine (intervals)	Vacc. route	Vacc. Temp. (°C)	Interval vaccine challenge	Challenge route	RPS	Clinical symptoms 1	Viral load 1
[38]	SPDV (subtype 3)	Atlantic salmon (46 g)	Glycoprotein E1 (+GFP) DNA plasmid or Glycoprotein E2 (+GFP) DNA plasmid	na	20 µg/fish	2 (6 week interval)	i.m.	10–14	9 weeks post prime/3 weeks post booster	i.m. (subtype 3)	similar to controls	+ + +	No difference in heart and pancreas, reduced viral load in kidney compared to non-vaccinated controls (14 dpi*).
			Recombinant E1 or E2 proteins	water in oil emulsion	50 µg/fish		i.p.				94.7/90	+ + +	No difference in viral load in heart, reduced viral load in pancreas and kidney (14 dpi).
			Inact. whole vaccine (commercial) (subtype 3)	water in oil emulsion	0.05 ml		i.p.				100	+	Reduced viral load in heart, pancreas and kidney.
[37]	SPDV (subtype 3)	Atlantic salmon (30 g)	Structural polyprotein (C-E3-E2-6K-E1) plasmid	na	15 µg/fish	1	i.m.	10	10 weeks	i.p.(subtype 3)	na	+	+ +
			Glycoprotein E2 DNA plasmid	na	15 µg/fish		i.m.					+ + +	+ + +
			Inact. whole vaccine (commercial) (subtype 1)	/	0.1 ml/fish		i.p.					+ +	+ +
[41]	ISAV	Atlantic salmon (20 g)	EGFP-HE (hemagglutinin-esterase) surface protein plasmid	na	15 µg/fish	3 (3 week intervals)	i.m.	8–10	3 weeks	i.p.	39.5–60.5	–	+ +
			EGFP-NP (internal nucleoprotein) plasmid	na			i.m.				similar to controls		+ + +
[43]	ISAV	Atlantic salmon (30–45 g)	EGFP-HE (hemagglutinin-esterase) surface protein plasmid	na	15 µg (+15 µg adjuvant)/fish	1	i.m.	10	7 weeks	i.p.	14–28	na	na
				IFNa plasmid							64		
				IFNc plasmid							74		
				IFNb plasmid							76		
				na					8 weeks	co-hab	60		+ + +
				IFNa plasmid							94		+
				IFNc plasmid							90		+
			Inact. whole virus (formalin)	water in oil adjuvant			ip				66		na

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Table 1 (*continued*)

Reference	Virus	Host (size)	Vaccine target	Adjuvant	Vaccine amount	No. Vaccine (intervals)	Vacc. route	Vacc. Temp. (°C)	Interval vaccine challenge	Challenge route	RPS	Clinical symptoms 1	Viral load 1
[45]	IPNV	Atlantic salmon (20 g)	EGFP-Polyprotein plasmid+ EGFP-VP2 plasmid	na	15 μg each plasmid/fish	1	i.m.	10	68 days	immersion	84	na	na
			VP2 plasmid		25 μg/fish						29		
			EGFP-VP2 (central region) plasmid		25 μg/fish						−21		
			N, C-terminals and centre part EGFP-VP2 plasmids		25 μg each plasmid/fish						12.5		
			N, C-terminals and centre part of EGFP-VP2 plasmids + the EGFP-VP3 plasmid		25 μg each plasmid/fish						−14		
[65]	IPNV	Rainbow trout (4–5 g)	VP2 plasmid	na	2, 5, 10 μg/fish	1	i.m.	15	30 days	i.p.	76, 88, 88	na	+
[46]	IPNV	Rainbow trout (4–12 g)	Polyprotein (NH2-VP2-VP4VP3-COOH) - His plasmid	na	1 μg/fish	1	i.m.	14	30 days	i.p.	na	na	+ (pervaence/ load)
[48]	IPNV	Brown trout (1.5 g)	VP2-His plasmid	na	10 μg/fish	1	oral (alginate microparticles via pipette)	15	15 days	immersion	83	na	++
									30 days	immersion	84	na	na
		Rainbow trout (1 g)			10 μg/fish				15 days	immersion	78–82	na	na
									30 days	immersion	83–67	na	na
[49]	IPNV	Rainbow trout (1.5 g)	VP2 plasmid	na	oral - 10 μg/fish/ day for 3 days	once over 3 days	oral-feed (sodium alginate microparticles)	15	15 days	immersion	na	na	na
									30 days	immersion	na	na	++
[66]	IPNV	Rainbow trout (3 g)	VP2 plasmid	na	5% of body weight/ 10 μg/fish	2 (15 days)	oral-feed (sodium alginate microparticles)	15	30 days	i.p.	na	–	++
					5% of body weight/ 25 μg/fish		oral-feed (sodium alginate microparticles)						+
					5% of body weight/ 10 μg/fish		oral-feed (CS/ TPP nanoparticles)						++
					5% of body weight/ 25 μg/fish		oral-feed (CS/ TPP nanoparticles)						+
[52]	IPNV	Atlantic salmon (20 g)	Central immunogenic region of VP2 capsid protein plasmid	na	500 μg/fish	1	i.m.	9–14	68 days	i.p.	47.8	na	na
			Central immunogenic region of VP2 capsid protein plasmid		1 μg/g fish, 2 μg/g fish over 5 days		Oral feed: plasmid in liposome				58.2 (1 μg/g fish), 66.7 (2 μg/g fish)	na	na
[67]	IPNV	Rainbow trout (5 g)	Bi-valent: IHNV glycoprotein-IPNV VP2-3 plasmid	na	1 μg/g fish	1	i.m.	15	30 or 60 days	i.p. (IPNV, IHNV+IPNV)	86.7 (IPNV + IHNV challenge)	na	+
[31]	SVCV	Common carp (11.4 g)	Glycoprotein plasmid + truncated glycoproteins plasmids	na	16.6 μg each plasmid/fish	1	injection	20	6 weeks	i.p.	similar to controls	na	na
			G protein + His/galactosidase/kanR gene/Ig kappa chain leader plasmids		16.6 μg each plasmid/fish						3		
			G protein plasmids under different promoters		16.6–25 μg each plasmid/fish						33–48		

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Table 1 (*continued*)

Reference	Virus	Host (size)	Vaccine target	Adjuvant	Vaccine amount	No. Vaccine (intervals)	Vacc. route	Vacc. Temp. (°C)	Interval vaccine challenge	Challenge route	RPS	Clinical symptoms 1	Viral load 1
[33]	SVCV	Koi carp (1.5–4.3 g)	Glycoprotein plasmid	na	10 μg	1	i.m.	19–20	28 days	i.p.	50-88* dependent on viral dose and size of fish	na	-/+ (prevalence/ load)
[34]	SVCV	Common carp (1.5–2 g)	Glycoprotein plasmid	na	1 μg/g fish 0.1 μg/g fish	1	i.m.	20	2.5 months	immersion	83.3 100	na	– na
[32]	SVCV	Common carp (5 g)	EGFP-G protein plasmid	na	1, 5, 10, 20, 40 mg/L with/without SWCNT**	1	immersion	17	28 days	i.m.	2.5–40 (increasing with dose) 5–57.5 (+SWCNT - increasing with dose)	na	na
					1, 4, 8, 12, 20 μg with/without SWCNT		i.m.				7.5–45 (increasing with doses 4–20 μg) 7.5–65 (+SWCNT - increasing with dose 1–20 μg)		
[54]	KHV	Common carp (10–15 g)	Glycoprotein plasmid	na	2.5, 7.5, 12.5 μg/fish	1	i.m.	–	42 days	/	96.7 (12.5 μg only)	na	na
[55]	KHV	Koi carp (200–300 g)	ORF25 - structural protein plasmid	na	1, 10, 50 μg/fish	3 (3 week intervals)	i.m.	25	2 weeks	into thoracic cavity (i.p.)	77.6 (1 μg), 80.4 (10 μg), 86 (50 μg)	- (all vacc. Groups)	na
[56]	KHV	Common carp (30 days)	Glycoprotein 25 plasmid	In killed *E. coli*			immersion		28 days	injection	47	na	na
[57]	KHV	Common carp (0.19 g)	G protein plasmid	In killed *E. coli*	1.3 × 108 CFU/ml (800 fish per L) 1.3 × 108 CFU/ml (1200 fish per L)	1	immersion	28	30 days	i.m.	63 10		
[60]	NNV	European sea bass (6 g)	RNA2 gene plasmid	na	10 μg in chitosan nanoparticles/fish/day for 2 days	1	oral feed	na	3 months	i.m.	45 (plus delayed onset of mortalities)	++	na
[58]	AHNV	Turbot (2.2 g)	RNA2 capsid protein plasmid	na	/	1	i.m.	15	10 weeks	i.m.	similar to controls	na	virus detected in all fish
			recombinant capsid protein	oil adjuvant	10 μg, 50 μg						57-67 (10 μg), 7–17 (50 μg)	++(10 μg) +++(50 μg)	
[59]	Betanodavirus	Asian sea bass (10–15 g)	Capsid protein plasmid	na	20 μg	1	i.m.	24	21 days	i.m.	77.33	na	na
[43]	MGNNV	Orange spotted grouer (1.2 g)	Capsid protein plasmid	na	0, 0.01, 0.1 and 1 μg plasmid/g of fish	1	i.m.	/		na	na	na	na
				CpG ODN1214: 0, 1, 10, 50 pg	1 μg/g fish				1 week 2 weeks	i.m.	36-59 (no effect CpG) 53-71 (no effect CpG)		

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Table 1 (*continued*)

Reference	Virus	Host (size)	Vaccine target	Adjuvant	Vaccine amount	No. Vaccine (intervals)	Vacc. route	Vacc. Temp. (°C)	Interval vaccine challenge	Challenge route	RPS	Clinical symptoms 1	Viral load 1
[61]	RSIV	Red sea bream (5–10 g)	Capsid protein plasmid	na	25 μg	1	i.m.	23–25	30 days	i.p.	42.8–68.7	na	na
			Transmembrnce domain protein plasmid	na							67.7–71.4		
			formalin inactivated RSIV virus (commercial)	/	100 μl		na				85.7–89		
[62]	IHV-1	Channel catfish (6–10 months)	ORF 59 (predicted glycoprotein), ORFs 6,7,8a,10 51 (predicted membrane/associated proteins), ORF 53 (predicted capsid protein) plasmids	na	50 μg/g fish	1	i.m.	20	4–6 weeks	immersion	15 (ORF 6), 38 (ORF 59), 46 (ORF 6 + ORF 59) < 46 (other ORFs)	na	–
[63]	CCV	Channel catfish (1 g)	ORFs 1, 3, 6, 7, 8, 10, 19, 39, 46, 51, 59 plasmids: immediate early, membrane, major capsid, major membrane glycoprotein. Individually tested	na	5, 25, 50 μg/g fish	1	i.m.	30	5 weeks	immersion	similar to controls	na	na
			ORFs 1, 3, 6, 7, 8, 10, 19, 39, 46, 51, 59 plasmids. Different combination tested		1 μg each plasmid in pool/fish								
			ORF 6 59 plasmids individual and combined, from current study and from Ref. [62].		50 μg each plasmid/fish								
[64]	LCDV	Japanese flounder (50–100 g)	ORF 0147 L major capsid protein plasmid	na	30 μg plasmid/fish	1	oral (stomach syringe)	/	28 days	i.m.	na	+	na
[50]	IPNV	Rainbow trout (3 g)	VP2 plasmid loaded on CS-TPP nanoparticles	na	5% boday weight, pellets with 10, 25 μg	2 (15 days)	oral	15	30 days	i.p.	47–70	na	–
			VP2 plasmid loaded on alginate microparticles								59–82	na	–

1 + + +: similar to controls, + + reduction in symptoms/viral load, + significant reduction in symptoms/viral load, - no clinical signs/no virus detected./: information not detailed. CS/TPP: Chitosan tripolyphosphate. SWCNT: single walled carbon nanotube. na: none added/not analysed. RPS: relative percentage survival. Salmon Pancreas Disease Virus (SPDV). Infectious Salmon Anaemia Virus (ISAV). Infectious Pancreatic. Necrosis Virus (IPNV). Spring Viraemia of Carp Virus (SVCV). Koi Herpes Virus (KHV). Nodavirus/Nervous Necrosis Virus (NNV). Atlantic halibut nodavirus (AHNV). Malabar grouper nervous necrosis virus (MGNNV). Red seabream iridovirus (RSIV). Channel catfish herpes virus (IHV-1). Channel catfish herpes virus (CCV). Lymphocystis disease virus (LCDV).

mechanisms were found to be upregulated by the DNA vaccines as evaluated by gene expression and antibody reactivity respectively. Since no heterologous virus challenge was included, it cannot be excluded that protection was partly due to innate mechanisms. This may count for many vaccination trials in teleost fish, where innate protection tends to play a more important and long-lasting role compared to mammals [51]. However, a subsequent oral vaccination study by Reyes et al. [52] including a 725 bp long VP2 gene fragment as antigen gene, was performed in 20 g sized Atlantic salmon pre-smolts challenged 68 days post vaccination by i.p. injection of IPNV. At this time, with this size of fish, and with a temperature of 9–14 °C, the major part of innate protection would be expected to have waned. The reported RPS values of 67% in combination with the used doses of 1–2 mg DNA vaccine/kg fish support the idea that it will be realistic to use oral DNA vaccination for protection against IPN in cultured salmonids. Interestingly, Reyes et al. [52] compared i.m. delivery of the naked VP2 plasmid with oral delivery of the plasmid in liposomes incorporated in feed (2 mg/kg fish). RPSs of 47.8% and 66.7% were observed respectively following IPNV challenge. These results reflect the trend for better protection following oral administration of IPN DNA (single gene target) vaccines. As suggested for the rhabdovirus G DNA vaccines [27], Ballesteros et al. [53] reported that the transcriptional response to successful oral VP2 DNA vaccination reflected the response occurring after IPNV infection.

2.5. DNA vaccines against other viral pathogens

A number of experimental DNA vaccines, expressing the viral G protein (ORF25 PG), have been reported for the Koi Herpes Virus (KHV, alloherpesviridae). RPSs of up to 96.7% were recorded by Nuryati et al. [54] in common carp, and of up to 86% by Zhou et al. [55] in koi. Nuswantoro et al. [56] and Aonullah et al. [57] reported a more moderate protection level, with RPSs of 47% and up to 63%, respectively. These latter studies delivered the plasmid in killed *E. coli* by immersion whereas the former studies used i.m. injection. All studies used injection as challenge route. However, it is difficult to compare outcomes of the studies due to differences in vaccine route and composition which could have given rise to variations in vaccine uptake and immune response.

Four studies on DNA vaccination against nodaviruses have been reported. Three administered a plasmid expressing the capsid gene by i.m. injection, and one incorporated the plasmid in chitosan nanoparticles in feed. All vaccinations were followed by i.m. injection of virulent virus. These resulted, respectively, in no protection in turbot [58], an RPS of 77.3% in Asian seabass [59] and 45% RPS in European seabass [60]. Chen et al. [42] reported RPSs of up to 59% and up to 71% in Orange-spotted grouper challenged i.m. at one or two weeks respectively, post vaccination (i.m.) with a DNA plasmid expressing the capsid protein of Malabaricus Grouper Nervous Necrosis Virus (MGNNV, nodaviridae). Such a short time between vaccination and challenge could imply that protection was mainly due to innate mechanisms. Again, differences in terms of vaccine constructs, formulation, delivery route, fish host species and experimental setup make comparison of these results difficult.

RPSs of 43–69% and 68–71% were reported in red sea bream after Red Seabream IridoVirus (RSIV, iridoviridae) i.p. challenge, following DNA vaccination (i.m.) with iridovirus capsid and transmembrane domain proteins respectively [61]. This was in comparison to an RPS of 86–89% using an inactivated whole virus vaccine but its administration route was not indicated. DNA vaccination (i.m.) against Channel Catfish Virus (CCV or Ictalurid Herpes Virus 1, IHV-1, alloherpesviridae) in channel catfish has also been tested, using a number of different IHV-1 viral genes. RPSs of up to 46% were obtained dependent on the plasmid insert, or combination of plasmids with different inserts [62]. Using the same plasmids, in addition to a number of other CCV ORFs, individually or in combinations, Harbottle et al. [63] repeated the DNA vaccine experiments in channel catfish and found no differences in protection compared to control fish. These fish were held at 30 °C compared to 20 °C in the Nusbaum et al. [56] study. Lower temperature in the latter might thus have prolonged innate protective mechanisms. Tian et al. [64] found significant reduction in clinical disease signs in Japanese flounder following oral DNA vaccination with the major capsid protein gene of Lymphocystis Disease Virus (LCDV, iridoviridae) and i.m. challenge.

An overview of DNA vaccination experiments, and outcomes, in non novirhabdovirus models is provided in Table 1. Summaries of novirhabdovirus DNA vaccination experiments are provided in Kurath [16].

2.6. Synthesis

When considering the variation in reported protection for these different antiviral fish DNA vaccines, illustrated in Table 1, it is difficult to draw a conclusion on their potential protective efficacy in general, and relative to other vaccine types, with the DNA vaccines generating a range of RPS values, from poor to high. There are few studies that include conventional killed virus vaccines as references for comparing efficacy, and no study that included their administration intramuscularly to allow a rigorous evaluation of the intramuscular injection as a vaccine delivery route. It is also difficult to draw conclusions on aspects giving rise to successful DNA vaccination against viral fish diseases, in general, or caused by specific virus families, due to the wide range of approaches and parameters used in the DNA vaccination-challenge experiments. Among others, these include formulation and dose of vaccine, size and age of fish, duration and specificity of protection, temperature, route of vaccination and challenge procedure. The interval between vaccination and challenge, preferably expressed as degree-days (product of temperature x days), is important for evaluating whether a protective effect may be due to innate or adaptive mechanisms – or both. It has been widely reported that the salmonid rhabdovirus G-gene DNA vaccines can generate an initial innate protective anti-viral response. The duration is temperature dependent and inclusion of a suitable heterologous vaccine (or heterologous pathogen challenge) in a time-course setup is the ultimate way to determine whether a vaccine mediated protection is due to non-specific or specific mechanisms (or both). This aspect was considered by Embregts et al. [34] who chose to leave a longer interval between vaccination and challenge to avoid interference. Specifically, how long a time is needed between vaccination and challenge to ensure that mainly adaptive mechanisms are prevalent, depends on the fish species and water temperature. In applied terms, the time of Onset Of Immunity (OOI) and the Duration Of Immunity (DOI) following vaccination are important. For classical oil-adjuvanted i.p. injected bacterial vaccines 400-500 degree-days are often recommended between vaccination and transfer of the fish to an infected environment. For the VHS and IHN DNA vaccines far less time appears to be needed, taking the protective rapid IFN response into account. However, early innate responses to vaccination are not necessarily protective, and time of OOI will have to be determined individually for each vaccine. Few studies of DOI for fish DNA vaccines have been reported, and although the salmonid VHS and IHN DNA vaccines appear to induce “life-long” protection [6,68], this will also have to be determined on a case per case basis. Having said this, costs for long term vaccine testing in experimental facilities prevent many research groups from addressing this point, and identification of early generic correlates of long-term protection would be very valuable [13,69].

The combination of vaccine delivery route and challenge model may further influence the experimental results and their interpretation. As discussed below, the optimal setup may vary, depending on the fish host and nature of the viral disease/pathogen as suggested by results observed for birnaviradae, rhabdoviridae, and orthomyxoviridae. An IPNV VP2 DNA vaccine, delivered orally encased in nanoparticles, protected rainbow trout against IPNV challenge delivered by immersion

or i.p. [48–50], whereas similar DNA vaccines delivered i.m. failed to protect Atlantic salmon against an immersion challenge [45] but gave good protection in rainbow trout i.p. challenge [65]. Mutoloki et al. [70] discuss the compartmentalisation of the immune response seen in mammalian models where both mucosal and systemic responses are generated by oral vaccination but systemic responses for the most part by parenteral vaccination. There is some evidence for this also in fish, though not full compartmentalisation [70–72]. Assuming that the DNA vaccination response is likewise compartmentalised, i.m. vaccination against IPN, followed by immersion/co-hab challenge may result in viruses gaining an initial foothold in the absence of a strong adaptive mucosal response, potentially resulting in lowered efficacy compared to that seen with challenge by injection. However, it is important not to generalize about these aspects, since progression of an infection in vaccinated animals does not only depend on the immune response profile induced by the vaccine but to a large extent on the individual pathogen, its route of infection/propagation and main target tissues in the host. Also, it should be kept in mind that while good vaccines protect against disease, they often do not protect against infection, as recently demonstrated for DNA vaccination of rainbow trout against VHS [68].

Efforts should be made to make the vaccine induce an immune response profile similar to that induced by a sublethal viral infection. In terms of applied perspectives, earlier work has demonstrated that DNA vaccination against VHS and IHN in rainbow trout can be combined and that a single injection hereby provides protection against both diseases [73]. Good protection in terms of viral load reduction was also observed by Xu et al. [67] following IPNV challenge of fish i.m. vaccinated with a bi-valent DNA vaccine expressing both the IHNV G and IPNV VP2 proteins. Although the beneficial effect of combining the two vaccines was not analysed, it is tempting to speculate that the good effect of i.m. DNA vaccination against IPN in this case might be due to an adjuvant effect of the IHN DNA vaccine triggering an early IFN-related innate response.

Fish rhabdoviruses DNA vaccines give very effective anti-viral protection, yet for other virus types much optimisation is still required, with vaccination dose, temperature, mode of delivery, adjuvants and target antigen all influencing the outcome. Remarkably, also in the case of experimental DNA vaccines for mammals, the rhabdovirus G gene constructs appear superior to many other DNA vaccines [74]. An advantage of DNA vaccines is the potential ease with which they can be modified to incorporate changes in antigen, cell/tissue targeting elements [75–77], and molecular adjuvants [10,42,78–80] to improve antigenicity and immune response. This potential can only grow as knowledge on immune mechanisms increases. Although single stranded RNA viruses such as VHSV are known to be rather variable and able to adapt to new host conditions [81], repeated passaging of VHSV in DNA vaccinated fish did not result in the virus bypassing the protective immune response [68]. Related to applied aspects, this suggests that once developed, the DNA vaccine could maintain potency for several years since the results imply that the vaccine can be used repeatedly without losing the protective effect against VHS.

3. A mechanism of action not fully understood

Although the immune responses generated following DNA vaccination have been well documented [7,16,19,82], the mechanisms underlying the responses still remain to be understood in order to improve DNA vaccination efficacy in disease models where it is currently suboptimal. There are a number of specific attributes to DNA vaccines that stimulate the innate and adaptive responses [83,84]. As discussed further below, Fig. 3 illustrates how expression of the rhabdovirus G protein by transfected cells, i.e. cells taking up the DNA vaccine plasmid, stimulate both arms of the immune response.

3.1. Interferon induction by DNA vaccination

The induction of innate immune mechanisms by DNA vaccination can be due to the recognition of DNA motifs, the mRNA transcripts and/or the expressed protein antigen. The empty plasmid itself has been reported in some cases to illicit a partial protective immune response [45,60]. Unmethylated CpG motifs, common in bacterial DNA, have been shown to act as Pathogen Associated Molecular Patterns (PAMPS), activating macrophages, dendritic cells, B cells, and stimulating a Th1 response through binding to Toll Like receptors (TLRs) [82,196]; and may contribute to the stimulating effect of DNA.

Double stranded DNA, independent of its methylation, has also been shown to stimulate the antiviral IFN response following activation of cytosolic or nuclear receptors such as the cGAS-STING pathway, as well as stimulating the inflammasome [85]. These pathways have been suggested to be important for DNA vaccine action [9,78].

In vitro studies in Rainbow Trout Gonad (RTG) 2 cell lines suggested that the VHSV G gene DNA vaccine construct (but not the empty vector), following transfection, can induce type I IFN in neighbouring cells, [86,87]. These results need to be confirmed in other cell lines, ideally myocyte-derived, and with other highly protective DNA vaccine constructs. If confirmed, then the pathway(s) involved in IFN induction should be examined in greater detail. Certain TLRs such as TLR-4 have been shown in human infection models to interact with viral proteins, stimulating type I IFN expression through a TRAM-TRIF-IRF3 pathway, and downstream activation of ISGs [88]. The G protein of the mammalian rhabdovirus VSV has been demonstrated to trigger an IFN response via recognition by TLR4 [89]. TLR-4 can be activated through binding with ligands intracellularly, or at the cell membrane surface [90,91]. TLR-4 orthologs have been found in cyprinid and in salmonids fish but their precise function remains to be determined [92].

The following examples support the positive role of type I IFN in generating protection following DNA vaccination in fish. Lazarte et al. [93] demonstrated that the incorporation of a sequence encoding for DDX41, a member of the DEAD-like helicases superfamily DNA helicase family, induced type I IFN and increased the protection to VHSV in Olive flounder. However, since the expression of the DDX41 gene alone induced a strong type I IFN response and some protection, the results did not allow for the discrimination between additive or adjuvant effect of this gene.

A DNA vaccine expressing the ISAV HE surface protein provided only a moderate protection [41]. The HE antigen appeared to be an IFN suppressor potentially impairing the early IFN response [40]. When combined with a plasmid producing any one of the type I IFN subtypes, a significantly higher level of protection was obtained [42]. A similar adjuvant effect of an IFN type I encoding plasmid was earlier reported for a DNA vaccine against cytomegalovirus in mice [94].

The early type I IFN response is transient but has been shown in murine models to be critical for the later development of antigen-specific protection [94–96]. In fish, the early response to the VHS/IHN DNA vaccines is followed by the appearance of specific antibodies, although the frequency of positive individuals varies [7,34,97–100]. Similarly, when analysed, a specific CTL response has been reported [34,42,101]. While the link between the innate and adaptive responses to DNA vaccines remains to be demonstrated directly, several observations suggest it also exists in fish.

Chang et al. [43] reported that the effect of IFN plasmid adjuvant in salmon at the injection site resulted in increased *igm* and *cd8* gene expression (the latter further thought to be from cytotoxic T cells due to elevated expression of perforin 1-2 and Granzyme A), and enhanced antibody titres. Rainbow trout B-cells have been shown to have phagocytic capacity and are assumed to be able to act like Antigen Presenting Cells (APC) [102,103]. In this context, it is interesting that studies in mice have shown that deletion of the type I IFN response abrogates the APC activation and hereby the adaptive response to DNA vaccination [14], and that B-cells cells as reviewed by Zahm et al. [11]

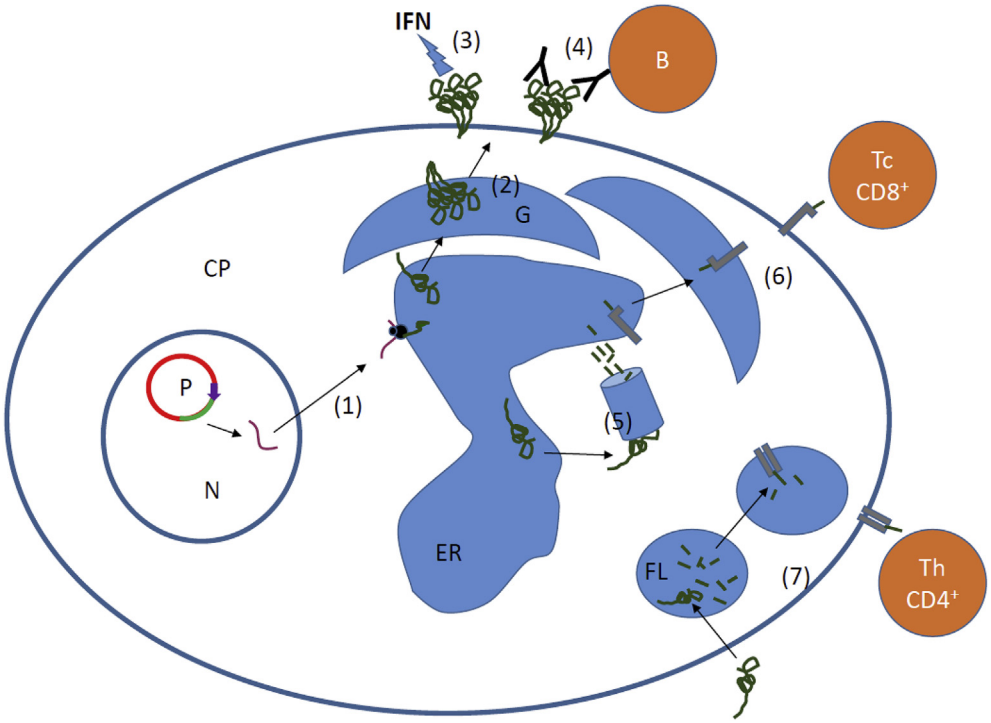

Fig. 3. Schematic illustration of immune mechanisms expected to be activated in the vaccinated fish by expression of the VHSV G protein in transfected cells harboring the DNA vaccine plasmid in their nucleus. Following uptake of the vaccine plasmid (P) in the nucleus (N), the CVM promoter (purple arrow) will drive transcription of the G gene (light green), and the resulting mRNA transcripts will be exported to the cytoplasm (CP). Ribosomes binding to the mRNA will mediate translation of the polypeptide into the endoplasmatic reticulum (ER) (1). Following translation, the transmembrane G protein will be folded and glycosylated and associate into non-covalently associated trimers while on its way from the ER to the Golgi cisternae (G) before it finally reaches the cell membrane in its mature form, protruding as spikes into the extracellular space and hereby making the transfected cell appear like a virus infected cell. Here, the G protein can be recognized by TLRs or other pattern recognition receptors on neighbouring cells, and hereby initiate an IFN type I response (3). Also, the native G can be recognized by B-cell receptors and circulating antibodies (4). Driven by the ER export system, some G protein molecules will be translocated to the cytoplasm and here be processed into peptides by the proteaosome. The peptides will be transported back into the ER where some will bind to MHC I molecules (5). Following translocation via the Golgi cisternae presentation on the cell surface will take place and mediate activation of cytotoxic T-cells (Tc) (6). Some G protein molecules will be released into the extracellular space, either by secretion or via degradation of transfected cells. Following endocytosis or phagocytosis by antigen presenting cells (transfected or not), such exogenous molecules will end up in endo/phago-lysosomes and be degraded into peptides. Some of these peptides will be bound to MHC II molecules and subsequently be presented on the cell surface, where activation of T helper cells (Th) will take place (7). Alternative routes, particularly related to antigen presenting cells, include cross presentation of exogenous G molecules being directed into the MHC I presentation pathway as well as direct presentation of endogenous peptides on MHC II molecules. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

may act as direct APCs after DNA vaccination of mice. Castro et al. [104] also reported an influx of B cells at the site of i.m. injection following DNA vaccination of rainbow trout with the VHSV *g* gene (Fig. 4), while Embregts et al. [34]; saw an upregulation of IgT, and elevated IgM following SVCV G DNA vaccination in carp, though the latter not significantly different from empty vaccine control. Though a challenge was not performed by Castro et al. [104], the protective effect of the VHSV G-DNA vaccine is well established and a high level of protection was generated in the Embregts et al. [34] study. This supports the idea discussed by Zahm et al. [11], that specifically recruiting B cells to the site of injection or direct delivery of the DNA vaccines to B-cells may be a strategy to improve efficacy. Further studies are needed to confirm whether B cell activation is important for protection in fish following DNA vaccination. Embregts et al. [34] also observed antigen stimulated host T cell (T helpers or CTL) proliferation *in vitro*, following DNA vaccination in carp.

The dual phase response observed following DNA vaccination of rainbow trout with the VHSV and IHNV G genes [28] was extended by Kurath et al. [6] to include a late response phase at which no specific antibodies are detectable, despite RPS values above 60. Protection has been observed against VHS at 6, 9 and 17 months and against IHN at 24 months after vaccination. In both cases, no antibodies were detected [35,68,105,106]. It remains to be determined whether the induction of type I IFN by the DNA vaccine plays an important part in generating long term memory B and T cells, or high affinity antibody production, as has been seen in mammalian models [107,108].

3.2. Antigen presentation

3.2.1. MHC class I and class II presentation

Activation and proliferation of cytotoxic T cells (CTLs), and B cells, depends on the presentation of processed antigenic peptides by MHC class I (intracellular) or class II (extracellular) molecules on the cell surfaces [21]. The former is typical of endogenously expressed antigens such as viral and DNA vaccine antigens. The processing of antigens through MHC class I presentation in APCs capable of activating naïve T cells, distinguishes DNA vaccines from the conventional vaccine types (inactivated virus or recombinant protein) where the APC lysosomal system/MHC class II presentation pathway is mainly activated [109,110]. The presentation through MHC class I may be one of the reasons why DNA vaccines are considered more effective against viral diseases. The influence of secreted versus membrane bound DNA vaccine antigens should therefore be considered, though Ulmer et al. [111] reported that influenza virus nucleoprotein, without signals for membrane localisation nor secretion was still released from transfected mice myoblasts *in vitro*, in the absence of cell lysis. It is thought that APCs are attracted to the site of vaccine administration and may be responsible for the CTL activation through being directly transfected and expressing the antigen internally for processing, or by cross priming [112] whereby APCs ingest extracellular antigen or antigen released from damaged/apoptotic cells and present it via MHC class I. Evidence, from i.m. vaccinations in mammalian models, suggests that CTL activation by DNA vaccines may be predominantly due to cross-priming [111,113].

Most nucleated cells can present via MHC class I but it is generally considered that only professional APCs (such as macrophages, B cells and dendritic cells) can present via both MHC class I and II [114]. However, as also outlined below, there is an ongoing discussion as to the ability of skeletal muscle, under different conditions, to express MHC class II molecules and present antigen via this pathway [115–118].

Naive $CD4^+$ T helper cells interact with specific antigen-MHC class

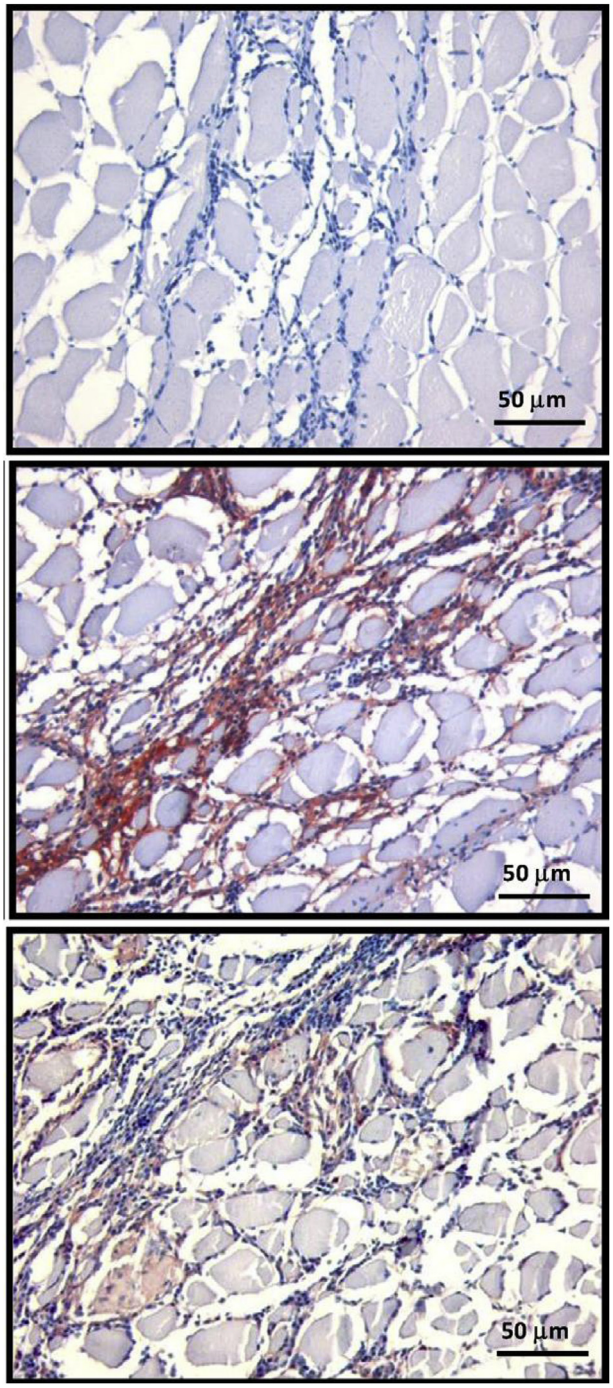

Fig. 4. Immuno-histochemical detection of presumed B cells at the injection site in the muscle of rainbow trout given the VHSV G-encoding DNA vaccine. Infiltrating IgM (Middle) and IgT (Bottom) positive cells were detected at day 7 post-vaccination using specific monoclonal antibodies in muscle sections from fish vaccinated with 20 μg of a VHSV DNA vaccine. The specificity of the reactions was determined by omitting the primary antibodies (Top). Castro et al. [104].

II complexes on APCs leading to the differentiation of B cells into antibody producing plasma cells [119].

It remains to be confirmed that the scenario outlined above also counts for teleost fish, but at the effector level both specific antibody and CTL responses have been observed following DNA vaccination in mammals and in fish [34,101,120].

Though both MHC class II restricted $CD4^+$ T cell activation and MHC I-restricted or perforin-mediated lysis have been suggested to orchestrate destruction of antigen-expressing mouse myocytes following DNA vaccination, it was also demonstrated that destruction occurred in part due to perforin-independent mechanisms, namely Antibody-Dependent Cellular Cytotoxicity (ADCC), such as complement or Fc-receptor mediated antibody-dependent cell cytotoxicity [121]. Therefore, other response types, independent of MHC class I presentation may play important roles in DNA vaccine protection.

3.2.2. Myocytes as antigen presenting cells (i.m. vaccine administration)

Though still debated, it has been found by some researchers that muscle cells can act as APCs in mammalian systems, processing antigen and expressing co-factors necessary for MHC class I $CD8^+$ T-cell activation [122]. It was previously stated that muscle cells do not possess MHC molecules, and this is largely true of unstimulated muscle cells [123]. However, transfection with DNA vaccines was shown to upregulate MHC class I and co-stimulating factors in mice myocytes in the presence of IFN-γ, with siRNA knockdown of the Interferon Regulatory Factor (IRF) 3 significantly reducing MHC class I presentation [122]. Other non-immune cells have been converted to facultative APCs following transfection with dsDNA, or in a cytokine environment characteristic of an inflammatory response and type I IFN, both characteristics of DNA vaccination [122]. Of note is that Shirota et al. [122] reported that different muscle cell lines exhibited these characteristics more frequently than other cell types, again supporting i.m. injection as an optimal delivery route for DNA vaccination in terms of promoting APC presentation and generation of an adaptive response. MHC class II antigen (endogenous and exogenous) presentation to $CD4^+$ T cells has also been reported in human muscle cells following exposure to the pro-inflammatory cytokines IFN-y and TNF-α [117], or in inflammatory myopathies [116].

In initial studies, MHC class I and II expression were absent from carp and Atlantic salmon muscle [124]. However, these studies were performed on unstimulated tissues. Subsequently, MHC class I expression was found upregulated *in vitro* in Atlantic salmon myocytes, stimulated with Interleukin-1 beta. MHC class II was not upregulated, but the published report did not state whether it was present at basal levels [125]. It would be worthwhile to assay for MHC class II expression in fish myocytes following stimulation with DNA vaccine relevant molecules – e.g. dsDNA, type I and II IFN, or following transfection. Early studies demonstrated both upregulated expression and increased presence of MHC II at the injection site of fish vaccinated with the VHSV G DNA vaccine [120,126]. While this was assumed to be associated with infiltrating cells, expression by myocytes might also have contributed.

3.2.3. Conformation of antigen proteins, and potential influence on endosomal processing and epitope presentation

As the production and processing of the antigen encoded by the vaccine plasmid reflects that of a natural viral infection, transmembrane glycoprotein vaccine antigens will be exposed in their native form/conformation at the surface of transfected cells. This was clearly illustrated by the expression of correctly folded VHSV G protein in transfected fish cell cultures (Fig. 5). Due to the 6 intramolecular disulphide bonds, correct folding of this protein in prokaryotic expression systems remained a challenge in early approaches towards recombinant vaccine development [128,129]. Although correct protein conformation may not be required for presentation of antigen peptides in the MHCI and MHCII context, it may be important in some circumstances to optimise presentation (see below) and is probably more crucial for the stimulation of innate mechanisms as well as by recognition by B-cells and production of protective antibodies (Fig. 3 and [127]). Folding into their native conformation may require interaction between multiple viral proteins for some viral antigens, therefore, requiring a polyprotein encoding DNA vaccine for expression of antigenically and immunogenically correct antigen. In addition, the sequence and expression of the polyprotein transcripts may be important, with post-translational proteolytic cleavage enhancing immunogenic effect [45]. It has

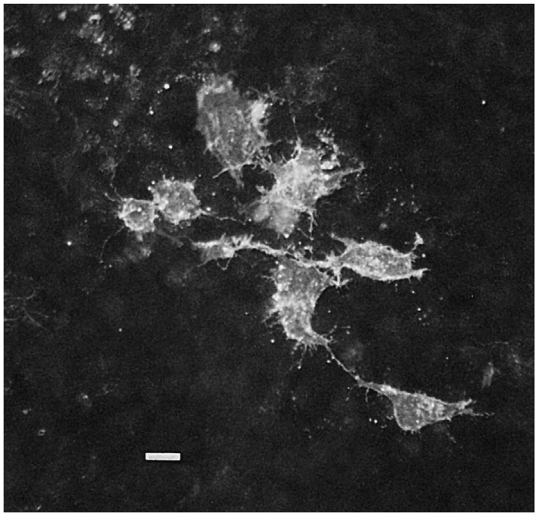

Fig. 5. DNA vaccination mediates expression of correctly folded antigen. EPC cells were transfected with a VHSV G gene DNA vaccine plasmid construct and stained by indirect immunofluorescence using a primary monoclonal antibody recognizing a disulfide bond dependent conformational neutralization epitope on the viral G protein. Bar corresponds to 20 μm. Lorenzen et al., [127].

also been recently shown in human and mammalian models that some presented antigens result from peptide splicing of different regions of a protein, that the splicing is not random, that spliced antigens can represent up to 25% of the antigens presented, and that some are important for induction of protective mechanisms [130,131]. Fusion peptides from two different proteins, presented via MHC II have also been reported, but only in relation to host endogenous proteins to date [132]. Therefore, though Proteasome-Catalyzed Peptide Splicing (PCPS) has not been demonstrated in fish, it is possible that incomplete or modified proteins as DNA vaccines may result in loss of potentially important immunogenic antigens, found in natural infections.

The presence of fusion partners, incorporated in many of the experimental fish vaccines may also influence antigen presentation. Mikalsen et al. [45] speculated that the EGFP fusion protein may have unmasked epitopes resulting in immunostimulation. Alternatively, a fusion partner preventing correct folding of the fusion protein may stimulate increased proteasome processing, or autophagy when protein aggregates build up in cell [133]. Autophagy plays a role in numerous immune functions, including promotion of MHC II antigen presentation and T cell polarisation [134], whereas proteasomes, in a pro-inflammatory environment as described for i.m. DNA vaccination, convert to immunoproteasomes, increasing antigen presentation on MHC I molecules [135]. However, when correct antigen conformation is critical for immunogenicity, fusion partners may have negative impact on vaccine efficacy (Authors unpublished observations).

This latter hypothesis, of increased MHC I peptide presentation resulting from misfolded proteins (DRiP: Defective Ribosomal Products) has recently been questioned, with studies indicating that the antigen peptide repertoire arises from the entire cell proteome i.e. newly synthesised misfolded and stable long-lived proteins [136]. Though specific sequence has been added to vaccines to improve immunogenicity or targeting of different cell compartments involved in immune response, it has also been shown in mammals, in the case of MHC II antigen presentation, that inclusion of linkers or spacers can result in changes to the profiles of naturally immunodominant antigenic epitopes [137].

One additional aspect of antigen presentation, related to the conformation and stability of the translated vaccine target, may be important for the CTL response associated with DNA vaccination. It has been reported for mammalian models that MHC class I presentation by APCs in muscle, or other non-APCs following DNA vaccination may be for the most part via cross presentation [111,138–140] although other studies dispute this and indicate that direct transfection of APC cells, or direct activation of CTL by transfected non-APC in a suitable environment, may be equally important [141,142]. In relation to cross-presentation and activation of a CTL response, it has also been demonstrated in mice that while unstable/short lived proteins are efficiently processed via MHC class I direct presentation pathways, stable proteins are required for cross presentation by MHC class I [143]. Studies have also indicated that antigen produced in a particulate as opposed to soluble form, and potentially associated with cell membrane, may induce a stronger cross-priming response [140]. Therefore if cross-presentation is found to represent the/an important pathway for generating CTL responses following DNA vaccination, then the preference for stable protein/expressed in particulate form may offer one explanation of why i.m. DNA vaccination with IPNV polyprotein can offer protection following immersion challenge whereas VP2 only cannot (birnavirus VP2 requiring interaction with the VP3 protein for particle assembly) – i.e. it results in generation of a stronger $CD8^+$ response in a cross-priming environment of muscle tissue. In addition, protection generated by oral delivery of VP2-only expressing DNA vaccine in alginate, chitosan or liposome particles [48–50], may be due to more efficient direct uptake of these particles by APCs in the absence of stable protein particulate formation. Further comparative vaccination trials with parenteral vs oral vaccine delivery followed by different challenge routes in the same fish species are needed to clarify these aspects.

The above observations in relation to cross-presentation are based on studies in mammalian models, and even within these there are disagreements and there remains much still to be understood. The occurrence of cross presentation in fish is unknown. The existence of dendritic cell types, a major cell type associated with cross presentation [144], in fish also remains uncertain even though there is increasing evidence for their presence [145–149]. This provides a basis from which to further investigate antigen presentation in fish, and potential to improve vaccination strategies.

3.3. Understanding long lasting protective mechanisms through improved analysis of the adaptive immune responses

Protection following DNA vaccination against IHN and VHS have been demonstrated at 2 years and at 17 months post vaccination, respectively [35,68]. However, even though T cell activation and antibody production have been observed in fish following DNA vaccination, the generation of long lived activated T or B cell populations (immune memory), is far from being established. Some of these issues are due to lack of fish immunological tools, such as well established cytotoxicity assays, and markers for identification of different T and B cell subpopulations. However, some improvements could be considered in terms of experimental design and analysis, in particular the analysis of the antibody response. Within the studies discussed in this paper, antibody analysis consisted of total antibody titres, antigen specific antibody assays (ELISA), neutralising antibody assays (most common), and/or complement activated antibody assays. While neutralising antibodies are protective [120] and have been found elevated in protected fish they are not consistently detected in all individuals [16,105], and non-neutralising antibodies may also confer protection [150–153]. In terms of immunoglobulin gene expression, analyses focus on IgM and IgT, and analyses for both membrane forms and secreted forms are not always performed. Likewise, IgD is rarely assayed for, despite its role now being better understood in mammalian systems [154], secretory IgD identified in rainbow trout, and IgM^-/IgD^+ B cell populations being defined in carp and trout [155–157]. IgD specific antibodies may contribute to long term immunity through interacting with and stimulating basophils to produce proinflammatory and B cell activating cytokines [158] earlier in the response. Antibody affinity maturation has not been confirmed in fish. There is an assumption that it does not occur, based largely on the absence of germinal centres, but a number of studies have

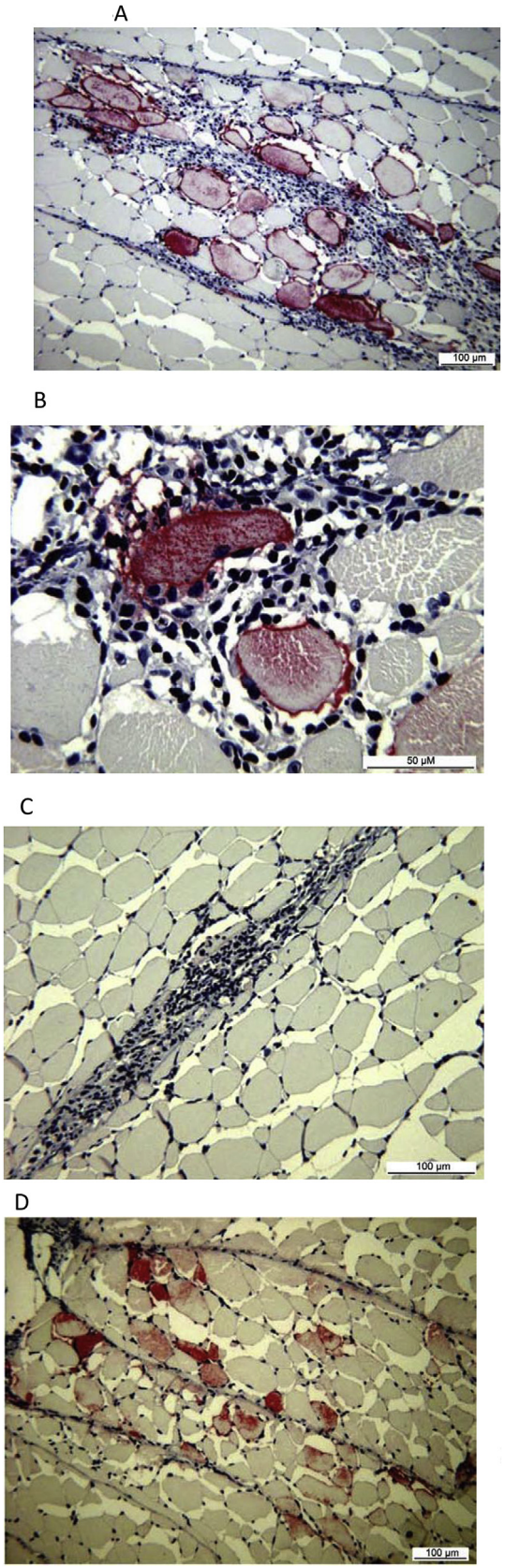

Fig. 6. Visualization of the local inflammatory response in DNA vaccinated rainbow trout by immune-histochemical staining of tissue sections of muscle injection site. The fish had been injected i.m. either with a plasmid encoding the VHSV G protein (A, B, C), or with a plasmid encoding the VHSV N protein (D). Tissue was sampled at 21 (A, B), 31 (C), or 38 days post vaccination (D). Staining was performed with monoclonal antibodies specific for the VHSV G protein (A, B, C) or for the VHSV N protein (D) respectively [126]. Massive leucocyte infiltration is seen in response to muscle cells expressing the G protein resulting in their elimination within 5 weeks post vaccination (A-C). In contrast, only a modest reaction is evident to muscle cells expressing the N protein, still being detected at this time (D).

shown features in fish consistent with the process [159]. Ye et al. [160] highlighted the kinetics of changes in rainbow trout B cell subpopulations, based on their antibody affinities post vaccination. Trout B cell populations secreting low affinity antibodies (to keyhole limpet hemocyanin) occurred first and decreased early on, while high affinity antibodies only became apparent at later stages. It was suggested that as in mammalian models, high affinity antibodies in trout may be indicative of plasma cells, long lasting and producing high titres, while low affinity antibody B cells do not progress from the plasmablast stage [160]. The differentiation to high affinity B cell populations at later stages may be due to antigen-driven selection when available antigen decreases. These rainbow trout B cell populations were distinguished using an ELISA based affinity partitioning assay, and it was observed that high affinity antibody producing B cell populations only developed around week 15 post vaccination, beyond the period assayed for in many of the reported fish (DNA) vaccination trials. Furthermore, such a shift in affinity/B cell populations would easily be masked using classical affinity assays. Cain et al. [161] and Kaattari et al. [162] also saw increases in antibody affinity over time in rainbow trout following antigen administration. Robertson et al. [79] monitored antibody response, using ELISA, for a period of 22 weeks post vaccination with a DNA vaccine encoding the ISAV HE protein + IFNa or IFNc adjuvant constructs. He found that at week 22, pHE + IFNa antibody levels had declined, while those for pHE + IFNc were still high. These late antibodies may have been representative of higher affinity B cell populations. However, the pHE +IFNc antibody levels also started later, and antibody affinity was not analysed. A better understanding of the antibody response generated may shed more light on the mechanisms giving rise to their generation, and to responses to DNA vaccination.

4. Vaccine delivery route

In comparison to conventional i.p. delivered whole inactivated vaccines, DNA vaccines have generally been delivered i.m. in fish although oral delivery of DNA vaccines in fish is now being investigated with mixed results [52,60,66]. Therefore, to date, a simple intramuscular injection of the purified plasmid has been often demonstrated as an effective route to generate an immune response leading to protection in fish. The ability of the myofibrils to act as an efficient “protein factory” may explain some of this success, with muscle cells in mammalian models proving the most efficient cell type analysed with respect to plasmid uptake and protein production [163]. In addition, the increased hydrostatic pressure in the muscle tissue at the time and site of injection may also explain the efficient cellular intake of the plasmid [164,165], while Wolff et al. [166] observed cellular uptake of the plasmid DNA through membrane invaginations and T tubules, found on muscle cells. Trout myocytes have been shown to express antigens encoded by the DNA vaccine up to approx. 1 month and half after administration by i.m. injection [120,126], though longevity of transfected muscle cells may be dependent on the immunogenicity of the expressed target [34,167]. A number of authors have suggested that damage to the muscle tissue/cells at the injection site gives rise to a pro-inflammatory response, which in turn promotes the migration of leukocytes, including APCs to the site. Both control and DNA plasmid

injected fish demonstrated upregulation of pro-inflammatory genes and leucocyte influx, supporting the idea that the local response to DNA vaccination was due to a combination of injection damage and antigen expression (see Fig. 6) [34,126].

Attempts to use routes other than intramuscular injection of the rhabdovirus DNA vaccines have shown limited success. Following their successful oral DNA vaccination against IPN in rainbow trout, Ballesteros et al. [168] report some protection against IHN in rainbow trout following prime-boost oral delivery of 2 × 25 μg or a single delivery 100 μg of an alginate formulated IHNV DNA vaccine to 3–4 g sized rainbow trout fingerlings. However, although a systemic response with early upregulation of innate type I IFN related genes and a later somewhat elevated antibody reactivity was found, the highest obtained RPS value was 56% compared to an RPS of 70% obtained with a 20 times lower dose delivered by i.m. injection. Furthermore, since the authors did not include control fish receiving similar high amounts of DNA plasmid without the IHNV G gene inserted, the protection could at least partly be due to non-specific effects.

DNA vaccine incorporated into nanoparticles [169] may be a more promising approach as the size of particle influences the delivery to a particular type of immune cells ensuring optimal antigen presentation, for example nanoparticles tends to be phagocytized preferentially by dendritic cells [170]. This in turn may result in presentation via MHC class I [171] and activation of the specific CTL response typical of viral infections. Microparticles are more likely to be phagocytosed by macrophages and the antigens presented via MHC class II, generating a humoral response [171]. Particle size can also influence the process of cross-presentation [172].

5. Molecular adjuvants

Molecular adjuvants are plasmid encoded molecules producing signalling molecules, cytokines or other factors added to the DNA vaccine to enhance its immunogenicity and efficiency [173]. There are examples of DNA vaccines against bacterial diseases exhibiting higher efficiency in combination with molecular adjuvants such as Myd88 [174] or IL8 [80]. The most impressive adjuvant effect was seen with a DNA vaccine against ISA, where the ISAV HE gene construct alone provided moderate (calculated RPS = 43%) compared to high protection (RPS = 91%) when combined with an Atlantic salmon IFNa encoding plasmid. Importantly the IFNa plasmid alone gave minimal protection (RPS = 9%, [42]).

6. Safety aspects

Some potential risks have been associated with DNA vaccination. With respect to the vaccinated host, these include integration into genome and disruption of biological processes, and potential unwanted immune responses such as auto-immunity or tolerance to the pathogen [175,176]. Limited data is available for fish, but no significant adverse effects on the host have been identified in initial safety testing in humans [177].

The risks to the consumer concerns the potential ingestion of any residual plasmid from food products, containing elements such as human viral promoter regions (such as the CMV promoter) or antibiotic resistance genes that could potentially have harmful consequences if integrating into the consumers' genome or taken up by their gut microflora. However, this risk is considered negligible since the consumer is one step removed from the presentation of vaccine to the vaccinated animal, and at the site of vaccine injection there is a rapid degradation of the plasmid, within 90 min after vaccination in mice [178]. Fast degradation of the plasmid has also been observed in fish [82]. Considering the time between injection and harvest in a normal fish farming production cycle, the residual plasmid in the fillet reaching the consumer would be extremely low. Moreover, after stomach degradation, antibiotic resistant genes reaching the gut flora would be even lower [177]. This combined with the fact that natural integration rates are very low, makes the overall risk nearly negligible.

In addition, there is a risk of release of the plasmid into the environment. Intramuscular injection route results in plasmid leakage due to the compactness of the muscular tissue and the hydrostatic pressure increasing at the end of the needle [179]. On a local scale, this can represent a significant amount of plasmid released into the environment. There is also a possibility of plasmid shedding from the animal after vaccination [52]. The environmental risks of DNA plasmid release again relate to components contained in the plasmid backbone. A main concern is that elements such as antibiotic resistance gene markers recombine and propagate in the environmental bacteria flora [180,181]. Similarly, bacterial populations in/on the host – e.g. in intestine or on skin or gill, may also encounter plasmids released externally or disseminated internally from vaccine delivery site. However, plasmid DNA will begin to degrade once released into the crude environment. Also, most plasmids have been engineered to be able to propagate only in limited bacterial hosts [182] and are modified to render them mobilisation defective and reduce the risk of uptake by bacterial microorganisms in the wild [177]. A very small number of bacteria have been found to date to be able to incorporate plasmids from which specific uptake signals have been removed. If a concern at all, it may need to be investigated in relation to oral vaccination more so than injection, due to greater exposure of plasmids in general to bacterial populations via this route. A further safety precaution could be taken by carrying out DNA vaccination in a dedicated facility away from natural water sources. However, safety issues relating to transfer to environmental organisms was considered to be negligible by scientists surveyed for their expert opinions [176,183]. Alternative strains of *E. coli* with selection mechanisms by trophic complementation would allow the isolation of antibiotic-free DNA vaccine [184,185] with the possibility to reduce any environmental risk. Replacement of the CMV promoter by fish promoter has also been investigated [198].

7. Marketing authorisation of DNA vaccines within the EU

Directive 2001/82/EC regulates veterinary medicinal products (VMPs) where a VMP is defined as (a) any substance or combination of substances presented as having properties for treating or preventing disease in animals; or (b) any substance or combination of substances which may be used in or administered to animals with a view either to restoring, correcting or modifying physiological functions by exerting a pharmacological, immunological or metabolic action, or to making a medical diagnosis. With respect to human medicinal products, falling under Directive 2001/83/EC, a distinction is made at the regulatory level between DNA vaccines against infectious disease, and those against non-infectious disease e.g. cancer cells, with the latter falling under gene therapy and a different set of regulations. This distinction is not made in relation to veterinary medicines. All veterinary DNA vaccines are assessed under the same set of EU regulations and relevant additional guidelines.

Key issues concerning approval, marketing and administration of DNA vaccines for veterinary use, are whether they, or subsequently the recipient host, are considered genetically modified organisms (GMOs). In the scenario where both vaccine and vaccinated species were defined as GMOs, EU *Directive 2001/18 relating to the deliberate release into the environment of GMOs, and/or labelling *regulations under GM Food and Feed Regulation (EC) No. 1829/2003 and Traceability and Labelling of GMOs (EC) No. 1830/2003, would need to be applied in addition to Directive 2001/82/EC [186]. Not only would this make marketing authorisation more onerous to obtain, but as public opinion is divided on the subject of GMOs, classification of DNA vaccines or recipient hosts as GMOs would therefore have a detrimental impact on acceptance of the DNA vaccines, and their commercial uptake. Good summaries of these and other issues in relation to regulatory approval for fish DNA vaccines can be found in Lorenzen and La Patra [7];

Evensen and Leong, [19]; Holvold et al. [15]; Myhr [176]; Dalmo [13]; The Norwegian Advisory Technology Board [197] and Gomez-Casado et al. [187].

However, under EU legislation, DNA vaccines appear not to be considered as GMOs given the recent example of CLYNAV, a DNA vaccine against SPDV (see below). EU Directive 2001/18/EC defines "organisms" as any biological entity capable of replication or of transferring genetic material. GMOs are defined as organisms, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination. These definitions do not unambiguously exclude a plasmid, given that plasmids can replicate in bacterial cells and can transfer genetic material between bacteria, and that modified viral vectors, which also are incapable of replicating on their own, can be considered as GMOs. Nevertheless, the EU Commission has ratified the Cartagena Protocol (biosafety of GMOs in the environment) where it is stated that plasmids or naked genetic material are not considered as organisms [197] based on the criteria that the plasmid cannot replicate on its own. Given the decision that the DNA vaccine CLYNAV is not a GMO, then, unless a plasmid is deliberately modified to promote integration into a host genome, or to replicate in a eukaryotic host, it is unlikely to be considered a GMO under EU regulations.

The next consideration is whether DNA vaccinated animals are considered GMOs. Under Directive 2001/18/EC, Annex 1A, Part 1 lists techniques of genetic modification. Among others, this includes the insertion of nucleic acid material into plasmid vector systems, followed by administration of these into a host organism in which they do not naturally occur and where they are capable of continued replication. Secondly, techniques involving the direct introduction into an organism of replicating heritable material prepared outside the organism by micro- and macro-injection and microencapsulation. Therefore, the wording of EU directive 2001/18/EC does not specifically exclude the classification of DNA vaccinated fish as GMOs. However, in relation to DNA vaccines the plasmid will not replicate in the eukaryotic host, unless specifically modified to do so. Also, integration of the vaccine DNA into host cell (somatic or germinal) genomes is considered an unlikely event, as long as the plasmid is not specifically designed for this ([188]; Danish Medical Agency). Among European countries, only the UK, Denmark and Norway have so far stated that DNA vaccinated animals are not genetically modified. This statement is qualified by the pre-requisite that the foreign DNA is not expected to integrate into the host's genome. It seems likely, based on the successful outcome of the Marketing Authorisation Application (MAA) for the CLYNAV DNA vaccine, that the EU may take a similar stance on future DNA vaccines – i.e. if the assessment shows risk of integration is negligible, then the vaccinated animals will not be considered as GMOs.

Despite the decision on CLYNAV it must be remembered that each new DNA vaccine submitted for marketing authorization will be evaluated on a case by case basis. Changes to the vaccine may or may not affect the designation of the plasmid as a GMO (e.g. incorporation of viral ORFs and associated systems for eukaryotic replication) or the recipient host as GMO (vaccine/insert or delivery modifications which increase likelihood of host genome integration).

7.1. Marketing Authorisation Applications (MAAs)

The route for authorisation of a veterinary DNA vaccine in the EU is through the European Medicines Agency (EMA), which operates the centralised system for Marketing Authorisation Applications (MAAs) (Regulation (EC) No 726/2004). The centralised route for authorisation is mandatory for products developed by biotechnology.

The application is assessed by the Committee for Veterinary Medicinal Products (CVMP) applying the technical requirements laid down in Directive (2001)/82 and associated guidelines. A specific guideline to consider when preparing an application is "DNA vaccines non-amplifiable in eukaryotic cells for veterinary use" EMA/CVMP/IWP/07/98 http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/general/general_content_001629.jsp&mid=WC0b01ac058002ddc5. This guideline is under revision with a target date for completion in 2018.

The additional guideline includes requirements for assessments of genomic integration (quantitative as well as qualitative), consideration (assessment not currently required; Klug et al. [189]), of undesirable immune effects including autoimmunity or allergic response, perhaps in particular with respect to genes encoding host immune-stimulatory molecules, and any undesired biological activity of the expressed antigen. The guidelines also set out requirements for design details (justification for use of all plasmid elements, in particular selection markers such as antibiotic resistance genes and long retroviral-like terminal repeats should be avoided if possible), efficacy, and quality control during production. With respect to safety testing of the DNA vaccine itself, this should be carried out in line with 2001/82/EC Title II Part 3. Uptake, distribution and localisation of the plasmid must be analysed, at different time points after administration, taking into account both the route of administration and the amount of plasmid inoculated. Sampling times should be based on duration of gene expression and persistence of the DNA in the vaccinated animal. In terms of the distribution studies, gonadal tissues should be included if necessary to assess the risk of DNA integration into germ line cells of vaccinated animals, and any welfare or environmental impacts this might have. Given the reports of high levels of introgression from interaction of farmed Atlantic salmon escapees with wild salmon [190], then this is an important consideration for DNA vaccinated fish in open pens.

In addition to the standard requirements for assessing a non-GMO biotech veterinary vaccine, for the first authorised DNA vaccine in Europe, CLYNAV, an extended environmental risk assessment in line with those required for GMOs as outlined in Directive (2001)/18/EC was provided to address the potential safety issues for the host, consumer and environment.

* EU Regulation: "binding legislative act which is applied across the EU, such that each government does not have to take individual action to implement the regulations". EU Directive: "legislative acts but up to individual countries to decide the corresponding action needed" (EU Regulations). Scientific guidelines: no legal enforcement (EMA Scientific Guidelines).

7.2. CLYNAV example

The DNA vaccine CLYNAV, (Elanco), generated against infection with the SPDV subtype 3, was approved for marketing authorisation by the EU Commission in 2017. This is the first DNA vaccine approved for use within the EU, and 12 years after the first and only other DNA vaccine (Apex®-IHN) for use in fish, licensed by the Canadian Food Inspection Agency (CFIA). Norway, as a member of the European Economic Area, and one of the main target markets for the CLYNAV vaccine, independently approved the MA, following the EU decision.

In the case of CLYNAV, the CVMP recommended marketing authorisation based on submitted quality, safety and efficacy data, concluding an overall positive benefit-risk. This decision by the CVMP members was not unanimous. The concerns raised by CVMP members voicing a negative opinion focused on risk to vaccinated salmon health and welfare, duration of protection, interaction with other vaccines, and adverse reactions at site of injection reducing fillet quality but did not relate to integration nor consideration of GMO status nor risk of environmental impact. The main issue was that Atlantic salmon currently fall under the MUMs list (EMA/CVMP/IWP/123243/2006-Rev.2 http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/04/WC500089628.pdf accessed in Feb 2018) and therefore data from laboratory experiments are sufficient, without the need for corresponding data from field trials. It is sufficient to carry out trials at two premises (CVMP guidelines) and with a defined number of fish within the premises. Those against supporting marketing

authorisation felt that, with such a novel vaccine technology, field trials should have been included. These points were also raised by the Norwegian Medicines Agency (NoMA), who has given approval for use of CLYNAV in Norway, following the European Commission decision, but recommend that the vaccine should not be in general use until sufficient clinical evidence is acquired on safety and efficacy. Consequently, the use was initially limited to 500,000 doses (https://legemiddelverket.no/nyheter/legemiddelverket-anbefaler-ikke-alminnelig-bruk-av-ny-laksevaksine accessed in Feb 2018). However, to carryout a field trial prior to the CVMP assessment and the EU decision, may have entailed consideration of whether it constituted deliberate release of GMOs into the environment, as, at that stage, a decision on status of DNA vaccinated fish was not clear.

In April 2016, the EU Commission mandated the European Food Standards Agency (EFSA) to review data provided by Elanco on potential for integration of CLYNAV into the salmon genome [191]. No integration event in vaccinated fish was detected using a probe capture enrichment- Next Generation Sequencing approach and, based on this and additional theoretical modelling of potential integration rates, the submitted data report concluded that the integration rates were negligible. However, no quantitative values were placed on the term "negligible" and due to no limit of detection (LOD) being defined, and a number of highlighted issues with the approach, the EFSA could not evaluate the term "negligible" with respect to integration rates [191].

The EFSA did however, construct worst case scenarios leading to upper estimates of integration for the CLYNAV DNA vaccine. These are based on knowledge of homologous and non-homologous integration rates, and on levels of QPCR CLYNAV plasmid detection in muscle and gonadal samples at termination of experiments, or LOD of QPCR plasmid detection assays where no plasmid was detected. The worst-case scenario assumed that all plasmid detected at termination of the experiment was considered integrated even though data indicated that "free" non-integrated plasmid was still likely to be present in samples. Based on this, predictions were that 1 in 31,250 gonadal cells would have one copy integrated, while integration would occur in 1 in 915–1524 diploid muscle nuclei in salmon injected with 2–10 times recommended commercial CLYNAV doses. Therefore, the EFSA predicted that true values of integration could be expected to be orders of magnitudes less than this, though no value was provided. It should be noted that the study termination in this case was 822 days post vaccination, considerably longer than most farmed Atlantic salmon production cycles. Assessing worst case scenarios for integration at later time points must make the assumption that any integration into tissue is stable over time – i.e. genetically modified cells are not lost over time, so that end point also represents integration levels at relevant earlier time points. In the CLYNAV study, detection of plasmid in gonadal tissue was below LOD from 14 dpv onwards for both 2× and 10× vaccination doses [191].

Even though in this instance the EFSA used QPCR biodistribution data to estimate worst case scenarios for integration, QPCR data alone was not acceptable in a previous data assessment on CLYNAV integration [192]. The US Food and Drug Administration has stated that integration studies are only required when greater than 10,000 plasmid copies per μg host DNA are present by study termination [193]. Again, a value for termination timepoint is not specified.

In July 2017, the EU Commission granted marketing authorisation. Therefore, the above integration levels for worst case integration scenarios could potentially be used as a benchmark for future vaccine assessments. It is likely however, that EU regulatory bodies will still require some form of integration study included in MAAs, beyond QPCR detection of plasmids, for the immediate future. What would happen in terms of EU authorisation if one or few integration events were detected and confirmed, while residual plasmid levels based on QPCR suggested negligible risk, remains to be seen. Irrespective of the data for the CLYNAV vaccine, it is clearly stated in both EU and NoMA guidelines that each DNA vaccine must be considered on a case by case basis as the DNA vaccine design including features in the plasmid backbone or the inserted transgene, may change the risk and the impact of any integration into the host, or in organisms in the environment outwith the host.

Overall, the granting of marketing authorisation for CLYNAV is a positive step forward in the development of DNA vaccines for commercial use in aquaculture. It has helped to clarify aspects related to the GMO status of the vaccine and vaccinated hosts, and it has established approaches for generating appropriate data for MAAs, including indication of what would be acceptable values for these data to support granting of authorisation.

Nevertheless, as mentioned, every DNA vaccine will be assessed on its own characteristics, moreover as modifications are relatively easy, and concepts and technology are continuously advancing. Policy and scientific advice on authorisation must advance in timely fashion alongside these, not only to reduce uncertainty and encourage commercial investment in research, but also to help alleviate any public concern through proactive rather than reactive provision of information [7,176,187].

8. Concluding remarks and future perspectives

The recent approval of the first DNA vaccine for the aquaculture sector in Europe will test the public acceptance of such a vaccine. Given the still very negative public opinion of GMOs and GMO containing food, in both the EU and indeed elsewhere, and mistrust of scientists and commercial interests, it is important for those most likely to be involved in discussing the use of DNA vaccines to consider how DNA vaccines are presented. However, as long as neither the plasmids nor the vaccinated host are considered GMOs, and no related labelling of the final product is required, it may be expected that use of DNA vaccines may receive limited attention among consumers. Response to any public concerns or queries regarding the CLYNAV vaccine may determine attitudes to the use of this and future veterinary DNA vaccines in Europe. All information regarding evaluation of risks versus benefits should therefore be publicly available, including the rationale for considering DNA vaccines like CLYNAV safe.

Nevertheless, future efforts towards an even higher safety level by e.g. replacing plasmid CMV promoter and antibiotic resistance genes should still be considered important, to alleviate any public concern despite their minimal risk to consumer or environmental safety. Moreover, as antimicrobial resistance, including occurrence in food chains and environment, is now of major global concern and increasingly in the public eye, such initiatives can contribute to a better public acceptance. Lastly, the recent revival of RNA vaccines in the medical research [194,195] may challenge the long-term development of DNA vaccines should they prove more efficacious, commercially viable and less controversial in terms of safety.

It is reasonable to assume that the industry will be willing to invest into R&D for solving these issues as well as in improvement of delivery, efficiency and safety of DNA vaccines that may be authorised in the future. Vaccination machines, improvement of formulation and adjuvants have the potential to decrease the costs of DNA vaccination and in turn to improve the health status of European finfish aquaculture. For this purpose, the comparison between the mammalian and fish models with respect to delivery and efficiency, the identification of the mechanisms generating early innate response and its interaction with the long term adaptive response are important. More focus should be placed on the vaccine-antigen characteristics and mode/route of delivery in stimulating those mechanisms underlying interferon induction, antigen presentation, and B and T cell activation. Importantly, experimental vaccine testing should include dose-response, time-course and specificity of protection aspects. Furthermore, DNA vaccine testing should preferably include comparison with more traditional forms of vaccines (inactivated or attenuated virus vaccines), and efforts should be made to develop correlates for evaluation of long term protection. As

for testing of other vaccines, challenge trials should be designed to reflect natural conditions as far as possible, in order to achieve results reflecting vaccine potency under farming conditions.

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Opinions given in section 7 relating to the marketing authorisation of DNA vaccines within the EU represent the views of the authors and not EU agencies, expert working groups or legislators.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fsi.2018.07.012.

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